Endogenous phenolics and starch modifying enzymes as determinants of sorghum for food use in Burkina Faso

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Abstract

The objective of this thesis is to screen for biochemical determinants in sorghum varieties cultivated in Burkina Faso to identify the best sorghum varieties to be used as source of bioactive components or for specific local foods, e.g. "tô", thin porridges for infants, granulated foods "couscous", and local beers "dolo". The results revealed that sorghum varieties have different contents of starch, amylose, amylopectin and that germination decreased more the content of amylose than amylopectin. While on average α-amylase activity increased in all varieties by 2-20 fold, β-amylase activity did not uniformly increase after germination and even decreased in some varieties. The majority (82%) of sorghum varieties cultivated in Burkina Faso are low proanthocyanidins (condensed tannins) containing sorghums. Some varieties had relatively high content (> 0.4%, w/w), of phenolic compounds such as 3-deoxyanthocyanidins and flavan-4-ols, which are of particular interest for food, colorant and pharmaceutical industries. Independent of grain germination, all sorghum varieties are among the most prominent natural sources of antioxidants due essentially to their phenolic contents. Sorghum varieties are highly polymorph in their expression of phenolic biosynthesizing enzymes, e.g. phenylalanine ammonia lyase, and phenolic modifying enzymes, e.g. peroxidases and polyphenol oxidases. Several peroxidase isoenzymes are expressed both before and after germination, and display a high activity *in vitro*. These enzymes are the main potential oxidases involved in the oxidation of endogenous phenolic compounds in sorghum grain. The major sorghum grain peroxidase isoenzyme representing more than 80% of total peroxidase activity was characterized at the molecular level. The enzyme, localized in chromosome 1 of sorghum, is a monomeric glycoprotein containing a non-covalently bound type-b heme. The catalytic properties and primary structure of the enzyme are similar to cereal peroxidases, in particular to barley peroxidase 1. Agronomic characteristics of grains (presence of absence of pigmented testa layer and color of the glumes) and plants (red or tan) could be linked to sorghum grain contents in phenolics and starch modifying enzymes. On average, varieties resistant to stresses have greater content and diversity in phenolic compounds and higher oxidative enzyme activities than stress susceptible varieties. The screened biochemical parameters could be linked to the preferences of varieties for specific local foods. For instance, among varieties used for "tô", "dolo", couscous and thin porridge preparation, the "dolo" varieties had the highest average content and diversity in phenolics as well as the highest antioxidant activities. Varieties good for infant porridges preparation have low amylose content and high α-amylase activity after germination.

Keywords: sorghum, starch, amylose, amylopectin, α-amylase, β-amylase, phenolic compounds, proanthocyanidins, 3-deoxyanthocyanidin, apigeninidin, luteolinidins, flavan-4-ols, phenylalanine ammonia lyase, peroxidase, polyphenol oxidase, antioxidant, germination.

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CHAPTER 1

General introduction

1. 1. *Sorghum bicolor* (L.) Moench

Description

Sorghum bicolor (L.) Moench (**Figure 1**) is the fifth most important cereal crop after wheat, rice, maize, and barley in terms of production (FAO, 2005). The total world annual sorghum production is about 60 million tons from a cultivated area of 46 millions ha. Sorghum is a plant belonging to the tribe of Andropogoneae and the family of Poaceae. In 1753, Linnaeus described three species of cultivated sorghum: *Holcus sorghum*, *Holcus saccaratus* and *Holcus tricolor*. In 1794, Moench distinguished the genus *Sorghum* from the genus *Holcus*, and in 1805 Person suggested the name *Sorghum vulgare* for *Holcus sorghum* (L.). In 1961, Clayton proposed the name *Sorghum bicolor* (L.) Moench as the correct name for cultivated sorghum and this is currently the accepted one (Doggett, 1988).

Figure 1. *Sorghum bicolor* (L.) Moench

As most angiosperm (flowering plant) lineages, sorghum is thought to be ~200 million years old (Paterson et al., 2003). Sorghum, maize, rice and wheat diverged from a common ancestor only 50-70 million years ago (Paterson et al., 2003). The main races of cultivated sorghum are: bicolor, vulgare, caudatum, kafir, guinea, and durra (Deu et al., 1994; BSTID-NRC, 1996). Common names of sorghum vary from continent to country levels. The most encountered names are: kafferkoren, soedangras, suikergierst, or suiker-sorghum (The Netherlands), kaoliang (China), mtatam, shallu or

feterita (East Africa), durra (Egypt), chicken corn, sorghum or guinea corn (United Kingdom), jola, jowar, jawa, cholam, bisinga, durra or shallu (India), kaffir corn (South Africa), milo, sorgo, sudangrass or sorghum (USA), milo (Middle East Africa) and great millet, guinea corn, feterita, sorghum or sorgho (West Africa). Sorghum is C4 crop, whom certain varieties also posses "stay green" genes that enable them to perform photosynthesis permanently. Sorghum is particularly adapted to drought prone areas: hot, semi-arid tropical environments with 400-600 mm rainfall-areas that are too dry for other cereals. Sorghum is also found in temperate regions (France) and at altitudes of up to 2300 meters in the tropics. It is well suited to heavy soils commonly found in the tropics, where tolerance to water logging is often required.

Sorghum is a vigorous grass that varies between 0–6 m in height. It has deep and spread roots with a solid stem. Leaves are long (0.3-1.4 m) and wide (1-13 cm), with flat or wavy margins. The flower is a panicle, usually erect, but sometimes recurved to form a goose neck (**Figure 1** and cover picture). Grain or caryopse is usually covered by glumes. Glumes are the maternal plant tissues in the panicle that holds the developing caryopses after pollination. The caryopse is rounded and bluntly pointed, from 4–8 mm in diameter and varying in size, shape and color with variety (**Figure 2**). Caryopse color is an important trait that affects grain quality in sorghum.

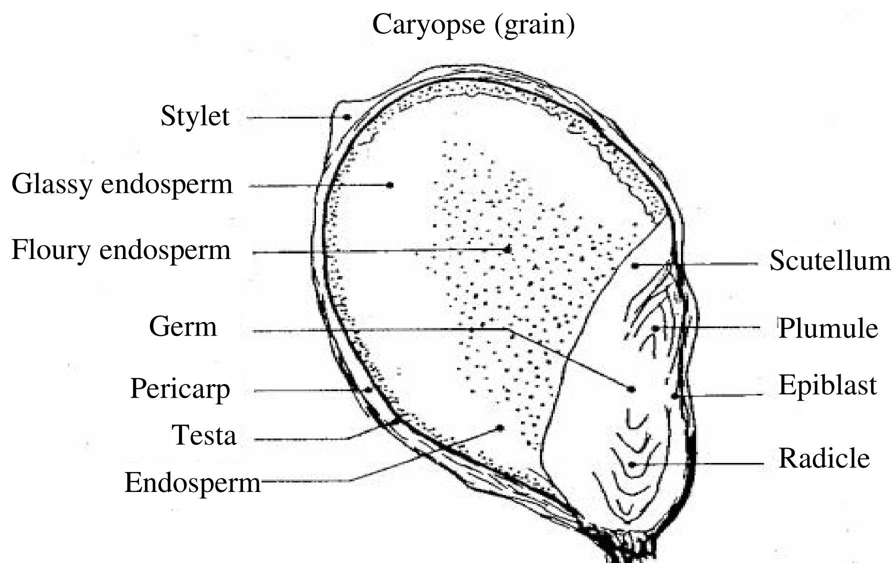


Figure 2 . Structure of sorghum grain (Sautier and O'Deye, 1989). Reproduced with the permission of Harmattan Editions, Paris

Sorghum caryopse is composed of three main parts: seed coat (testa or pericarp), germ (embryo) and endosperm (storage tissue). In some sorghum genotypes the testa is highly pigmented. The presence of pigment and the color is a genetic character controlled by the R and Y genes (Waniska, 2000). The thickness of the testa layer is not uniform and is governed by the Z gene. In some genotypes there is a partial testa, while in others it is not apparent or is absent.

Distribution

It is believed that sorghum originated in Africa, more precisely in Ethiopia, between 5000 and 7000 years ago (ICRISAT, 2005). From there, it was distributed along the trade and shipping routes around the African continent, and through the Middle East to India at least 3000 years ago. It then journeyed along the Silk Route into China. Sorghum was first taken to North America in the 1700-1800's through the slave trade from West Africa. It was re-introduced in Africa in the late 19th century for commercial cultivation and spread to South America and Australia. Sorghum is now widely found in the dry areas of Africa, Asia (India and China), the Americas and Australia.

Sorghum is genetically diverse. The world sorghum germplasms are deposited at the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT, Patancheru) in India. ICRISAT holds about 36,000 germplasm accessions of this crop. The varieties are distinguished on the basis of morphological traits, differences in isoenzyme patterns and DNA polymorphism (Chantereau and Nicou, 1991; Ollitraut et al., 1989a, 1989b; Zongo, 1991; Tao et al., 1993; Vierling et al., 1994, Deu et al., 1994). The sorghum genome is currently sequenced (Paterson et al., 2003; http://fungen.org/Sorghum.htm). Sorghum has 2n=20 chromosomes and is estimated to contain 750 Mb being twice the genome of rice and six times the genome of *Arabidopsis* (Passardi et al., 2004). Thus, a rough estimate of the total number of genes in sorghum based upon the currently 107 652 known expressed sequence tag (EST) data would be between 35 000 and 40 000 (http://fungen.org/Sorghum.htm).

Worldwide utilization

Sorghum, is grown in the United States, Australia, and other developed nations essentially for animal feed. However, in Africa and Asia the grain is used both for human nutrition and animal feed. It is estimated that more than 300 millions people from developing countries essentially rely on sorghum as source of energy (Godwin and Gray, 2000). The main foods prepared with sorghum are: tortillas (Latino America), thin porridge, e.g. “bouillie” (Africa and Asia), stiff porridge, e.g. tô

(West Africa), couscous (Africa), injera (Ethiopia), nasha and kisra (Sudan), dolo (Africa), baked products (USA, Japan, Africa), etc. Tortillas are a kind of chips prepared from sorghum alone or by mixing sorghum with maize and cassava (Anglani, 1998). Nasha is a traditional weaning food (infant porridge) prepared by fermentation of sorghum flour (Graham et al., 1986). Injera is a local fermented pancake-like bread prepared in Ethiopia from sorghum (Yetneberk et al., 2004). Kisra is traditional bread prepared from a fermented dough of sorghum (Mahgoub et al., 1999).

Sorghum alone is not considered as a bread making cereal because of the lack of gluten, but addition of 20-50% sorghum flour to wheat flour produces excellent bread (Anglani, 1998; Carson et al., 2000; Hugo et al., 2000, 2003). Among interesting features of sorghum utilization is biscuits and other cooked products (Olatunji et al., 1989). In the USA and Japan, sorghum utilization as human food is increasing because of its use in snacks and cookies (Rooney and Waniska, 2004). The future promise of sorghum in the developed world is for wheat substitution for people allergic to gluten (Fenster, 2003). In addition, pasta products, such as spaghetti and macaroni made from semolina or wheat could be made with mixtures of composite flour consisting of 30-50% sorghum in wheat (Hugo et al., 2000; 2003). Pre-cooked sorghum flours mixed with vitamins and exogenous sources of proteins (peanuts or soybeans) are commercially available in many African countries for the preparation of instant soft porridge for infants. Sorghum can be puffed, popped, shredded and flaked to produce ready-to-eat breakfast cereals.

Sorghum starch is successfully applied for the production of bio-ethanol (Suresh et al., 1999; Aggarwal et al., 2001). In Nigeria and South Africa, sorghum is industrially used for the production of lager beer (Taylor and Dewar, 2001). More information on sorghum utilization for human nutrition can be found elsewhere (FAO, 1995; Anglani, 1998; Taylor and Dewar, 2001; Awika and Rooney, 2004a, Rooney and Waniska, 2004).

Production and utilization in Burkina Faso

Current annual sorghum production in Burkina Faso is about 1.4 million tons; the country being the world leader in sorghum production and consumption per inhabitant (FAO, 2005). In Burkina Faso, sorghum alone accounts for 51% of the total cereal crop land area. Therefore, true food security will be hard to achieve in this country without a significant improvement in the production, use, and marketing of this major staple cereal. The yield is 950 kg/ha, while in the USA it is 3310 kg/ha (FAO, 2005). The low production in Burkina Faso is essentially due to biotic (insects, fungal diseases, weeds, etc.) and abiotic stresses (drought, logging, photoperiod, soil

quality, etc.). Most of the cultivated varieties in Burkina Faso have white or red caryopses. An investigation of sorghum diversity in Burkina Faso based on agro-morphological traits and isoenzyme patterns has revealed (Zongo, 1991; Tenkouano, 1995) the presence of more than 800 ecotypes. This number increases every year because of the introduction of newly improved varieties (Tenkouano, 1995; Séréme et al., 1994, Trouche et al., 2000, 2001). In Burkina Faso, sorghum is essentially used for thin porridges, tô, couscous, and dolo.

Tô is prepared by cooking a slurry of sorghum flour. Thin porridges (usually used as weaning food) are also prepared in the same manner with less amount of flour to obtain a fluid end-product. Often sorghum porridges are characterized by thick pastes that may form rather stiff gels depending on variety used.

Couscous is a steamed and granulated traditional African food originating from North Africa. The traditional method of preparing couscous is a steam-cook process in a special pot called "couscoussière". Couscous is prepared by mixing flour with water to obtain agglomerated flour-water mixtures. The agglomerates are then put on top of the "couscoussière". The stew cooks in the bottom pot while the granules are steamed on top. Sorghum varieties differ in their couscous-making ability. White sorghum varieties from tan plants yield the best couscous product (Galiba et al., 1988). Couscous quality criteria include size uniformity, color, stickiness, and mouth-feel (Aboubacar and Hamaker, 1999).

Dolo is a reddish, cloudy or opaque local beer prepared exclusively from red sorghum malt (Hilhorst, 1986). The primary quality criterion of selection of sorghum varieties for beer is their potential to produce malt with high α-amylase and β-amylase activities (Verbruggen, 1996; Taylor and Dewar, 2001). The sorghum malting process starts by immersing the grain in water to activate hydrolytic enzymes. Traditional germination involves seedling growth in warm water-saturated air for 3 to 5 days (Hilhorst, 1986). The germinated grain is then dried to moisture content of 10-12%. The malt obtained is used to prepare dolo. Briefly, dolo preparation starts by mixing sorghum malt flour with water (1:10, w/v). The mixture is decanted and the supernatant (containing hydrolytic enzymes) is separated from the precipitate (containing starch). Water is added to the precipitate and the mixture is boiled to gelatinize starch, but the supernatant is not boiled. It is interesting to note that people, who originally prepare dolo ("dolotières"), empirically know that the supernatant contains "some things", e.g. enzymes that are thermolabile so they should not be boiled. After cooling, the precipitate is filtered to separate soluble components (starch, sugars, proteins, etc.) and the spent (used as animal feed). The mashing step (incubation of hydrolytic enzymes with their substrates) consists of combining the previous supernatant and the filtrate at 50-60ºC for 12-16h, to

obtain the wort. The wort is cooked, and then re-cooled overnight to room temperature (35-40ºC). The cooled wort is a sweet non-fermented beverage highly appreciated by children. It is traditionally called “soft dolo”. The fermentation (1-2 days) is initiated by addition of dried yeasts to the wort. The final product, dolo, is separated from yeasts by filtration. Characteristics of dolo are: alcohol content (2-4%, v/v), pH 4-5, stability at room temperature (12-16h), red color, and opaque appearance. In Burkina Faso, the government has encouraged since 1984 the research on the preparation of lager beer from sorghum. However, until now it is not successful because of the lack of real financial support.

Sorghum grain composition and nutritive value

Starch is the main component of sorghum grain, followed by proteins, non-starch polysaccharides (NSP) and fat (**Table 1**). The average energetic value of whole sorghum grain flour is 356 kcal/100g (BSTID-NRC, 1996). Sorghum has a macromolecular composition similar to that of maize and wheat (BSTID-NRC, 1996). However, sorghum contains resistant starch, which impairs its digestibility, notably for infants (FAO, 1995). This resistance is desired in other applications to fight human obesity. Foods prepared from high tannin sorghums varieties have a longer passage in the stomach (Awika and Rooney, 2004a). Edible products incorporating slowly digestible starch are known to exhibit a low glycemic index and increase satiety (Shin et al., 2004).
Sorghum contains non-starch polysaccharides (NSP), mainly located in the pericarp and endosperm cell walls, with proportions in the kernel ranging from 2 to 7% depending on variety (Knudsen and Munck, 1985; Verbruggen et al., 1993). The NSP in sorghum grain are essentially constituted of arabinoxylans and other β-glucans representing 55% and 40% of the total NSP (Verbruggen et al., 1993; Hatfield et al., 1999). Verbruggen and co-workers (1993, 1998) found arabinoxylans from sorghum to be glucuronoarabinoxylans containing ferulic acid and *p*-coumaric acid. Arabinoxylans, being one of the major NSP present in sorghum cell walls, play an important role in the processing of sorghum for baking and brewing (Rouau, 1993; Verbruggen et al., 1998). The other β-glucans comprise cellulose (1,4-β-D-glucans), curdlan-type glucans (1,3-β-D-glucans), and lichenan-type glucans (1,3; 1,4-β-D-glucans) (Knudsen and Munck, 1985; Verbruggen et al., 1993, 1996, 1998). These β-glucans are predominantly water-unextractable, and form viscous and sticky solutions. In brewing, together with arabinoxylans, they are associated with processing problems like poor wort and beer filtration rates and the occurrence of haze (Aisien and Muts, 1987; Dufour et al., 1992).

Sorghum also contains non-carbohydrate cell-wall polymers such as lignins with proportions constituting up to 20% of the total cell wall materials (Hatfied et al., 1999).

The protein content in whole sorghum grain is in the range of 7 to 15% (FAO, 1995; Beta et al., 1995). Using the solubility-based classification (Jambunatan et al., 1975), sorghum proteins have been divided into albumins, globulins, kafirins (aqueous alcohol-soluble prolamins), cross-linked kafirins and glutelins. The kafirins comprise about 50-70% of the proteins (Hamaker et al., 1995; Oria et al., 1995; Duodu et al., 2003). α-Kafirins (23 and 25 kDa) make up about 80% of the total kafirins and are considered the principal storage proteins of sorghum, whereas β-kafirins (16, 18, and 20 kDa), and γ-kafirin (28 kDa) comprise about 5% and 15% of total kafirins, respectively. The nutritional quality of sorghum proteins is poor because these kafirins are protease resistant (Badi et al., 1990; Oria et al., 1995; Anglani, 1998). However, a wide variability according to variety has been observed with respect to the levels of proteins in sorghum (Reddy and Eswara, 2002). The protein digestibility of sorghum may decrease upon cooking (Axtell et al., 1981; Taylor and Taylor, 2002), but pre-fermentation may increase the digestibility (Taylor and Taylor, 2002). The low digestibility is due to protein-protein, protein-carbohydrate, protein-(poly)phenol and carbohydrate-(poly)phenol interactions (Knudsen et al., 1988; Axtell, 1981, Hamaker et al., 1987; Cherney, et 1992, Taylor and Taylor, 2002).

The fat in sorghum grain (mainly present in the germ) is rich in polyunsaturated fatty acids (Glew et al., 1997). The fatty acid composition of sorghum fat (linoleic acid 49%, oleic 31%, palmitic 14%, linolenic 2.7%, stearic 2.1%, etc.) is similar in content to that of corn fat, but it is more unsaturated (Knudsen et al., 1988; Adeyeye and Ajewole, 1992; FAO, 1995).

Sorghum is a good source of vitamins, notably the B vitamins (thiamin, riboflavin, pyridoxine, etc.), and the liposoluble vitamins A, D, E and K.

Sorghum is reported to be a good source of more than 20 minerals (BSTID-NRC, 1996). Sorghum is also rich in phosphorus, potassium, iron and zinc (Glew et al., 1997; Anglani, 1998). Zinc (an important metal for pregnant women) deficiency is more common in corn and wheat than in sorghum (Hopkins et al., 1998).

Table 1. Proximate composition of sorghum grain[a]

Macro-components (g/100g f. m.)		Essential amino-acids (mg/100g, d.m.)		Vitamins (mg/100g d. m.)		Mineral (mg/100g d. m.)	
Carbohydrates	65 - 80	Leu	832 - 1480	Vit.-A	21 RE**	Ca	21
Starch	60 - 75	Ile	215 - 460	Thiamin	0.35	Cl	57
Amylose	15 - 30	Met/Cys	190 - 244	Riboflavin	0.14	Cu	1.8
Amylopectin	45 - 55	Lys	126 - 277	Niacin	2.8	I	0.029
Non starch polysaccharides	2 - 7	Phe/Tyr*	567 - 386	Pyridoxine	0.5	Fe	5.7
Low M_w carbohydrates	2 - 4	Thr	189 - 425	Biotin	0.007	Mg	140
Proteins	7 - 15	Trp	63 - 187	Pantothenate	1.0	P	368
α-Kafirins	4 - 8	Val	313 - 607	Vitamin C	<0.001	K	220
ß-Kafirins	0.2 - 0.5	Arg*	500 - 537			Na	19
γ-Kafirins	0.7 - 1.6	His*	200 - 234			Zn	2.5
Other proteins	2 - 5						
Fat	1.5 - 6						
Ash	1 - 4						
Moisture	8 - 12						

[a]Sources: Verbruggen et al. (1993, 1996); FAO (1995), Hamaker et al., (1995), BSTID-NRC (1996), Glew et al. (1997), Duodu et al. (2003). *Not strictly essential amino-acids,**RE = retinol equivalent; f.m. = fresh matter, d. m. = dry matter; NSP = non starch polysaccharides.

Effect of germination on sorghum composition

The physiological maturity of sorghum grain generally occurs 50 days after anthesis, and marks the end of nutrient delivery and the beginning of senescence, and caryopse desiccation (Waniska, 2000). The mature grain is then harvested and stored. In a dormant stage, it is characterized by dehydration and a dramatic decrease of metabolic activity. Germination is induced by rehydration of the seed, which increases both respiration and metabolic activity thus allowing the mobilization of primary and secondary metabolites (Limami et al., 2002). Therefore, the biochemical composition between ungerminated and germinated kernels is different. Germination induces the synthesis of hydrolytic enzymes, e.g. starch degrading enzymes, and proteases. The reduction of phytic acid, some flavonoids and proanthocyanidins has been observed during germination (FAO, 1995; Traoré et al., 2004). The breakdown of protease resistant prolamins (Mazhar and Chandrashekar, 1993) and the increase of the availability of minerals (iron, zinc, etc.) and essential amino acids (principally Lys, Tyr and Met) upon germination has also been reported (FAO, 1995; Anglani, 1998). Germination of sorghum is important for the preparation of weaning foods with low paste viscosity and high energy density (Malleshi and Desikachar, 1988). While

germination usually has positive aspects, it is important to note that it increases the content of nitrilosides (cyanogenetic β-glycosides, e.g. dhurrin) of the grain (Ahmed et al., 1996; Traoré et al., 2004). These compounds release cyanide (prussic acid) which may be removed either by heating the flour or removing shoots, roots and the germs, but removing the latter reduced the content in α-amylase (Uvere et al., 2000; Traoré et al., 2004). Upon germination, the initially low content of vitamin C is strongly increased (Taur, et al., 1984).

1.2 Starch and starch degrading enzymes

Starch

Starch is the primary source of stored energy in cereal grains. Starch is deposited as granules in the endosperm cells, being the main constituent of the endosperm. Sorghum starch granules have diameters ranging from 5 to 25 µm (average 15 µm). Sorghum starch has a specific particularity because of its high gelatinization temperature (70-75ºC), which decreases its industrial application (Dufour et al.; 1992; Taylor, 1992). Native starch granules are essentially insoluble in cold water. The term "gelatinization" is used to describe the swelling and hydratation of granular starches (Zobel, 1984). Starch gelatinization is the disruption of molecular orders within the starch granule manifested in irreversible changes in properties such as granular swelling, native crystalline melting, loss of birefringence, and starch solubilization. These changes render all or part of the material in granules soluble and consequently enable to contribute to food properties such as texture, viscosity, and moisture retention (Whistler and BeMiller, 1997). The point of initial gelatinization and the range over which it occurs is governed by the starch structure. Sorghum starch is classified as type-B, e.g. a moderate swelling starch compared to type-A starches (potato, tapioca, waxy sorghum, etc.), which are high swelling starches (Beta and Corke, 2001). The retrogradation involves reassociation of the molecules and occurs when the starch is cooled, and this is dependent on the ratio of amylose and amylopectin. Enzymatic sorghum starch hydrolysis or chemical treatment can improve its technological properties (Zhang et al., 1999).

Regardless of the botanical source, starch is structurally composed of two high molecular weight homopolysaccharides known as amylose and amylopectin (**Figure 3**). Amylose constitutes approximately 20-30% of starch, and is composed of essentially homogenous linear units of α-(1→4)-D-glucopyranose, which can form helicoidal structures in solution (Manners, 1974; Jarvis

and Walker, 1993). The interior of the helix is hydrophobic, allowing amylose to form a complex with free fatty acids, iodine, etc. (Fennema, 1985).

Amylopectin is constituted of short chains of α-(1→4)-D-glucopyranose (majority 10-20 units in sorghum starch) branched to α-(1→6)-D-glucopyranoses to form a highly ramified structure (Blennow et al., 2001).

α-Amylase and β-amylase

Starch degradation in plants is accomplished by α-amylase [α-1,4-D-glucan 4-glucanohydrolase, EC 3.1.1.1], β-amylase [α-1,4-D-glucan maltohydrolase, EC 3.1.1.2], amyloglucosidase [α-1,4-glucan-glucohydrolase, EC 3.1.1.3] and starch phosphorylase [1,4-α-D-glucan:phosphate α-D-glucosyltransferase, EC 2.4.1.1] action on α-(1→4)-linkages (Manners, 1974). Amylases are hydrolytic enzymes, which depolymerize starch according to a classic acid-base mechanism. α-Amylases are endo-enzymes that randomly split α-(1→4)-linkages in starch with retention of anomeric configuration of glucose residues. β-Amylase is an exoglucosidase acting from the non-reducing end, releasing β-maltose units from starch, hence the name β-amylase (Kaplan and Guy, 2004). The β-maltoses released undergo mutarotation into α-maltose (Robyt and Akerman, 1973). Both α-amylase and β-amylase cannot split the α-(1→6)-linkages in amylopectin. Therefore, the degradation of starch by these enzymes is incomplete. In addition, plant amylases scarcely hydrolyze raw starch: their action is lower than 5% hydrolysis (Dicko et al., 1999).

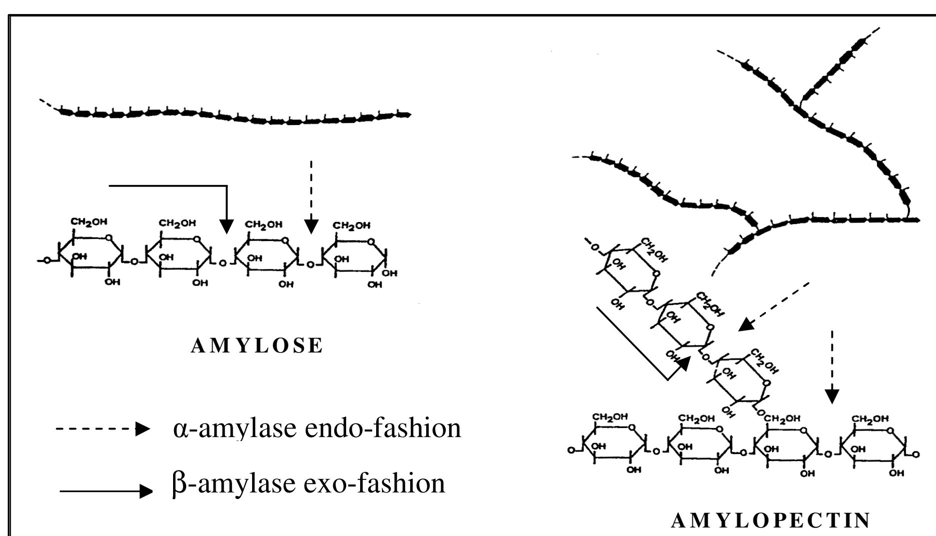


Figure 3. Structures of amylose and amylopectin. The mode of starch hydrolysis by α-amylase and β-amylase is illustrated.

Sorghum amylases were first detected in 1928 and partially purified since that time by solvent fractionation (Patwardhan and Norris, 1928). The starch-liquefying or dextrinizing power is referred to α-amylase activity, while the starch-saccharifying or saccharolytic power is referred to as β-amylase activity. Sorghum α-amylases and β-amylases occur as glycosylated (2-3 glycoforms), anionic (p*I* 4-5) isoenzymes of different molecular weights (Mundy, 1982; Okon and Uwaifo, 1984). Two α-amylase isoenzymes with molecular masses of 41.5 and 42.7 kDa (Mundy, 1982) and three β-amylases with molecular masses of 20, 40 and 60 kDa were purified from sorghum grain (Okon and Uwaifo, 1984).

One of the constraints of utilizing sorghum varieties in industrial brewing is the low activity of starch degrading enzymes. For instance, in Nigeria, sorghum has become the predominant cereal for industrial scale malting and brewing of beer, following legislation banning the importation of barley and wheat (Hug et al., 1991). The major disadvantage encountered using sorghum in brewery is its low content of β-amylase (Taylor and Robbins, 1993; Swanston et al. 1993; Verbruggen, 1996).

1. 3. Sorghum phenolic compounds

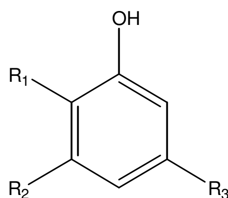
Phenolic compounds, of which more than 8000 are known, embrace a wide range of plants secondary metabolites possessing in common an aromatic ring substituted by one or more hydroxyl groups (Harborne, 1994; Pietta, 2000). Phenolic compounds are the most widely distributed secondary metabolites, ubiquitously present in the plant kingdom. Located in the vacuole, they are found in free form or linked to carbohydrates (glucose, galactose, rhamnose, mannose, rutinose etc.), and tend to be soluble in water or organic solvents (methanol, HCl-methanol, acetone, dimethylformamide, etc.). Among cereals, sorghum has the highest content of phenolic compounds reaching up to 6% (w/w) in some varieties (Deshpande et al., 1986; Beta et al., 1999, Awika and Rooney, 2004a). The main classes are simple phenols, hydroxybenzoic acids, hydroxycinnamic acids, flavonoids (flavanols, flavones, flavanones, isoflavones and anthocyanins), chalcones, aurones (hispidol), hydroxycoumarins, lignans, hydroxystilbenes and polyflavans (proanthocyanidins and pro-deoxyanthocyanidins) (Chung et al., 1998; Krueger et al., 2003). Sorghum does not contain tannic acid and hydrolysable tannins (Waniska, 2000, Awika et al., 2004a). Lignans and hydroxystilbenes are not yet detected (Awika et al., 2004a). **Table 2** gives the approximate range of phenolic compounds content in sorghum varieties.

Table 2. Phenolic contents in sorghum grain

Phenolic compounds	Content µg/g (dry weight)	Reference
Hydroxybenzoic acids		
p-Hydroxybenzoic	15 - 36	Hahn et al. (1983)
Gallic	26 - 46	Hahn et al. (1983)
Protocatechuic	24 141	Hahn et al. (1983)
Vanillic	8 - 50	Hahn et al. (1983)
Hydroxycinnamic acids		
p-Coumaric	100 - 200	Verbruggen et al. (1993)
Caffeic	25 - 52	Hahn et al. (1983)
Ferulic	300 - 500	Verbruggen et al. (1993)
Sinapic (sinapinic)	50 140	Hahn et al. (1983)
Flavonoids		
Anthocyanins	0 - 2 800	Sérémé et al. (1992); Awika et al. (2003, 2004b)
Flavan-4-ols	0 - 1 300	Bate-Smith (1969) ; Audilakshmi et al. (1999)
Proanthocyanidins	0 - 68 000	Beta et al. (1999); Awika and Rooney (2004a)

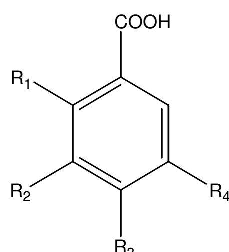
Simple phenols and phenolic acids

Simple phenols are relatively rare in plants. Catechol and resorcinol were reported in sorghum grain; however their concentrations were not given (Watt and Breyer-Brandwijk, 1962; Czarnota et al., 2003; Towo et al., 2003). These compounds are undesired in food products because they are carcinogenic, hepatotoxic and goitrogenic (Gaitan et al., 1989, Reed, 1995). Interestingly, if present they can be removed by food processing like heating (Gaitan et al., 1989). The phenolic acids in sorghum include hydroxybenzoates and hydroxycinnamates (**Figure 4**). They are found in free form or bound as esters, and are concentrated in the outer layers of the grain (Waniska, 2000; Awika et al., 2004a). The most abundant phenolic acids in sorghum are ferulic acid and *p*-coumaric acid (Hahn et al., 1983; Verbruggen et al., 1993).



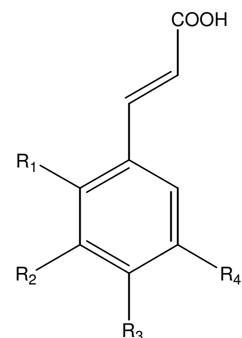
Simple phenols

Phenol: $R_1 = R_2 = R_3 = H$
Catechol: $R_1 = OH, R_2 = R_3 = H$
Resorcinol: $R_2 = OH, R_1 = R_3 = H$
Phloroglucinol: $R_2 = R_3 = OH, R_1 = H$



Hydroxybenzoic acids

Gallic: $R_1 = H, R_2 = R_3 = R_4 = OH$
Gentisic: $R_1 = R_4 = OH, R_2 = R_3 = OH$
Salicylic: $R_1 = OH, R_2 = R_3 = R_4 = H$
p-(OH)-Benzoic: $R_1 = R_2 = R_4 = H, R_3 = OH$
Protocatechuic: $R_1 = R_4 = H, R_2 = R_3 = OH$
Syringic: $R_1 = H, R_2 = R4 = OCH_3, R_3 = OH$
Vanillic: $R_1 = R_4 = H, R_3 = OH; R_2 = OCH_3$

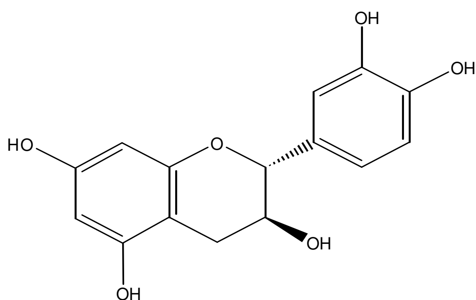


Hydroxycinnamic acids

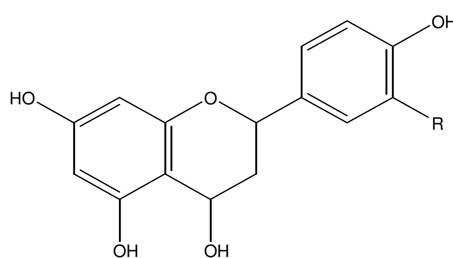
p-Coumaric: $R_1 = R_2 = R_4 = H, R_3 = OH$
Caffeic : $R_1 = R_4 = H, R_2 = R_3 = OH$
Ferulic: $R_1 = R_2 = H, R_4 = OCH_3, R_3 = OH$
Sinapic : $R_1 = H, R_2 = R_4 = OCH_3, R_3 = OH$

Flavonoids

Flavonoids *sensu latu* constitute the largest class of phenolic compounds with more than 3000 structures, possessing in common a flavylium unit (C_6-C_3-C_6) (Iacobucci and Sweeny, 1983). Sorghum contains flavonoids such as flavanols (flavan-3-ols, flavan-4-ols, etc., **Figure 5**), flavanones, flavones and anthocyanins (Haslam, 1998; Awika, 2004a). The flavan-4-ols apiforol (pro-apigeninidin or leuco-apigeninidin) and tuteoforol (proluteolinidin or leuco-luteolinidin) are abundant in sorghum, and precursors of apigeninidin and luteolinidin, respectively (Ferreira and Desmond and Slade, 2002; Haslam, 1998; Hagerman, 2005).



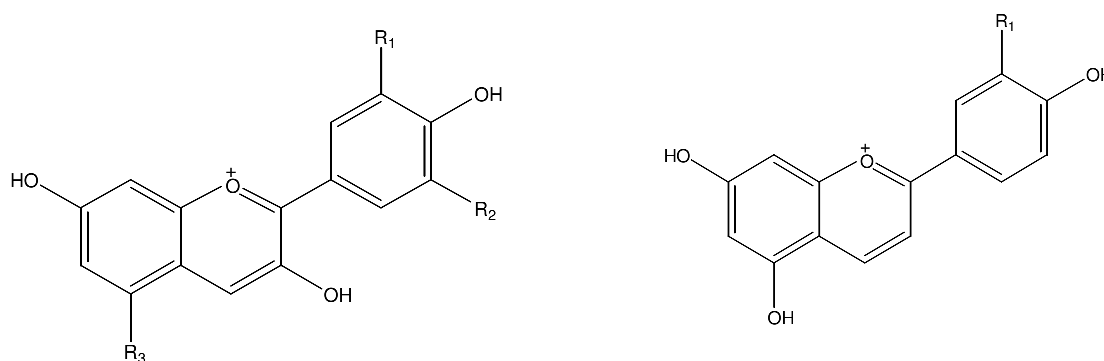
Flavan-3-ol
catechin



Flavan-4-ols
Apiforol (leucoapigeninidin) : R = H
Luteoforol (leucoluteolinidin) : R = OH

Figure 5. Structure of some sorghum flavanols (Melake-Berhan et al., 1996; Hagerman, 2005)

The most abundant anthocyanins in sorghum grain are 3-deoxyanthocyanidins, e.g. apigeninidin and luteolinidin (**Figure 6**) (Bate-Smith, 1969; Kouda et al., 1994, 1996; Palé et al., 1997; Awika et al., 2004b). The red color of the grain's pericarp is essentially due to the presence of 3-deoxyanthocyanidins (Bate-Smith, 1969, see cover picture). Like most anthocyanidins, these compounds are used as natural food colorants (Morazzoni and Magistretti, 1990; Coultate, 1996; Awika et al., 2004a). As a food (E163) or pharmaceutical (Myrtocyan) additive, these phenolic compounds represent a world market of 250 millions US dollars (Morazzoni and Magistretti, 1990; Coultate, 1996). 3-Deoxyanthocyanidins have interesting food applications because of their thermal and color stability (Iacobucu and Sweeny, 1983, Awika et al., 2003, 2004b).



Anthocyanidins

Fisetinidin : $R_1 = R_2 = OH, R_3 = H$
Cyanidin : $R_1 = OH$; $R_2 = H, R_3 = OH$
Pelargonidin : $R_1 = R_2 = H, R_3 = OH$
Peonidin : $R_1 = OCH3$; $R_2 = H, R_3 = OH$
Malvidin : $R_1 = R_2 = OCH_3, R_3 = OH$
Delphinidin $R_1 = R_2 = R_3 = OH$
Petunidin $R_1 = OCH3$; $R_2 = R_3 = OH$

3-Deoxyanthocyanidins

Apigeninidin : $R_1 = H$
Luteolinidin : $R_1 = OH$

Figure 6. The main sorghum anthocyanidins and 3-deoxyanthocyanidins (Kouda-Bonafos et al., 1996; Palé et al., 1997; Awika et al., 2004a, 2004b).

Polyflavans

The term polyflavan is referred to phenolic compounds formed by polymers of flavylium units substituted with hydroxyl groups (Krueger et al., 2003, Awika and Rooney, 2004a). Most polyflavans are often called condensed tannins, but this generic name is sometimes confusing because it does not give a structural definition of the compounds. The polyflavans found in sorghum are essentially polymers of flavan-3-ols (proanthocyanidins) and pro-3-deoxyanthocyanidins (**Figure 7**). Sorghum proanthocyanidins consist of flavan-3-ol units linked by C-C (type B proanthocyanidins) and occasionally C-O-C (type A proanthocyanidins) bonds ranging from one to

fifteen (Krueger et al. 2003, Awika and Rooney, 2004a). The most abundant polyflavans in sorghum are homopolymers of catechin/epicatechin with uniform B-type interflavan bonds (Krueger et al., 2003). Not all sorghum varieties contain these polyflavans because their content is genetically governed by B1-B2 genes (Serna-Salvidor and Rooney, 1995; Butler, 1992; Waniska, 2000). In general, varieties with pigmented testa layers contain proanthocyanidins (Waniska, 2000).

The main pro-deoxyanthocyanidins found in sorghum are pro-apigeninidins and pro-luteolinidins (**Figure 7**). Stafford (1990) has suggested that although present in sorghum, these polyflavans are very rare in other plants. Hydrolysis of pro-apigeninidins and pro-luteolinidins yields apigeninidins and luteolinidins, respectively (Hagerman, 2005).

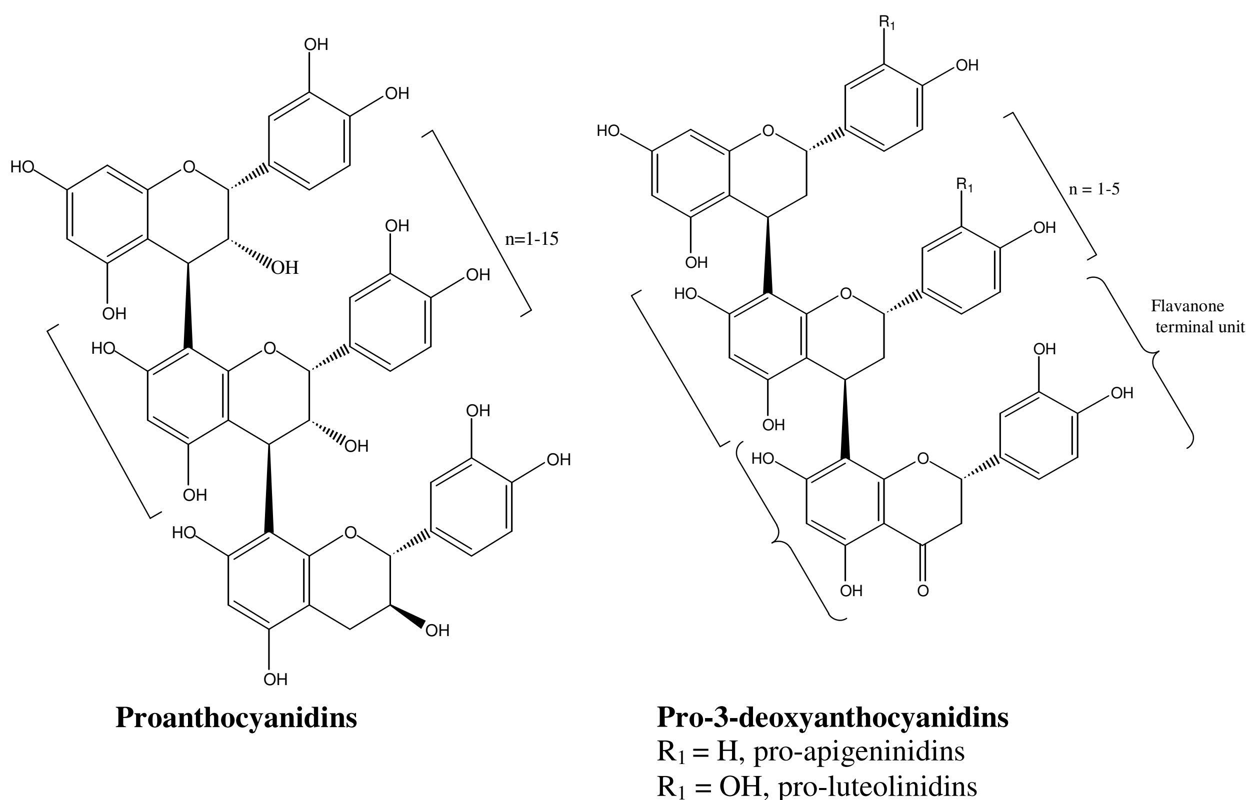


Figure 7. Structure of sorghum polyflavans (Krueger et al. 2003; Chen and Hagerman, 2004; Awika and Rooney, 2004a).

Role of phenolic compounds in plants

Phenolics play an important role in plant metabolism, but also protect the plant against stresses. For instance, it has been recently shown that flavonoids, such as catechin, regulate the auxin transport in plants, and, therefore, play an important role in plant development (Brown et al., 2001). Several studies have shown that the plant resistance to both biotic (pathogens and predators)

and abiotic (UV-radiation, drought, etc.) stresses is related to phenolic compounds (Parr, 2000). All classes of phenolic compounds (hydroxyl benzoic acid and hydroxycinnamic acid derivatives, flavonoids, polyflavans, etc.) are involved in the resistance mechanisms. Sorghum 3-deoxyanthocyanidins are phytoalexins (plant-microbe interaction) or allelochemicals (plant-plant interaction), involved in plant resistance to biotic stresses such as fungi and parasitic plant invasion (Weiergang et al., 1996; Lo et al., 1999; Parr and Bolwell, 2000; Weir et al., 2004). Proanthocyanidins, 3-deoxyanthocyanidins and flavan-4-ols prevent losses from premature germination and damage due to mold (Waniska, 2000). Hydroxycinnamic acids are constituent of plant cell-wall polymers such as lignin, suberin and cutin. These polymers are physical barriers against invading predators, drought and several other stresses (Parr and Bolwell, 2000).

Health related properties of phenolic compounds

Condensed tannins, e.g. proanthocyanidins, may bind to proteins, carbohydrates and minerals, thereby affecting the nutritional and functional value of the bound constituents. Of major nutritional concern is the ability of proanthocyanidins to bind strongly to large proteins and to proline-rich proteins, thereby reducing their digestibility (Butler, 1992). Proanthocyanidins may be antinutritional through direct interference within the animal body of the digestive processes or inhibition of hydrolytic enzymes through formation of complexes (Nguz et al., 1998). One of the most undesired effects of phenolic compounds is their pro-oxidant activity which can lead to mutagenicity and carcinogenicity (Stoewsand et al., 1984; Morton, 1992). This pro-oxidant activity depends strongly on the type of phenolic compounds (Awad et al., 2000; Rietjens et al., 2001; Awad, 2002; van der Woude et al., 2002).

The earlier idea of classification of proanthocyanidins exclusively as antinutritional factors is now questioned because they also are believed to have health benefits for humans (Hagerman et al., 1998; Waniska, 2000, Parr and Bolwell, 2000; Clifford, 2000; Awika, 2004a). Sorghum proanthocyanidins are unlikely to bind minerals (Waniska, 2000) and high molecular weight proanthocyanidins (DP >3) do not cross the gastroinstinal cell monolayer (Deprez et al., 2001). Bioavailability of iron in sorghum for human subjects was found to be affected more by phytic acid than by the proanthocyanidins content of the grains (Radhakrishnan and Sivaprasad, 1980). Gomez-Cordoves and co-workers (2001) demonstrated the effective therapeutic effect of sorghum proanthocyanidins against human melanoma. The binding of proanthocyanidins with proteins participates in their antibacterial activity (Murdiati and McSweeney, 1987; Scalbert, 1991). Proanthocyanidins have been shown to inhibit the growth of human immunodeficiency virus 1

(HIV-1), influenza virus, and herpes simplex virus by blocking their entry in the host cells (Chan and Kim, 1998; Okuda et al.; 1991, Lu et al., 2004; Hamauzu et al., 2005). The anti-HIV-1 activity is of high interest in Africa, and in Burkina Faso in particular, where HIV-1 prevalence is a major concern. The mechanism of proanthocyanidins toxicity against microbes is related to inhibition of hydrolytic enzymes, interactions to inactivate microbial adhesions and cell envelope transport proteins, and non-specific interaction with carbohydrates (Cowan, 1999). Among sorghum flavanols, the flavan-4-ols (**Figure 5**) have particular therapeutic interest because of their antitumor activity (Ferreira and Slade, 2002). Flavan-4-ols revealed strong host mediated antitumor activity, which is due to the enhancement of immune response of the host animals through the actions on tumor cells and some immunocytes (Okuda et al., 1991; Ferreira and Slade, 2002).

A number of highly reactive oxygen species such as singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), nitric oxide radical ($NO^{\bullet}$), and alkyl peroxyl ($ROO^{\bullet}$) are regularly produced in the human body (Langseth, 1995). These radicals can damage lipids, proteins and DNA and participate in pathogenesis and ageing (Ryan and Robards, 1998; Santos-Buelga and Scalbert, 2000; Parr and Bolwell, 2000). Phenolic compounds, together with other natural compounds (vitamins C and E, and carotenoids), contribute to the defense by scavenging free radicals, by inhibiting oxidative enzymes such as lipoxygenase and cyclooxygenase and by chelating metal ions (Shi et al., 2001). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals (Bors et al., 2001). In general, phenolic compounds possessing *ortho*-hydroxyls, found for instance in caffeic acid and in the B-ring of some flavonoids (catechin, quercetin, luteolinidin, etc.), have higher antioxidant activities than the others (Natella et al., 1999; Shi et al., 2001). Antioxidant activities of phenolic compounds have been suggested to exert beneficial pharmacological effects on neurological disorders on the basis of *in vitro* observations (Moosmann and Behl, 1999). Epidemiological studies have shown that consumption of some phenolic compounds is associated with a reduced risk for developing chronic diseases, such as coronary heart disease, cancer, diabetes, and Alzheimers's disease, linked to their free radical scavenging activities (Ames et al., 1993; Block et al., 1992; Hertog et al., 1993; Temple, 2000; Joshipura et al., 2001; Willett, 2002; Khokhar and Magnusdottir, 2002; Yang et al., 2004). Another interesting property of phenolic compounds, notably hydroxyanthraquinones and hydroxynapthoquinones, is their cathartic effect (Clifford, 2000). Cathartic compounds are believed to give a better feeling and help to deal with difficult emotions and eliminate them.

1.4. Phenolic enzymes in sorghum

Importance of phenylalanine ammonia lyase in biosynthesis of phenolic compounds

The biosynthesis of phenolic compounds in plant (**Figure 8**) is initiated by the shikimic acid pathway (Tomas-Barberan and Espin, 2001; Heldt, 2005). This pathway continues with the production of phenylalanine, which is subsequently deaminated by the enzyme phenylalanine ammonia lyase [EC 4.3.1.5, PAL]. PAL can deaminate both L-phenylalanine and L-tyrosine into cinnamate derivatives (Hodgins, 1971, Rosler et al., 1997; Heldt, 2005). The released ammonia is refixed by glutamine synthetase [L-glutamate-ammonia ligase, EC 6.3.1.2] to produce glutamine (Singh et al., 1998; Heldt, 2005). PAL is inhibited by its products, e.g. *trans*-cinnamates (Heldt, 2005). Apart from PAL, the main other important enzymes in phenolic synthesis are cinnamate-4-hydroxylase [EC 1.14.13.11]; 4-coumarate CoA ligase [EC 6.2.1.12], acetyl CoA carboxylase [EC 6.4.1.2], chalcone synthase [EC 2.3.1.74); and chalcone-flavanone isomerase (EC 5.5.1.6] (Hrazdina, 1992, Haslam, 1998). PAL is indirectly associated with the synthesis of phenol polymers, including lignin and suberin (Parr and Bolwell, 2000; Heldt, 2005). In several fruits and vegetables, a high plant PAL activity has been associated with the accumulation of anthocyanins and other phenolic compounds (Tovar et al., 2002). In barley, PAL activity has been associated with the response to pathogen challenge (Shiraishi et al., 1995) and to light (Baztan and Torres, 1988). The inhibition of PAL activity in barley induces the susceptibility to fungal attack (Carver et al., 1994). PAL activity has been detected in the green shoots and leaves (Stafford, 1969, Mohan et al., 1988) of sorghum. In sorghum, the infection of the plant with pathogen involved a very rapid accumulation of PAL mRNA (Cui et al., 1996).

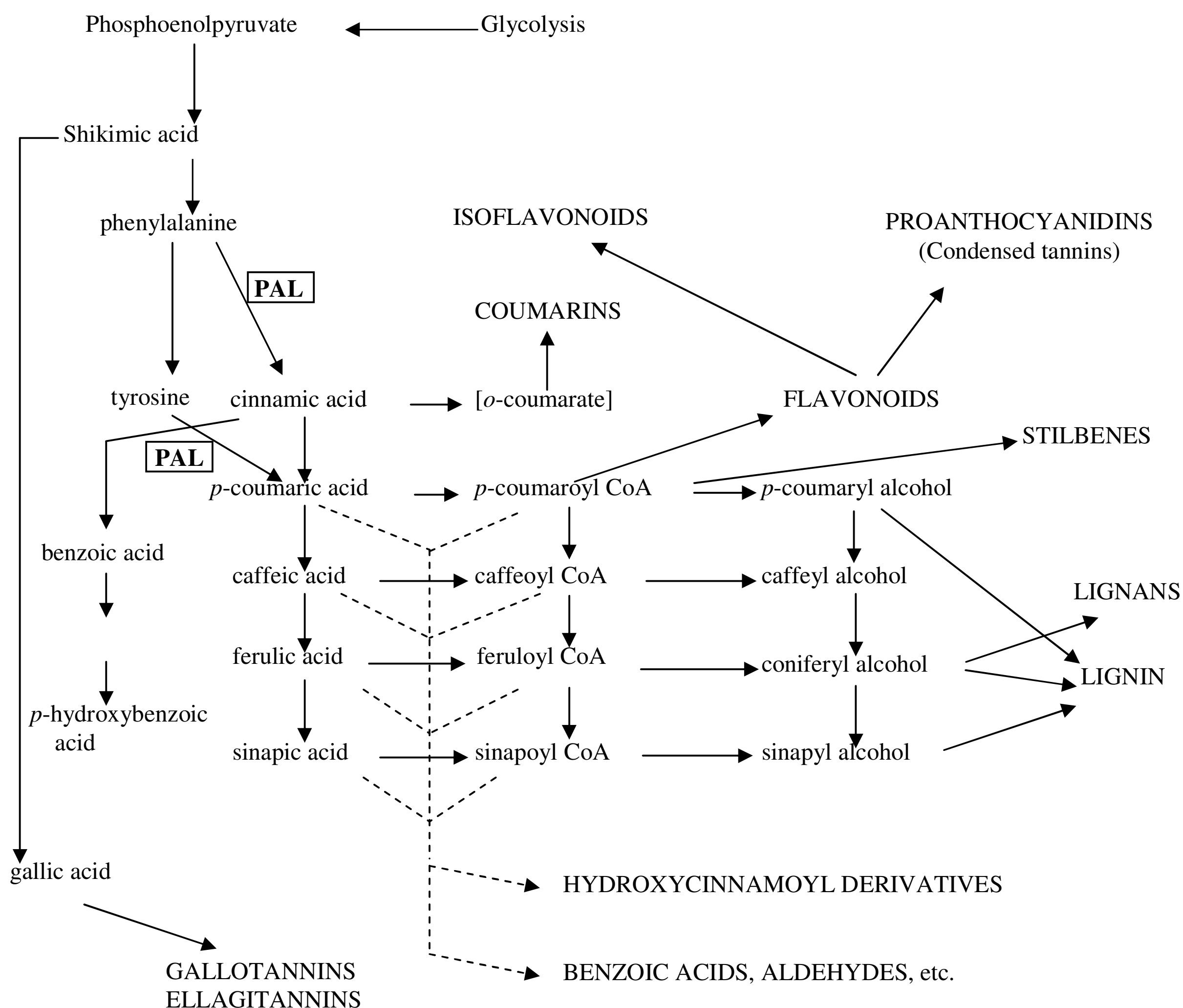


Figure 8. Schematic illustration of the biosynthesis of plant phenolic compounds (Ryan and Robards 1998; Parr and Bolwell, 2000). PAL= phenylalanine ammonia lyase

Polyphenol oxidases

Polyphenol oxidases [monophenol, *o*-diphenol: oxygen oxidoreductase, EC 1.14.18.1, PPOs] are type-3 copper containing oxidases that occur in plants as monomeric, dimeric and tetrameric structures, and as several isoforms (Kowalski et al., 1992; Martinez and Whitaker, 1995; Sheptovitsky and Bruvig, 1996; Jolivet et al., 1998; Klabunde et al., 1998; Timothy et al., 2001; Chazarra et al., 2001). In wheat, PPO isoenzymes displaying monophenolase activity were found exclusively in the endosperm while those having only *o*-diphenolase activity were localized in the pericarp (Taneja and Sachar, 1974; Mayer and Harel, 1979; Marsh and Galliard, 1986; Hatcher and Kruger, 1993). The crystal structure of a potato PPO has been solved (Klabunde et al., 1998). The

active site of PPO is constituted of a binuclear copper-cluster (**Figure 9**). The copper atoms are linked to each other through oxygen atoms and bound to the polypeptide chain through three histidine residues. PPO may catalyze a regioselective aerobic two electrons transfer oxidation of monophenols (monophenolase or cresolase activity) to *o*-diphenols and their subsequent dehydrogenation to the corresponding *o*-quinones (diphenolase or catecholase activity) (Martinez and Whitaker, 1995; Timothy et al., 2001). The *o*-quinones produced may undergo non-enzymatic cyclization or polymerization reactions to yield melanin-like pigments (Martinez and Whitaker, 1995; Rodakiewicz-Nowak and Ito, 2003). Not all PPO isoenzymes can perform the hydroxylation step and even those possessing that activity may loose it during extraction or storage (Sanchez-Ferrer et al., 1995). The catecholase activity is a shared property of all PPOs.

PPOs are involved in various protection mechanisms, including human pigmentation and the browning of fruits and vegetables. The defense function of PPO in plants is attributed to the modification of endogenous phenolic compounds, notably allelochemicals or phytoalexins into *o*-quinones which are toxic to the invading pathogens and pests (Mayer and Harel, 1979; Lagrimini and Rothstein, 1987; Luthra et al., 1988; Kowalski et al., 1992; Dowd et al., 2000, Weir et al., 2004). PPO is suggested to be indirectly involved in auxin biosynthesis because the *o*-quinones produced by PPO can react with tryptophan to form indole-3-acetic acid (Mayer and Harel, 1979). PPO was also found both in latent and active forms in the photosystem-II of the thylacoid membrane-protein complex, suggesting a function in photosynthesis (Sheptovitsky and Brudvig, 1996).

In sorghum, PPO is present in the leaves (Stafford and Dresler, 1972; Vaughn and Duke, 1981) and in the grain (Glennie, 1981). Three PPO isoforms with different molecular masses were found in the leaves, although no molecular masses were given (Stafford, 1972). PPO activity in sorghum leaves has been associated in response to fungal invasion (Luthra et al., 1988; Gowda et al., 1989). However, until now no further purification and characterization of sorghum PPO has been performed.

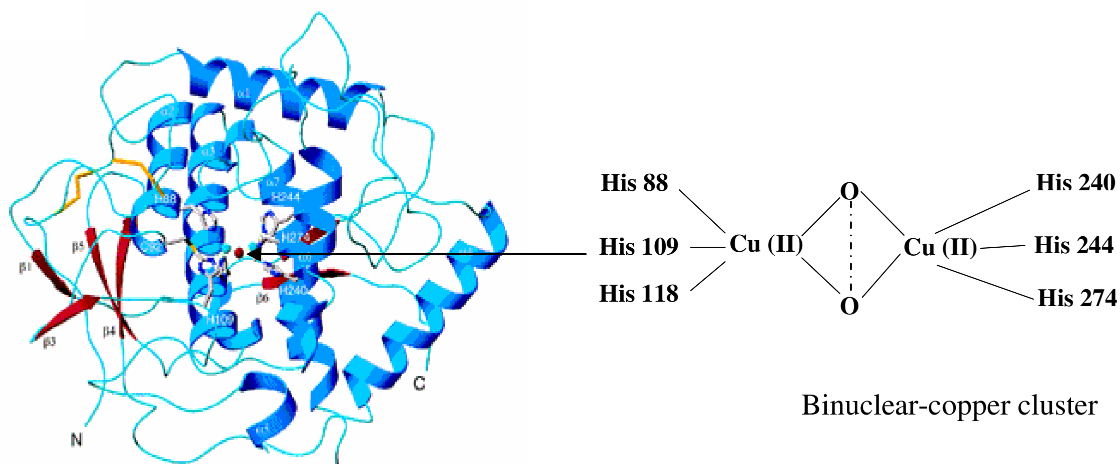


Figure 9. Structure of potato PPO and its binuclear copper-cluster (Klabunde et al., 1998).

Peroxidases

Peroxidases [EC 1.11.1.7, POXs] are ubiquitous enzymes found in bacteria, fungi, plants and animals (Krylov and Dunford, 1996, Dunford, 1999). They have in common that they accept hydrogen peroxide or hydroperoxide analogs as oxidant by forming water as by-product. Most POXs studied to date contain ferric protoporphyrin IX as prosthetic group and act through a high-valence iron-oxo species. Besides these heme-containing POXs, selenium-, manganese-, vanadium- and flavin-containing POXs are known (Adam et al., 1999). Based on sequence similarity, number of calcium ions and origins, POXs have been divided in three major classes (Welinder, 1992). Class I includes yeast cytochrome c POXs, ascorbate POXs and bacterial catalase-POXs. Cytochrome c POXs are found in the mitochondrial electron transport chain, where they probably protect against toxic peroxides. Ascorbate POXs are the main enzyme responsible for hydrogen peroxide removal in chloroplasts and cytosol of higher plants (Dalton, 1993). Bacterial catalase-POXs have both classical POX and catalase activities (Welinder, 1991; Fraaije et al., 1996). Class II comprises secretory fungal POXs such as lignin POXs and manganese-dependent POXs (Reddy and Souza, 1994). Class III consists of the secretory plant POXs found in e.g. horseradish, barley, and wheat.

Plant POXs have many different physiological functions including the removal of hydrogen peroxide from chloroplasts and cytosol; the oxidation of toxic compounds; the biosynthesis of cell walls (lignin and suberin); defense responses towards wounding and other stresses; indole-3-acetic acid regulation; ethylene biosynthesis; etc. (Dunford, 1999; Welinder et al., 2002; Duroux and

Welinder, 2003). POXs occur in plants as several isoenzymes or glycoforms, with different cell localization (Dunford, 1999; Duroux and Welinder, 2003). While in the five chromosomes of *Arabidopsis* 73 POX genes were found (Duroux and Welinder, 2003), up to 138 POX genes and 14 POX pseudogenes were annotated in the twelve chromosomes of rice (*Oryga sativa* japonica) (Passardi et al., 2004). Considering the phylogenetic linkage between rice and sorghum (Paterson, 2003), and the fact that the genome of sorghum (750 Mb) is twice as large as that of rice, even more POX genes are expected in sorghum.

Plant secretory POXs generally have the following structural properties in common (Henriksen et al., 1998; Dunford, 1999; Welinder et al., 2002; Duroux and Welinder, 2003; Veitch, 2004):

- monomeric glycoprotein structure of 300-310 amino-acids
- putative N-glycosylation sites: Asn-X-Ser/Thr (X≠Pro)
- non-covalently bound iron(III)-protoporphyrin-IX (type-b heme)
- key catalytic amino-acid residues around the heme prosthetic group: His^{42}, His^{170}, Arg^{38}, Asn^{70}, Asp^{238}, Phe^{41} and Pro^{139} (horseradish peroxidase numbering)
- four conserved disulphide bridges: Cys^{11-91}, Cys^{44-49}, Cys^{97-301}, and $Cys^{177-209}$ (horseradish peroxidase numbering)
- two structural calcium ion binding sites
- structural water molecules extending from the heme pocket to the distal calcium-binding site

The crystal structures of POXs from peanut (Schuller et al., 1996), horseradish (Gajhede et al., 1997); barley (Henriksen et al., 1998), and Arabidopsis (Mirza et al., 2000; Østergaard et al., 2000) have been elucidated. The cationic horseradish (HRP-C) isoenzyme is the most studied POX (**Figure 10**), because of its availability and its catalytic performance.

POXs oxidize reducing substrates (AH) either in presence of hydrogen peroxide (classical POX cycle) or molecular oxygen (oxidase cycle) (**Figure 11**). Recently, it has been shown (Berglund et al., 2002) that HRP-C is essentially in five oxidation states (**Figure 11**) during catalysis (native enzyme, compound-I, compound-II, Compound-III, and ferro-peroxidase). In each of these states, the enzyme has a different conformation, especially at the heme environment

(Berglund et al., 2002). Compound-I is obtained by two-electron oxidation of the resting enzyme by H_2O_2 and is stable (Hiner et al., 2002). In contrast to the generally assumed concept of irreversible formation of Compound–I, Rietjens and co-workers proposed that the formation of Compound I can be a reversible reaction via an uptake of a water molecule (van Haandel et al., 1998, van Haandel, 2000). The one-electron-reduction of Compound-I by AH produces Compound-II. Another electron from AH is required to reduce Compound-II to the native enzyme (Dunford, 1999). Formation of Compound-I (k_1) and the regeneration of the native enzyme (k_3) are generally rate-limiting steps in POXs catalysis (Dunford, 1999). Compound-III is formed from Compound-II with excess of H_2O_2 or upon reaction of the ferrous-enzyme (oxyperoxidase) with O_2 (Dunford, 1999, Berglund et al., 2002). It is important to note that an excess of H_2O_2 can irreversibly inactivate POX because Compound-III is unstable (van Haandel, 2000). In addition, Compound-III can also react with an excess of H_2O_2 to produce verdohemoprotein called P-670; in this case H_2O_2 acts as a suicidal substrate (Dunford, 1999; Hernandez-Ruiz et al., 2001; Sakharov and Sakharova, 2002). The POX catalytic cycle yields free radicals ($A^{\bullet}$), which can undergo polymerization reactions, coupling with molecular oxygen, etc. In the oxidase cycle, POX transfers one electron to molecular oxygen, which in turn is transferred to a substrate; this is termed the monooxygenase activity (Dawson, 1988). The monooxygenase catalytic route of POX is performed via the formation of the ferrous form (Fe^{2+}) of the enzyme (ferro-POX) (Gazarian et al., 1998). Native POX or Compound III may also perform catalase or dismutase reactions (EC 1.11.1.6) by decomposition of H_2O_2 to water and oxygen (Hiner et al., 2001).

Some POXs are able to oxidize auxin in the absence of H_2O_2, thus display indole-3-acetic acid (IAA)-oxidase activity (Christensen et al., 1998, Gazarian et al., 1998; Dunford, 1999). POXs from non-plant sources are unable to oxidize IAA in the absence of H_2O_2, because they are lacking the IAA-binding site localized in the distal domain near the heme pocket (Savitsky et al., 1999). The IAA-oxidase activity of POX isoforms is related to their physiological role in auxin metabolism (Hiner et al., 2001; Marco et al., 1999). Comparison of IAA-oxidase activity among barley and HRP isoenzymes revealed that only strongly basic isoenzymes possessed this activity (Hiner et al., 2001; Marco et al., 1999). The indole-3-acetic acid oxidase mechanism is complex; one of the possible routes is depicted in **Figure 11**, which is different from the POX and oxidase cycles (Gazarian et al., 1998; Savitsky et al., 1999).

POX activities have been detected in the leaves (Stafford and Bravinder-Bree, 1972; Vaughn and Duke, 1981; Luthra et al., 1988) and grains (Glennie, 1981) of sorghum, but a sorghum POX enzyme has never been purified and characterized before.

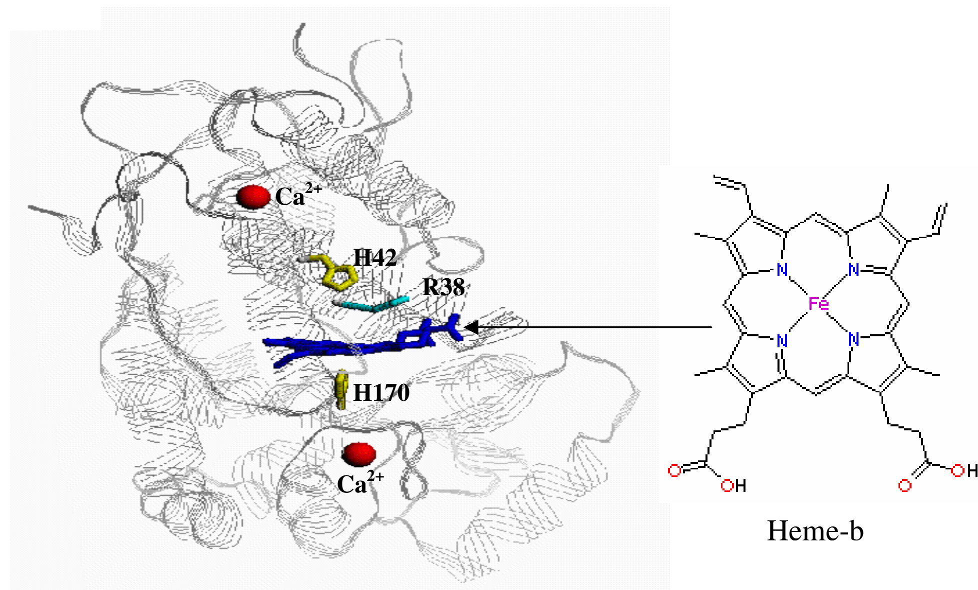


Figure 10. Three-dimensional structure of horseradish peroxidase. Shown are the heme, the structural calcium ions, and the three key amino-acid residues of the active site: Arg + 2 His. Source: Dunford (1999) and Silaghi-Dumitrescu (2005).

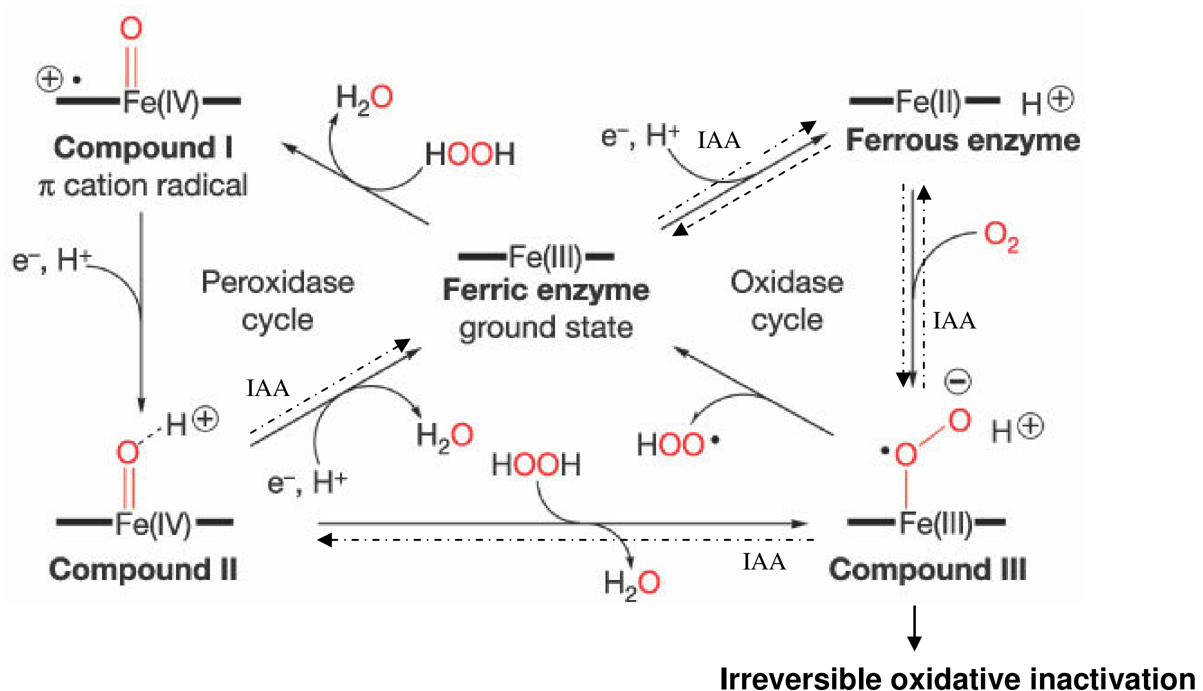


Figure 11. Mechanism of the peroxidases (POX) catalysis. The solid lines show the peroxidases and oxidase cycles as proposed by Berglund and co-workers (2002). The dotted lines show the route of the indole-3-acetic acid oxidase activity which can be performed by some plant POXs (Gazarian et al., 1998; Dunford, 1999; Savitsky et al., 1999). IAA= indole-3-acetic acid.

Role of phenolic enzymes in food

The contents of phenolic compounds and the phenolic oxidizing enzymes are strongly associated with food quality (Deshpande et al., 1986; Hilhorst et al., 1999; 2002; Tomas-Barberan and Espín, 2001). Phenolic compounds are potential substrates of POX and PPO and their oxidation products (benzoquinones and polymeric compounds) affect food quality (Matheis and Whitaker, 1984, Martinez and Whitaker, 1995). PPO and POX work synergistically because PPO may generate H_2O_2 during the course of catalysis (Richard-Forget et al., 1997) and POX on other hand can generate O_2 (Matheis and Whitaker, 1984). Both PPO and POX generate benzoquinones. Because of their electrophilic nature, these quinones undergo secondary reactions, such as polymerization with amino acid side groups in proteins (Anderson and Morris, 2001). PPO and POX are known to influence product properties during and after food processing (Matheis and Whitaker, 1984; Haslam, 1998, Feillet et al., 2000). They affect post harvest degradation of food by causing browning and the development of off-flavors in raw and unblanched cereals (March and Galliard, 1986; Hatcher and Kruger, 1993). In wheat the appearance of colored products in the flour is attributed to the oxidation of endogenous phenolic compounds by PPO (March and Galliard, 1986; Hatcher and Kruger, 1993). PPO and POX are generally admitted to be the most determinant enzymes for the preservation and organoleptic qualities of fruits and vegetables (Matheis and Whitaker, 1984; Parr, 2000, Tomas-Barberan and Espin, 2001).

1.5. Objective of the thesis

Several improved sorghum varieties adapted to semi-arid and tropic environments are released every year by sorghum breeders. Selection of varieties meeting specific local food and industrial requirements from this great biodiversity is of high importance for food security. In Africa in general and in Burkina Faso in particular, demand for sorghum is increasing. This is due to not only the growing population, but also to the country policy to enhance its processing and industrial utilization. In Burkina Faso, although more than 800 sorghum ecotypes have been described, at the present, none of the varieties have been characterized at the molecular level with respect to food quality. However, the acquisition of good quality grain is fundamental to produce acceptable food products from sorghum. In this thesis, 50 sorghum varieties originating from all over the world, which are cultivated in Burkina Faso, will be analyzed for their (bio)chemical and food processing properties. Varieties are selected on the basis of their current utilization in several local foods and because of their adapted agronomic properties.

The major goal of this thesis is to screen for biochemical markers in sorghum to identify the best suited sorghum varieties to be used according to the targeted utilization as a source of food ingredient or utilization for specific foods.

To reach these objectives, the content of starch and starch degrading enzymes as well as phenolics and phenolic related enzymes in ungerminated and germinated sorghum varieties will be determined. In particular, sorghum grain peroxidases were purified and characterized to the molecular level.

1.6. Outline of the thesis

In **Chapter 1** an introduction is presented on the importance of sorghum on food security and the relevance of the screened biochemical constituents for food quality, in order to give an insight in the food quality properties of sorghum varieties.

In **Chapter 2**, fifty sorghum varieties were selected according to their agronomic and food properties. The contents of phenolic compounds and the activities of polyphenol oxidase and peroxidases in ungerminated sorghum varieties were determined. This allowed to compare contents in total phenolic compounds and proanthocyanidins (condensed tannins) and to identify phenolic oxidizing isoenzymes.

In **Chapter 3** the effect of germination on proanthocyanidins, 3-deoxyanthocyanidins and flavan-4-ols is described. The varieties were compared with respect to their antioxidant activities and the best varieties as novel natural sources of free radical scavenger were identified.

Chapter 4 describes the effect of germination on the contents of starch and the activities of α-amylase, β-amylase, phenol biosynthesizing enzyme phenylalanine ammonia lyase and the phenol modifying enzymes peroxidase and polyphenol oxidase in sorghum varieties.

In **Chapter 5** the impact of the content of phenolic compounds and related enzymes for the resistance and susceptibility of sorghum varieties to biotic and abiotic stresses is discussed.

Chapter 6 focuses on a new and universal zymography method for the detection of both monophenolase and *o*-diphenolase activities of polyphenol oxidase as well as peroxidases in crude (plant) extracts.

Chapter 7 describes the purification and characterization of the major peroxidase from sorghum grain. This highly basic, heme-containing glycoprotein is active with a wide range of phenolic compounds and catalyzes the oxidation of auxin (indole-3-acetic acid) in the absence of hydrogen peroxide.

Chapter 8 deals with the concluding remarks and discussion of the findings.

REFERENCES

Aboubacar, A.; Hamaker, B. R. *Cereal Chem*. **1999**, *76*, 308–313.
Adam, W.; Lazarus, M., Saha-Moller, C. R.; Weichold, O.; Hoch, U.; Haring, D. and Schreider, P. **1999**, 63, 73-107.
Adeyeye, A.; Ajewole, K. *Food Chem*. **1992**, *44*, 41-44.
Aggarwal, N. K.; Nigam, P.; Singh, D.; Yadav, B. S. *World J. Microbiol. Biotechnol*. **2001**, *17*, 411-415.
Ahmed, S. B.; Mahgoub, S. A.; Babiker, B. E. *Food Chem.* **1996**, 56, 159-162.
Aisien, A. O.; Muts, G. C. J. *J. Inst. Brew.* **1987**, *93*, 328–331.
Ames, B. N.; Shigenaga, M. K.; Hagen, T. M. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 7915-7922.
Anderson, J. V.; Morris, C. F. *Crop. Sci*. **2001**, *41*, 1697-1705.
Anglani, C. *Plant Foods Hum. Nutr*. **1998**, *52*, 85-89.
Asquith, T. N.; Butler, L. G. *Phytochemistry* **1986**, *25*, 1591-1593.
Audilakshmi, S.; Stenhouse, J. W.; Reddy, T. P.; Prasad, M. V. R. *Euphytica* **1999**, *107*, 91-103.
Awad, H. M. *Studies on the pro-oxidant chemistry of flavonoids*. PhD Thesis, Wageningen University, Wageningen, The Netherlands, **2002**.
Awad, H. M.; Boersma, M. G.; Vervoort, J.; Rietjens, I. M. *Arch. Biochem. Biophys*. **2000**, *378*, 224-233.
Awika, J. M.; Rooney, L. W. *Phytochemistry*. **2004a**, *65*, 1199-1221.
Awika, J. M.; Rooney, L. W.; Waniska, R. D. *J. Agric. Food. Chem*. **2004b**, *52*, 4388-4394.
Awika, J. M.; Rooney, L. W.; Wu, X.; Prior, R. L.; Cisneros-Zevallos, L. *J. Agric. Food Chem*. **2003**, *51*, 6657-6662.
Axtell, J. D.; Kirleis, A. W.; Hassen, M. M.; D'Croz Mason, N.; Mertz, E. T.; Munck, L. *Proc. Nat. Acad Sci.* USA. **1981**, *78*, 1333-1335.
Badi, S.; Pedersen, B.; Monowar, L.; Eggum, B. O. *Plant Foods Hum. Nutr*. **1990**, *40*, 5-19.
Bate-Smith, E. C. *Phytochemistry* **1969**, *8*, 1803-1810.
Baztan, J.; Torres, M. *Rev. Esp. Fisiol*. **1988**, *44*, 73-80.
Berglund, G. I.; Carlsson, G. H.; Smith, A. T.; Szoeke, H.; Henriksen, A.; Hajdu, J. *Nature* **2002**, *417*, 463-468.
Beta, T.; Corke, H. *J. Cereal Sci*. **2001**, *34*, 261-268.
Beta, T.; Rooney, L. W.; Waniska, R. D. *Cereal Chem*. **1995**, *72*, 533-538.
Beta. T.; Rooney. L. W.; Marovatsanga. L. T.; Taylor. J. R. N. *J. Sci. Food Agric*. **1999**, *79*, 1003-1010.
Billau, C.; Louarme, L.; Nicolas, J. *J. Food Biochem*. **1999**, *23*, 145-172.
Blennow, A.; Bay-Smidt, A. M.; Bauer, R. *Int. J. Biol. Macromol*. **2001**, *28*, 409-420.
Block, G.; Patterson, B.; Subar, A. *Nutr. Cancer* **1992**, *18*, 1-29.
Brown, D. E.; Rashotte, Aaron M.; Murphy, A. S.; Normanly, J.; Tague, B. W.; Peer, W. A.; Taiz, L.; Muday, G. K. *Plant Physiol.* **2001**, *126*, 524-535.
BSTID-NRC (Board on Science and Technology for International Development- National Research Council). *Lost crops of Africa*. Academic Press, Washington DC, **1996**. pp. 127-213.
Butler. L. G. *Plant polyphenols: synthesis. properties and significance*; Hemingway. R. W.; Laks. P. E. Eds.; Plenum press: New York. **1992**, pp. 693-698.
Bvochora, J. M.; Reed. J. D.; Read. J. S.; Zvauya. R. *Process Biochem*. **1999**, *35*, 21-25.
Cai, T.; Ejeta, G.; Butler, L. G. *Theor. Appl. Genet*. **1995**, *90*, 211-220.
Carver, T. L. W.; Zeyen, R. J.; Bushnell, W. R.; Robbins, M. P. *Physiol. Mol. Plant Pathol*. **1994**, *44*, 261-272.
Carson, L.; Setser, C.; Sun, X. S. *Int. J. Food Sci. Technol.* **2000**, 35, 465-471.
Chaman, M. E.; Copaja, S. V.; Argandeña, V. H. *J. Agric. Food Chem*. **2003**, *51,* 2227-2231.
Chan, D. C.; Kim, P. S. *Cell* **1998**, *3*, 681-684.
Chantereau, J.; Nicou, R. *Le sorgho* Vol. 18 *;* Maisonneuve et Lavose ed. ; Agence de Coopération Culturelle et Technique (CTA), Paris, Wageningen, **1991.**
Chazarra, S.; Garcia-Carmona, F.; Cabanes, J. *J. Agric. Food Chem.* **2001**, *49*, 4870-4875.
Chen, Y.; Hagerman, A. E. *J. Agric. Food Chem.* **2004**, *52*, 6061–6067.
Cherney, D. J. R.; Cherney, J. H.; Patterson, J. A.; Axtell, J. D. *Ani. Feed Sci. Technol.* **1992**, *39*, 79-93.
Christensen, J. H.; Bauw, G.; Welinder, K. G.; Montagu, M. V.; Boerjan, W. *Plant Physiol*. **1998**, *118*, 125-135.
Chung, K.T.; Wong, T.Y.; Wei, C.I.; Huang, Y.W.; Lin, Y. *Crit. Rev. Food Sci. Nutr*. **1998**, *38*, 421–464.
Clifford, M. N. *J. Sci. Food Agric*. **2000**, *80*, 1126-1137.
Coultate, T. *Food: The chemistry of its compounds*. 3rd Ed, Edition Lavoisier : Paris. **1996.**
Cowan, M. M. *Clin. Microbiol. Rev*. **1999,** *12*, 564-582.
Cui, Y.; Frederiksen, R.; Magill, C. Physiol. Mol. *Plant Pathol*. **1996***, 49*, 187-199.
Czarnota, M. A.*;* Rimando, A. M.; Weston, E. L. *J. Chem. Ecol*. **2003**, *9*, 2073-2083.
Dalton, D.A. *Peroxidases in Chemistry and Biology,* Vol. II, Everse, J.; Everse, K.E.; Grisham, M.B. Eds; CRC Press: Boca Raton, **1991,** pp. 139-153.
Dawson, J. *Science* **1988**, *240*, 433-439.

Deprez, S.; Mila, I.; Huneau, J. F.; Tome, D. ; Scalbert, A. *Antio. Redox Signal*. **2001**, *3*, 957-967.
Deshpande, S. S.; Cheryan. M.; Salunkhe. D. K. CRC *Crit. Rev. Food Sci. Nutr*. **1986**, *24*, 401-449.
Deu, M.; Gonzalez de Leon, D.; Glaszman, J. C.; Degremont, I.; Chantereau, J.; Lanaud, C.; Hamon, P. *Theor. Appl. Gen*. **1994**, *88*, 838-844.
Dicko, M. H.; Searle-van Leeuwen, M. J. F.; Beldman, G.; Ouédraogo, O. G.; Hilhorst, R.; Traoré, A. S. *Appl. Microbiol. Biotechnol.* **1999**, *52*, 802-805.
Doggett, H. *Sorghum*, 2nd Ed, Longman Scientific and Technical, London, **1988**.
Dowd, P. F.; Herms, D. A.; Berhow, M. A.; Lagrimini, L. M. *Plant Peroxidases Newsletter*. **2000**, *14*, 93-101.
Dufour, J. P.; Melotte, L.; Srebrnik, S. *ASBC J*. **1992**, *50*, 110-119
Dunford; H. B. *Heme peroxidases*. Dunford, H. B., Ed.; Wiley-VCH, New York, USA, **1999**.
Duodu, K. G.; Taylor, J. R. N.; Belton, P. S.; Hamaker, B. R. *J. Cereal Sci*. **2003,** *38*, 117-131.
Duroux, L.; Welinder K. G. *J. Mol. Evol*. **2003,** *57*, 397-407
Feillet, P.; Autran, J-C.; Icard-Verniere, C. *J. Cereal Sci*. **2000**, *32*, 215-233.
Fennema, O. R. in: *Food Chemistry.* O. R., Fennema, Ed.; Marcel Dekker, New York. **1985,** pp. 23-67.
Fenster, C.; US Grains Council 43rd Board of Delegates' Meeting, July 2003, Minneapolis, MN. **2003**. http://www.grains.org/news/latest_news/43_delegates_meeting_updates.html
Ferreira, D.; Slade, D. *Nat. Prod. Rep.* **2002**, *19*, 517-541.
Food and Agricultural Organization (FAO). *FAO Food and Nutrition Series*, No. 27. ISBN 92-5-103381-1. 1995. consulted on 27 September 2004 at: http://www.fao.org/DOCREP/T0818e/T0818E00.htm#Contents
Food and Agricultural Organization (FAO). FAOSTAT **2005**. http://faostat.fao.org/faostat/
Fraaije, M. W.; Roubroeks, H. P.; Hagen, W. R.; van Berkel, W. J. H. *Eur. J. Biochem.* **1996**, *235*, 192-198.
Gaitan, E.; Lindsay, R.H.; Reichert, R.D.; Ingbar, S. H.; Cooksey, R. C.; Legan, J.; Meydrech, E. F.; Hill, J.; Kubota, K *J. Clin. Endocrinol. Metab*. **1989**. *68*, 707-714.
Gajhede, M.; Schuller, D. J.; Henriksen, A.; Smith, A. T.; Poulos, T. L. *Nature Struct. Biol*. **1997**, *4*, 1032-1038.
Galiba, M.; Waniska, R. D.; Rooney, L. W.; Miller, F. R. *J. Cereal Sci*. **1988**, *7*, 183-193.
Gazarian, I. G.; Lagrimini, L. M.; Mellon, F. A.; Naldrett, M. J.; Ashby, G. A.; Thorneley, R. N. F. *Biochem. J*. **1998**, *333*, 223-232.
Glennie, C. W. *J. Agric. Food Chem.* **1981**, *29*, 33-36.
Glew, R. H.; Vanderjagt, D. J.; Lockett, C.; Grivetti, L. E.; Smith, G. C.; Pastuszyn, A.; Millson, M. *J. Food Comp. Anal*. **1997**, *10*, 205-217.
Godwin, I. D.; Gray, S. J. *Transgenic cereals* (O'Brien L.; Henry R. J.; Eds.) AACC, St Paul, Minnesota, USA; **2000**; pp. 153-177.
Gomez-Cordoves, C.; Bartolome, B.; Vieira, W.; Virador, V. M. *J. Agric. Food Chem.* **2001**, *49*, 1620-1624.
Gowda, P.; Bhavanishankara, S.; Bhat, S. G.; Bhat, S. Shankara. *Cur. Sci*. **1989**, *58*, 1037-1039.
Graham, G. G.; MacLean, W. C. Jr; Morales, E.; Hamaker, B. R.; Kirleis, A. W.; Mertz, E. T.; Axtell, J. D. *J. Nutr*. **1986**, *116*, 978-984.
Hagerman, A. E.; Riedl, K. M.; Jones, G. A.; Sovik, K. N.; Ritchard, N. T.; Hartzfeld, P. W.; Riechel, T. L. *J. Agric. Food Chem*. **1998**, *46*, 1887-1892.
Hagerman, A. E. *Tannin chemistry*; Hagerman, A. E. *Ed.;* Department of Chemistry and Biochemistry. Miami University Oxford, OH 45056 USA. **2005**. http://www.users.muohio.edu/hagermae/
Hahn, D. H.; Faubion, J. M.; Rooney, L.W. *Cereal Chem*. **1983**, *60*, 255–259.
Hahn, D. H.; Rooney, L. W. *Cereal Chemistry* **1986**, *63*, 4–8.
Hamaker, B. R.; Kirleis, A. W.; Butler, L. G.; Axtell, J. D.; Mertz, E. T. *Proc. Nat. Acad. Sci. USA*. **1987**, *84*, 626-638.
Hamaker, B. R.; Mohamed, A. A.; Habben, J. E.; Huang, C. P.; Larkins, B. A. *Cereal Chemistry* **1995**, *72*, 583-588.
Hamauzu, Y.; Yasui, H.; Inno, T.; Kume, C.; Omanyuda, M. *J. Agric. Food. Chem*. **2005,** *53*, 928-934.
Harborne, J. B. *Acta Hort.* **1994**, *381*, 36-45.
Haslam. E. *Practical polyphenolics: from structure to molecular recognition and physiological action.* Haslam, E. Ed.; Cambridge University Press. Cambridge. UK. **1998**.
Hatcher, D. W.; Kruger, J. E. *Cereal Chem*. **1993**, *70*, 51-55.
Hatfield, R. D.; Wilson, J. R.; Mertens, D. R. *J. Sci. Food. Agric*. **1999**, *79*, 891-899.
Heldt, H. W. Plant Biochemistry; Elsevier: Amsterdam. **2005**.
Henriksen, A.; Welinder, K. G.; Gajhede, M. *J. Biol. Chem.* **1998**, *273*, 2241-2248.
Hernandez-Ruiz J; Arnao M. B.; Hiner, A. N.; Garcia-Canovas, F.; Acosta, M. *Biochem. J*. **2001**, *15*, 107-114.
Hertog, M. G. L.; Hollman, P. C. H.; Katan, M. B.; Kromhout, D. *Nutr. Cancer* **1993**, *20*, 21-29.
Hilhorst, R. *Bierbereiding in Burkina Faso.* PtIProcestechniek **1986**, *41*, 93-95.
Hilhorst, R.; Dunnewind, B.; Orsel, R.; Stegeman, P.; Vliet, T. van, Gruppen, H.; Schols, H. *J. Food Sci.* **1999,** *64*, 808-813.

Hilhorst, R.; Gruppen, H.; Orsel, R.; Laane, C.; Schols, H. A.; Voragen, A. G. J. *J. Food Sci.* **2002**, *67*, 497-506.
Hiner, A. N. P.; Hernandez-Ruiz, J.; Rodriguez-Lopez, J. N.; Arnao, M. B.; Varon, R.; Garcia-Canovas, F.; Acosta, M. *J. Biol. Inorg. Chem.* **2001**, *6*, 504-516.
Hiner, A. N. P.; Hernandez-Ruiz, J.; Williams, G. A.; Arnao, M. B.; Garcia-Canovas, F.; Acosta, M. *Archiv. Biochem. Biophys*. **2001**, *392*, 295-302.
Hiner, A. N. P.; Raven, E. L.; Thorneley, R. N. F.; Garcia-Canovas, F.; Rodriguez-Lopez, J. N. *J. Inorg. Biochem*. **2002**, *91*, 27-34.
Hopkins, B. G.; Whitney, D. A.; Lamond, R. E.; Jolley, V. D. *J. Plant Nutr*. **1998**, *21*, 2623-2637.
Hrazdina, G. *Plant polyphenols: synthesis. properties. significance* Vol 59, Hemingway R. W. and Laks. P. E. Ed.; Plenum Press: New York. **1992**, pp. 61-72.
Hug, H.; Strozyk, F.; Borowczak, M. P. *Brauwelt Int*. **1991**, *2,* 114-122.
Hugo, L. F.; Rooney, L. W.; Taylor, J. R. N. *Cereal Chem*. **2000**, *77*, 428-432.
Hugo, L. F.; Rooney, L. W.; Taylor, J. R. N. *Cereal Chem*. **2003**, *80*, 495-499.
Iacobucci, G. A.; Sweeny, J. G. *Tetrahedron* **1983**, *39*, 3005-3015.
ICRISAT, Patancheru, India. **2005**. http://www.icrisat.org/text/research/grep/homepage/sorghum/sorghumhomepage.htm
ICRISAT, Sorghum report, Patancheru, India. **1982**, pp 294-311.
Jambunathan, R.; Mertz, E. T.; Axtell, J. D. *Cereal Chem*. **1975**, *52*, 119-121.
Jarvis. C. E.; Walker. J. R. L. *J. Sci. Food Agric*. **1993**, *63*, 53-57.
Jolivet, S.; Arpin, N.; Wichers, H. J.; Pellon, G. *Mycol. Res*. **1998,** *102*, 1459-1483.
Joshipura, K. J.; Hu, F. B.; Manson, J. E.; Stampfer, M. J.; Rimm, E. B.; Speizer, F. E.; Colditz, G.; Ascherio, A.; Rosner, B.; Spiegelman, D.; Willett, W. C. *Ann. Intern. Med*. **2001**, *134*, 1106-1114.
Kaplan, F.; Guy, C. L. *Plant Physiol*. **2004**, *135*, 1674-1684.
Khokhar, S.; Magnusdottir, S. G. M. *J. Agric. Food Chem*. **2002**, *50*, 565-570.
Klabunde, T.; Eicken, C.; Sacchettini, J. C.; Krebs, B. *Nat. Struct. Biol*. **1998**, *5*, 1084-1090.
Knudsen, B.; K .E.; Kirleis, A.W.; Eggum, B. O.; Munck, L. *J. Nutr.;* **1988***, 118*, 588-597.
Knudsen, B.; K. E.; Munck, L. *J. Cereal Sci*. **1985**, *3*, 153-164.
Kock, J. L. F.; Groenewald, E. G.; Kruger, G. H. J.; Eloff, J. N.; Lategan, P. M. *J. Sci. Food. Agric*. **1985***, 36*, 1140-1144.
Kouda-Bonafos, M.; Czyzewska, E.; Nacro, M.; Oehschager, A. C. *J. Chemical Ecol.* **1994**, *20*, 2123-2125.
Kouda-Bonafos, M.; Nacro, M.; Ancian, B. *Magn. Reson. Chem.* **1996**, *34*, 389-394.
Kowalski, S. P.; Eannetta, N. T.; Hirzel, A. T.; Steffens, J. C. *Plant Physiol*. **1992**, *100*, 677-684.
Krueger, C. G.; Vestling, M. A.; Reed, J. D. *J. Agric. Food Chem*. **2003**, *51*, 538–543.
Krylov, S.; Dunford, H. B. *Plant peroxidases: Biochemistry and Physiology*. Obinger, C.; Burner, U.; Ebermann, R.; Penel, C.; Greppin, H.; Eds; Geneva, University of Geneva, **1996,** pp. 59-69.
Lagrimini, L. M.; Rothstein, S. *Plant Physiol*. **1987**, *84*, 438-442.
Langseth, L. *Oxidants, antioxidants, and disease prevention*; Bracco U. Ed.; ILSI Press: Belgium, **1995**, 24p.
Limami, A. M; Rouillon, C.; Glevarec, G.; Gallais, A.; Hirel, B. *Plant Physiol*. **2002**, *130*, 1860-1870.
Lo, S. C. C.; De Verdier, K.; Nicholson, R. L. *Physiol. Mol. Plant Pathol.* **1999**, *55*, 263-273.
Lu, L.; Liu, S.; Jiang, S.; Wu, S. *Acta Pharma.* **2004**, *25*, 213-218.
Luthra, Y. P.; Ghandi, S. K.; Joshi, U. N.; Arora, S. K. *Acta Phytopathol. Entomol. Hung.* **1988**, *23*, 393-400.
Mahgoub, S. E. O.; Ahmed, B. M.; Ahmed, M. M. O.; El Agib, N. A. A. *Food Chem*. **1999,** *67*, 129-133.
Malleshi, N. G.; Desikachar, H. S. R. *J. Food Sci. Technol*. **1988**, *25*, 1-3.
Mangan, J. L. *Nutr. Res. Rev*. **1981**, *1*, 209-231.
Manners, D. J. *Plant carbohydrate biochemistry*. Brian, J. B. Ed.; Academic Press: London, **1974**.
Marco, A.; Guzzardi, P.; Jamet, E. *Plant Physiol*. **1999**, *120*, 371-381.
Marsh, D. R.; Galliard, T. *J. Cereal Sci.* **1986**, *4*, 241-248.
Martinez, M. V.; Whitaker, J. R. *Trends Food Sci. Technol.* **1995**, *6*, 195-200.
Matheis. G.; Withaker. J. R. *J. Food Biochem*. **1984**, *8*, 137-162.
Mayer, A. M.; Harel, E. *Phytochemistry* **1979**, *18*, 193-215.
Mazhar, H.; Chandrashekar, A. *Cereal Chem*. **1993**, 70, 667-671.
Melake-Berhan, A.; Butler. L. G.; Ejeta. G.; Menkir. A. *J. Agric. Food Chem*. **1996**, *44*, 2428-2434.
Mirza, O.; Henriksen, A.; Ostergaard, L.; Welinder, K. G.; Gajhede, M. *Acta Crystal*. **2000**, *56*, 372-375.
Mohan, S.; Purushothaman, D.; Jayaraj, S.; Rangarajan, A. V. *Curr. Sci***. 1988**, *57*, 492-493.
Moosmann. B.; Behl, C. *Proc. Natl. Acad. Sci.* USA. **1999**, *96*, 8867-8872.
Morazzoni, P.; Magistretti, M. J. *Fitoterapia* **1990**, *61*, 13-20.
Morton, J. F. In *Plant polyphenols: synthesis, properties and significance*; Hemingway, R. W.; Laks, P. E., Eds.; Plenum press, New York, **1992**; pp. 739-765.
Mundy, J. *Carl. Res. Comm*, **1982**, *47*, 263-274.

Murdiati, T. B.; McSweeney, C. S.; *Proceedings of the 2nd International Symposium on the Nutrition of Herbivores.* Rose, M. Ed.; University of Queensland, Brisbane, **1987**, pp 41-42.
Natella, F.; Nardini, M.; Di Felice, M.; Scaccini, C. *J. Agric. Food Chem.* **1999,** *47,* 1453-1459.
Nguz, K.; van Gaver, D.; Huyghebaert, A. *Sci. Aliments* **1998**, *18*, 507-514.
Ninfali, P.; Bacchiocca. M. *J. Agric. Food Chem.* **2003**, *51*, 2222-2226.
Nissum, M.; Schiodt, C. B.; Welinder, K. G. *Bioch. Biophys. Acta* **2001**, *1545*, 339-348.
Nwanguma, B. C.; Eze, M. O. *J. Sci. Food Agric*. **1996**, *70*, 162-166.
Okon, E. U.; Uwaifo, A. O. *J. Agric. Food Chem*. **1984**, *32*, 11-14.
Okuda, T.; Yoshida, T.; Hatano, T.; *Economic and Medicinal Plant Research, Vol 5,* Wagner, H.; Farnsworth, N. R. Eds.; Academic Press: London, **1991,** 129-165.
Olatunji, O.; Adesina, A. A.; Koleoso, O. A. Symposium on the current status and potential of industrial uses of sorghum in Nigeria. Kano, Nigeria, 4-6 décembre, **1989**.
Ollitrault, P.; Arnaud, M.; Chantereau, J. *Agron. Trop*. **1989a**, *44*, 211-222.
Ollitrault, P.; Escoute, J. ; Noyer, J. L. *Agron. Trop*. **1989b,** *44*, 203-210.
Oria, M. P.; Hamaker, B. R.; Shull, J. M. *J. Agric. Food Chem*. **1995**, *43*, 2148-2153.
Østergaard, L.; Teilum, K.; Mirza, O.; Mattsson, O.; Petersen, M.; Welinder, K. G.; Mundy, J.; Gajhede, M.; Henriksen, A. *Plant Mol. Biol***. 2000**, 44, 231-243.
Palé, E.; Kouda-Bonafos, M.; Nacro, M.; Vanhaelen-Fastré, R.; Ottinger, R. *Phytochemistry* **1997**, *45*, 1091-1093.
Parr, A. J.; Bolwell, G. P. *J. Sci. Food Agric*. **2000**, *80*, 985-1012.
Passardi, F.; Longet, D.; Penel, C.; Dunand, C. *Phytochemistry* **2004**, *65*, 1879-1893.
Paterson, A. H.; Bowers, J. E.; Peterson, D. G.; Estill, J. C.; Chapman; B. *Curr. Opin. Gen. Dev*. **2003**, *13*, 644-650.
Patwardhan, V. N.; Norris, R. V. *J. Indian Inst. Sci*. **1928**, A11, 121-133.
Pietta, P. G. *J. Nat. Prod*. **2000,** *63,* 1035-1042.
Radhakrishnan, M. R.; Sivaprasad, J. *J. Agric. Food Chem*. **1980**, *28*, 55-57.
Reddy, C. A.; D'Souza, T. M. *FEMS Microbiol. Rev.* **1994**, *13*, 137-152.
Reddy, N. P.; Eswara, J. M. *Acta Physiol. Plant*. **2002**, *24*, 285-290.
Reed, J. D. *J. Ani. Sci*. **1995.** *73*, 1516-1528.
Richard-Forget, F.; Gaulliard, F. A. *J. Agric. Food Chem*. **1997**, *45*, 2472-2476.
Rietjens, I. M.; Awad, H. M; Boersma, M. G.; van Iersel, M. L.; Vervoort, J.; van Bladeren, P. J. *Adv. Exp. Med. Biol.* **2001**, *500*, 11-21.
Robyt, J. F.; Ackerman, R. J. *Archiv. Biochem. Biophys*. **1973**, *155*, 445-451.
Rodakiewicz-Nowak, J.; Ito, M. *J. Chem. Technol. Biotechnol.* **2003**, *78*, 809–816.
Rodriguez-Lopez, J. N.; Lowe, D. J.; Hernandez-Ruiz, J.; Hiner, A. N.; Garcia-Canovas, F.; Thorneley, R. N. *J. Am. Chem. Soc*. **2001**, *123*, 11838-11847.
Rooney, L. W.; Waniska, R. D. Food and Nutritional Quality of Sorghum and Millet. Report Project TAM-226. Department, Texas A&M University, College Station, Texas 77843-2474, USA, **2004.**
Rösler, J.; Krekel, F.; Amrhein, N.; Schmid, J. *Plant Physiol*. **1997**, *113*, 175–179.
Rouau, X. *J. Cereal Sci.* **1993**, *18*, 145–157.
Ryan, D.; Robards, K. *Analyst* **1998**, *123*, 31-44.
Sachdev, A.; Johari, R. P.; Mehta, S. L. *Ind. J. Plant Physiol*. **1997**, *2*, 1-4.
Sae, S. W.; Kadoum, A. M.; Cunningham, B. A. *Phytochemistry* **1971**, *10*, 1-10.
Sakharov, I. Y.; Sakharova, R. V. *Biochim. Biophys. Acta*. **2002**, *1598*, 108-114.
Sanchez-Ferrer, A.; Rodriguez-Lopez, J. N.; Garcia-Canovas, F.; Garcia-Carmona, F. *Biochim. Biophys. Acta* **1995**, *1247*, 1-11.
Santos-Buelga, C.; Scalbert, A. *J. Sci. Food Agri***c. 2000**, *80*, 1094-1117.
Santos-Buelga, C.; Williamson, G. *Methods in Polyphenol Analysis.* Royal Society of Chemistry, Ed.; Cambridge, UK, **2003**, 383p.
Sautier, D.; O'Deye, M. *Mil, Mais, Sorgho-Techniques et alimentation au Sahel.* Harmattan. Paris, France. **1989**.
Savitsky, P. A.; Gazaryan, I. G.; Tishkov, V. I.; Lagrimini, L. M.; Ruzgas, T.; Gorton, L. *Biochem. J.* **1999**, *340*, 579-583.
Scalbert, A. *Phytochemistry* **1991,** *30*, 3875-3883.
Schuller, D. J.; Ban, N.; van Huystee, R. B.; McPherson, A.; Poulos, T. L. *Structure* **1996**, *4*, 311-321.
Serafini, M.; Ghiselli, A; Ferro-Luzzi, A. *Lancet* **1994**, *344*. 626.
Sérémé, A.; Kouda-Bonafos, M.; Nacro, M. *Plant Foods Hum. Nutr.* **1994**, *46*, 331-334.
Serna-Saldivar, S.; Rooney, L. W. Sorghum and Millets: Chemistry and Technology'; D.A.V. Dendy, Ed.; American Association of Cereal Chemists, St. Paul, USA, **1995**, pp. 69-124.
Sheptovitsky, Y. G; Brudvig, G. W. *Biochemistry* **1996**, *35*, 16255-16263.
Shi, H.; Noguchi, N.; Niki, E. Flavonoids and other polyphenols. *Methods Enzymol*. **2001**, *335*, 157-166.
Shin, S. I.; Choi, H. J.; Chung, K. M.; Hamaker, B. R.; Park, K. H.; Moon, T. W. *Cereal Chem.* **2004**, *81*, 404-408.

Shiraishi, T.; Yamada, T.; Nicholson, R. L.; Kunoh, H. *Physiol. Mol. Plant Pathol*. **1995**, *46*, 153-162.

Silaghi-Dumitrescu, R. L. *HRP: A Summary of its Structure, Mechanism and Substrate Diversity*. Department of Chemistry, University of Georgia in Athens, Georgia. **2005**, Available at**:** http://www.unige.ch/LABPV/publications/silaghi/Dumitrescu.html

Singh, S.; Lewis, N. G.; Towers, G. H. *J Plant Physiol*. **1998**, *153*, 316-23.

Stafford, H. A. *Phytochemistry* **1969**, *8*, 743-752.

Stafford, H. A. *Flavonoids metabolism*. CRC Press: Boca Raton, USA; **1990**.

Stafford, H. A.; Bravinder-Bree, S. *Plant Physiol.* **1972**, *49*, 950-956.

Stafford, H. A.; Dresler, S. *Plant Physiol*. **1972**, *49*, 590-495.

Stoewsand, G. S.; Anderson, J. L.; Boyd, J. N.; Hrazdina, G.; Babish, J. G.; Walsh, K. M.; Losco, P. *J. Toxicol. Env. Health* **1984**, *14*, 105-114.

Suresh, K.; Kiransree, N.; Venkateswar Rao, L. *Bioprocess Engin*. **1999**, *21*, 165-168.

Swanston, J. S.; Taylor, K.; Rao, N. S.; Subramanian, V.; Murty, D. S. *J. Inst. Brew*. **1993**, *99*, 483-486.

Taneja, S. R.; Abrol, Y. P.; Sachar, R. C. *Cereal Chem*. **1974**, *51*, 457-465.

Tao, Y.; Manners, J. M.; Ludlow, M. M.; Henzell, R. G. *Theor. Appl. Gen.* **1993**, *86*, 679-688.

Taur, A.T.; Pawar, V. D.; Ingle, U. M. *Indian J. Nutr. Diet* **1984**, *21*, 129-136.

Taylor, J. R. N. *ASBC J*. **1992**, *50*, 13-18.

Taylor, J. R. N.; Robbins, D. J. *J. Inst. Brew.* **1993**, *99*, 413-416.

Taylor, J.; Taylor, J.R.N. *Int. J. Food Sci. Techno.* **2002**, *37*, 129-138.

Taylor, J. R. N.; Dewar, J. *Advances in Food and Nutrition Research*, Vol. 43; S.L. Taylor, ed.; Academic Press, San Diego, USA, **2001**.

Temple, N. J. *Nutr. Res.* **2000**, *20*, 449-459.

Tenkouano, A. SICNA and ICRISAT, Ed. *INTSORMIL*, **1995**, *36*, 41-44.

Timothy, E. M.; Quintanar, L.; Palmer, A. E.; Hasset, R.; Severance, S.; Kosman, D. J.; Solomon, E. I. *J. Am. Chem. Soc*. **2001**, *123*, 5507-5517.

Tomas-Barberan, F.; Espín, J. C. *J. Sci. Food Agric.* **2001**, *81*, 853-876.

Towo, E. E.; Svanberg, U.; Ndossi, G. D. *J. Sci. Food Agric*, **2003**, *83*, 980-986.

Tovar, M. J.; Romero, M. P.; Girona, J.; Motilva, M. J. *J Sci Food Agric.* **2002***,* *82*, 892-898.

Traore, T.; Mouquet, C.; Icard-Verniere, C.; Traore, A. S.; Treche, S. *Food Chem*. **2004**, *88*, 105-114.

Trouche, G. ; Da, S. ; Pale, G. ; Sohoro, A.; Ouedraogo, O. ; Den Gosso, G. In Sélection participative. Hocdé, H.; Lançon, J.; Trouche, G. Eds. ; CIRAD : Montpellier. **2001**. pp. 36-55.

Trouche, G.; Fliedel, G.; Chantereau J.; Barro, C. *Agric. Dev*. **2000**, *23*, 94-107.

Uriyo, M.; Eigel, W. E. *Process Biochem*. **2000**, 35, 433-436.

Uvere, P. O.; Adenuga, O. D.; Mordi, C. *J. Sci. Food Agric*. **2000**, *80*, 352–358.

van Haandel, M. J. H.; Primus, J. L.; Teunis, C.; Boersma, M. G.; Osman, A. M.; Veeger, C.; Rietjens, I. M. C. M. *Inorg. Chim. Acta* **1998**, *275-276*, 98-105.

van Haandel, Marjon J. H. *Structure, function and operational stability of peroxidases*. PhD thesis, Wageningen University, Wageningen, The Netherlands**, 2000**.

Vaughn, K.C.; Duke, S.O. *Protoplasma* **1981**, *108*, 319-327.

Veitch, N. C. *Phytochemistry Rev*. **2004**, *3*, 3-18.

Verbruggen, M. *Glucuronoarabinoxylans from sorghum grain*. PhD thesis. Wageningen University, Wageningen, The Netherlands, **1996**.

Verbruggen, M. A.; Beldman, G.; Voragen, A. G. *Carbohydr. Res*. **1998**, *306*, 275-282.

Verbruggen. M. A.; Beldman. G.;Voragen. A. G. J.; Hollemans. M. *J. Cereal Sci*. **1993**, *17*, 71-82.

Vierling, R. A.; Xiang, Z.; Joshi, C. P.; Gilbert, M. L.; Nguyen, H. T. *Theoret. Appl. Gen*. **1994**, *87*, 816-820.

Vliegenthart, J. F. *Carbohydr. Res.* **1998**, *306*, 265-274.

Waniska, R. D. Technical and institutional options for sorghum grain mold management: proceeding of an International consultation. Chandrashekar, A.; Bandyopadhyay, R.; Hall, A. J. Eds.; ICRISAT, Patancheru, India. **2000**. pp. 72-106. http://www.icrisat.org/text/research/grep/homepage/sgmm/chap4.pdf

Waniska, R. D.; Poe, J. H.; Badyopadhyay, R. *J. Cereal Sci*. **1989**, *10*, 217-225.

Watt, J. M.; Breyer-Brandwijk, M. G. *The medicinal and poisonous plants of southern and eastern Africa*. 2nd ed. E.&S. Livingstone, Ltd., Edinburgh and London, **1962.**

Weiergang, I.; Hipskind, J. D.; Nicholson R. L. *Physiol. Mol. Plant Pathol.* **1996**, *49*, 377-388.

Weir, T. L.; Park, S. W.; Vivanco, J. M. *Curr. Opin. Plant Biol.* **2004**, *7*, 472-479.

Welinder, K. G. *Biochim. Biophys. Acta* **1991**, *1080*, 215-220.

Welinder, K. G. *Curr. Opin. Struct. Biol.* **1992**, *2*, 388-393.

Welinder, K. G.; Justesen, A. F.; Kjaersgard, I. V. H.; Jensen, R. B.; Rasmussen, S. K.; Jespersen, H. M.; Duroux, L. *Eur. J. Biochem*. **2002**, *269*, 6063-6081.

Whistler, R. L.; BeMiller, J. N. **1997**. *Carbohydrate Chemistry Scientists*. American Association of Cereal Chemists, St. Paul, Minnesota.

Willett, W. C. *Science* **2002**, *296*, 695-698.

Woude, van der H.; Awad, H. M.; Boersma, M. G.; Boeren, S.; van Zanden, J.; Van Bladeren, P. J.; Vervoort, J.; Rietjens, I. M. C. M. *Free Rad. Res.* **2002**, *36*, 103-104.

Yang, J.; Meyers, K. J.; van der Heide, J.; Liu, R. H. *J. Agric. Food Chem*. **2004**, *52*, 6787-6793.

Yetneberk, S.; De Kock, H. L.; Rooney, L. W.; Taylor, J. R. N. *Cereal Chem*. **2004**, *81*, 314-321.

Zhang, G.; Hamaker, B. R. Starch/Staerke **1999**, *51*, 21-25.

Zobel, H. F. *In Starch chemistry and technology*, 2nd Edidtion. Academic Press, Inc. Orlando, San Diego, New York, London, Toronto, Montreal, Sydney, Tokyo. **1984**, pp. 285-309.

Zongo, J. D. Thèse de Doctorat ès-Sciences, Université Nationale de Côte d'Ivoire, Abidjan, Côte d'Ivoire, **1991.**

CHAPTER 2

Comparison of content in phenolic compounds, polyphenol oxidase and peroxidase in grains of fifty sorghum varieties from Burkina Faso

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ABSTRACT

Analysis of fifty sorghum [*Sorghum bicolor* (L.) Moench] varieties used in Burkina Faso showed that they have different contents of phenolic compounds, peroxidase (POX), and polyphenol oxidase (PPO). Most of the varieties (82%) had a tannin content less than 0.25% (w/w). POX specific activity was higher than the monophenolase and *o*-diphenolase specific activities of PPO. For POX, there was a diversity of isoforms among varieties. No clear correlation could be made between the quantitative composition of the grain in phenolics, PPO and POX, and resistance of plant to pathogens. In general, varieties good for a thick porridge preparation ("tô") have low phenolic compounds content and a medium POX activity. From the red varieties, those used for local beer ("dolo") had a high content in phenolic compounds and PPO, and a low POX activity. The variety considered good for couscous had a low POX content. The characteristics might be useful as selection markers for breeding for specific applications.

Keywords: sorghum, peroxidase, polyphenol oxidase, tannin, phenolic compounds

INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench] is the staple cereal in sub-Saharan Africa and India where 300 million people rely on its grains (Goodwin and Gray, 2000). Burkina Faso is the World leader in sorghum consumption per inhabitant (FAO, 2001). Sorghum represents in this country 48-57% of the total cereal production (FAO, 2001; Diawara et al., 1995). The grain is mainly used for human nutrition, with an average consumption of 200 kg per person per year (Diawara et al., 1995). White grains are used for local foods such as "tô" or other porridges, couscous, etc. "Tô" is a thick porridge prepared by cooking a slurry of cereal flour in boiling water. Couscous is a steamed and granulated food with different type of texture, also prepared from cereal flour. Malt from red sorghum grains is widely used for the preparation of a cloudy local beer ("dolo") and for non-fermented beverages. The knowledge about the content of phenolic compounds, polyphenol oxidase (PPO), and peroxidase (POX) within cereal grains is scarce. However, these biochemical constituents have been shown to be determinants in food quality (Parr and Bolwell, 2000; Tomas-Barberan and Espín, 2001).

PPOs [monophenol, 3,4-L-dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1] are copper-containing oxidases that catalyze the O_2-dependent oxidation of catechols to the corresponding quinones (*o*-diphenolase or catecholase activity). They may also catalyze the regioselective aerobic hydroxylation of monophenols to *ortho*-diphenols and their subsequent oxidation to *o*-quinones (monophenolase or cresolase activity). In most plants POXs [donor: H_2O_2 oxidoreductase, EC 1.11.1.7] are heme-containing enzymes that catalyze the conversion of H_2O_2 to water using phenolic compounds as hydrogen donors. POX and PPO activities have been detected in the leaves (Stafford and Dresler, 1972; Stafford and Bravinder-Bree, 1972; Vaughn and Duke, 1981; Luthra et al., 1988) and grains (Sae et al., 1971; Glennie, 1981; Ollitrault et al., 1989) of sorghum.

POX and PPO may act synergistically in enzymatic browning, because PPO may promote POX activity by generating H_2O_2 from the oxidation of phenolic compounds (Richard-Forget and Gaulliard, 1997). They play an important role in plant defense by the oxidation of endogenous phenolic compounds into quinones, which are toxic to the invading pathogens and pests (Luthra et al., 1988; Dow et al., 2000). The resulting quinones may undergo non-enzymatic auto-polymerization or covalent hetero-condensation with proteins and carbohydrates to produce colored compounds (Parr and Bolwell, 2000). These compounds may also constitute a physical barrier against biotic and abiotic stresses (Dowd et al., 2000; Abdel-Aal et al., 2001). In food, the

reaction products of these enzymes may not only affect taste, bitterness, astringency and color, but when interacting with proteins, these products may hinder digestibility and palatability, thereby reducing the nutritional value of foods (Matheis and Withaker, 1984; Tomas-Barberan and Espín, 2001). In barley, POXs and PPOs have been found to be involved in oxygen scavenging during the mashing process (Clarkson et al., 1992). Interestingly, POX could modify the functional properties of food macromolecules, notably hydroxycinnamic acids containing polysaccharides (pectins, arabinoxylans, etc.) and tyrosine containing proteins, via the mediation of their homo and hetero-crosslinking (Oudgenoeg et al., 2001). This property may be desired in bakery since it results in a better quality of both the dough and the baked product (Hilhorst et al., 1999).

Sorghum and barley are the two important food grains reported to contain significant quantities of phenolic compounds (Butler, 1992; Billau and Nicolas, 2001). Phenolic compounds are plant secondary metabolites biosynthesized through the shikimic acid pathway, in which phenylalanine ammonia-lyase is the key enzyme (Parr and Bolwell, 2000; Tomas-Barberan, and Espín, 2001). Phenolic compounds are believed to be involved in plant growth and reproduction, protection against UV radiation, and resistance to pathogens and predators (Parr and Bolwell, 2000). Some phenolic compounds present in food may have dietary and therapeutic effects (Parr and Bolwell, 2000; Tomas-Barberan, and Espín, 2001). However, tannins are often considered as antinutritional factors, since they inhibit hydrolytic enzymes and link with macronutrients to form indigestible complexes (Butler, 1992). Further, vicinal hydroxyl groups of phenolic compounds (caffeic acid, chlorogenic acid, quercetin, etc.) may chelate metal ions and reduce their bioavailability (Parr and Bolwell, 2000; Layrisse et al., 2000). Possible carcinogenic effect (Morton, 1992) and pro-oxidant activity (Awad et al., 2001) of phenolic compounds are also reported. Nevertheless, the enzymatic oxidation of phenolic compounds considerably enhances their enzyme inhibitory effect and toxicity, and also reduces their health promoting properties (Field and Lettinga, 1992; Awad et al., 2001).

The objective of this study was first to determine the content of phenolic compounds and their oxidative enzymes in fifty sorghum varieties that are produced in Burkina Faso and used for food processing, second to relate the determined biochemical properties to food use as these are reported to be determinants of food quality.

MATERIALS AND METHODS

Chemicals and reagents

Electrophoresis gels (IEF, pH 3-9) were purchased from Amersham Pharmacia Biotech. 4-Hydroxyanisole (4HA) and gallic acid (3,4,5-trihydroxybenzoic acid) were from Aldrich. Catechin and 3,4-dihydroxyphenylpropionic acid (DHPPA) were from Across Organics. Folin-Ciocalteu's reagent, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS), insoluble polyvinylpyrrolidone (PVP), 3,3'-diaminobenzidine (DAB), bovine serum albumin (BSA) and 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) were from Sigma Chemicals Co. Hydrogen peroxide and ferric ammonium citrate (FAC) were from Merck. All other chemicals were of analytical grade.

Sorghum grains

Grains of 50 sorghum [*Sorghum bicolor* (L.) Moench] varieties were kindly provided by the Centre de Coopération International en Recherche Agronomique pour le Développement (CIRAD) at Ouagadougou (Burkina Faso) and Montpellier (France), and the Centre National de Recherche Scientifique et Technique (CNRST) of Burkina Faso. The selection included local varieties as well as internationally improved varieties in order to represent the most widely cultivated sorghums in Burkina Faso (**Table 1**). Varieties were grown (1996-1998) in the natural season, in the semi-arid environment of West Africa (temperature: 30-42°C; precipitation: 600-800 mm/year), at the Breeding Station in the village of Saria (Burkina Faso). Of the varieties 90% were white and 10% red, which reflects the actual figure of sorghum production in Burkina Faso. The varieties were chosen according to their resistance or susceptibility to pests (anthracnose, sorghum midge, sooty stripe and the parasitic plant striga), sensitivity or insensitivity to photoperiod, drought tolerance or susceptibility, grain hardness or softness and suitability or not for traditional processing into local foods ("tô" and couscous) or beer ("dolo"). All the outlined known properties of the plant and grain processing were either obtained from literature (Trouche et al., 1998; Chantereau et al., 1998; Neya and Normand, 1998; Sharma et al., 1999; Trouche et al., 2000; Ouattara et al., 2001) or from CIRAD and CNRST. For convenience, the sorghum varieties were designated with Arabic numbers from 1 to 50 preceded by V. The order was used as it occurred in the collection of breeders. The mature grains (ungerminated) were surface-sterilized by washing with 5% (v/v) aqueous sodium hypochlorite for 5 min with stirring.

Table 1. List of local and improved sorghum varieties used in Burkina Faso[a]

Variety number	Name	Genetic type	Country of origin	Race	Grain testa	Color of grain/plant	Known particular properties: Plant/grain	Food[b]
1	CEF 322/53-1-1	IL	Burkina Faso	C	-	W/R	Post-flowering drought resistant	good for "tô"
2	Sariaso 10	IL	Burkina Faso	C	-	W/Tan	Post-flowering drought resistant	poor for "dolo"
3	IRAT 204	IL	Senegal	C	-	W/Tan	Sooty stripe and anthracnose susceptible	/
4	BF 89-18/139-1-1	IL	Burkina Faso	C	-	W/Tan	Post-flowering drought susceptible	good for "tô"
5	BF 88-2/31-3	IL	Burkina Faso	C	-	W/Tan	Pre-flowering drought susceptible	/
6	SRN 39	IL	Sudan	C	-	Y/Tan	Striga resistant	/
7	Framida	IL	South Africa	KC	+	R/R	Striga resistant	good for "dolo"
8	IS 15401	LR	Cameroon	GC	-	W/R	Striga resistant	/
9	S 29	LR	Burkina Faso	G	-	W/R	Striga susceptible	good for "tô"
10	F2-20	IL	Burkina Faso	C	-	W/Tan	Leaf anthracnose resistant	/
11	CE 180-33	IL	Senegal	C	+	W/Tan	Leaf anthracnose susceptible	/
12	ICSV 1049	IL	Burkina Faso	C	-	W/Tan	Sooty stripe resistant	good for "tô"
13	ICSV 745	IL	India	C	-	W/Tan	Sooty stripe susceptible Sorghum midge resistant	/
14	IRAT 174	IL	Burkina Faso	C	-	W/R	Photoperiod sensitive	/
15	Cauga 22-20	IL	Burkina Faso	GC	+	W/R	Photoperiod sensitive	/
16	G 1414	LR	Burkina Faso	G		W/R	Photoperiod sensitive	/
17	Cauga 108-15	IL	Burkina Faso	GC	-	W/R	Photoperiod insensitive	/
18	Magadji 1-509	LR	Burkina Faso	GC	-	R/R	Photoperiod insensitive	/
19	ICSV 1002	IL	Burkina Faso	C	-	W/Tan	Leaf anthracnose resistant	good for "tô"
20	BC1 S29/2-2	IL	Burkina Faso	G	-	W/R	Sorghum midge susceptible	good for "tô"
21	Kaapelga	LR	Burkina Faso	G	-	W/Tan	Hard grains (PSI< 10)	good for "tô"
22	IRAT 277	IL	Burkina Faso	C	-	W/Tan	Soft grains (PSI >16)	poor for "tô"
23	BF 88-2/31-1	IL	Burkina Faso	C	-	W/Tan	-	poor for "tô"
24	CEM 326/11-5-1-1	IL	Mali	GC	-	W/Tan	Hard grains (PSI< 10)	good for "tô"
25	CEF 396/12-3-1	IL	Burkina Faso	GC	-	W/R	Hard grains (PSI< 10)	good for "tô"
26	G 1636	LR	Burkina Faso	G	-	W/Tan	Soft grains (PSI >16)	/
27	Nazongala tan	IL	Burkina Faso	G	-	W/Tan	Soft grains (PSI >16)	good for "tô"
28	Nongomsoba	LR	Burkina Faso	G	-	W/Tan	Soft grains (PSI >16)	good for "tô"
29	CGM 19/9-1-2	IL	Mali	G	-	W/R	-	good for"dolo"
30	Kokologho	LR	Burkina Faso	C	+	W/R	Post-flowering drought resistant	/
31	IRAT 202	IL	Senegal	C	+	W/Tan	Pre-flowering drought resistant	good for couscous
32	Tiamassie 289	LR	Burkina Faso	G	+	W/R	-	poor for "tô"
33	Kapla-57	LR	Burkina Faso	G	+	R/R	Sorghum midge susceptible	good for "dolo"
34	IRAT 9	IL	Cameroon	C	+	R/R	Grain mold resistant	good for "dolo"
35	Sariaso 9	LR	Burkina Faso	G	-	W/R	Sooty stripe resistant	good for "tô"
36	IRAT 10	IL	Niger	C	-	W/R	Grain mold susceptible	/
37	CEF 395/9-2-3	IL	Burkina Faso	GC	-	W/Tan	Hard grains (PSI < 10)	good for "tô",
38	G 1296	LR	Burkina Faso	GC	-	R/R	Good for dyeing	/
39	Nafo-Natogué 775	LR	Burkina Faso	G	-	R/R	-	good for "dolo"
40	Farkakofsi 781	LR	Burkina Faso	G	+	R/R	-	good for "dolo"
41	Sariaso 808	LR	Burkina Faso	G	+	R/R	-	good for "dolo"
42	Zugilga	LR	Burkina Faso	G	+	R/R	-	good for "dolo"
43	90L1235	IL	USA	GC	-	W/R	Sorghum midge resistant	/
44	CCGM 1/19-1-1	IL	Mali	G	-	W/R	Sorghum midge susceptible	/
45	CK 60	IL	USA	K	-	W/R	Striga susceptible	/
46	CGM 19/9-1-1	IL	Mali	G	-	W/R	Striga susceptible	/
47	B 35	IL	USA	D	-	W/R	Post-flowering drought resistant	/
48	Tx 7000	IL	USA	C	-	W/R	Post-flowering drought susceptible	/
49	Segaolane	IL	Botswana	C	-	W/R	Pre-flowering drought resistant	/
50	Ajabsido	LR	Sudan	C	+	W/R	Pre-flowering drought resistant	/

[a]Abbreviations: C, *Caudatum*; G, *Guinea*; CG, *Guinea-Caudatum*; D, *Durra*; K, *Kafir*; KC, *Kafir-Caudatum*, R, red; W, white; Y, yellow; IL, Inbred line; LR, Landrace; PSI, particle size index. Grain with (+) or without (-) pigmented testa layer. /, not known. [b]Note that all white varieties are generally used for tô and porridge preparation and the red ones are used for brewing "dolo".

The grains were dried by ventilation at room temperature (20-25°C) to a moisture content of 12-14% (w/w), and ground into flour in a microanalytical mill (Fritsch, Marius Instruments, The Netherlands) to pass a screen of 0.5 mm. Milling was done at 4°C and carefully to avoid overheating. The flours were stored at -80°C prior to analysis to prevent endogenous enzymatic or non-enzymatic reactions.

Determination of total phenolic compounds content

Total content of phenolic compounds was extracted from 50 mg of sorghum flour by continuous stirring with 1.5 mL of 1% (v/v) HCl in methanol at 25°C, for 20 min (Cai et al., 1995). The suspension was centrifuged (5000g, 10 min, 25°C) and the supernatant collected. The residue was re-extracted with HCl-methanol as described above, and the two supernatants were pooled. Total phenolic compounds content was determined using Folin-Ciocalteu's method (Singleton et al., 1999) adapted to a 96-well plate assay. To 10 µL of extract was added 25 µL of Folin-Ciocalteu's reagent (50%, v/v). After 5 min of incubation, 25 µL of 20% (w/v) sodium carbonate solution and water were added to the mixture to have a final volume of 200 µL. Blanks were prepared for each sorghum sample by replacing Folin-Ciocalteu's reagent with water. Gallic acid was used as a standard and results were expressed as gallic acid equivalent per gram of flour (w/w). The standard was always freshly prepared. The absorbances (after 30 min) were measured at 760 nm using a multiwell plate reader (EAR 400, Labinstruments, Australia).

Determination of tannin content

Tannins were extracted from 50 mg of sorghum flour with 75% DMF and quantified by the FAC method as described by Beta and co-workers (1999), using catechin (flavan-3-ol monomer) as a standard. The assay was monitored at 525 nm. The standard was always freshly prepared. Tannin content was expressed as catechin equivalent per gram of flour (w/w).

Extraction of enzymes

Enzyme extracts were prepared by mixing 250 mg of sorghum flour with 1.2 mL of 50 mM Tris-HCl buffer pH 7.3 containing 0.5 M $CaCl_2$ and 2% (w/v) PVP, at 4°C for 1 hour. The homogenate was centrifuged (14000g, 4°C, 45 min) and the resulting supernatant was used as crude extract of both PPOs and POXs. Total protein was quantified by the linearized method of Bradford (Zor and Selinder, 1996) using the ratio of A_{620}/A_{450} versus protein concentration. BSA was used as standard.

Enzyme assays

The spectrophotometric assay for PPO was performed as described by Espín and co-workers (1995, 1998). 4HA and DHPPA were used as phenolic substrates to determine the monophenolase and *o*-diphenolase activities of PPO, respectively. The enzyme extract (10 µL) was incubated with 150 µL 50 mM sodium acetate pH 5.5, 10 µL 40% (v/v) DMF and 10 µL 50 mM MBTH, at 25°C, for 5 min. The reaction was started by addition of 20 µL 100 mM of the phenolic substrate (prepared in 0.15 mM phosphoric acid). The reaction was monitored at 500 nm. Control assays, in which the enzyme extract or substrates were replaced by buffer were performed. One unit of PPO activity (U) is defined as the amount of enzyme producing 1 µmol of MBTH-quinone-adducts per min resulting from the oxidation of 4HA or DHPPA. POX activity was measured spectrophotometrically by monitoring the H_2O_2-dependent oxidation of ABTS, at 25°C. The reaction mixture consisted of 10 µL of 200-fold diluted crude enzyme extract, 20 µL of 100 mM ABTS, 10 µL of 100 mM H_20_2 and 160 µL of 50 mM sodium acetate pH 5.0. Control assays in which the enzyme extract or substrates were replaced by buffer were performed. The reaction was monitored at 405 nm. One unit of POX activity (U) is defined as the amount of enzyme releasing 1 µmol of ABTS radical/min under the assay conditions.

All enzyme assays were monitored with a multiwell plate reader SLT340 ATTC (Labinstruments, Australia) on-line interfaced to a computer (Macintosh Performa 450). Kinetic data were determined in the linear phase of reaction traces using DeltaSoftII version 4.1S (Biometallic, Inc.). The reactions were monitored over 3 min. The initial slopes of the reaction traces caused by enzyme activities were corrected with the slopes of the blanks.

Zymography of POX and PPO

For enzyme zymography, IEF was carried out with a PhastSystem unit (Amersham Pharmacia Biotech) using pH 3-9 gradient gels, according to the manufacturers instructions. The crude extract (2 µL) from each variety was loaded onto the gel. After IEF the gels were stained separately for POX and PPO activities. For the zymography of POX the gel was incubated in 50 mM sodium citrate buffer pH 5.0, containing 1 mM DAB and 10 mM H_2O_2, for 30 min. The zymography of *o*-diphenolase activity of PPO was performed as described previously (Dicko et al., 2002; this thesis, **Chapter 6**).

Statistical analysis

All assays were carried out in triplicate, and the means and standard deviations are reported. Differences in mean performance for each composition among sorghum varieties were tested by the Student's *t*-test. Pearson linear correlation coefficients were used to assess relationships among total phenolic compound, tannin, PPO, POX and protein contents.

RESULTS

Phenolic compounds in sorghum varieties

The 50 sorghum varieties varied significantly ($P< 0.01$) in their total phenolic compound and tannin contents (**Figure 1**). Total phenolic compound and tannin contents were highly positively correlated among varieties (**Table 2**).

Table 2. Pearson correlation (r values) matrix between phenolic compounds, oxidative enzymes and proteins of sorghum grains

	Total phenolic compounds	Tannins	Monophenolase	*o*-Diphenolase	Peroxidase
Tannins	0.88[a]				
Monophenolase	0.12	0.17			
o-Diphenolase	-0.04	-0.19	0.35		
Peroxidase	-0.24	-0.19	-0.01	0.07	
Proteins	0.29	0.39	0.05	-0.23	-0.26

[a]Significant ($P< 0.001$), n=50.

The mean values of total phenolic compounds and tannin in the varieties were 0.60 and 0.27%, respectively. The average content of phenolic compounds was higher in the red grains (1.39 and 0.90% of total phenolic compounds and tannin, respectively) than in the white grains (0.40 and 0.11% of total phenolic compounds and tannin, respectively). The average content of total phenolic compounds is lower in the hard grains (0.31%) than in the soft grains (0.59%). The highest concentration of total phenolic compounds and tannin was found in variety V7 (2.72 and 1.97%, respectively) and variety V38 (2.64 and 1.97%, respectively). All the varieties contained phenolic compounds, but some of them (V1, V8, V26, V43, V44 and V49) contained almost no tannin (**Figure 1**). Tannin content-based classification of varieties (**Table 3**) shows that the majority (82%) has low tannin content (≤0.25%). Varieties with relatively high tannin content (≥0.75%) accounted

for 10%. Among tannin containing varieties, the variation was 6-fold. The inter-varietal difference in total phenolic compounds content was a factor 20.

Table 3. Classification of Burkina Faso sorghum grain varieties based on tannin content

Tannin content (%)	Group	Percentage of varieties
≤ 0.25	Low	82
0.26-0.5	Medium	4
0.51-0.75	High	4
≥ 0.75	Very high	10

Oxidative enzymes in sorghum varieties

The mean values of the monophenolase and *o*-diphenolase specific activities of PPO in sorghum varieties were 0.9 mU/mg and 45.3 mU/mg, respectively. The *o*-diphenolase activity of PPO was 50-100 times higher than the monophenolase activity among varieties (**Figure 2**). Inter-varietal difference in monophenolase activity was a factor of three, but the varieties exhibited almost similar *o*-diphenolase activity (**Figure 2**). There was no significant ($P<0.001$) difference between the monophenolase and *o*-diphenolase activities of PPO in the group of red and white grains. The mean value of the POX specific activity in the varieties was 1.90 U/mg. Average POX activity was higher in the group of white grains (2.0 U/mg) than the red grains (1.6 U/mg). Inter-varietal difference of POX specific activity was at most a factor of five. Variety V17 (Cauga 108-15) displayed the highest POX specific activity (4.23 U/mg). Lowest POX specific activity (0.81 U/mg) was found in the variety V31 (IRAT 202). Specific activity of POX was twenty (V31) to ninety (V17) times higher than the PPO *o*-diphenolase activity. No significant correlation was found between phenolics, monophenolase and *o*-diphenolase activities of PPO, and POX activity of all varieties (**Table 2**).

Zymography of PPO and POX in the grain extracts allows the detection of isoenzymes present in the varieties. A band of PPO was found at p*I* ≥ 9 for all varieties. As an illustration (**Figure 4**), PPO is shown for eight varieties. Cationic (p*I* ≥9) POX isoenzymes were ubiquitous in all varieties (**Figure 5**). However, in some varieties neutral and anionic POX isoenzymes could be detected in the pH range of 4 to 8 (**Figure 5**).

Comparison of the average content of phenolic compounds, PPO and POX in groups of varieties according to their food properties

The average content of PC, PPO and POX in varieties grouped according to their known properties and food processing is illustrated in **Table 4**. The group of varieties that is less suitable for tô has a low average content of POX (1.2 U/mg) compared to the group of varieties good for "tô" (1.8 U/mg).“Tô” varieties have, interestingly, less total phenolic compounds (0.32%) and tannin (0.11%) than the other groups good for "dolo" and couscous. Varieties that are good for "dolo" have noticeably high total phenolic compounds (1.18 %) and tannin (0.71%) contents. Those that are poor for "dolo" differ in all aspects (phenolic compounds, PPO and POX) from those that are good for “dolo”. The variety used for couscous had the lowest POX activity (0.8 U/mg) of all the varieties investigated (**Table 4**).

Table 4. Comparison of the average content (value ± standard error) of phenolic compounds and their oxidative enzymes in groups of sorghum varieties of known food properties

	Group properties	Total phenolics (%)	Tannins (%)	Monophenolase (mU/mg)	*o*-Diphenolase (mU/mg)	Peroxidase (U/mg)
Food	good for "tô" (n=12)	0.32 ± 0.03	0.11 ± 0.01	0.8 ± 0.1	43.4 ± 1.3	1.8 ±0.1
	poor for "tô "(n=2)	0.31 ± 0.02	0.09 ± 0.01	0.6 ± 0.1	42.1 ± 3.4	1.2 ± 0.1
	good for "dolo" (n=8)	1.18 ± 0.05	0.71 ± 0.03	1.1 ± 0.1	47.0 ± 3.8	1.5 ± 0.1
	poor for "dolo" (n=2)	0.29 ± 0.01	0.25 ± 0.01	0.7 ± 0.1	39.7 ± 3.2	3.0 ± 0.2
	good for couscous (n=1)	0.79 ± 0.04	0.19 ± 0.01	1.1 ± 0.1	42.7 ± 3.4	0.8 ± 0.1
Grain	hard grain (n=4)	0.31 ± 0.02	0.12 ± 0.01	0.8 ± 0.1	42.2 ± 3.4	1.3 ± 0.1
	soft grain (n=4)	0.59 ± 0.03	0.08 ± 0.01	0.9 ± 0.1	46.1 ± 3.7	1.7 ± 0.1
	white color (n=40)	0.40 ± 0.02	0.11 ± 0.01	0.9 ± 0.1	45.2 ± 3.5	2.0 ± 0.1
	red color (n=10)	1.39 ± 0.07	0.90 ± 0.01	1.0 ± 0.1	45.7 ± 3.6	1.6 ± 0.1
	Mean value (n=50)	0.60 ± 0.03	0.27 ± 0.01	0.9 ± 0.1	45.3 ± 2.3	1.9 ± 0.1

DISCUSSION

Phenolic compounds and oxidative enzymes in sorghum grain

Although, total content of phenolic compounds, tannin and oxidative enzyme may change according to age/harvesting-time and environmental conditions, it was shown for wheat, barley and other plants that mainly genetic factors are responsible for differences among varieties (Park et al., 1997; Billau and Nicolas, 2001; Parr and Bolwell, 2000; Tomas-Barberan and Espín, 2001). Since grains were not dehulled prior to surface-sterilization, and the incubation time was kept short (5

min), sodium hypochlorite is not likely to react with polyphenols. Best extraction results for total phenol and tannins in sorghum grain were obtained with HCl-methanol (Deshpande et al., 1988; Cai et al., 1995) and DMF (ISO, 1988), respectively. Whereas some polyphenol assays (FAC, vanillin-HCl, butanol-HCl, prussian blue, etc.) are supposedly specific for different types of polyphenols (Deshpande et al., 1986), Folin-ciocalteu's assay for total phenol has been recommended because of its wide applicability for biological materials (Singleton et al., 1999).

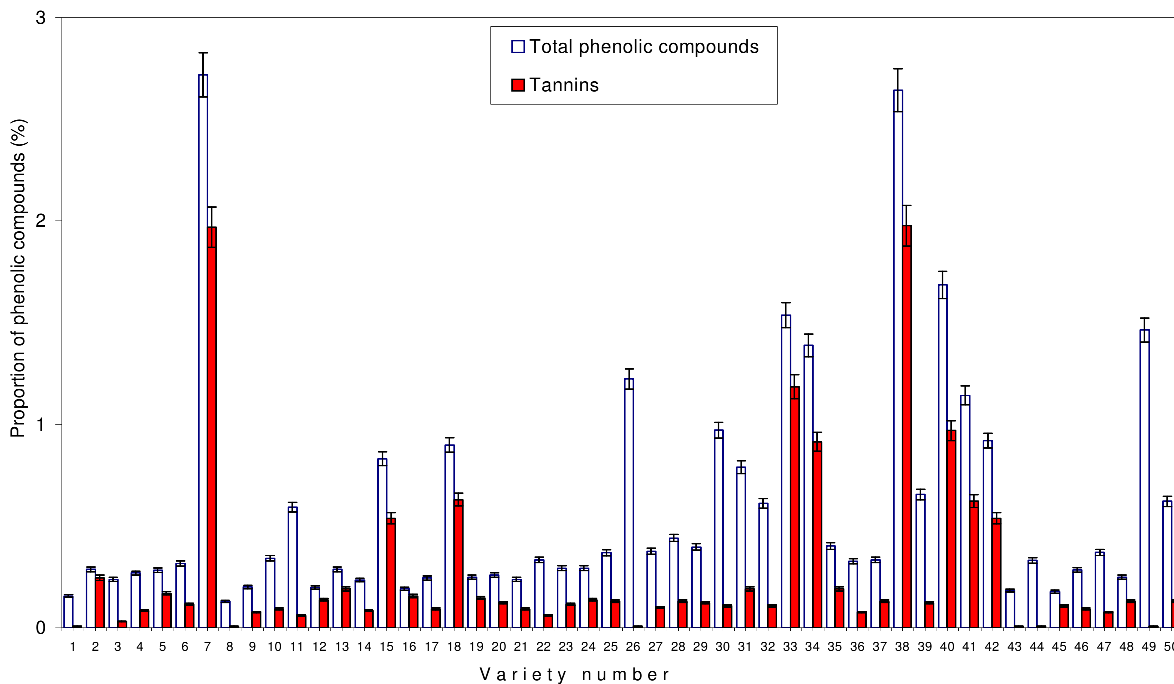

Figure 1. Total phenolic compound and tannin contents of sorghum varieties. The standard error bars represent the average standard error for each experiment.

Although the FAC method for tannins quantitation may include non-tannins phenolic compounds, it has been chosen as standard assay for sorghum tannins (ISO, 1988). It may allow distinguishing between sorghum varieties containing free and condensed-polyphenols (tannin). In agreement with earlier studies (Cai et al., 1995; Beta et al., 1999), several sorghum varieties were found to contain no tannin. A high tannin content may be a positive agronomic trait but less suitable for food usage due to its alleged anti-nutritional properties (Butler, 1992; Morton, 1992; Layrisse et al., 2000). In line with the general observation, sorghum grains with high phenolic content were found to be soft (Waniska et al., 1989). In order to prevent the reaction of oxidative enzymes with endogenous

polyphenols, insoluble PVP, was used in the enzyme's extraction buffer. The occurrence of POX and PPO in mature sorghum grains found in the present study disagrees with those of Glennie (1981) who found that for *Sorghum bicolor* var. NK-300, PPO and POX activities could not be detected in mature grain. Since cereals do not contain laccase (EC 1.10.3.2) (Billau and Nicolas, 2001), and knowing that 4HA and DHPPA are not oxidized by POX in absence of hydrogen peroxide (Dicko et al., 2002, this thesis, **Chapter 6**), it may be inferred that the O_2-dependent oxidase activity is essentially due to PPO. The higher *o*-diphenolase of PPO (as compared to the monophenolase activity) is due to the lower turnover rate of the monophenolase activity of plant PPOs (Sanchez-Ferrer et al., 1995). The protein band at p*I* ≥9 may be due to several cationic PPO isoenzymes having a high p*I*. Thus, the difference in monophenolase activity among the varieties might originate from the heterogeneity of cationic PPO isoenzymes (Stafford and Dresler, 1972). The varieties are highly polymorph in their POX composition, in agreement with the results of Ollitrault and co-workers (1989) who also found varietal differences of POX isoenzymes in germinated sorghum grains. Cationic POXs were also the only detectable isoenzymes in the grain of *Sorghum* var. Frontier 400 (Sae et al., 1972) and the major isoenzymes in the first internodes of *Sorghum vulgare* var. Wheatland milo (Stafford and Bravinder-Bree, 1972). Similar results were reported for barley and wheat kernels, where 93% and 98% of the POX activities, respectively, are cationic isoenzymes (Billau et al., 1999). The average specific activity of POX in the fifty sorghum varieties (1.90 U/mg) is higher than the POX specific activity in both barley (1.35 U/mg) and wheat (1.35 U/mg) kernels (Billau et al., 1999). The inter-varietal difference in POX activity (5-fold) among Burkina Faso sorghums is higher than that found (1.2-fold) between Nigeria sorghums (Nwanguma and Eze, 1995). The enzyme assays and zymogram studies showed that POXs in sorghum grain have more isoforms and a higher activity than PPOs. Luthra and co-workers (1988) analyzed sorghum leaves and also showed that POX activity was several times higher than PPO activity. From this, it may be tentatively concluded that POX is more involved in the *in vivo* oxidation of phenolic compounds than PPO.

Although the average POX specific activity was higher in the group of white grains than for red, the color of the grain could not be significantly linked to its content in POX and PPO. Therefore, the appearance of the grain may be controlled by other physiological parameters than the content in oxidative enzymes. Indeed, several interacting factors (pericarp color and thickness, presence or absence of testa, endosperm texture, etc.) are known to influence the appearance of the grain.

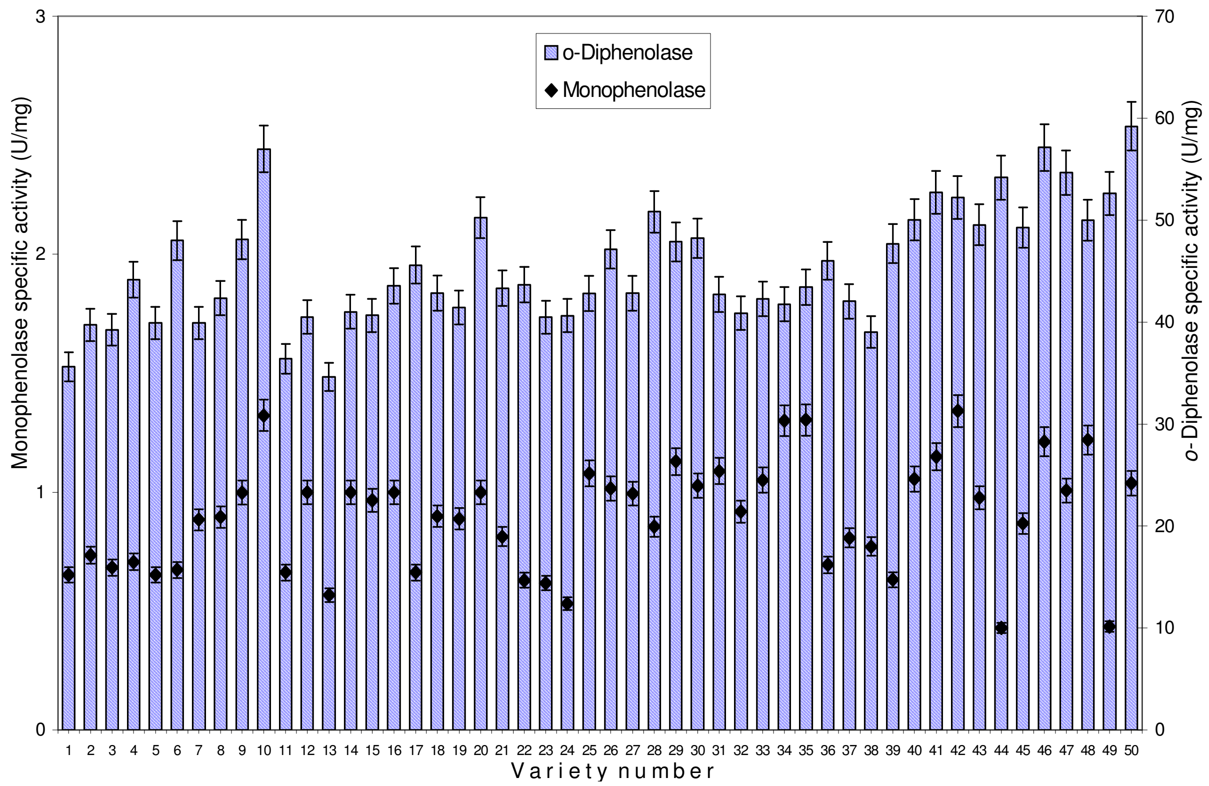

Figure 2. Monophenolase and *o*-diphenolase specific activities of PPO in sorghum varieties. The standard error bars represent the average standard error for each experiment.

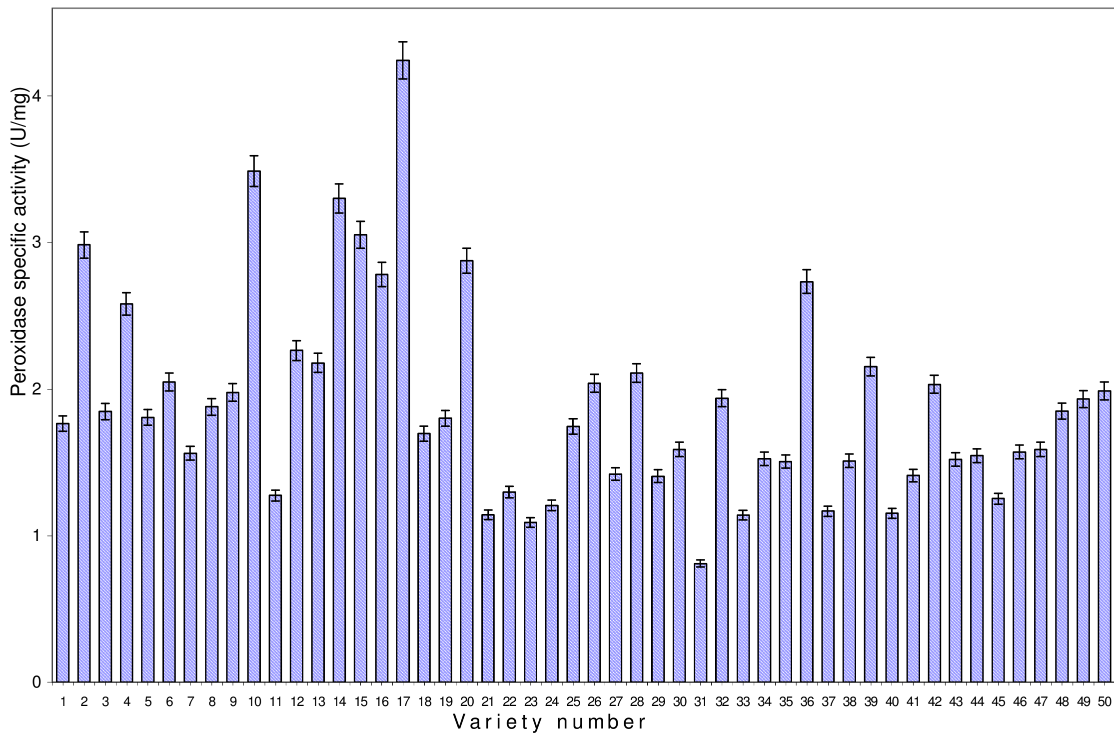

Figure 3. POX specific activities in sorghum varieties. The standard error bars represent the average standard error for each experiment.

The phenolic, POX and PPO contents of the grains could not be simply related to the susceptibility or resistance of individual sorghum plant to striga, anthracnose, sorghum midge or sooty stripe. This also holds true for the abiotic factors such as drought and photoperiod.

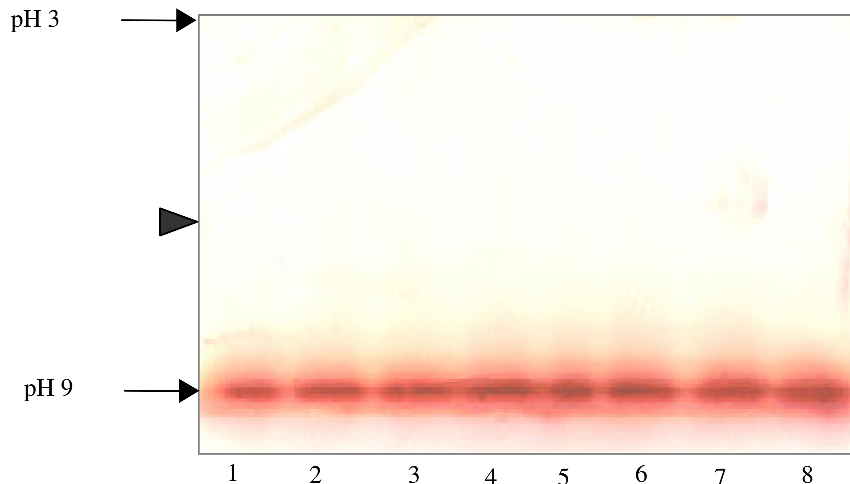

Figure 4. Zymograms of *o*-diphenolase activity of PPO in sorghum varieties. Since all varieties presented the same PPO pattern, eight of them were arbitrarily selected as examples. Lanes 1 to 8 are crude extracts from grains of sorghum varieties V1, V5, V10, V15, V20, V25, V30 and V35, respectively. The samples were applied in the middle of the gels (►).

Relationship between phenolic compounds, PPO and POX contents in grain and food application

It is known in industrial brewing that high POX activity may not be desired because POX may catalyze the oxidative polymerization of endogenous phenolic compounds of malt, notably the anthocyanidins, leading to flavor deterioration and the occurrence of haze (Clarkson et al., 1992; Billau and Nicolas, 2001). "Dolo" varieties have a higher content in total phenolic compounds ($P<0.05$) and tannin ($P<0.1$), and higher monophenolase activity ($P<0.05$) than varieties that are poor for "dolo". Their content in phenolic compounds is also higher than in varieties used for "tô" and couscous, as well as the mean value. PPO may be involved in the oxidation of endogenous phenolic compounds to yield the desired opaque color of "dolo" and influence its organoleptic properties.

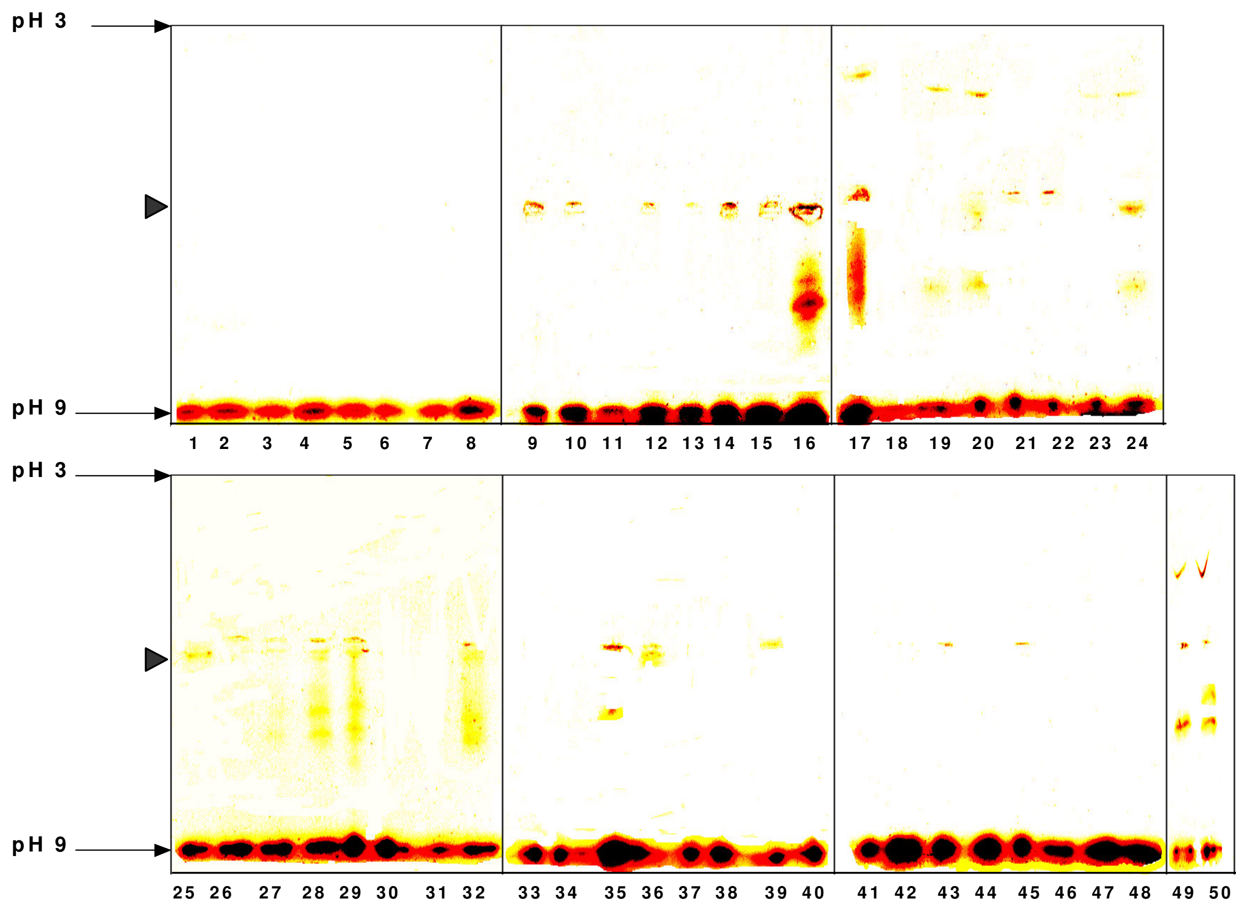

Figure 5. Zymograms of POX activity in sorghum varieties. Each Arabic number represents a sorghum variety. Samples were applied in the middle of the gel (►).

Thus, red varieties with high phenolic compounds and PPO contents and a low POX activity may be the best for "dolo" preparation. Among "dolo" varieties, V40 (Zouobdo) and V39 (Nafo-Natogué) have high (0.97%) and low (0.12%) tannin content, respectively. The latter also has a medium POX activity (2.15 U/mg), therefore, it may be the most suitable for industrial brewing purposes. The group of varieties poor for "tô" differs from the group good for "tô", exclusively with respect to its low POX content. In "tô" preparation, the formation of a thick paste is necessary. Thus, in analogy with what was found in wheat dough (Hilhorst et al., 1999) POX may mediate the gelatinization of sorghum flour during "tô" preparation. Among “tô” varieties, V1 (CEF-322/53-1-1) and V21 (Kaapelga) have white flour and low phenolic compound and PPO contents, and a suitable POX activity. Hence, these varieties may be the most suitable for this local food. The variety V31 (IRAT 202) reputed to be the best sorghum variety for couscous preparation in West Africa, notably in Senegal, deviates from the other varieties in its low content of POX (0.8

U/mg). That may be justified by the fact that for couscous preparation the formation of a gel mediated by POX via the crosslinkage of macromolecules is not desired. Therefore, couscous varieties might have a low POX content. It is known that the suitability of sorghum varieties for food and beverages is a function of the chemical and physical properties of kernels, and process conditions. In addition to these parameters, the composition of the grain in oxidative enzymes may also play an important role in grain quality.

Although phenolic compounds and their oxidative enzymes are thought to be involved in plant responses to physical stress and to pathogens the present study shows that these constituents of the grains could not be used as a marker for the described agronomic properties of the fifty varieties. It remains to be investigated whether these constituents in the whole plant are useful indicators, or whether specific polyphenols and their oxidative enzymes are induced upon challenging the plant with a pathogen. The present work has given some indications for the use of biochemical properties as criteria to select for varieties most suited for a specific food application.

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REFERENCES

Abdel-Aal, E. S. M.; Hucl, P.; Sosulski, F. W.; Graf, R.; Gillott C.; Pietrzak L. Screening spring wheat for midge resistance in relation to ferulic acid content. *J. Agric. Food Chem.* **2001**, *49*, 3559-3566.

Awad, H. M.; Boersma, M. G.; Boeren, S.; van Bladeren, P. J.; Vervoort, J.; Rietjens, I. M. C. M. Structure-activity study on the quinone/quinone methide chemistry of flavonoids. *Chem. Res. Toxicol.* **2001**, *14*, 398-408.

Beta, T.; Rooney, L. W.; Marovatsanga, L. T.; Taylor, J. R. N. Phenolic compounds and kernel characteristics of Zimbabwean sorghums. *J. Sci. Food Agric.* **1999**, *79*, 1003-1010.

Billau, C., Louarme, L.; Nicolas, J. Comparison of peroxidases from barley kernel (*Hordeum vulgare* L.) and wheat germ (*Triticum aestivum* L.): isolation and preliminary characterization. *J. Food Biochem.* **1999**, *23*, 145-172.

Billau, C.; Nicolas, J. Main oxidoreductases involved in the malting and brewing technological processes. *Sci. Aliments.* **2001**; *21*, 83-131.

Butler, L. G. Antinutritional effects of condensed and hydrolysable tannins. In *Plant polyphenols: synthesis, properties and significance*; Hemingway, R. W.; Laks, P. E., Eds.; Plenum press, New York, **1992**; pp. 693-698.

Cai, T.; Ejeta, G.; Butler, L. G. Screening for grain polyphenol variants from high-tannin sorghum somaclones. *Theor. Appl. Genet.* **1995**, *90*, 211-220.

Chantereau J.; Ag Hamada M.; Bretaudeau A.; Tembely, S. O. Etude de nouvelles variétés de sorgho en milieu paysan dans la zone cotonnière Cmdt au Mali (1995-1996). In *Amélioration du sorgho et de sa culture en Afrique de l'Ouest et du Centre*; Ratnadass A., Chantereau J., Gigou J., Eds.; CIRAD-ICRISAT, **1998**; pp. 199-210.

Clarkson, S. P.; Large, P. J.; Bamforth, C. W. Oxygen-scavenging enzymes in barley and malt and their effects during mashing. *J. Inst. Brew.* **1992**, *98*, 111-115.

Deshpande, S. S., Cheryan, M. and Salunkhe, D. K. Tannin analysis of food products. CRC *Crit.Rev. Food Sci. Nutri.* **1986**, *24*, 401-449.

Diawara, B.; Kaboré, I. Z; Sawadogo, L. Essai de stockage en vrac du sorgho rouge et niébé: amélioration des structures de stockage traditionnelles. In *Processing and Industrial Utilization of Sorghum and Related Cereals in Africa*; Menyonga, J. M.; Bezuneh, T.; Nwasike, C. C.; Sedego, P. M.; Tenkouano, A., Eds.; OAU/STRC-SAFGRAD regional symposium, Agence Internationale de Communication pour le Développement, Ouagadougou, Burkina Faso; **1995**; pp. 207-214.

Dicko, M. H.; Hilhorst, R.; Gruppen, H.; Laane, C.; van Berkel, W. J. H.; Voragen, A. J. G. Zymography of monophenolase and *o*-diphenolase activities of polyphenol oxidase. *Anal. Biochem*. **2002**, *360*, 336-339.

Dowd, P. F.; Herms, D. A.; Berhow, M. A.; Lagrimini, L. M. Mechanisms of insect resistance in transgenic plants (over) expressing a tobacco anionic peroxidase. *Plant Peroxidase Newsletter*. **2000**, *14*, 93-101.

Espín, J. C.; Morales, M.; Varón, R.; Tudela, J.; García-Cánovas, F. A continuous spectrophotometric method for determining the monophenolase and *o*-diphenolase activities of apple polyphenol oxidase. *Anal. Biochem.* **1995**, *231*, 237-246.

Espín, J. C.; Tudela, J.; Garcia-Cánovas, F. 4-hydroxyanisole : the most suitable monophenolic substrate for determining spectrophotometrically the monophenolase activity of polyphenol oxidase. *Anal. Biochem.* **1998**, *259*, 118-126.

Field, J. A.; Lettinga, G. Toxicity of tannic compounds to microorganisms. In *Plant polyphenols: synthesis, properties and significance*; Hemingway, R. W.; Laks, P. E., Eds.; Plenum press, New York, **1992**; pp 673-692.

Food and Agricultural Organisation (FAO). FAOSTAT Agricultural database **2001**. web: http://apps.fao.org/cgi-bin/nph-db.pl?subset=agriculture

Glennie, C. W. Preharvest changes in polyphenols, peroxidase, and polyphenol oxidase in Sorghum grain. *J. Agric. Food Chem*. **1981**, *29*, 33-36.

Godwin, I. D.; Gray, S. J. Overcoming productivity and quality constraints in sorghum: the role for genetic engineering. in *Transgenic cereals*; O'Brien L.; Henry R. J., Eds.; St. Paul : American Association of Cereal Chemists, St Paul, Minnesota, USA; **2000**; pp. 153-177.

Hilhorst, R.; Dunnewind, B.; Orsel, R.; Stegeman, P.; van Vliet, T.; Gruppen, H.; Schols, H. A. Baking performance, rheology and chemical composition of wheat dough and gluten affected by xylanase and oxidative enzymes. *J. Food Sci.* **1999**, *64*, 808-813.

ISO Sorghum. Determination of tannin content, International Organization for Standardization, ISO 9648. **1988**

Layrisse, M.; Garcia-Casal, M. N.; Solano, L.; Baron, M. A.; Arguello, F.; Llovera, D.; Raminrez, J.; Leets, I.; Tropper, E. Iron bioavailibility in humans from breakfasts enriched with iron bis-glycine chelate, phytate and polyphenols. *J. Nutr.* **2000**, *130*, 2195-2199.

Luthra, Y. P.; Ghandi, S. K.; Joshi, U. N.; Arora, S. K. Total phenolic compounds and their oxidative enzymes in sorghum leaves resistant and susceptible to *Ramulispora sorghicola* Harris. *Acta Phytopathol. Entomol. Hung.* **1988**, *23*, 393-400.

Matheis, G.; Withaker, J. R. Modification of proteins by polyphenol oxidase and peroxidase and their products. *J. Food Biochem*. **1984**, *8*, 137-162

Morton, J. F. Astringency masticatories. In *Plant polyphenols: synthesis, properties and significance*; Hemingway, R. W.; Laks, P. E., Eds.; Plenum press, New York, **1992**; pp. 739-765.

Neya A.; Le Normand, M. Responses of sorghum genotypes to leaf anthracnose (*Collelotrichum graminicola*) under field conditions in Burkina Faso. *Crop Prot*. **1998**, *17*, 47-53.

Nwanguma, B. C.; Eze, M. O. Heat sensitivity, optimum pH and changes in activity of sorghum peroxidase during malting and mashing. *J. Inst. Brew.* **1995**, *101*, 275-276.

Ollitrault, P.; Escoute, J.; Noyer, J. L. Polymorphisme enzymatique des sorghos. *Agron. Trop*. **1989**, *44*, 203-222.

Ouattara L.; Trouche G.; Fliedel G.; Diawara B. Potentialités d'utilisation des sorghos guinéa tan. In *Vers une production, utilisation et commercialisation durables du sorgho en Afrique occidentale et centrale*; Akintayo, I., Sedgo, J., Eds.; *ROCARS-ICRISAT*, **2001**, pp. 24-41.

Oudgenoeg, G.; Hilhorst, R.; Piersma, S. R.; Boeriu, C. G.; Gruppen, H.; Hessing, M.; Voragen, A. G. J.; Laane C. Peroxidase-mediated cross-linking of a tyrosine-containing peptide with ferulic acid. *J. Agric. Food Chem.* **2001**, *49*, 2503-2510.

Park, W. J.; Shelton, D. R.; Peterson, C. J.; Martin, T. J.; Kachman, S. D.; Wehling, R. L. Variation in polyphenol oxidase activity and quality characteristics among hard white wheat and hard red winter wheat samples. *Cereal Chem*. **1997**, *74*, 7-11.

Parr, A. J.; Bolwell, G. P. Phenols in plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. *J. Sci. Food Agric*. **2000**, *80*, 985-1012.

Richard-Forget, F.; Gaulliard, F. A. Oxidation of chlorogenic acid, catechins and 4-methylcatechol in model solutions by combinations of pear (*Pyrus communis* cv Williams) polyphenol oxidase and peroxidase: a possible involvement of peroxidase in enzymatic browning. *J. Agric. Food Chem*. **1997**, *45*, 2472-2476.

Sae, S. W.; Kadoum, A. M.; Cunningham, B. A. Purification and some properties of sorghum grain esterase and peroxidase. *Phytochemistry* **1971**, *10*, 1-10.

Sanchez-Ferrer, A.; Rodriguez-Lopez, J. N.; Garcia-Canovas, F.; Garcia-Carmona, F. Tyrosinase: A comprehensive review of its mechanism. *Biochim. Biophys. Acta* **1995**, *1247*, 1-11.

Sharma, H. C.; Mukuru S.Z.; Hari-Prasad, K. V.; Manyasa, E.; Pande, S. Identification of stable sources of resistance in sorghum to midge and their reaction to leaf diseases. *Crop Prot*. **1999**, *18*, 29-37.

Singleton, V. L.; Orthofer, R.; Lamuela-Raventos, R. M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol*. **1999**, *299*, 152-177.

Stafford, H. A.; Bravinder-Bree, S. Peroxidase isozymes of first internodes of sorghum: tissue and intracellular localization and multiple peaks of activity isolated by gel filtration chromatography. *Plant Physiol.* **1972**, *49*, 950-956.

Stafford, H. A.; Dresler, S. 4-hydroxycinnamic acid hydroxylase and polyphenolase activities in *Sorghum vulgare*. *Plant Physiol.* **1972**, *49*, 590-595.

Tomas-Barberan, F.; Espín, J. C. Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. *J. Sci. Food Agric.* **2001**, *81*, 853-876.

Trouche, G.; Fliedel G.; Chantereau J.; Barro, C. Productivité et qualité des grains de sorgho pour le tô en Afrique de l'Ouest: les nouvelles voies d'amélioration. *Agric. Dev*. **2000**, *23*, 94-107.

Trouche, G.; Vaksmann, M.; Chantereau, J.; Kouressy, M.; Barro, C.; Rami, J. F. Déterminisme génétique mendélien de la sensibilité à la photopériode des sorghos Ouest-Africains. *In Le futur des céréales photopériodiques pour une production durable en Afrique tropicale semi-aride*; Florence, B. L., Reyniers J. F. Eds.; Ce.S.I.A./CIRAD, Montpellier, **1998**; pp 225-246.

Vaughn, K.C.; Duke, S. O. Tissue localization of polyphenol oxidase in *Sorghum Bicolor*, Bundle sheath plastids, mesophyll plastids, guard cells. *Protoplasma* **1981**, *108*, 319-327.

Waniska, R. D.; Poe, J. H.; Badyopadhyay, R. Effects of growth conditions on grain molding and phenols in sorghum. *J. Cereal Sci*. **1989**, *10*, 217-225.

Zor, T.; Selinger, Z. Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. *Anal. Biochem.* **1996**, *236*, 302-308.

CHAPTER 3

Evaluation of the effect of germination on phenolic compounds and antioxidant activities in sorghum varieties

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ABSTRACT

The screening of fifty sorghum varieties showed that, on average, germination did not affect the content in total phenolic compounds, but decreased the content of proanthocyanidins, 3-deoxyanthocyanidins and flavan-4-ols. Independent of germination, there are inter-varietal differences in antioxidant activities among sorghum varieties. Phenolic compounds and antioxidant activities were more positively correlated in ungerminated varieties than in germinated ones. Sorghum grains with pigmented testa layer, chestnut color glumes and red plants had higher contents, larger diversity of phenolic compounds and higher antioxidant activities than other sorghums. Some red sorghum varieties had higher antioxidant activities (30-80 µmol of Trolox equivalents/g) than several sources of natural antioxidants from plant foods. Among varieties used for "tô", "dolo", couscous and porridge preparation, the "dolo" (local beer) varieties had the highest average content and diversity in phenolic compounds as well as the highest antioxidant activities. The biochemical markers determined are useful indicators for the selection of sorghum varieties for food and agronomic properties.

Keywords: sorghum, germination, antioxidant, proanthocyanidins, 3-deoxyanthocyanidins, apigeninidins, flavan-4-ols

INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench is one of the most important cereal crops in the world with a current annual production over 60 million tons, of which USA and Africa produce 20% and 40%, respectively (FAO, 1995, 2005). Sorghum is the staple food in several countries, notably in Africa (FAO, 1995; Anglani, 1998; Taylor and Dewar, 2001; Awika and Rooney, 2004a). In West Africa, ungerminated sorghum grains are generally used for the preparation of "tô" (thick porridge) and couscous (granulated food). Malted sorghum is often used in the production of local beer ("dolo"), infant food (thin porridge) and non-fermented beverages.

Sorghum is a cereal reported to contain simple phenols, hydroxybenzoic acids, hydroxycinnamic acids (ferulic acid being the most abundant), anthocyanins, proanthocyanidins (PAs) and several other flavonoids (Schutt and Netzly, 1991; Melake-Berhan et al., 1996; Palé et al., 1997; Kouda-Bonafos et al., 1996; Haslam, 1998; Audilakshmi et al., 1999; Awika and Rooney, 2004a, Awika et al., 2004b). The 3-deoxyanthocyanidins (3-DAs), namely apigeninidins and leucoluteolinidins, are particularly abundant in sorghum grain, but rare or absent in other plants (Palé et al., 1997; Awika and Rooney, 2004a; Awika et al., 2004b). 3-DAs are of interest because they are more stable in organic solvents as well as in acidic solutions than anthocyanidins commonly found in fruits, vegetables and other cereals (Kouda-Bonafos et al., 1996; Palé et al., 1997; Awika et al., 2004b). This has been suggested as a potential advantage of sorghum as a viable commercial source of anthocyanins (Awika and Rooney, 2004a). An agronomic interest exists in sorghum grains containing flavan-4-ols, such as leucoapigeninidin (apiforol) and leucoluteolinidin (luteoforol), because they may confer a high resistance of the grain to molding (Schutt and Netzly, 1991; Melake-Berhan et al., 1996; Audilakshmi et al., 1999). A new interest in flavan-4-ols is linked to their anticarcinogenic activity (Ferreira and Slade, 2002).

Nowadays, phenolic compounds are generally regarded as desirable components of human food, because of their antioxidant activity. Therefore, they are considered to be of nutraceutical importance (Parr and Bolwell, 2000; Awika and Rooney, 2004a). Even for proanthocyanidins (condensed tannins), their earlier classification as essentially antinutritional factors, throughout formation of complexes with proteins and carbohydrates, could be balanced against their potential to serve as biological antioxidants (Hagerman et al., 1998). All phenolic compounds are able to scavenge free radicals through electron-donating properties, generating a relatively stable phenoxyl radical or non radical species (Hagerman et al., 1998; Parr and Bolwell, 2000; Santos-Buelga and

Scalbert, 2000; Lu and Foo, 2001; Pellegrini et al., 2003). Some phenolic compounds protect against neurological disorders and exert anticarcinogenic, antimutagenic and cardioprotective effects linked to their free radical scavenging activities (Parr and Bolwell, 2000; Santos-Buelga and Scalbert, 2000; Lu and Foo, 2001; Pellegrini et al., 2003). Research on discovering naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants has attracted much attention (Parr and Bolwell, 2000; Santos-Buelga and Scalbert, 2000). Recently, Awika and co-workers (2003) reported methods to determine antioxidant activities of sorghums, their brans, baked and extruded products. For red sorghum the bran had 3-5 times more antioxidant activity than the whole grain (Awika et al., 2003). These antioxidant activities were higher than those of blueberries (*Vaccinium* species), one of the most important natural sources of antioxidants (Awika et al., 2003). In a previous study on fifty sorghum varieties (Dicko et al., 2002; this thesis, **Chapter 2**), we showed that the varieties are highly diverse in their contents in phenolic compounds and oxidative enzymes. Although phenolic antioxidant activities are detected in sorghum (Awika et al., 2003), it is not known whether germination affects the content of these phenolic compounds and their antioxidant activities.

Germination is an essential step in the preparation of sorghum grains for several products. In one study, a several-fold increase in total phenolic compounds and PAs after germination of sorghum was reported (Nwanguma and Eze, 1996). In other studies, both an increase or a decrease of total phenolic compounds and PAs upon germination has been observed (Subramanian et al., 1992; Iwuoha et al., 1997; Bvochora et al., 1999). However, these studies were performed with only a limited number of sorghum varieties. In the present work, the effect of germination on phenolic compounds and antioxidant activities in 50 sorghum varieties is studied and correlations between these properties and local food applications are determined.

MATERIALS AND METHODS

Chemicals and reagents

2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Folin-Ciocalteu reagent for phenol, and ascorbic acid (vitamin C) were from Sigma-Aldrich. Gallic acid (3,4,5-trihydroxybenzoic acid) was from Aldrich. Sorghum apigeninidin, isolated and characterized by Kouda-Bonafos and co-workers. (1996), was a gift of Dr. Eloi Palé from the Laboratory of Natural Substances, University of

Ouagadougou, Burkina Faso. Cyanidin-chloride was from Extrasynthèse (Lyon, France). Apple ciders procyanidin oligomers (average degree of polymerization of 7.4) were kindly provided by Stephanie Prigent (Wageningen University, Wageningen, The Netherlands) and Dr. Catherine M. G. C. Renard (INRA, Rennes, France). These procyanidins were purified by RP-HPLC and characterized by thiolysis-HPLC as described by Guyot and co-workers (Guyot et al., 1998, 2001). All other chemicals were of analytical grade.

Sorghum bicolor L. (Moench) varieties

Sorghum varieties were grown during the rainy season of 2002 at the experimental station of Saria, in Burkina Faso (West Africa). Growth conditions were as described previously (Trouche et al., 2001; Ouattara et al., 2001). The varieties were chosen according to criteria described previously (Dicko et al., 2002; this thesis, **Chapter 2**). Information as to which varieties were "good" or "poor" for the preparation of specific dishes was collected from sorghum breeders familiar with the preference of the local population after a participatory survey (Trouche et al., 2001). For convenience, the sorghum varieties were classified in alphabetic order of their name followed by Arabic numbers from 1 to 50 preceded by V (**Table 1**). Mature grains (> 60 days after anthesis) were harvested, surface-sterilized and germinated as described previously (Subramanian et al., 1992; Dicko et al., 2002; this thesis, **Chapter 2**). Prior to germination, grains were steeped in water in the dark at 20-25°C, for 16 h. Germination was performed at 27 ± 2°C for 72 h. The appearance of primary shoots and roots was observed in all the varieties at the end of germination. Germinated and ungerminated sorghum grains were dried, ground and stored as described previously (Dicko et al., 2002; this thesis, **Chapter 2**). The moisture contents of the flours as determined by heating in oven ate 110ºC, for 5-6h were 5-7% (w/w).

Total phenolic compounds extraction and assay

Sorghum phenolic compounds were extracted from sorghum flour as described previously (Dicko et al., 2002; this thesis, **Chapter 2**). The total phenolic extract was analyzed directly or kept in the dark, at –30°C for less than 48 h to avoid oxidation. The total phenolic compounds content was determined using a miniaturized Folin-Ciocalteu method (Dicko et al., 2002; this thesis, **Chapter 2**). Results were expressed as gallic acid equivalents per gram of flour (w/w, dry matter basis). The same extract was used for quantification of PAs, 3-DAs and flavan-4-ols.

Proanthocyanidins (PAs) and dlavan-4-ols Assay

The total phenolic extract was assayed for PAs and flavan-4-ols essentially as described by Melake-Berhan and co-workers (1996) with miniaturization to adapt the assay to a 96-well plate format as follows. To determine flavan-4-ols, 50 µL of the extract was added to 700 µL of reagent A (30%, v/v 12N HCl in butan-1-ol) or to 700 µL of reagent B (15 %, v/v 0.1 N acetic acid; 15%, v/v, methanol and 70%, v/v, butan-1-ol). The sample in reagent A was mixed by vortex and left at 25°C, for 1 h, to allow formation of anthocyanidin pigments derived from flavan-4-ols (Melake-Berhan et al., 1996). Aliquots of the mixture (150 µL) were put in duplicate in a 96-multiwell plate and the absorbance read at 550 nm to quantify anthocyanidins formed from flavan-4-ols (Melake-Berhan et al., 1996). Cyanidin was used as standard to estimate the total amount of the anthocyanidins derived from flavan-4-ols.

For PAs quantification, the remaining sample in the tube with reagent A was further heated at 100°C, for 2 h. Under these conditions, PAs are converted to anthocyanidins, and the unstable pigments formed from flavan-4-ols are destroyed (Melake-Berhan et al., 1996). After cooling, 200 µL of the sample was put in duplicate in a 96-multiwell plate and the absorbances of anthocyanidin compounds derived from PAs were read at 550 nm (Melake-Berhan et al., 1996). Sample mixtures with reagent B, which were not heated served as blanks for the quantification of both PAs and flavan-4-ols. Apple procyanidins with an average DP ≈ 7.4, treated as indicated above were used as standard for sorghum PAs quantification (**Figure 1**).

3-Deoxyanthocyanidins (3-DAs) assay

For direct spectrophotometric quantification of 3-DAs, 50 µL of the total phenolic extract was mixed with 150 µL of methanol and the absorbances were read at 475 nm (Melake-Berhan et al., 1996). Sorghum apigeninidin (Kouda-Bonafos et al., 1996) was used as standard.

Determination of antioxidant activity

The antioxidant activity of sorghum phenolic compounds was determined both by the ability to scavenge 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid radical ($ABTS^{\bullet+}$) (Riedl and Hagerman, 2001) and by reduction of phosphomolybdenum Mo(VI) to Mo(V) (Prieto et al., 1999; Lu and Foo, 2001).

The stock of $ABTS^{\bullet+}$ radical was chemically generated by incubation of 10 mL of 100 mM ABTS with 10 mL of 50 mM $(NH_4)_2S_2O_8$, in 5 mM Na_2HPO_4 buffer pH 7.4. The decolorization was initiated by incubation of 250 µL of $ABTS^{\bullet+}$ radical with 10 µL of total phenolic extract. The

decrease in absorbance at 734 nm was monitored at 1 min intervals for 30 min. The absorbances of control and sample were monitored simultaneously in a 96-well plate. The amount of $ABTS^{\bullet+}$ radical scavenged was calculated from the difference in absorbance between the control and the total phenolic extract containing sample, using the extinction coefficient (ε_{734}= 12 867 M^{-1} cm^{-1}) of the radical.

To evaluate the antioxidant activity by the phosphomolybdenum assay, the reagent was prepared by constituting a mixture of 0.6 M H_2SO_4, 28 mM Na_2HPO4 and 4 mM $(NH_4)_6Mo_7O_{24}.4H_2O$. Oxido-reduction assay was performed by incubating 50 µL of phenolic extract with 500 µL of the reagent, in an 1.5 mL Eppendorf vial, at 95°C in oven, for 90 min. The blank was prepared by replacing the phenolic extract with the extraction solution (acidified methanol). After cooling, 200 µL of the formed green phosphate/Mo(V) complex was put in a microtiter plate, and the absorbances were read at the maximum absorption (820 nm).

Calibration curves were made using Trolox and vitamin C. Trolox antioxidant equivalents (TAE) and vitamin C antioxidant equivalents (VCAE) were expressed on weight basis (µmol/g of flour, dry matter).

Statistical analysis

All spectrophotometric assays were carried out in 96-well microtiter plates (Nunclon, Denmark) using a multiwell plate reader (µQuant Bio-Tek Instrument, Inc) on line interfaced with a personal computer. The absorbances and slopes of absorbances (OD/min) were automatically recorded using KC junior software version 1.31.5 (Bio-Tek Instrument, INC, USA). All assays were carried out at least in duplicate. Mean values, standard deviations and standard errors are reported. Significant differences in mean performance for each composition among sorghum varieties were tested by Student's t-test, $P < 0.05$ implies significance. Pearson linear correlation coefficients were used to assess relationships among biochemical constituents.

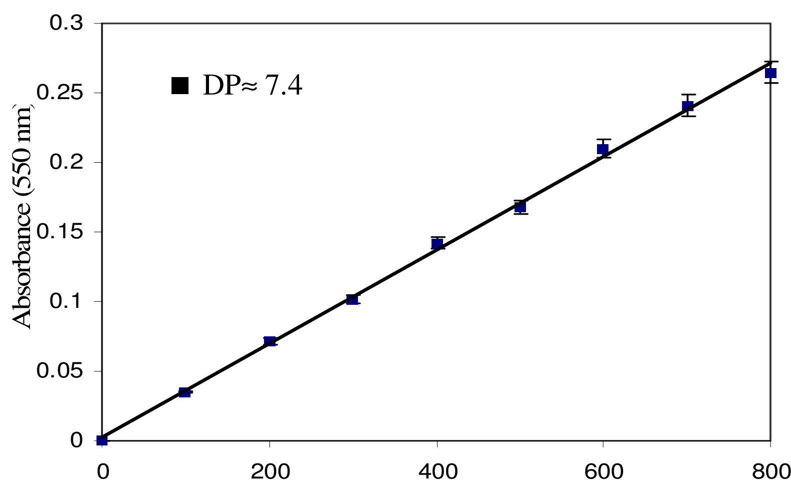

Figure 1. Calibration curve of procyanidins of DP≈7.4. The phenolic extract (50 µL) was incubated with 700 µL of reagent A (30%, v/v 12N HCl in butan-1-ol) at 100°C for 2 h. The blank was prepared by mixing 50 µL of the sample with 700 µL of reagent B (15 %, v/v 0.1 N acetic acid; 15%, v/v, methanol and 70%, v/v, butan-1-ol) without heating. Vertical bars indicate the average standard error for each experiment.

RESULTS AND DISCUSSION

Effect of germination on phenolic compounds in sorghum varieties

It is important to note that the levels of phenolic compounds within varieties found are of more interest for inter-varietal comparison than as absolute levels because of the lack of universal assays and standards, and because of differences in reactivity among phenolic compounds within a particular class. The apple procyanidins (DP≈7.4) used as standard could underestimate the level of PAs with low degree of polymerization or overestimate the level of PAs with high degree of polymerization (Guyot et al., 1998). The wavelength (475 nm) used to measure the amount of 3-DAs may mostly quantify apigeninidins over the other 3-DAs such as luteolinidins (Melake-Berhan et al., 1996). The used assays may not reveal all the complexity of the chemistry of phenolic compounds that occurs in the plant tissue during germination, but they are useful in assessing the effect of germination on the selected classes of phenolic compounds.

Although grown under the same conditions, the sorghum grains screened varied significantly in their levels of total phenolic compounds, PAs, 3-DAs and flavan-4-ols (**Table 1**). The average total phenolics content of sorghum varieties used in this study (harvested in 2002) was on average 40% higher than that of the same sorghum varieties harvested in 1998 (Dicko et al., 2002; this thesis, **Chapter 2**). This increase in total phenolic content was observed in 80% of the

varieties. However, in some varieties the total phenolic content decreased by a factor 1.2 to 2.5. Nevertheless, varieties containing the highest amounts of total phenolic compounds (V18 and V19) remained the same over the years. These results indicate that there is an inter-seasonal variation of total phenolic compounds content among sorghum varieties. This is in agreement with the observation that environment may affect the biosynthesis of phenolic compounds in sorghum (Mueller-Harwey and Reed, 1992). Germination affected the content in phenolics according to variety (**Table 1**). Nwanguma and Eze (1996) found an increase of several-fold in total phenolic compounds in all screened four sorghum varieties after germination. In contrast, the results of the present study showed that on average germination does not generally affect the total phenolic compounds content in sorghum. However, in line with previous observations (Subramanian et al., 1992; Iwuoha and Aina, 1997; Bvochora et al., 1999), depending on variety, sorghum total phenolic compounds decreased or increased upon germination. The presence of PAs (condensed tannins) in sorghum varieties was previously (Dicko et al., 2002; this thesis, **Chapter 2**) assessed using the ferric ammonium citrate (International Standard Organization method). In the present study, it was confirmed using the acid-butanol assay that the majority (more than 80%) of the screened sorghums from Burkina Faso are not high tannins (<0.5%, w/w) containing sorghums. This is observed for worldwide sorghums (FAO, 1995; Nwanguma and Eze, 1996; Anglani, 1998; Bvochora et al., 1999; Beta et al., 1999; Awika and Rooney, 2004a). Some of the screened varieties (32%) were PAs free. Overall, the PAs content decreased after germination. The decrease in extractable PAs after germination could be due to leaching of water-soluble PAs which are located in the pericarp and testa (Beta et al., 1999; Waniska, 2000; Awika and Rooney, 2004a) or due to formation of insoluble complexes with proteins (Riedl and Hagerman, 2001). Quantifying PAs content in two sorghum varieties, Iwuoha and Aina (1997) also found a decrease in PAs content after germination. This indicates that the effect of germination on total phenolic compounds content is dependent on variety.

Among the varieties 42% contained 3-DAs before and after germination. On average, germination did not induce the synthesis of 3-DAs among varieties but decreased their content. Variety V19 displayed a higher content of 3-DAs (0.42%, w/w of apigeninidin equivalent) than sorghum variety Monome Kaya found by Sérémé and co-workers (1994). This sorghum variety Monome Kaya was determined to have the highest content (0.25%, w/w of apigeninidin) among 30 sorghum varieties from Burkina Faso which were screened for apigeninidin (Sérémé et al., 1994). Because 3-DAs are anthocyanidins with industrial importance in food, varieties V19 and V37, which were found to be rich in these compounds in this study, can be considered as important

sources. The apigeninidin content is also an indicator of grain resistance to fungi such as *Colletrichum graminicola*, *Fusarium oxysporum*, *Gibberelle zeae*, and *Gliocladium roseum* (Schutt and Netzly, 1991). The screening for 3-DAs is, therefore, of great interest for breeders.

Only 28% of varieties displayed detectable amounts in flavan-4-ols. After flavan-4-ol contents were screened in 22 sorghum varieties, Audilakshmi and co-workers (1999) detected these compounds in only four varieties (18%). This supports that flavan-4-ols are quite rare in sorghum. On average, germination reduced the content in flavan-4-ols by 33% in varieties containing those compounds, and even led to the complete disappearance in some varieties (V2, V34, and V35). The decrease of flavan-4-ol content in all varieties upon germination may be related to their conversion into other flavonoids (anthocyanidins and flavan-3-ols), which is stimulated by enzymes that are produced during germination and that are involved in the biosynthesis of the latter compounds (Haslam, 1998).

Effect of germination on antioxidant activities in sorghum varieties

Because the ranking obtained by the comparison of antioxidant activities of food plants is dependent on the assays used (Pellegrini et al., 2003), the evaluation of the antioxidant activities of sorghum phenolic compounds was performed in this study using two different methods. While the ABTS assay monitored the capacity of phenolic compounds to scavenge free radicals (Riedl and Hagerman, 2001), the phosphomolybdenum measured their reducing power (Prieto et al., 1999). Phenolic extracts of non-germinated sorghum varieties evaluated using the ABTS assay showed antioxidant activities ranging from 16 to 80 µmol of TAE/g or 6 to 28 µmol of VCAE/g (**Figures 2A** and **2B**). The evaluation of the antioxidant activity using the phosphomolybdenum assay gave values ranging from 17 to 85 µmol TAE/g or 9 to 96 µmol VCAE/g. The mean value of antioxidant activities in sorghum varieties before germination was 42 µmol TAE/g or 15 µmol VCAE/g, with the ABTS assay and 45 µmol TAE/g or 24 µmol VCAE/g with the phosphomolybdenum assay. Antioxidant activities changed differently in varieties upon germination, but on average, they were not affected by germination. The inter-varietal differences of the antioxidant activities of sorghum varieties could be related to the difference in their total phenolic compounds content and to the diversity of phenolic compounds. Indeed, several studies of structurally-related phenolic compounds have revealed differences in their antioxidant capacity (Rice-Evans, 1999; Bors et al., 2001). The antioxidant activity of phenolic compounds depends on the number of conjugated unsaturated bonds, the redox properties, and the presence of vicinal hydroxyls in the aromatic ring (Rice-Evans, 1999; Bors et al., 2001; Lu and Foo, 2001).

Table 1. Comparison of phenolics composition of ungerminated (g-) and germinated (g+) sorghum varieties*

code	name	color of grain/glumes/ plant	testa[a]	main food property	total phenolics (%)[b]		PAs (%)[c]		3-DAs (%)[d]		flavan-4-ols (%)[e]	
					g-	g+	g-	g+	g-	g+	g-	g+
V1	Ajabsido	W/R/R	+	good for "tô"	0.58	0.96	0.11	0.19	0.04	0.05	nd	nd
V2	BF 88-2/31-1	W/P/tan	-	poor for "tô"	0.85	0.60	nd	nd	nd	nd	0.17	nd
V3	BF 88-2/31-3	W/R/tan	-	good for couscous	0.72	0.69	nd	nd	nd	nd	nd	nd
V4	BF 89-18/139-1-1	W/P/tan	-	good for "tô"	0.66	0.41	nd	nd	nd	nd	nd	nd
V5	Cauga 108-15	W/P/R	-	good for "tô"	0.72	0.63	0.17	0.18	0.04	0.02	nd	nd
V6	Cauga 22-20	W/P/R	+	good for "tô"	1.38	1.22	1.50	1.21	0.02	0.02	0.22	0.13
V7	CE 180-33	W/R/tan	+	good for couscous	0.59	0.87	0.11	0.06	nd	nd	nd	nd
V8	CEF 322/53-1-1	W/P/R	-	good for "tô"	0.71	0.67	0.07	nd	nd	nd	nd	nd
V9	CEF 395/9-2-3	W/P/tan	-	good for "tô"	0.87	0.91	nd	nd	nd	nd	nd	nd
V10	CEF 396/12-3-1	W/P/R	-	good for "tô"	0.68	0.88	0.06	0.17	0.02	0.02	nd	nd
V11	CEM 326/11-5-1-1	W/P/tan	-	good for "tô"	0.69	0.73	nd	nd	nd	nd	nd	nd
V12	CGM 1/19-1-1	W/P/R	-	good for "tô"	0.64	0.81	0.06	0.07	nd	0.02	nd	nd
V13	CGM 19/9-1-1	W/B/R	-	good for "tô"	0.61	0.82	nd	nd	nd	0.02	nd	nd
V14	CGM 19/9-1-2	W/P/R	-	good for “dolo”	0.55	0.81	0.08	nd	nd	nd	nd	nd
V15	CK 60	W/P/R	-	good for "tô"	0.76	1.22	0.24	0.28	0.04	0.07	nd	nd
V16	F2-20	W/R/tan	-	good for couscous	0.55	0.86	nd	nd	0.02	nd	nd	nd
V17	Farkakofsi 781	R/B/R	+	good for “dolo”	1.28	1.47	0.66	0.72	0.14	0.12	0.38	0.30
V18	Framida	R/C/R	+	good for “dolo”	1.74	1.85	1.13	0.58	0.03	0.05	0.37	0.29
V19	G 1296	R/R/R	+	good for “dolo”	3.01	2.95	2.18	1.85	0.42	0.24	0.42	0.37
V20	G 1414	W/P/R	-	good for "tô"	0.71	0.84	0.08	0.15	nd	nd	nd	nd
V21	G 1636	W/P/tan	-	poor for "tô"	0.76	0.74	0.07	0.08	nd	nd	nd	nd
V22	ICSV 1002	W/P/tan	-	good for "tô"	0.82	0.51	nd	nd	0.02	nd	nd	nd
V23	ICSV 1049	W/P/tan	-	good for porridge	0.64	0.78	nd	nd	nd	nd	nd	nd
V24	ICSV 745	W/P/tan	-	poor for "tô"	0.66	0.46	nd	nd	nd	nd	nd	nd
V25	IRAT 10	W/B/R	-	good for "tô"	0.82	0.72	0.06	0.06	nd	nd	nd	nd
V26	IRAT 174	W/C/R	-	good for “tô”	0.92	0.75	0.09	0.08	0.06	0.04	nd	nd
V27	IRAT 202	W/R/tan	+	good for couscous	1.20	1.22	0.62	0.33	nd	nd	nd	nd
V28	IRAT 204	W/P/tan	-	good for couscous	0.96	0.61	nd	nd	nd	nd	nd	nd
V29	IRAT 277	W/R/tan	-	poor for "tô"	0.87	0.79	nd	nd	nd	nd	nd	nd
V30	IRAT 9	R/C/R	+	good for “dolo”	1.50	1.18	0.90	0.54	0.07	0.03	0.41	0.38
V31	IS 15401	W/P/R	-	good for couscous	0.65	0.63	0.09	nd	nd	nd	nd	nd
V32	Kaapelga	W/P/tan	-	good for "tô"	0.73	0.83	nd	nd	nd	nd	nd	nd
V33	Kapla-57	R/P/R	+	good for “dolo”	0.60	0.97	0.53	0.41	nd	nd	nd	nd
V34	Kokologho	W/B/R	+	poor for “dolo”	0.81	1.02	0.66	0.41	0.02	0.06	0.19	nd
V35	90L1235	W/B/R	-	good for couscous	1.28	0.97	0.53	0.40	0.07	0.07	0.19	nd
V36	Magadji 1-509	R/B/R	-	good for “dolo”	0.70	0.99	0.07	0.11	nd	nd	nd	nd
V37	Nafo-Natogué 775	R/B/R	+	good for “dolo”	1.47	1.75	1.16	1.17	0.18	0.17	0.34	0.24
V38	Nazongala tan	W/B/tan	-	good for porridge	0.66	1.16	0.09	0.13	0.04	0.06	nd	nd
V39	Nongomsoba	W/B/tan	-	good for porridge	0.82	0.76	nd	0.08	nd	nd	nd	nd
V40	S 29	W/R/R	-	good for "tô"	0.72	1.01	0.07	0.12	nd	nd	nd	nd
V41	Sariaso 10	W/R/R	-	good for porridge	0.73	0.70	0.08	nd	nd	nd	0.23	0.22
V42	Sariaso 11	W/P/tan	-	good for "tô"	0.74	0.55	nd	nd	nd	nd	nd	nd
V43	Sariaso 12	W/B/R	-	good for "tô"	0.83	1.05	0.17	0.14	0.05	0.03	0.18	0.13
V44	Sariaso 14	W/P/tan	-	good for porridge	0.45	0.63	nd	nd	nd	nd	nd	nd
V45	Sariaso 9	W/B/R	-	good for "tô"	0.98	0.77	0.26	0.17	0.06	0.04	0.22	0.14
V46	Segaolane	W/P/R	-	poor for "tô"	0.63	0.71	0.12	0.06	nd	nd	nd	nd
V47	SRN 39	Y/P/tan	-	poor for “dolo”	1.10	0.96	0.17	0.09	0.06	0.03	0.19	0.12
V48	Tiamassie 289	W/B/R	+	poor for "tô"	0.58	0.61	0.06	0.06	0.03	0.02	nd	nd
V49	Tx 7000	W/P/R	-	poor for "tô"	0.46	0.81	0.08	0.07	nd	nd	nd	nd
V50	Zugilga	R/B/R	+	good for “dolo”	1.71	1.01	0.91	0.42	0.05	0.02	0.20	0.18
Mean Value (n=50)					0.88	0.92	0.26	0.21	0.03	0.02	0.07	0.05
SE (n=2)					0.05	0.05	0.01	0.01	0.002	0.002	0.003	0.003

*Abbreviations: W = white; R= red; Y= yellow; P = pale, C = chestnut; B= black; nd = not detected; g- = ungerminated sorghum; g+ = germinated sorghum. [a]Pigmented testa layer present (+) or absent (-); [b]Gallic acid equivalents (%, w/w, dry matter basis); [c]PAs = proanthocyanidins (%, w/w , procyanidins DP≈7.4, dry matter basis); [d]3-DAs = 3-deoxyanthocyanidins (%, w/w, apigeninidin); [e]anthocyanidin equivalents derived from flavan-4-ols (%, w/w , cyanidin, dry matter basis).

Although the antioxidant activities of phenolic compounds could be beneficial, the major nutritional concern is the ability of PAs to bind strongly to large proteins and to proline-rich proteins, thereby reducing their digestibility (Butler, 1992). PAs display free radical scavenging activity even when forming complexes with proteins (Riedl and Hagerman, 2001), and they may be degraded in the digestive system into low molecular weight monomers which could be absorbed by the intestine (Awika and Rooney, 2004a). This supports the idea that the potential of PAs to diminish nutrient digestibility may be compensated by their *in vivo* antioxidant activity, even when bound to proteins (Hagerman et al., 1998).

Correlations between antioxidant activities and phenolic compounds

In line with the report of Awika and co-workers (2003), antioxidant activities were positively correlated with the level of total phenolic compounds, both before and after germination (**Table 2**). A relatively strong correlation was found between antioxidant activities and PAs when compared to those for 3-DAs and flavan-4-ols. The weaker correlation between antioxidant activity and phenolic compounds upon germination could be due to the synthesis of other antioxidant compounds such as vitamin C and tocopherols in sorghum during germination (Malleshi and Klopfenstein, 1998; Simontacchi et al., 2003). Germination lowered the correlations between different phenolic compounds and antioxidant activities. The difference found between Trolox and vitamin C on scavenging the $ABTS^{\bullet+}$ radical is related to the difference in their mechanism of radical scavenging (Rice-Evans, 1999). Indeed, it has been found that α-tocopherol, an analogue of Trolox, is a much more effective chain-breaking antioxidant in scavenging lipid peroxyl radicals than vitamin C (Rice-Evans, 1999).

Table 2. Pearson correlation (r values) matrix between phenolic compounds and their antioxidant activities in ungerminated (g-) and germinated (g+) sorghum varieties

	antioxidant activities[a]		total phenolics		PAs[b]		3-DAs[c]	
	g-	g+	g-	g+	g-	g+	g-	g+
Total phenolics	0.78	0.64						
PAs	0.79	0.63	0.92	0.87				
3-DAs	0.66	0.56	0.80	0.86	0.68	0.78		
Flavan-4-ols	0.67	0.07*	0.68	0.65	0.61	0.52*	0.60	0.57

[a]Trolox antioxidant equivalents using the ABTS (2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) assay. [b]PAs = proanthocyanidins, [c]3-DAs = 3-deoxyanthocyanidins; g- = ungerminated sorghum; g+ = germinated sorghum. *Not significant (P<0.05); all others are significant.

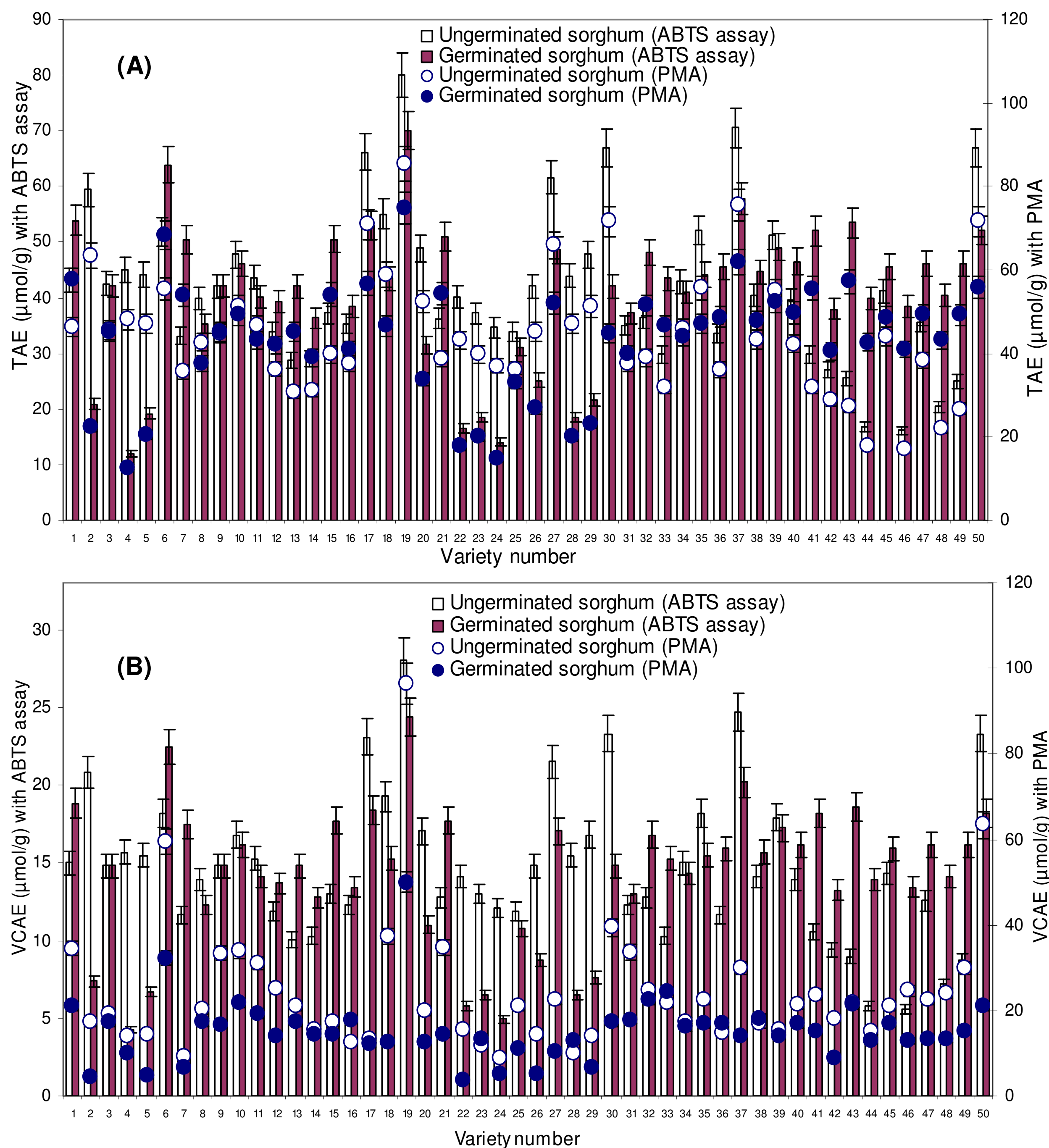

Figure 2. Comparison of the effect of germination on antioxidant activities of sorghum varieties. (**A**), evaluation of the antioxidant activities as Trolox antioxidant equivalents (TAE). (**B**), evaluation of the antioxidant activity as vitamin C antioxidant equivalents (VCAE). ABTS= (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); PMA= phosphomolybdenum assay. Vertical bars indicate the average standard error for each experiment.

On weight/weight basis, the total phenolic compounds (gallic acid equivalents) of whole sorghum grains have an antioxidant capacity comparable to that of pure sorghum PAs (Riedl and Hagerman, 2001). This may explain the good correlation found between antioxidant activity and the content in PAs (**Table 2**). PAs are reported to have higher antioxidant activity than other phenolic compounds because they have little or no pro-oxidant activity and because of the presence of *ortho*-hydroxyl groups in their structures (Hagerman et al., 1998). In addition, oxidized PAs undergo phenolic coupling reactions, generating hydroxyls, which govern their antioxidant activity and which are higher than the other phenolic compounds (Bors et al., 2001).

Phenolic compounds and antioxidant activities of sorghum varieties grouped according to plant or grain properties

Independent of germination, red varieties contained on average significantly more total phenolic compounds, PAs, 3-DAs and flavan-4-ols than white varieties (**Table 3**). Nevertheless, some white varieties (V6, V27 and V35) have a relatively high contents of total phenolic compounds (1.2-1.4%, w/w). Investigations carried out by others also showed phenolic content in white varieties to be as high as 3% (w/w) (Beta et al., 1999). All red varieties contained 3-DAs, whereas among white varieties only 35% possessed trace amounts of 3-DAs (0-0.07%, apigeninidin equivalents), if no interference of other phenolics in the assay is assumed. Red sorghum grains had on average significantly higher antioxidant activities (30-80 µmol TAE/g) than white grains (16-62 µmol of TAE/g) both before and after germination. These results are in line with those of Awika and co-workers (2003) who found the same trend for ungerminated sorghum grains. Sorghum grains with pigmented testa layer have higher levels of total phenolic compounds (2 fold), PAs (9-11 fold), 3-DAs (8-10-fold), and antioxidant activities (1.5 fold) than sorghum without pigmented testa. Sorghum grains covered with glumes possessing the chestnut color have a higher content in total phenolic compounds, PAs, 3-DAs, flavan-4-ols and antioxidant activities than the other red, black and pale glume colors. Audilakshmi and co-workers (1999) also found correlations between glume colors and the content of the grains in total phenols and flavan-4-ols. **Table 3** shows that the grains from red sorghum plants have higher phenolic compounds and antioxidant activities than grains from tan sorghum plants. The levels of phenolic compounds in plant tissues are not only dependent on the genotype but they are also dependent on the biotic and abiotic stresses. Because the varieties were grown in the same environment and no visible effect of stress was observed, these correlations between the levels of phenolic compounds and the plant and grain characteristics may

be essentially attributed to the genetic diversity of sorghum varieties (Doherty et al., 1987; Audilakshmi et al., 1999; Waniska et al., 2000).

Phenolic compounds and antioxidant activities of sorghum varieties grouped according to food properties

The groups of sorghum varieties used for specific local foods displayed significant differences in their contents of total phenolic compounds, PAs, 3-DAs, flavan-4-ols and antioxidant activities before and after germination (**Table 3**). Varieties good for "dolo" had, before and after germination, on average the highest total phenolic content (1.40-1.44%, w/w), PAs (0.61-0.79%, w/w), 3-DAs (0.06-0.09%, w/w), flavan-4-ols (0.20-0.24%, w/w) and antioxidant activities (49-55 µmol TAE/g) of all groups.

Table 3. Comparison of the average content of phenolics and antioxidant activities in groups of ungerminated (g-) and germinated (g+) sorghum varieties of known properties

Group properties		total phenolics (%)[a]		PAs (%)[b]		3-DAs (%)[c]		flavan-4-ols (%)[d]		antioxidant activities[e]	
		g-	g+	g-	g+	g-	g+	g-	g+	g-	g+
food	good for "tô" (n = 20)	0.78	0.81	0.15	0.14	0.02	0.02	0.03	0.02	40	39
	poor for "tô" (n = 7)	0.69	0.68	0.05	0.04	< 0.01	< 0.01	0.02	< 0.01	34	33
	good for "dolo" (n = 9)	1.40	1.44	0.79	0.61	0.09	0.06	0.24	0.20	55	49
	poor for "dolo" (n = 2)	0.96	0.99	0.41	0.25	0.04	0.05	0.19	0.06	39	44
	good for couscous (n = 7)	0.85	0.84	0.19	0.11	< 0.01	<0.01	0.03	< 0.01	43	40
	good for porridge (n = 5)	0.66	0.81	0.04	0.04	< 0.01	< 0.01	0.05	0.04	35	41
	mean value (n=50)	0.88	0.92	0.26	0.21	0.03	0.02	0.07	0.05	42	41
plant/grain	red grains (n = 9)	1.46	1.46	0.80	0.62	0.11	0.07	0.26	0.21	56	50
	white grains (n = 41)	0.76	0.80	0.13	0.11	< 0.01	< 0.01	0.03	< 0.01	39	38
	grains without testa (n=37)	0.75	0.78	0.07	0.07	< 0.01	< 0.01	0.03	0.02	38	37
	grains with testa (n=13)	1.27	1.31	0.81	0.61	0.10	0.08	0.32	0.27	53	51
	glumes chestnut (n= 3)	1.39	1.26	0.71	0.40	0.05	0.04	0.26	0.22	55	37
	glumes red (n= 9)	1.00	1.12	0.35	0.28	0.05	0.03	0.07	0.07	46	47
	glumes black (n= 13)	0.97	1.01	0.36	0.30	0.05	0.05	0.13	0.08	44	46
	glumes pale (n=25)	0.74	0.78	0.16	0.13	< 0.01	< 0.01	< 0.01	< 0.01	37	37
	red plants (n=30)	0.96	1.03	0.41	0.32	0.04	0.04	0.11	0.08	43	44
	tan plants (n=20)	0.77	0.75	0.05	0.04	< 0.01	< 0.01	< 0.01	< 0.01	41	35
	SE (n=2)	0.05		0.01		0.002		0.003		2	

[a]Gallic acid equivalents (%, w/w, dry matter basis). [b]PAs = proanthocyanidins (%, w/w of apple procyanidins DP≈7.4, dry matter basis); [c]3-DAs = 3-deoxyanthocyanidins (%, w/w, apigeninidin, dry matter basis); [d]anthocyanidin equivalents derived from flavan-4-ols (%, w/w, cyanidin, dry matter basis); [e]Trolox antioxidant equivalents (µmol/g of flour, dry matter basis) using the ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) assay. g- = ungerminated sorghum; g+ = germinated sorghum.

To obtain the desired reddish or opaque color of “dolo”, local manufacturers tend to use red varieties that contain, even after malting, a high content of colored polyphenols such as 3-DAs. In addition, the fact that varieties good for “dolo” had more flavan-4-ols and 3-DAs than varieties poor for “dolo” may be an advantage for grain molding resistance (Schutt and Netzly, 1991; Melake-Berhan et al., 1996; Audilakshmi et al., 1999) during the traditional malting of sorghum. The high antioxidant activities found in varieties used for “dolo” (49-55 µmol TAE/g) indicates that “dolo” varieties contain antioxidant activity levels higher than red wines (9-12 µmol TAE/g) (Pellegrini et al., 2003). From our data, it can be inferred that the whole grains of (for example) the sorghum varieties V17, V19, V30, V37 and V50 have antioxidant activities (66-80 µmol TAE/g) higher than most other plant foods (Pellegrini et al., 2003), e.g. blackberry (20 µmol TAE/g). Furthermore, it is important to stress that a high antioxidant activity is expected to be found in sorghum brans (Awika et al., 2003), which are commercially available in West Africa and are used as animal feed (Akintayo and Sedgo, 2001).

Germination did not significantly affect the content of PAs in groups of varieties both good and poor for tô. The group of varieties good for porridge had on average low contents of total phenolic compounds, PAs, DAs and flavan-4-ols. Apparently, varieties with moderate or high PAs and colored flavonoid levels are already avoided for porridge preparation. The high PA contents in varieties may impair the nutritional quality of their final products with respect to the possible inhibitory effect of PAs on hydrolytic enzymes. Therefore, varieties good for porridge, which do not contain PAs after germination (V41 and V44), may be the best for the preparation of infant porridges from a nutritional stand-point. For infant porridges, the low PAs content is presumably more desired than high antioxidant activity. Varieties good for couscous did not contain 3-DAs before and after germination, in line with the preparation of this dish for which a colorless final product is often preferred.

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REFERENCES

Akintayo I.; Sedgo J. In *Towards sustainable sorghum production and utilization in West and Central Africa*; Akintayo I.; Sedgo J. Eds.; WASRN/ICRISAT, 19-22 April 1999, Lomé, Togo, **2001.**

Anglani, C. Sorghum for human food: a review. *Plant Foods Hum. Nutr*. **1998**, *52*, 85-89.

Audilakshmi, S.; Stenhouse, J. W.; Reddy, T. P.; Prasad, M. V. R. Grain mould resistance and associated characters of sorghum genotypes. *Euphytica*, **1999**, *107*, 91-103.

Awika, J. M.; Rooney, L. W. Sorghum phytochemicals and their potential impact on human health. *Phytochemistry* **2004a**, *65*, 1199-1221.

Awika, J. M.; Rooney, L. W.; Waniska, R. D. Properties of 3-deoxyanthocyanins from sorghum. *J. Agric. Food. Chem.* **2004b**, *52*, 4388-4394.

Awika, J. M.; Rooney, L. W.; Wu, X., Prior, R. L.; Cisneros-Zevallos, L. Screening methods to measure antioxidant activity of sorghum and sorghum products. *J. Agric. Food Chem*. **2003**, *51*, 6657-6662.

Beta, T.; Rooney, L. W.; Marovatsanga, L. T.; Taylor, J. R. N. Phenolic compounds and kernel characteristics of Zimbabwean sorghums. *J. Sci. Food Agric.* **1999**, *79*, 1003-1010.

Bors, W.; Michel, C.; Stettmaier, K. Structure-activity relationships governing antioxidant capacities of plant polyphenols. *Methods Enzymol*. **2001,** *335*, 166-181.

Butler, L. G. Antinutritional effects of condensed and hydrolysable tannins. In *Plant polyphenols: synthesis. properties and significance*; Hemingway. R. W.; Laks. P. E. Eds.; Plenum press: New York. **1992**; pp. 693-698.

Bvochora, J. M.; Reed, J. D.; Read, J. S.; Zvauya, R. Effect of fermentation processes on proanthocyanidins in sorghum during preparation of Mahewu: a non-alcoholic beverage. *Process Biochem*. **1999**, *35*, 21-25.

Dicko, M. H.; Hilhorst. R.; Gruppen, H.; Traoré, A. S.; Laane, C.; van Berkel, W. J. H.; Voragen. A. G. J. Comparison of content in phenolic compounds, polyphenol oxidases and peroxidases in grains of fifty sorghum varieties from Burkina Faso. *J. Agric. Food Chem.* **2002**, *50*, 3780-3788.

Doherty, C. A.; Waniska, R. D.; Rooney, L. W.; Earp, C. F.; Poe, J. H. Free phenolic compounds and tannins in sorghum caryopsis and glumes during development. *Cereal Chem*. **1987**, *64*, 42-46.

Ferreira, D.; Slade, D. Oligomeric proanthocyanidins: naturally occurring O-heterocycles. *Nat. Prod. Rep.* **2002**, *19*, 517-541.

Food and Agricultural Organization of the United Nations (FAO). Sorghum and millets in human nutrition. *FAO Food and Nutrition Series*, No. 27, **1995**. http://www.fao.org/DOCREP/T0818e/T0818E00.htm#Contents; http://faostat.fao.org/faostat/

Guyot, S.; Marnet, N.; Drilleau, J. F. Thiolysis-HPLC characterization of apple procyanidins covering a large range of polymerization states. *J. Agric. Food Chem*. **2001**, *49*, 14-20.

Guyot, S.; Marnet, N.; Laraba, D.; Drilleau, J. F. Reversed-phase HPLC following thiolysis for quantitative estimation and characterization of the four main classes of phenolic compounds in different tissue zones of a French cider apple variety (*Malus domestica* var. Kermerrien). *J. Agric. Food Chem*. **1998**, *46*, 1698-1705.

Hagerman, A. E.; Riedl, K. M.; Jones, G. A.; Sovik, K. N.; Ritchard, N. T.; Hartzfeld, P. W.; Riechel, T. L. High molecular weight plant polyphenolics (tannins) as biological antioxidants. *J. Agric. Food Chem*. **1998**, *46*, 1887-1892.

Haslam, E. Practical polyphenolics: from structure to molecular recognition and physiological action; Haslam, E. Ed., Cambridge University Press: Cambridge, UK, **1998**; pp. 178-225.

Iwuoha, C. I.; Aina, J. O. Effect of steeping condition and germination time on the alpha-amylase activity, phenolics content and malting loss of Nigerian local red and hybrid short Kaura sorghum malts. *Food Chem.* **1997**, *58*, 289-295.

Kouda-Bonafos, M.; Nacro, M.; Ancian, B. Total assignment of 1H and 13C NMR chemical shifts of a natural anthocyanidin, apigeninidin, using two-dimensional COLOC and HMBC techniques. *Magn. Reson. Chem*. **1996**, *34*, 389-394.

Lu, Y.; Foo, L. Y. Antioxidant activities of polyphenols from sage (*Salvia officinalis*). *Food Chem.* **2001**, *75*, 197-202.

Malleshi, N. G.; Klopfenstein, C. F. Nutrient composition, amino acid and vitamin contents of malted sorghum, pearl millet, finger millet and their rootlets. *Int. J. Food Sci. Nutr*. **1998**, *49*, 415-422.

Melake-Berhan, A.; Butler. L. G.; Ejeta. G.; Menkir, A. Grain mold resistance and polyphenol accumulation in sorghum. *J. Agric. Food Chem*. **1996**, *44*, 2428-2434.

Mueller-Harvey, I.; Reed, J. D. Identification of phenolic compounds and their relationships to in-vitro digestibility of sorghum leaves for bird-resistant and non-bird-resistant varieties. *J. Sci. Food Agric.* **1992**, *60*, 179-196.

Nwanguma, B. C.; Eze, M. O. Changes in the concentrations of the phenolic constituents of sorghum during malting and mashing. *J. Sci. Food Agric.* **1996**, *70*, 162-166.

Ouattara, L.; Trouche, G.; Fliedel , G. ; Diawara, B. Potentialités d'utilisations des sorghos guinea tan au Burkina Faso. In *Towards sustainable sorghum production and utilization in West and Central Africa*; Akintayo I.; Sedgo J. Eds., WASRN/ICRISAT, 19-22 April 1999, Lome, Togo, **2001a;** pp. 24-41.

Palé, E.; Kouda-Bonafos, M.; Nacro, M.; Vanhaelen-Fastré, R.; Ottinger, R. 7-O-methylapigeninidin, an anthocyanidin from *Sorghum caudatum*. *Phytochemistry* **1997**, *45*, 1091-1093.

Parr, A. J.; Bolwell, G. P. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. *J. Sci. Food Agric*. **2000**, *80*, 985-1012.

Pellegrini, N.; Serafini, M.; Colombi, B. ; Del Rio, D.; Salvatore, S.; Bianchi, M.; Brighenti, F. Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three in vitro assays. *J. Nutr*. **2003**, *133*, 2812-2819.

Prieto, P.; Pineda, M.; Aguilar, M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. *Anal. Biochem.* **1999**, *269*, 337-341.

Rice-Evans, C. Screening of phenolics and flavonoids for antioxidant activity. In *Antioxidant food supplements in human health*; Packer, L.; Hiramatsu, M.; Yoshikawa, T. Eds.; Academic Press: San Diego, London, Boston, New York, Sydney, Tokyo, Toronto, **1999**; pp. 239-253.

Riedl, K. M.; Hagerman, A. E. Tannin-complexes as radical scavengers and radical sinks. *J. Agric. Food Chem.* **2001**, *49,* 4917-4923.

Santos-Buelga, C.; Scalbert, A. Proanthocyanidins and tannin-like compounds: nature, occurrence, dietary intake and effects on nutrition and health. *J. Sci. Food Agric*. **2000**, *80*, 1094-1117.

Schutt, C.; Netzly, D. Effects of apiforol and apigeninidin on growth of selected fungi. *J. Chem. Ecol*. **1991**, *17*, 2261–2266.

Sérémé, A.; Kouda-Bonafos, M.; Nacro, M. Tannins in utilization of sorghum in Burkina-Faso. *Plant Foods Hum. Nutr.* **1994**, *46*, 331-334.

Simontacchi, M.; Sadovsky, L.; Puntarulo, S. Profile of antioxidant content upon developing of *Sorghum bicolor* seeds. *Plant Sci.* **2003**, *164*, 709-715.

Subramanian, V.; Murty, D. S.; Rao, N. S.; Jambunatan, R. Chemical changes and diastatic activity in grains of sorghum (*Sorghum bicolor*) cultivars during germination. *J. Sci. Food Agric.* **1992**, *58*, 35-40.

Taylor, J. R. N.; Dewar, J. Developments in sorghum food technologies. *Adv. Food Nutr. Res*. **2001**, *43*, 217-264.

Trouche, G. ; Da, S. ; Palé, G. ; Sohoro, A.; Ouédraogo, O. ; Den-Gosso, G. Evaluation participative de nouvelles variétés de sorgho. In *Sélection participative du sorgho*; Hocdé, H.; Lançon, J.; Trouche, G. Ed.; CIRAD, Montpellier, **2001b**. pp. 36-55.

Waniska, R. D. Structure, phenolic compounds, and antifungal proteins of sorghum caryopes. In *Technical and institutional options for sorghum grain mold management: proceeding of an International consultation*; Chandrashekar, A.; Bandyopadhyay, R.; Hall, A. J. Eds.; ICRISAT, Patancheru, India. **2000**. pp. 72-106. http://www.icrisat.org/text/research/grep/homepage/sgmm/chap4.pdf

CHAPTER 4

Effects of germination on the activities of amylases and phenolic enzymes in sorghum varieties grouped according to food-end use properties

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ABSTRACT

Fifty sorghum varieties were screened to determine the effect of germination on levels of starch, α-amylase, β-amylase, phenylalanine ammonia lyase (PAL), peroxidase (POX) and polyphenol oxidase (PPO). Germination decreased starch content, with amylose being more degraded than amylopectin. In germinated grain, α-amylase activity increased several fold in all varieties, whereas β-amylase activity did not uniformly increase and even decreased in some varieties. Activity of the key enzyme in phenolic compounds biosynthesis, PAL, was detected only in half of the varieties before germination, but after germination PAL was detected in all of them. PPO was not activated in germinated sorghum grains, whereas POX activity increased up to 10 fold in some varieties. Zymography revealed that germination induced *de novo* synthesis of several POX isoenzymes, among which an anionic POX isoenzyme (p*I* 3.1) was ubiquitously present. Amylase and phenolic enzyme activities are correlated to grain and plant agronomic characteristics. The use of sorghum varieties for local dishes such as “tô”, “dolo”, couscous and thin porridges could be correlated with amylase and phenolic enzyme activities and in the contents of their substrates. The biochemical constituents determined are useful markers for selection of varieties for food utilization with special emphasis on infant porridges.

Keywords : sorghum, germination, starch, amylase, phenylalanine ammonia lyase, peroxidase, polyphenol oxidase, porridge.

INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench] plays a crucial role in food security in developing countries. In particular, in West Africa it is also a source of income on family level. Sorghum production is increasing due to the introduction of improved varieties and breeding conditions. It is, therefore, important to select varieties meeting specific agricultural and food requirements from this great biodiversity to insure food security.

Germination is one of the essential steps of sorghum processing into food because of the activation of enzymes, the decrease of antinutritional factors, the increase of bioavailability of minerals (iron, zinc, etc.), the increase of the content of essential amino acids (especially lysine, tryptophan and methionine), and the increase in digestibility of macronutrients (FAO, 1995; Anglani, 1998). These germination-related properties are dependent on sorghum variety (Nwanguma and Eze, 1995; Bvochora et al., 1999). Among the important reasons for germinating sorghum is the activation of starch degrading enzymes. During sorghum germination, α-amylase [α-1,4-D-glucan 4-glucanohydrolase, EC 3.2.1.1] isoenzymes are *de novo* synthesized (Marambe and Ando, 1995), whereas β-amylase [α-1,4-D-glucan maltohydrolase, E.C 3.2.1.2] isoenzymes may not be synthesized (Ziegler , 1999). The technological interest in high α-amylase activity-containing sorghum varieties is for brewing, and for preparation of low-viscosity weaning food (Malleshi and Desikachar, 1988; Thaoge et al., 2003; Traoré et al., 2004). Also, for β-amylase, the major disadvantage encountered using sorghum as brewing cereal is the low activity (Dufour et al., 1992; Taylor and Robbins, 1993) or total absence (Uriyo and Eigel, 2000) of this maltogenic enzyme, causing incomplete saccharification of starch.

Phenolic compounds and related enzymes are also important for the quality of plant-derived foods (Tomas-Barberan and Espín, 2001). Although sorghum grain contains a high proportion of phenolic compounds (Dicko et al., 2002a 2005; this thesis, **Chapters 2** and **3**) the activity of phenylalanine ammonia lyase [EC 4.3.1.5, PAL], the key enzyme in phenolic biosynthesis (Rösler et al., 1977; Parr and Bolwell, 2000; Tomas-Barberan and Espín, 2001) is scarcely studied. Furthermore, the effects of sorghum grain germination on PAL activity are unknown. PAL catalyzes the deamination of L-phenylalanine and L-tyrosine to *trans*-cinnamate derivatives (Rösler et al., 1977; Parr and Bolwell, 2000) and is involved in the formation of phenylpropanoids, hydroxycinnamates, flavonoids, proanthocyanidins, hydroxystilbenes, coumarins, lignans and lignins (Parr and Bolwell, 2000). Thus, PAL activity in plant-foods is important for the quality of

the final product (Thomas-Barberan and Espin, 2001; Parr and Bolwell, 2000). Peroxidases [donor: H_2O_2 oxidoreductase, EC 1.11.1.7, POXs] and polyphenol oxidases [monophenol, 3,4-L-dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1, PPOs] are the main enzymes involved in the oxidation of endogenous phenolic compounds and phenolic containing biomolecules. POX can modify the properties of food macromolecules, notably proteins and ferulic acid-containing polysaccharides, via oxidative-crosslinking (Boeriu et al., 2004). PPO is involved in the oxidation of endogenous cereal phenolic compounds resulting in browning of grain-flour and derived products (Baik et al., 1994). Recently, POX and PPO activities in sorghum kernels were analyzed (Dicko et al., 2002a; this thesis, **Chapter 2**). The POX activity was higher than the monophenolase and *o*-diphenolase activities of PPO, suggesting that POX may be more involved in endogenous phenolic compounds oxidation than PPO (Dicko et al., 2002a; this thesis, **Chapter 2**). Comparison of POX and PPO activities among sorghum varieties used for specific food applications such as "tô", porridge, couscous and "dolo", showed differences in these enzyme activities (Dicko et al., 2002a; this thesis, **Chapter 2**). However, the effect of germination on the activity of POX and PPO, and the occurrence of different isoenzymes in sorghum grain remains to be evaluated.

In the present study, the effect of germination on the content of starch and the activities of starch degrading enzymes is determined. A simultaneous comparison of the effect of germination on the occurrence of phenolic enzymes, e.g. PAL, PPO and POX in sorghum varieties was made for the first time. This information is useful in selecting varieties for specific local food applications, e.g. for instance the preparation of complementary infant food.

MATERIALS AND METHODS

Sorghum grains

Fifty sorghum [*Sorghum bicolor* (L.) Moench] varieties were grown during the rainy season of 2002 at the experimental station of Saria, in Burkina Faso (West Africa). The environment was semi-arid (temperature: 30-42°C; annual precipitation: 850 mm). Mature grains (≥ 60 days after anthesis) were harvested, surface-sterilized and germinated (Dicko et al., 2002a, 2005; this thesis, **Chapters 2** and **3**). Germinated and ungerminated sorghum grains were dried, ground and stored at -80°C, prior to analysis (Dicko et al., 2002a; this thesis, **Chapter 2**). The sorghum varieties were classified in alphabetic order of their name followed by Arabic numbers from 1 to 50 preceded by V (**Table 1**). Information as to which varieties were "good" or "poor" for the preparation of specific

dishes was collected from sorghum breeders. Hunter L (lightness), a* (green to red) and b* (blue to yellow) color values of flours were obtained with a tristimulus Hunter colorimeter (Microcolor II DR LANGE, The Netherlands) with a light excluder cap of 50 mm diameter and powder cuvette of 34 mm diameter.

Determination of protein content and enzyme activities

Enzymes extraction and total protein quantification were performed as described previously (Dicko et al., 2002a, this thesis, **Chapters 2**). Enzyme extracts were prepared by mixing 250 mg of sorghum flour with 1.2 mL of 50 mM Tris-HCl buffer pH 7.3 containing 0.5 M $CaCl_2$ and 2% (w/v) polyvinylpyrrolidone, at 4°C for 1 hour. The homogenate was centrifuged (14000g, 4°C, 45 min) and the resulting supernatant was used as crude extract of all screened enzymes. Total protein was quantified by the method of Bradford using the ratio of A_{620}/A_{450} versus protein concentration. Bovine serum albumin was used as standard. Enzyme activities were determined as follows. α-Amylase and β-amylase activities were determined using azurin-crosslinked amylose (MegaZyme, Australia) and *p*-nitrophenyl maltopentaoside (Betamyl kit, MegaZyme, Australia) as substrates, respectively (McCleary and Sheehan, 1989; Dicko et al., 1999, 2001). PAL activity was determined by measuring *trans*-cinnamic acid formation from L-phenylalanine (Tovar et al., 2002) using sodium *trans*-cinnamate (Aldrich) as standard. POX activity was determined by monitoring the H_2O_2-dependent oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Dicko et al., 2002a; this thesis, **Chapter 2**), in 50 mM sodium acetate pH 4. The monophenolase and *o*-diphenolase activities of PPO were determined as described previously (Dicko et al., 2002a, 2002b; this thesis, **Chapters 2** and **6**). Enzymes activities are expressed in term of specific activities (units/mg of protein).

Zymography of POX and PPO

Electrophoresis was performed with a PhastSystem (Amersham Pharmacia Biotech). Isoelectrofocusing (IEF) was performed using polyacrylamide pH 3-9 gradient gels calibrated with standard proteins (p*I* 3-10) as described previously (Dicko et al., 2001). After IEF, activities of POX and PPO were in-gel stained in conditions minimizing their mutual interference (Dicko et al., 2002b; this thesis, **Chapter 6**).

Viscosity measurement

Sorghum flours were compared for their viscosity at 20% (w/v) in water, taking into account the moisture content of the flour (5-7%, w/w), and water evaporation during heating. The heating device (Heidolph-MR 3001, 230V, 625 W) was preheated at 200°C. Sorghum flour was mixed with water, the slurry was stirred and then heated at a rate of 40°C/min, until gelatinization of starch (75 ± 2°C). The paste was stirred for 5 min, at 75 ± 2°C, then further heated to 95-98°C, for 5 min. Next, the paste was cooled on ice to 45 ± 2°C, with occasional stirring to measure the viscosity in a Brookfield viscometer (HBDV-II+ model) using a HB 6 spindle at 50-100 rpm.

Component analyses

Total starch and amylose contents were simultaneously determined using an iodine-binding spectrophotometric method (Jarvis and Walker, 1993), and amylopectin was deduced by difference. Total carbohydrate and soluble sugars were analyzed (Fox and Robyt, 1991; Subramanian et al., 1992), using glucose as standard. Total lipids, proteins, moisture and ash contents were determined as described previously (Verbruggen et al., 1993). Levels of components were expressed on fresh weight of the flour (w/w).

Statistical analysis

All spectrophotometric assays were carried out in 96-well microtiter plates (Nunclon, Denmark) using a multiwell plate reader (*µQuant* Bio-Tek Instrument, Inc) on line interfaced with a personal computer. The absorbances and slopes of absorbances (OD/min) were automatically recorded using KC junior software version 1.31.5 (Bio-Tek Instrument, INC, USA). All assays were carried out at least in duplicate. Statistical analyses were performed using the Student *t*-test as described previously (Dicko et al., 2005; this thesis, **Chapter 3**). To determine correlation coefficients between phenolic compounds and their related enzymes, data obtained from previous screening (Dicko et al., 2005; this thesis, **Chapter 3**) for total phenol, proanthocyanidins, 3-deoxyanthocyanidins, and flavan-4-ols of the flour of the same varieties were used.

RESULTS AND DISCUSSION

Enzymes related to food quality and their substrates in sorghum varieties

The selected sorghum varieties had different contents of starch, amylose and amylopectin (**Table 1**). The mean values of starch, amylose and amylopectin were 63.0%, 13.4% and 49.6%, respectively. Germination induced a reduction of the starch, amylose and amylopectin contents to 59.5%, 11.3% and 48.2%, respectively. The average proportion of amylose and amylopectin in sorghum starch before germination was 21.2% and 79.8%, respectively. After germination, the average proportion of amylose and amylopectin in sorghum starch slightly changed to 19.0% and 81.0%, respectively. The content of starch components found in this study is comparable to that reported for other sorghum varieties (FAO, 1995; Beta et al., 1995, 2000). Among the varieties screened, no waxy sorghum was found. This is because almost all sorghum varieties cultivated in Burkina Faso are primarily selected for “tô” preparation, for which high amylose content is needed (Bello et al., 1990; Trouche et al., 2000).

In contrast to the results of Ahmed and co-workers (1996), both α-amylase and β-amylase activities were detected in all fifty sorghum varieties before germination (**Table 1**). On average, α-amylase and β-amylase displayed similar activities in ungerminated sorghum grains. Upon germination, α-amylase activity increased several fold in all the varieties, whereas β-amylase activity either increased or decreased depending on variety. The increase of β-amylase activity in certain varieties may be explained by the release of “bound” forms of the enzyme, as *de novo* synthesis is not expected (Ziegler, 1999). The inter-varietal difference factors (ratio of highest to lowest values) of α-amylase activities among varieties were 56 and 18 before and after germination, respectively. The inter-varietal difference factor for β-amylase was 15, independent of germination. The inter-varietal difference factors for the α-amylase and β-amylase activities of the fifty sorghum varieties were higher than the corresponding values found in a previous study on sixteen sorghum varieties (Beta et al., 1995). This indicates a high polymorphism in amylase activities among sorghum varieties. Although α-amylase and β-amylase catalyze starch hydrolysis synergistically, their activities were not correlated ($r = 0.07$) in both ungerminated and germinated sorghum grains, in agreement with other findings (Beta et al., 1995). This supports the hypothesis that α-amylase and β-amylase may have different physiological functions in germinating cereal other than starch hydrolysis (Ziegler, 1999).

Table 1. Comparison of starch components[a] and amylase activities in ungerminated (g-) and germinated (g+) sorghum varieties

Code	Name	Grain/ Glumes/ Plant*	Testa	Main food property	Total starch (%)[b]		Amylose (%)[b]		Amylopectin (%)[b]		α-Amylase (U mg^{-1})[c]		β-Amylase (U mg^{-1})[c]	
					g-	g+	g-	g+	g-	g+	g-	g+	g-	g+
V1	Ajabsido	W/R/R	+	good for "tô"	66.1	58.1	21.5	14.8	44.6	43.3	1.4	12.8	3.5	5.4
V2	BF 88-2/31-1	W/P/tan	-	poor for "tô"	59.5	55.2	11.5	9.1	48.0	46.1	3.1	6.8	0.9	1.9
V3	BF 88-2/31-3	W/R/tan	-	good for couscous	59.5	56.2	10.2	9.2	49.3	47.0	0.6	1.7	1.2	2.8
V4	BF 89-18/139-1-1	W/P/tan	-	good for "tô"	66.0	63.2	17.1	17.0	48.9	46.2	1.1	1.4	0.6	1.1
V5	Cauga 108-15	W/P/R	-	good for "tô"	66.5	60.2	16.1	10.0	50.4	50.2	3.4	7.9	1.3	1.7
V6	Cauga 22-20	W/P/R	+	good for "tô"	66.2	58.1	16.8	12.0	49.4	46.1	3.9	7.2	1.7	1.1
V7	CE 180-33	W/R/tan	+	good for couscous	68.5	58.1	16.7	12.8	51.8	45.3	4.3	5.6	2.2	4.6
V8	CEF 322/53-1-1	W/P/R	-	good for "tô"	61.6	59.5	10.9	10.4	50.7	49.1	1.6	1.9	1.0	2.9
V9	CEF 395/9-2-3	W/P/tan	-	good for "tô"	65.2	61.6	12.3	9.2	52.9	52.4	0.2	1.9	1.7	3.9
V10	CEF 396/12-3-1	W/P/R	-	good for "tô"	63.7	58.1	11.6	10.7	52.1	47.4	0.3	4.9	1.8	3.8
V11	CEM 326/11-5-1-1	W/P/tan	-	good for "tô"	61.6	59.5	12.6	10.7	48.9	48.8	0.6	0.9	1.9	8.7
V12	CGM 1/19-1-1	W/P/R	-	good for "tô"	62.3	59.2	14.8	13.3	47.5	45.9	0.4	2.9	1.5	6.1
V13	CGM 19/9-1-1	W/B/R	-	good for "tô"	62.5	56.7	11.0	7.3	51.5	49.4	2.5	3.9	1.1	4.0
V14	CGM 19/9-1-2	W/P/R	-	good for "dolo"	68.5	61.6	14.2	9.7	54.3	51.9	0.8	3.2	0.9	2.7
V15	CK 60	W/P/R	-	good for "tô"	62.3	60.8	14.3	12.9	48.0	48.0	5.3	6.0	2.1	2.8
V16	F2-20	W/R/tan	-	good for couscous	61.6	59.5	11.5	9.5	50.1	50.0	1.6	1.7	1.6	2.1
V17	Farkakofsi 781	R/B/R	+	good for "dolo"	60.2	59.3	12.6	12.2	47.6	47.1	6.9	8.4	1.7	2.6
V18	Framida	R/C/R	+	good for "dolo"	64.2	59.2	15.5	11.4	48.7	47.8	1.4	1.6	1.1	3.1
V19	G 1296	R/R/R	+	good for "dolo"	61.6	57.5	10.9	10.1	50.7	47.4	11.3	11.5	2.0	4.7
V20	G 1414	W/P/R	-	good for "tô"	64.1	58.8	13.2	9.7	50.9	49.1	6.6	11.0	2.6	4.3
V21	G 1636	W/P/tan	-	poor for "tô"	62.5	61.6	10.8	10.1	51.7	51.5	1.2	8.7	1.3	8.7
V22	ICSV 1002	W/P/tan	-	good for "tô"	62.6	61.5	13.2	12.2	49.4	49.3	5.4	7.8	2.8	7.3
V23	ICSV 1049	W/P/tan	-	good for porridge	64.5	59.2	14.3	12.9	50.2	46.3	1.1	1.7	1.6	3.6
V24	ICSV 745	W/P/tan	-	poor for "tô"	67.6	60.2	17.2	13.8	50.4	46.4	4.3	7.5	2.5	3.2
V25	IRAT 10	W/B/R	-	good for "tô"	61.6	59.1	11.6	11.4	50.0	47.7	4.0	16.4	2.7	1.7
V26	IRAT 174	W/C/R	-	good for "tô"	65.2	60.2	14.8	11.1	50.4	49.1	3.5	5.5	2.5	3.0
V27	IRAT 202	W/R/tan	+	good for couscous	66.1	60.2	15.6	10.9	50.5	49.3	1.4	1.8	1.0	0.9
V28	IRAT 204	W/P/tan	-	good for couscous	62.0	61.6	11.2	10.2	50.8	51.4	10.2	16.3	0.9	2.1
V29	IRAT 277	W/R/tan	-	poor for "tô"	65.1	59.8	15.7	12.0	49.4	47.8	4.8	6.4	2.0	2.0
V30	IRAT 9	R/C/R	+	good for "dolo"	58.6	58.3	11.4	11.1	47.2	47.2	5.3	10.3	5.1	3.7
V31	IS 15401	W/P/R	-	good for couscous	57.2	55.2	11.5	10.0	45.7	45.2	4.9	13.8	2.0	5.4
V32	Kaapelga	W/P/tan	-	good for "tô"	63.5	61.0	11.9	10.2	51.6	50.8	1.4	12.2	2.2	5.7
V33	Kapla-57	R/P/R	+	good for "dolo"	65.5	61.6	12.5	9.9	53.0	51.7	2.2	13.5	1.2	3.4
V34	Kokologho	W/B/R	+	poor for "dolo"	59.5	58.5	11.0	10.1	48.5	48.4	4.1	5.9	2.1	2.3
V35	90L1235	W/B/R	-	good for couscous	65.0	62.0	14.8	12.1	50.2	49.9	3.4	4.9	3.1	1.9
V36	Magadji 1-509	R/B/R	-	good for "dolo"	65.7	60.1	15.1	14.2	50.6	45.9	0.7	10.1	1.5	2.4
V37	Nafo-Natogué 775	R/B/R	+	good for "dolo"	66.2	63.8	14.7	12.6	51.5	51.1	1.3	2.0	13.0	9.0
V38	Nazongala tan	W/B/tan	-	good for porridge	61.6	60.8	12.8	12.4	48.8	48.4	2.7	3.3	6.7	1.6
V39	Nongomsoba	W/B/tan	-	good for porridge	63.5	60.1	13.5	13.3	50.0	46.8	1.8	14.6	1.6	2.0
V40	S 29	W/R/R	-	good for "tô"	62.6	61.6	11.2	11.1	51.4	50.5	0.8	14.1	3.5	2.9
V41	Sariaso 10	W/R/R	-	good for porridge	63.5	60.2	14.2	11.4	49.3	48.8	3.7	13.7	3.4	4.8
V42	Sariaso 11	W/P/tan	-	good for porridge	64.2	61.7	12.7	10.6	51.5	51.1	5.1	14.3	1.8	2.2
V43	Sariaso 12	W/B/R	-	good for "tô"	64.4	59.5	15.3	11.1	49.1	48.4	1.7	12.1	1.2	2.4
V44	Sariaso 14	W/P/tan	-	good for porridge	62.5	57.4	15.0	10.3	47.5	47.2	3.1	13.2	1.1	1.1
V45	Sariaso 9	W/B/R	-	good for "tô"	59.5	58.7	13.5	13.0	46.0	45.7	0.8	16.1	2.9	3.4
V46	Segaolane	W/P/R	-	poor for "tô"	58.1	57.2	10.9	10.8	47.2	46.4	1.4	13.0	1.2	1.1
V47	SRN 39	Y/P/tan	-	poor for "dolo"	61.6	58.2	12.7	11.7	48.9	46.5	2.3	10.1	12.5	13.9
V48	Tiamassie 289	W/B/R	+	poor for "tô"	59.5	58.8	12.3	11.5	47.2	47.3	4.0	10.6	7.4	4.3
V49	Tx 7000	W/P/R	-	poor for "tô"	59.5	58.1	11.9	11.4	47.6	46.8	0.8	12.4	1.0	2.1
V50	Zugilga	R/B/R	+	good for "dolo"	60.5	58.8	11.4	11.4	49.1	47.5	3.5	13.3	1.5	1.5
		Mean			63.0 / 59.5		13.4 / 11.3		49.6 / 48.2		3.0 / 8.1		2.5 / 3.6	
		Overall range of variation			57-69 / 55-64		10-17/9-16		45-54/43-52		0.4-11/0.9-16		0.6-13/0.9-14	
		Standard experimental error			3.8		0.8		3		0.4		0.2	

[a]Abbreviations: R = red; W = white; Y = yellow; C = chestnut; B = black; g- = ungerminated sorghum; g+ = germinated sorghum. [a]Pigmented testa layer present (+) or absent (-). [b]Fresh weight basis (w/w). [c]Specific activities (Units/mg of protein).

PAL activity was detected in only 50% of the varieties before germination, whereas it was present in all varieties after germination (**Table 2**). PAL activity has been positively correlated with the content of total phenolics in developing olive (*Olea europaea* L. cv. Arbequina)(Tovar et al., 2002). Such a correlation is not observed in sorghum grains. This supports the notion that the total phenolics content in plants depends on a complex interplay of phenol (bio)synthesizing and modifying enzymes (Rösler et al., 1997).

POX activities increased by 1.2-10 fold in sorghum varieties upon germination (**Table 2**). The germination-induced increase of sorghum POX activities was higher than that found in barley, which ranged from 1.5 to 5 (Billau and Nicolas, 2001).

The increase of POX activities after germination can be related to the activation or *de novo* synthesis of POX, or both. To study this in more detail, a zymography analysis of POXs was performed. **Figure 1** shows an ubiquitous band of POX activity with p*I* ≥ 9 present both before and after germination. Due to the limited resolution of the pH gradient, POX isoenzymes (Cx) with p*I* ≥9 could not be resolved in the gel. Preliminary analysis by cation exchange chromatography of variety V5 showed that Cx consists of one major and two minor isoenzymes. Upon germination, an anionic isoenzyme (A1, p*I* 3.1) is induced only in germinated varieties, together with several other isoenzymes (A2-C4), which occurred differently according to variety. In summary, at least seven anionic isoenzymes: A1 (p*I* 3.1), A2 (p*I* 4.2), A3 (p*I* 4.8), A4 (p*I* 5.2), A5 (p*I* 6), A6 (p*I* 6.2) and A7 (p*I* 7.1) and five cationic isoenzymes: C1 (p*I* 7.8), C2 (p*I* 8.1), C3 (p*I* 8.3), C4 (p*I* 8.7) and Cx (p*I* ≥ 9), were clearly detected in sorghum grain. Although germination induces several new POX isoenzymes, it is difficult to correlate the spectrophotometric assays with in-gel enzyme staining because of the difference in the substrate specificity of the isoenzymes, the difference in their optimum pH of activity, and the in-gel inhibition of the oxidases by their products (Rescigno et al., 1997; Dicko et al., 2002b; this thesis, **Chapter 6**). In barley, germination activated essentially the cationic isoenzymes, and 20 isoenzymes were detected (Billau and Nicolas, 2001). Multigene families of POXs are described (Passardi et al., 2004), however it remains a challenge to assign a specific physiological role of each POX isoenzyme. The activation of POXs during germination may be related to their function in cell-wall (lignin and suberin) biosynthesis and the metabolism of the plant growth hormone auxin (Quiroga et al., 2000; Passardi et al., 2004). The anionic sorghum isoenzyme A1 expressed upon germination might play a role in plant development by participating in cell wall biosynthesis during the early stage of plant growth (Quiroga et al., 2000).

Table 2. Comparison of total phenolics* and their anabolic (PAL) and catabolic enzymes (POX and PPOs) in ungerminated (g-) and germinated (g+) sorghum varieties

Code	Name	Main food property	Total phenolics (%)*		PAL[a] (mU mg^{-1})**		POX[b] (U mg^{-1})		mono-PPO[c] (mU mg^{-1})		di-PPO[d] (mU mg^{-1})	
			g-	g+	g-	g+	g-	g+	g-	g+	g-	g+
V1	Ajabsido	good for "tô"	0.58	0.96	nd	10.3	68.8	81.2	0.7	1.0	35.6	16.3
V2	BF 88-2/31-1	poor for "tô"	0.85	0.61	3.9	1.7	11	49.9	0.7	0.2	39.7	18.7
V3	BF 88-2/31-3	good for couscous	0.72	0.69	2.3	9.9	17.4	79.9	0.7	0.8	39.2	24.4
V4	BF 89-18/139-1-1	good for "tô"	0.66	0.41	4.1	1.8	15.1	106.7	0.7	0.9	44.2	14.6
V5	Cauga 108-15	good for "tô"	0.72	0.63	nd	5.8	93.3	175.9	0.7	0.9	39.9	11.3
V6	Cauga 22-20	good for "tô"	1.38	1.22	1.1	11.1	31.7	100.7	0.7	0.7	48.0	14.1
V7	CE 180-33	good for couscous	0.59	0.87	nd	15.5	81.8	124.7	0.9	0.7	39.9	23.0
V8	CEF 322/53-1-1	good for "tô"	0.71	0.67	2.4	8.7	59.9	76.3	0.9	0.5	42.4	15.7
V9	CEF 395/9-2-3	good for "tô"	0.87	0.91	nd	2.5	56.5	75.8	1.0	0.1	48.1	14.0
V10	CEF 396/12-3-1	good for "tô"	0.68	0.88	nd	10.1	60.8	71.4	1.3	0.2	57.0	14.5
V11	CEM 326/11-5-1-1	good for "tô"	0.69	0.73	nd	7.1	50.9	81.6	0.7	0.2	36.4	20.7
V12	CGM 1/19-1-1	good for "tô"	0.64	0.81	nd	3.2	73.2	99.9	1.0	0.5	40.5	17.3
V13	CGM 19/9-1-1	good for "tô"	0.61	0.82	nd	8.8	44.2	95.4	0.6	0.5	34.6	15.1
V14	CGM 19/9-1-2	good for "dolo"	0.55	0.81	13.1	6.2	41.3	54.5	1.0	0.8	41.0	9.5
V15	CK 60	good for "tô"	0.76	1.22	9.0	11.2	59.7	144.3	1.0	0.8	40.7	0.9
V16	F2-20	good for couscous	0.55	0.86	9.2	1.2	42.8	184.0	1.0	0.3	43.6	15.1
V17	Farkakofsi 781	good for "dolo"	1.28	1.47	3.1	2.8	20.9	89.6	0.7	0.4	45.6	14.7
V18	Framida	good for "dolo"	1.74	1.85	6.2	6.0	8.8	89.2	0.9	0.7	42.9	18.1
V19	G 1296	good for "dolo"	3.01	2.95	2.9	6.6	15.2	109.7	0.9	1.0	41.4	20.3
V20	G 1414	good for "tô"	0.71	0.84	nd	0.9	22.6	105.3	1.0	1.4	50.3	9.7
V21	G 1636	poor for "tô"	0.76	0.74	nd	16.5	62.3	75.8	0.8	1.9	43.3	19.8
V22	ICSV 1002	good for "tô"	0.82	0.51	4.7	2.0	24.4	42.5	0.6	0.5	43.7	18.3
V23	ICSV 1049	good for porridge	0.64	0.78	5.1	0.6	23.2	83.0	0.6	1.2	40.5	20.5
V24	ICSV 745	poor for "tô"	0.66	0.46	nd	1.4	25.7	56.5	0.5	0.8	40.6	27.0
V25	IRAT 10	good for "tô"	0.82	0.72	5.6	2.3	21	98.5	1.1	0.8	42.8	17.2
V26	IRAT 174	good for "tô"	0.92	0.75	3.9	1.2	24.6	75.3	1.0	0.3	47.1	21.0
V27	IRAT 202	good for couscous	1.20	1.22	nd	3.8	12	15.6	1.0	0.6	42.8	15.3
V28	IRAT 204	good for couscous	0.96	0.61	2.2	9.2	18.9	16.4	0.9	0.8	50.8	16.9
V29	IRAT 277	poor for "tô"	0.87	0.79	4.1	1.9	21.4	49.1	1.1	0.6	47.9	13.0
V30	IRAT 9	good for "dolo"	1.50	1.18	4.4	8.1	31.5	82.4	1.0	2.3	48.3	18.5
V31	IS 15401	good for couscous	0.65	0.63	nd	0.1	86.6	107.5	1.1	1.9	42.7	24.4
V32	Kaapelga	good for "tô"	0.73	0.83	nd	13.7	40.2	76.5	0.9	0.9	40.9	39.5
V33	Kapla-57	good for "dolo"	0.60	0.97	2.7	13.6	37	92.6	1.1	1.4	42.3	40.7
V34	Kokologho	poor for "dolo"	0.81	1.02	5.1	16.9	18.4	26.1	1.3	1.3	41.8	62.5
V35	90L1235	good for couscous	1.28	0.97	3.6	19.7	36.3	45.7	1.3	1.9	43.4	54.6
V36	Magadji 1-509	good for "dolo"	0.70	0.99	nd	30.1	71.7	86.6	0.7	1.6	46.0	43.5
V37	Nafo-Natogué 775	good for "dolo"	1.47	1.75	2.0	6.0	35.9	88.3	0.8	1.1	42.0	42.1
V38	Nazongala tan	good for porridge	0.66	1.16	nd	7.4	75.8	180.3	0.8	1.4	39.0	45.0
V39	Nongomsoba	good for porridge	0.82	0.76	nd	13.7	17.9	37.4	0.6	1.6	47.7	47.3
V40	S 29	good for "tô"	0.72	1.01	nd	17.1	25.8	53.1	1.1	1.5	50.0	34.1
V41	Sariaso 10	good for porridge	0.73	0.7	7.7	8.7	31.7	39.5	1.1	2.1	52.7	46.3
V42	Sariaso 11	good for porridge	0.74	0.55	7.3	10.8	7.9	58.8	1.3	1.5	52.2	37.8
V43	Sariaso 12	good for "tô"	0.83	1.05	1.0	12.7	23.9	51.7	1.0	1.4	49.6	11.7
V44	Sariaso 14	good for porridge	0.45	0.63	6.1	11.9	18.3	53.8	0.4	1.5	54.2	42.1
V45	Sariaso 9	good for "tô"	0.98	0.77	nd	9.8	42.3	51.0	0.9	1.2	49.3	37.2
V46	Segaolane	poor for "tô"	0.63	0.71	nd	8.7	39.8	65.7	1.2	1.6	57.1	36.2
V47	SRN 39	poor for "dolo"	1.10	0.96	nd	13.1	47.1	96.2	1.0	2.3	54.7	33.1
V48	Tiamassie 289	poor for "tô"	0.58	0.61	nd	12.6	13.4	51.5	1.2	1.0	50.0	34.8
V49	Tx 7000	poor for "tô"	0.46	0.81	nd	12.7	67.5	82.6	0.4	1.3	52.6	28.3
V50	Zugilga	good for "dolo"	1.71	1.01	1.7	7.1	32.3	58.8	1.0	1.4	59.2	39.2
	Mean		0.88 / 0.92		2.49 / 8.49		38.21 / 79.90		0.90 / 1.05		45.3 / 25.5	
	Overall range of variation		0.5-3 / 0.5-2.9		0-13 /0.1-30		8-93 / 16-184		0.4-1.3 / 0.1-2		36-59/1-63	
	Standard experimental error		0.05		0.4		4		0.05		2	

*Data of total phenolics were obtained previously (Dicko et al., 2005; this thesis, **Chapter 3**). [a]PAL= phenylalanine ammonia lyase, [b]POX= peroxidase, [c]mono-PPO = monophenolase activity of polyphenol oxidase (PPO), [d]*o*-diphenolase activity of PPO. **Specific activities (Units/mg of protein).

The high expression of POXs in germinated sorghum grain indicates that these enzymes are involved in the oxidation of endogenous phenolic compounds in sorghum flour during the processing of foods requiring the germination step, e.g. beer, infant porridge, other beverages, etc.

Monophenolase (mono-PPO) and *o*-diphenolase (di-PPO) activities of PPO were present in sorghum varieties both before and after germination (**Table 2**). The mono-PPO activity was not markedly affected by germination, but, as found for barley (Billau and Nicolas, 2001), germination halved the di-PPO activity in most sorghum varieties. Mono-PPO and di-PPO activities were not correlated with total contents of phenolic compounds in both ungerminated and germinated grains.

Zymography of PPO isoenzymes in both ungerminated and germinated grains (results not shown) did not reveal any difference in isoenzymes pattern and intensity with previous results on ungerminated varieties (a single band at p*I* ≥ 9) (Dicko et al., 2002a; this thesis, **Chapter 2**). This observation is in good agreement with the lack of PPO activation during germination. In wheat, PPO isoenzymes displaying mono-PPO and di-PPO activities were localized in the endosperm and pericarp, respectively (Mayer and Harel, 1979; Hatcher and Kruger, 1993). The decrease of di-PPO activity during germination could be linked to the leaching of PPO present in the pericarp during the steeping step or formation of complex with phenolic compounds, which are concentrated in the pericarp. A positive correlation was found between mono-PPO and PAL (r = +0.53) in germinated grains. One possible hypothesis is that newly synthesized *p*-coumarates may easily undergo hydroxylation reactions to yield caffeic acid derivatives.

Amylases and phenolic enzymes in sorghum varieties grouped according to plant or grain agronomic characteristics

Table 3 shows that there is no significant difference between red and white grains in their starch contents. Red sorghum grains have higher amylase activities than white ones. The high amylase activities probably explains the preference for red sorghums for the preparation of “dolo”, a local sorghum beer. PAL, POX and PPO were more activated during germination in red grains than in white ones, indicating that the phenolic biosynthesis and catabolism process are relatively more important in red grains than in white grains. In wheat, the di-PPO activity was also higher in red grains than in white ones (Mayer and Harel, 1979; Hatcher and Kruger, 1993).

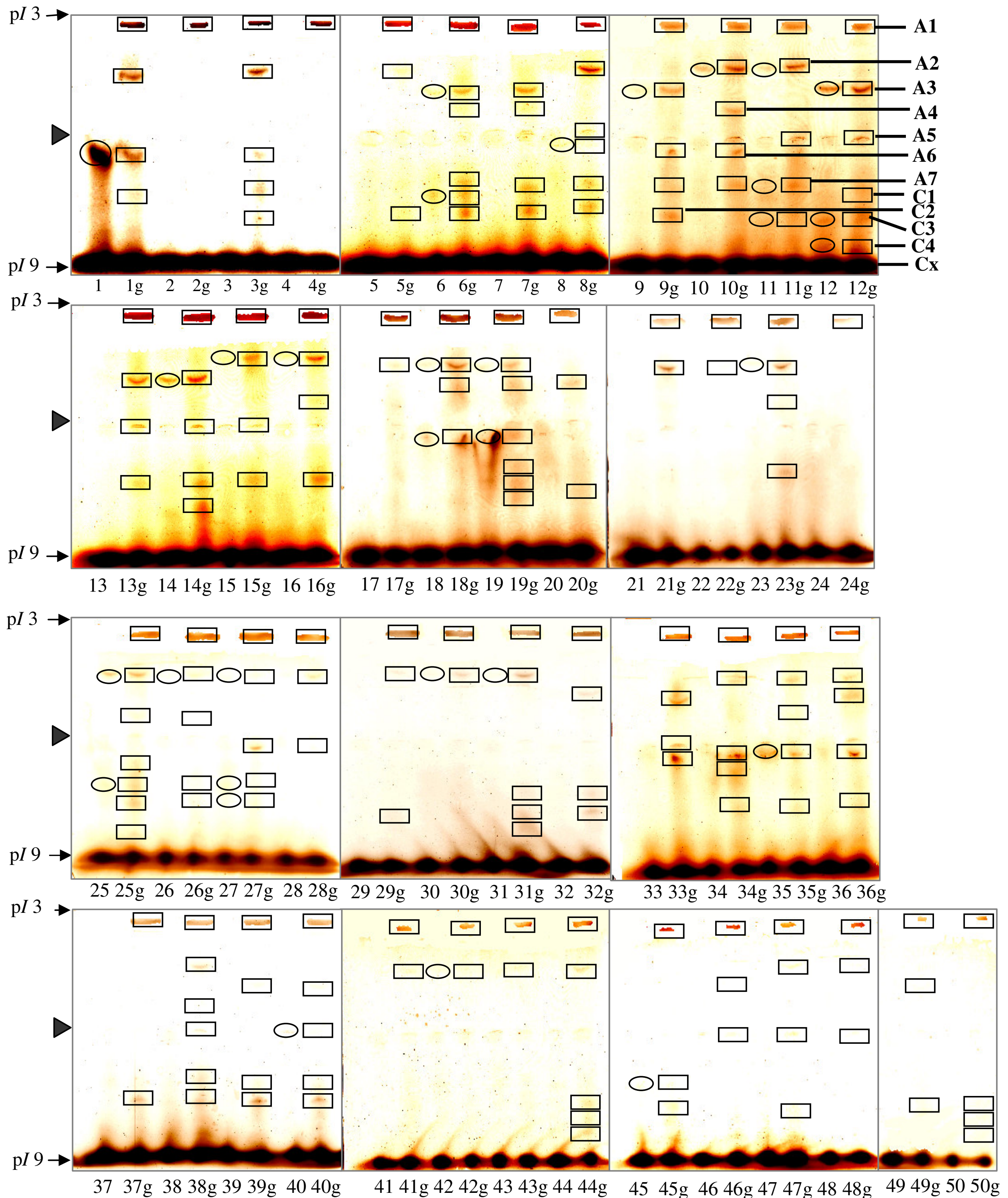

Figure 1. Zymograms of POX activity in ungerminated and germinated sorghum varieties. Samples were applied in the middle of the gels (▶). Each Arabic number represents a sorghum variety; the letter "g" stands for germinated sample. Symbols ⬭ and ▭ illustrate the isoenzymes detected before and after germination, respectively. *The main detected anionic isoenzymes are A1 (p*I* 3.1), A2 (p*I* 4.2), A3 (p*I* 4.8), A4 (p*I* 5.2), A5 (p*I* 6), A6 (p*I* 6.2) and A7 (p*I* 7.1). The main detected cationic isoenzymes are C1 (p*I* 7.8), C2 (p*I* 8.1), C3 (p*I* 8.3), C4 (p*I* 8.7) and Cx (p*I* ≥9).

None of the plant characteristics could be linked to the starch content (**Table 3**). α-Amylase and β-amylase activities were higher in ungerminated grains possessing pigmented testa layers than the grains without testa. However, after germination the presence of the testa could not be linked to these enzymes. PAL activity was higher in germinated grains with pigmented testa than in grains without testa, as expected by the high phenolic contents and their diversity found in the varieties with testa (Dicko et al., 2005; this thesis, **Chapter 3**). Germinated sorghum varieties covered by black glumes had remarkably higher mono-PPO and di-PPO activities than sorghums with other colors of glumes. This may indicate the involvement of PPO (present in the pericarp and in contact with glumes) in the oxidation of phenolic compounds into melanin-like pigments, which could contribute to the black color of glumes. In general, the results suggest that red plants produce grains with higher activities of amylases and phenolic related enzymes than tan plants.

Contents of food-grade biochemical markers in sorghum varieties grouped according to end-use properties

The average activities of amylases and phenolic enzymes and the contents of their substrates in sorghum varieties, grouped as function of food properties are also shown in **Table 3**. A relatively high amylose content was found in "tô" varieties, in line with the "tô" consistency conferred by amylose (FAO, 1995; Bello et al., 1990; Trouche et al., 2000).

One might speculate that the high POX activity found in tô varieties might be beneficial for the preparation of "tô" by increasing tô consistency through the homo and hetero cross-linking (Boeriu et al., 2004) of sorghum arabinoxylans that contain ferulic and *p*-coumaric acids (Verbruggen et al., 1993).

Interestingly, varieties good for "dolo" had a relatively higher α-amylase activity than the varieties poor for dolo. This is in agreement with the desired dextrinizing activity of α-amylase on starch (Dufour et al., 1992; Taylor and Robbins, 1993) during the mashing step in "dolo" processing. Although high activities of the maltogenic enzyme β-amylase are strongly desired in industrial brewing of sorghum beer (Dufour et al., 1992; Taylor and Robbins, 1993), the two varieties judged poor for "dolo" by local brewers had surprisingly more β-amylase than those good for "dolo". Thus, local brewers may tend to select varieties containing more starch liquefying activities rather than saccharolytic ones.

Table 3. Comparison of the average contents of (bio)chemical markers in ungerminated (g-) and germinated (g+) sorghum varieties grouped according to plant/grain characteristics or food end-use properties

	Starch (%)*		Amylose (%)*		Amylopectin (%)*		α-Amylase (U mg^{-1})		β-Amylase (U mg^{-1})		Total phenolics (%)[a]		PAL[b] (mU mg^{-1})		POX[c] (U mg^{-1})		mono-PPO[d] (mU mg^{-1})		di-PPO[e] (mU mg^{-1})	
	g-	g+	g-	g+	g-	g+	g-	g+	g-	g+	g-	g+	g-	g+	g-	g+	g-	g+	g-	g+
Plant and grain characteristics																				
White grains (n = 41)	63	59	14	11	50	48	2.7	7.8	2.1	3.3	0.8	0.8	5.0	8.0	40	79	0.9	1.0	45	24
Red grains (n = 9)	63	60	13	12	50	48	4.2	9.7	4.1	4.6	1.4	1.4	3.8	10	31	85	0.9	1.4	47	31
Grains without testa (n=37)	63	60	13	11	50	48	2.6	8.2	2.2	3.6	0.7	0.8	2.6	8.2	41	81	0.9	1.0	46	25
Grains with testa (n=13)	63	59	14	12	49	48	4.1	7.9	3.3	3.6	1.3	1.3	2.2	9.2	31	78	0.9	1.1	45	28
Glumes chestnut (n= 3)	63	59	14	11	49	48	3.4	5.8	2.9	3.3	1.4	1.3	4.8	5.1	22	82	1.0	1.1	46	19
Glumes red (n= 9)	64	59	14	11	50	48	3.4	7.6	2.3	3.4	1.0	1.1	2.9	8.3	35	82	0.9	1.0	44	23
Glumes black (n= 13)	62	60	13	12	49	48	3.0	9.2	3.6	3.0	1.0	1.0	1.7	12	35	74	0.9	1.2	45	36
Glumes pale (n=16)	63	60	13	11	50	48	2.8	8.0	2.0	4.0	0.7	0.8	2.5	7.4	43	82	0.9	1.0	46	22
Red plant (n=30)	63	59	13	11	49	48	3.1	9.0	2.6	3.3	1.0	1.0	2.5	9.3	41	82	0.9	1.1	46	26
Tan plant (n=20)	63	60	13	11	50	48	2.8	6.9	2.4	4.0	0.8	0.8	2.5	7.3	34	77	0.8	0.9	44	25
Food end-use properties																				
Good for "tô" (n = 20)	64	60	14	11	50	48	2.4	7.7	2.0	3.8	0.8	0.8	1.7	7.4	44	88	0.9	0.8	44	18
Poor for "tô" (n = 7)	62	59	13	11	49	47	2.8	9.3	2.3	3.3	0.7	0.7	4.0	7.9	34	62	0.9	1.0	47	25
Good for "dolo" (n = 9)	64	60	13	11	50	49	4.0	8.7	3.0	3.5	1.3	1.4	4.8	9.7	30	81	0.9	1.2	46	28
Poor for "dolo" (n = 2)	61	58	12	11	49	47	3.2	8.1	7.3	8.1	1.0	1.0	2.5	15	33	61	1.2	1.8	48	48
Good for couscous (n = 7)	63	59	13	11	50	48	3.8	6.5	1.7	2.9	0.9	0.8	4.3	8.5	42	82	1.0	1.0	43	25
Good for porridge (n = 5)	62	60	13	12	49	47	3.2	9.3	2.9	4.3	0.9	0.8	3.5	8.5	37	79	1.0	1.6	46	40
SE[f]	3.8		0.8		3		0.4		0.2		0.05		0.4		4		0.05		2	

*Fresh weight basis (w/w), [a]Data from this thesis, **Chapter 3** (gallic acid equivalent, %, w/w), [b]PAL= phenylalanine ammonia lyase, [c]POX = peroxidase, [d]mono-PPO = monophenolase activity of polyphenol oxidase (PPO), [e]di-PPO = *o*-diphenolase activity of PPO, [f]SE= standard experimental error.

High POX activity may not be desired in industrial beer processing because of the beer darkness and haze occurrence (Billau and Nicolas, 2001). For industrial brewing of sorghum, white

varieties displaying relatively high α-amylase and β-amylase activities and relatively low POX activity (V40, V41, V45, V47, V48) may be interesting.

Among the screened fifty sorghum varieties, ten (V18, V23, V28, V32, V39, V41, V42, V43, V44 and V45) were selected for further analysis for the preparation of infant porridge with low viscosity. These varieties were chosen because of their common availability in Burkina Faso. The mean values of the contents (w/w, fresh matter) of total carbohydrates, soluble sugars, proteins, lipids, ash, and moisture before germination were 75.2 ± 4.5%, 0.50 ± 0.04%, 11.5 ± 0.92 %, 2 ± 0.2%, 2.9 ± 0.2%, 7 ± 0.4%, respectively. Germination induced on average a reduction of the content of total sugars, lipids, and ash by 12%, 44% and 34%, respectively. Upon germination, protein contents increased by 10-20%, whereas soluble sugars increased up to 6 fold. The color hunter values L (range: 68-79), a* (range: 4-12), and b* (range: 8-14) varied between the flours of the selected varieties. Germination changed the color of the flours (L increased, a* and b* decreased), which became lighter. This color change is attributed to the decrease of 3-deoxyanthocyanidins (apigeninidins and luteolinidins) after germination (Dicko et al., 2005a; this thesis, **Chapter 3**). The potential energetic value of ungerminated sorghum flour was about 374 ± 22 kcal $100g^{-1}$. The average loss of potential energetic value after germination was 13% among the varieties analyzed. Before germination, the viscosity of all porridges was relatively high (128-202 Pa.s). Upon germination, the viscosities of porridges dropped to an acceptable range (1-3 Pa.s) for infant porridge preparation (Thaoge et al., 2003; Traoré et al., 2004). Amylose is more susceptible to retrogradation than amylopectin and waxy sorghum is less viscous than normal sorghum (FAO, 1995). Low amylose-containing sorghum varieties are also preferred for extrusion-cooking because they give better functional characteristics of the extrudates, such as enzyme-susceptibility and solubility (Gomez et al., 1988). Varieties (V2, V13, V23, V24, V28, V39 and V44) with low amylose content, high α-amylase activities and which do not contain proanthocyanidins may be recommended for infant porridges preparation.

Table 4 illustrates recommendations for selecting varieties adequate for specific foods. The recommendations are based on the above mentioned biochemical criteria, other findings (Gomez et al. 1988; Malleshi and Desikachar, 1988; Bello et al., 1990; Dufour et al., 1992; Taylor and Robbins, 1993; FAO, 1995; Nwanguma and Eze, 1995; Uriyo and Eigel, 2000; Trouche et al., 2000; Thaoge et al., 2003; Traoré et al., 2004; Dicko et al., 2002, 2005a, 2005b; this thesis, **Chapters 2, 3** and **4**), and known technological properties as judged by local users (Trouche et al.,

2000; Dicko et al., 2005a; this thesis, **Chapter 3**). Nevertheless, additional testing panels and technological processes are necessary to support these recommendations.

Table 4. Recommended sorghum varieties for local foods according to apparent determinant (bio)chemical markers

Use	Apparent determinant biochemical markers or process								Recommended varieties
	Grain color	Germi-nation	Starch content	Amylose content	α-Amylase and β-amylase	Phenolics content	POX[a]	PPO[b]	
"Tô"	white	no	high	high	low	medium	high	low	V1, V4, V5, V6, V8, V9, V10, V11, V12, V14, V15, V20, V21, V22, V25*, V26, V32*, V34, V38, V42*, V43*, V45*, V46, V48, V49
"Dolo"	red	yes	high	high	high	high	high	low	V17, V18*, V19*, V30*, V33, V36, V37, V50
Industrial beer	white	yes	high	low	high	low	low	low	V40, V41*, V45*, V47, V48
Couscous	white	no	medium	low	medium	low	medium	low	V3, V7, V16, V27*, V29, V31, V35
Infant porridge	white	yes	high	low	high	low	low	low	V2, V13, V23*, V24, V28*, V39*, V41*, V44*

*Varieties widely available in Burkina Faso. [a]POX = peroxidase activity, [b]PPO = polyphenol oxidase activity.

In conclusion, sorghum varieties differ both qualitatively and quantitatively in the activities of amylase and phenolic enzymes and in the contents of their substrates**.** Germination induced changes in the activities of amylases and phenolic enzymes in sorghum grain differently depending on variety. The characteristics of sorghum plant (tan or red), grain (red, white, presence of testa) and the end-use properties of the grain can be correlated with the biochemical composition of the grain. The screened biochemical markers have allowed recommendation of varieties as potential candidates for the preparation of specific local foods.

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REFERENCES

Ahmed, S. B.; Mahgoub, S. A.; Babiker, B. E. Changes in tannin and cyanide contents and diastic activity during germination and the effect of traditional processing on cyanide content of sorghum cultivars. *Food Chem*. **1996**, *56*, 159-162.

Anglani, C, Sorghum for human food: a review. *Plant Foods Hum Nutr*. **1998**, *52*, 85-89.

Baik, B. K.; Czuchajowska, Z.; Pomeranz, Y. Comparison of polyphenol oxidase activities in wheats and flours from Australian and U.S. cultivars. *J. Cereal. Sci*. **1994**, *19*, 291-296.

Bello, A. B.; Rooney, L. D.; Waniska, R-D. Factors affecting quality of sorghum tô, a thick porridge. *Cereal Chem*. **1990**, *67*, 20-25.

Beta, T.; Corke, H.; Rooney L.W.; Taylor, J. R. N. Starch properties as affected by sorghum grain chemistry. *J. Sci. Food Agric*. **2000**, *81*, 245-251.

Beta, T.; Rooney, L. W.; Waniska, R. D. Malting characteristics of sorghum cultivars. *Cereal Chem*. **1995**, *72*, 533-538.

Billau, C.; Nicolas, J. Les oxydoréductases intervenant en maltérie-brasserie. *Sci. Alim.* **2001**, *21*, 83-131.

Boeriu, C. G.; Oudgenoeg, G.; Spekking, W. T. J.; Berendsen, L. B. J. M.; Vancon, L.; Boumans, H.; Gruppen, H.; Van Berkel, W. J. H.; Laane, C.; Voragen, A. G. J. Horseradish peroxidase-catalyzed cross-linking of feruloylated arabinoxylans with β-casein. *J. Agric. Food Chem*. **2004**, *52*, 6633-6639.

Bvochora, J. M.; Reed. J. D.; Read, J. S.; Zvauya. R. Effect of fermentation processes on proanthocyanidins in sorghum during preparation of Mahewu, a non-alcoholic beverage. *Process Biochem*. **1999**, *35*, 21-25.

Dicko, M. H.; Gruppen, H.; Traoré, A. S.; van Berkel, W. J. H.; Voragen, A. G. J.; Evaluation of the effect of germination on content of phenolic compounds and antioxidant activities in sorghum varieties. *J Agric Food Chem.* **2005**, *53,* 2581-2588.

Dicko, M. H.; Hilhorst, R.; Gruppen, H.; Traoré, A. S.; Laane. C.; van Berkel, W. J. H.; Voragen, A. G. J. Comparison of content in phenolic compounds, polyphenol oxidases and peroxidases in grains of fifty sorghum varieties from Burkina Faso. *J. Agric. Food Chem*. **2002a**, *50*, 3780-3788.

Dicko, M. H.; Hilhorst, R.; Gruppen, H.; Laane, C.; van Berkel, W. J. H.; Voragen, A. G. J. Zymography of monophenolase and *o*-diphenolase activities of polyphenol oxidase. *Anal. Biochem*. **2002b**, *360,* 336-339.

Dicko, M. H.; Searle-van Leeuwen, M. J. F.; Beldman, G.; Ouédraogo, O. G.; Hilhorst, R.; Traoré A. S. Purification and characterization of β-amylase from *Curculigo pilosa. Appl. Microbiol. Biotechnol.* **1999**, *52*, 802-805.

Dicko, M. H.; Searle-van Leeuwen, M. J. F.; Hilhorst, R.; Traoré, A. S.; Beldman, G. Polysaccharide hydrolases from leaves of *Boscia senegalensis :* properties of the endo-(1→3)-ß-D-glucanase. *Appl. Biochem. Biotechnol*. **2001**, *94*, 225-241.

Dufour, J. P.; Melotte, L.; Sebrenik, S. Sorghum malts for the production of a lager. *ASBC Journal*. **1992**, *50*, 110-119.

Food and Agricultural Organization of the United Nations (FAO). Sorghum and millets in human nutrition. FAO Food and Nutrition Series, No. 27. ISBN 92-5-103381-1. 1995. Available at: http://www.fao.org/DOCREP/T0818e/T0818E00.htm#Contents

Fox, J. D.; Robyt, J. F. Miniaturization of three carbohydrate analyses using a microsample plate reader. *Anal. Biochem.* **1991**, *195*, 93-96.

Gomez, M. H.; Waniska, R. D.; Rooney, L. W.; Lucas, E. W. Extrusion-cooking of sorghum containing different amounts of amylose. *J. Food Sci*. **1988**, *53*, 1818-1822.

Hatcher, D. W.; Kruger, J. E. Distribution of polyphenol oxidase in flour millstreams of Canadian common wheat classes milled to three extraction rates. *Cereal Chem*. **1993**, *70*, 51-55.

Jarvis. C. E.; Walker. J. R. L. Simultaneous, rapid, spectrophotometric determination of total starch, amylose and amylopectin. *J. Sci. Food Agric*. **1993**, *63*, 53-57.

Malleshi, N. G.; Desikachar, H. S. R. Reducing the paste viscosity (dietary bulk) of roller dried weaning foods using malt or fungal amylase. *J. Food Sci. Technol.* **1988**, *25,* 1-3.

Marambe, B.; Ando, T. Physiological basis of salinity tolerance of sorghum seeds during germination. *J. Agro. Crop Sci*. **1995**, *174*, 291-296.

Mayer, A. M.; Harel, E. Polyphenol oxidases in plants *Phytochemistry* **1979**, *18*, 193-215.

McCleary, B. V.; Sheehan, H. Measurement of cereal α-amylase: a new assay procedure. *J. Cereal. Sci*. **1989**, *6*, 237-251.

Nwanguma, B. C.; Eze, M. O. Heat sensitivity, optimum pH and changes in activity of sorghum peroxidase during

malting and mashing. *J. Inst. Brew*. **1995**, *101*, 275-276.

Parr, A. J.; Bolwell, G. P. Phenols in plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. *J. Sci. Food Agric*. **2000**, *80*, 985-1012.

Passardi, F.; Longet, D.; Penel, C.; Dunand, C. The class III peroxidase multigenic family in rice and its evolution in land plants. *Phytochemistry* **2004**, *65*, 1879-1893.

Quiroga, M.; Guerrero, C.; Botella, M. A.; Barceló, A.; Amaya, I.; Medina, M. I.; Alonso, F. J.; Milrad de Forchetti, S.; Tigier, H.; Valpuesta, V. A tomato peroxidase involved in the synthesis of lignin and suberin. *Plant Physiol*. **2000**, *122*, 1119-1128.

Rescigno, A.; Sollai, F.; Rinaldi, A. C.; Soddu, G.; Sanjust, E. Polyphenol oxidase activity staining in polyacrylamide electrophoresis gels. *J. Biochem. Biophys. Methods* **1997**, *34*, 155-159.

Rösler, J.; Krekel, F.; Amrhein, N.; Schmid, J . Maize phenylalanine ammonia-lyase has tyrosine ammonia-lyase activity. *Plant Physiol*. **1997**, *113*, 175–179.

Subramanian, V., Murty, D. S., Rao, N. S. and Jambunatan, R. Chemical changes and diastatic activity in grains of sorghum (*Sorghum bicolor*) cultivars during germination. *J. Sci. Food Agric.* **1992**, *58*, 35-40.

Taylor, J.R.N.; Robbins, D. J. Factors affecting beta-amylase activity in sorghum malt. *J. Ins. Brew.* **1993**, *99,* 413-416.

Thaoge, M. L.; Adams, M. R.; Sibara, M. M.; Watson, T. G.; Taylor, J. R. N.; Goyvaerts, E. M. Production of improved infant porridges from pearl millet using a lactic acid fermentation step and addition of sorghum malt to reduce viscosity of porridges with high protein, energy and solids (30%) content. *World J. Microbiol. Biotechnol*. **2003**, *19*, 305-310.

Tomas-Barberan. F.; Espín. J. C. Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. *J. Sci. Food Agric.* **2001**, *81*, 853-876.

Tovar, M. J.; Romero, M. P.; Girona, J.; Motilva, M. J. *L*-Phenylalanine ammonia-lyase and concentration of phenolics in developing olive (*Olea europaea* L cv Arbequina) fruit grown under different irrigation regimes. *J. Sci. Food Agric.* **2002**, *82*, 892-898.

Traoré, T.; Mouquet, C.; Icard-Verniere, C.; Traoré, A. S.; Trèche, S. Changes in nutrient composition, phytate and cyanide contents and α-amylase activity during cereal malting in small production units in Ouagadougou (Burkina Faso). *Food Chem*. **2004**, *88*, 105-114.

Trouche, G.; Fliedel, G.; Chantereau J.; Barro, C. Productivité et qualité des grains de sorgho pour le "tô" en Afrique de l'Ouest: les nouvelles voies d'amélioration. *Agric. Dev*. **2000**, *23*, 94-107.

Uriyo, M.; Eigel, W. E. Duration of kilning treatment on α-amylase, β-amylase and endo-(1,3)(1,4)-β-D-glucanase activity of malted sorghum (*Sorghum bicolor*). *Process Biochem*. **2000**, *35*, 433-436.

Verbruggen, M. A.; Beldman, G.; Voragen, A. G. J.; Hollemans, M. Water-unextractable cell wall material from sorghum: isolation and characterization. *J. Cereal Sci*. **1993**, *17*; 71-82.

Ziegler, P. Cereal beta-amylases. *J. Cereal Sci.* **1999**, *29*, 195-204.

CHAPTER 5

Impact of phenolics and related enzymes in sorghum varieties for the resistance and susceptibility to biotic and abiotic stresses

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ABSTRACT

Contents of phenolic compounds and related enzymes before and after sorghum grain germination were compared between varieties either resistant or susceptible to biotic (sooty stripe, sorghum midge, leaf anthracnose, striga, and grain molds) and abiotic (logging, drought resistance and photo-period sensitivity) stresses. Independent of grain germination, sorghum varieties resistant to biotic and abiotic stresses had on average higher contents in proanthocyanidins (PAs), 3-deoxyanthocyanidins (DAs), and flavan-4-ols than susceptible varieties. Results show that content of 3-DAs is a good marker for sorghum resistance to both biotic and abiotic stresses because it is positively correlated with the resistance to all stresses except for photoperiod sensitivity. The second good marker for stress resistance is the content of PAs. Total phenolic compounds and the activities of related enzymes are not good markers for stress resistance in sorghum grains.

Keywords: sorghum, proanthocyanidins, 3-deoxyanthocyanidins, phenylalanine ammonia lyase, peroxidase, polyphenol oxidase, stress, biotic, abiotic

INTRODUCTION

Sorghum [*Sorghum bicolor* (L.)] Moench is a C4 plant grass of the hot, semi-arid tropics, genetically very diverse. It adequately takes the advantage of sunlight of tropical environment to perform photosynthesis in the most efficient way (Sheen, 1999). On the basis of genetic diversity, some sorghum varieties are more resistant to stresses than others (Deu et al, 1994; Tao et al, 1993; 2003). The constraints of sorghum production are both biotic and abiotic stresses. Among biotic stresses, leaf anthracnose caused by the fungus *Colletotrichum graminicola*, sooty stripe incited by the fungus *Ramulispora sorghi,* and striga caused by the parasitic plant *Striga hermonthica* (Del.) Bent are the most damaging pests in West-Africa in general, and particularly in Burkina Faso (Tenkouano, 1995; Neya et al., 1998; Trouche et al., 2001). In addition, sorghum midge caused by insects (*Contarinia sorghicola, Sitodiplosis moselana, Stenodiplosis sorghicola*, etc.), attacks developing sorghum grains (Sharma and Hariprasad, 2002; Tao et al., 2003). Also, grain mold is a high constraint of sorghum production, and several molds are associated with sorghum caryopse damage (Melake-Berhan et al., 1996; Waniska et al., 2001). In Africa, the absence of grain mold resistance has been cited as a constraint to adoption of improved cultivars (Audilakshmi et al., 1999).

Important abiotic stresses limiting sorghum production in West-Africa are drought, photoperiod sensitivity and logging (Tenkouano, 1995; Trouche et al., 2001). Logging may be indirectly related to fungal infections, which weaken the plant (Waniska et al., 2001).

Increase of activities of phenolic related enzymes and accumulation of phenolic compounds have been correlated with resistance of cereals to biotic stresses (Mohammadi and Kazemi, 2002). Plant resistance to biotic and abiotic stresses is often regulated by the metabolism of phenolic compounds. Sorghum phenolic compounds, e.g. phytoalexins (3-deoxyanthocyanidins) or allelochemicals (*p*-hydroxybenzoates, *p*-coumarates, and flavanols), are involved in plant resistance to all kind of stresses (Lo et al., 1999; Weston et al., 1999, Weir et al., 2004). The well known sorghum allelochemical, e.g. sorgoleone is also a phenolic derivative (Dayan et al., 2003; Weir et al., 2004).

It has been shown that both biotic (fungi, insects, viruses, etc.) and abiotic (drought, temperature, photoperiod, nutrient deficiencies, etc.) stresses induce phenylalanine ammonia lyase [EC 4.3.1.5, PAL] synthesis (Chalker-Scott and Fuchigami, 1989; Tovar et al., 2002). PAL activity has been detected in the green shoots and leaves (Stafford, 1969; Mohan et al., 1988) of sorghum.

In sorghum, the infection of the plant with pathogens involves a very rapid accumulation of PAL mRNA (Cui et al., 1996).

Also, peroxidases [donor: H_2O_2 oxidoreductase, EC 1.11.1.7, POXs] play an important role in stress related resistance. One of the important physiological roles of POXs is the synthesis of cell-wall polymers (lignin and suberin), which constitute physical barriers for both biotic and abiotic stresses (Cosgrove, 1997). In sorghum, POXs have been shown to be involved in thermal tolerance (Choudhary et al, 1993) and resistance to fungal infection (Luthra et al., 1988).

Polyphenol oxidases [monophenol, 3,4-*L*-dihydroxyphenylalanine: oxygen oxidoreductase, EC1.14.18.1, PPOs] play an important role in plant defence by the oxidation of endogenous phenolic compounds into *o*-quinones, which are toxic to the invading pathogens and pests (Mohammadi and Kazemi, 2002). The PPO activity in plants increases under abiotic stress conditions (Mayer and Harel, 1991) and upon fungal infections (Luthra et al, 1988).

Several studies in other plant species have shown that the levels of phenolic compounds, and the activities of PAL, POX and PPO are different between plants resistant and plants susceptible to stresses (Lo et al., 1999; Mohammadi and Kazemi, 2002). Comparing the effects of germination on the levels of phenolic compounds (Dicko et al., 2005a; this thesis, **Chapter 3**) and the activities of phenolic compounds related enzymes (Dicko et al., 2005b; this thesis, **Chapter 4**), it was found that sorghum varieties are highly variable in levels in these compounds. Germination affected these levels differently depending on variety. Whether the levels of phenolic compounds and related enzymes in sorghum grain could be linked to the grain or plant resistance or susceptibility to stress is unknown. The aim of the present study is to identify possible markers for the grain or plant resistance or susceptibility to these stresses. This is done by comparing the levels of endogenous phenolic compounds and related enzymes in ungerminated and germinated sorghum kernels of known grain or plant agronomic properties for resistance or susceptibility to biotic and abiotic stresses.

MATERIALS AND METHODS

Chemicals

4-Hydroxyanisole (4HA) and gallic acid (3,4,5-trihydroxybenzoic acid) were from Aldrich. 3,4-Dihydroxyphenylpropionic acid (DHPPA) was from Across Organics. Folin-Ciocalteu's reagent, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS), insoluble

polyvinylpyrrolidone (PVP), bovine serum albumin (BSA) and 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) were from Sigma Chemicals Co. Hydrogen peroxide was from Merck. Sorghum apigeninidin, isolated and characterized by Kouda-Bonafos and co-workers (1996), was a gift of Dr. Eloi Palé from the Laboratory of Natural Substances, University of Ouagadougou, Burkina Faso. Cyanidin-chloride was from Extrasynthèse (Lyon, France). Apple ciders procyanidin oligomers (average degree of polymerization of 7.4) were kindly provided by Stephanie Prigent (Wageningen University, Wageningen, The Netherlands) and Dr. Catherine M. G. C. Renard (INRA, Rennes, France). These procyanidins were purified by RP-HPLC and characterized by thiolysis-HPLC as described by Guyot and co-workers (Guyot et al., 2001). All other chemicals were of analytical grade.

Sorghum grains

Fifty sorghum varieties were grown during the rainy season of 2002 at the experimental station of Saria, in Burkina Faso (West Africa). The environment was semi-arid (temperature: 30-42°C; annual precipitation: 850 mm). Growth conditions have been described previously (Dicko et al., 2005a; this thesis, **Chapter 3**). For convenience, the sorghum varieties were classified in alphabetic order of their name followed by Arabic numbers from 1 to 50 preceded by V. Mature grains (≥60 days after anthesis) were harvested, surface-sterilized and germinated as described previously (Dicko et al., 2002, 2005a, this thesis, **Chapters 2** and **3**). Germinated and ungerminated sorghum grains were dried, ground and stored (Dicko et al., 2005a; this thesis, **Chapter 3**). Flours of both germinated and ungerminated sorghum varieties were analyzed. The list of varieties screened is shown in **Table 1**. The varieties were grouped according to their resistance or susceptibility to biotic stresses, e.g. sooty stripe, sorghum midge, leaf anthracnose, striga, and grain molds; and abiotic stresses, e.g. logging, drought resistance and photo-period sensitivity (**Table 2**). The information on resistance and susceptibility to these specific stresses was obtained from sorghum breeders from the experimental station of Saria and Farakoba (Institut National pour l'Environnement et la Recherche Agronomique, Burkina Faso).

Extraction and quantification of phenolic compounds

Sorghum phenolic compounds were extracted and quantified as described previously (Dicko et al., 2002, 2005a, this thesis, **Chapters 2** and **3**). Phenolic compounds were extracted from 50 mg of sorghum flour by continuous stirring with 1.5 mL of 1% (v/v) HCl in methanol at 25°C, for 20 min, followed by centrifugation (5000g, 10 min, 25°C) and supernatant collection.

The residue was re-extracted with 0.5 mL HCl/methanol as described above and the supernatants were pooled and denoted total phenolic extract. The total phenolic extract was used directly for analysis or kept in the dark at -30°C. The same extract was used for quantification of total phenolics, proanthocyanidins (PAs), 3-deoxyanthocyanidins (3-DAs) and flavan-4-ols. The total phenol content was determined using the Folin-Ciocalteu's method adapted to a 96-well plate assay. To 10 µL of extract, 25 µL of Folin-Ciocalteu's reagent (50%, v/v) was added. After 5 min of incubation, 25 µL of 20% (w/v) sodium carbonate solution and 165 µL water were added. Blanks were prepared for each sorghum sample by replacing Folin-Ciocalteu's reagent with water. Gallic acid was used as a standard and results were expressed as gallic acid equivalent per gram of flour (w/w). The standard was always freshly prepared. The absorbances were measured after 30 min at 760 nm.

PAs and flavan-4-ols were assayed essentially as described by Melake-Berhan and co-workers (1996) with miniaturization to adapt the assay to a 96-well plate format as follows. For PAs quantification, the sample remaining in the tube with reagent A was further heated at 100°C, for 2 h. Under these conditions, PAs are converted to anthocyanidins, and the unstable pigments formed from flavan-4-ols are destroyed. After cooling, 200 µL of the sample was put in duplicate in a 96 multiwell plate and the absorbances of anthocyanidin compounds derived from PAs were read at 550 nm. Sample mixtures with reagent B, which were not heated served as blanks for the quantification of both PAs and flavan-4-ols. Apple procyanidins with an average DP ≈ 7.4, treated as indicated above were used as standards for sorghum PAs quantification (Dicko et al., 2005a; this thesis, **Chapter 3**). For direct spectrophotometric quantification of 3-DAs, 50 µL of the total phenolic extract was mixed with 150 µL of methanol and the absorbances were read at 475 nm (Melake-Berhan et al., 1996). Sorghum apigeninidin was used as standard.

Enzyme extraction and determination of protein concentration

Enzyme extraction and total protein quantification were performed as described previously (Dicko et al., 2002, 2005b, this thesis, **Chapters 2** and **4**). Enzyme extracts were prepared by mixing 250 mg of sorghum flour with 1.2 mL of 50 mM Tris-HCl buffer pH 7.3 containing 0.5 M $CaCl_2$ and 2% (w/v) polyvinylpyrrolidone, at 4°C for 1 hour. The homogenate was centrifuged (14000g, 4°C, 45 min) and the resulting supernatant was denoted enzyme extract of PPO, POX, and PAL. Total protein was quantified by the linearized method of Bradford, using bovine serum albumin as standard.

Table 1. List of sorghum varieties and their agronomic properties[a]

Code	Variety name	Gen.[b]	Country of origin	Race	Grain testa	Color of grain/glume/plant	Known particular properties
V1	Ajabsido	LR	Sudan	C	+	W/R/R	Pre-flowering drought resistant
V2	BF 88-2/31-1	IL	Burkina Faso	C	-	W/P/tan	-
V3	BF 88-2/31-3	IL	Burkina Faso	C	-	W/R/tan	Pre-flowering drought susceptible, sooty stripe susceptible, grain mold susceptible, post-flowering drought resistant, logging resistant
V4	BF 89-18/139-1-1	IL	Burkina Faso	C	-	W/P/tan	Post-flowering drought susceptible
V5	Cauga 108-15	IL	Burkina Faso	GC	-	W/P/R	Photoperiod insensitive
V6	Cauga 22-20	IL	Burkina Faso	GC	+	W/P/R	Photoperiod sensitive
V7	CE 180-33	IL	Senegal	C	+	W/R/tan	Leaf anthracnose susceptible, photoperiod insensitive, logging resistant
V8	CEF 322/53-1-1	IL	Burkina Faso	C	-	W/P/R	Post-flowering drought resistant
V9	CEF 395/9-2-3	IL	Burkina Faso	GC	-	W/P/tan	Hard grains (PSI < 10)
V10	CEF 396/12-3-1	IL	Burkina Faso	GC	-	W/P/R	Hard grains (PSI< 10)
V11	CEM 326/11-5-1.1	IL	Mali	GC	-	W/P/tan	Leaf anthracnose resistant, photoperiod insensitive, post-flowering drought susceptible, logging resistant, Hard grains (PSI< 10)
V12	CGM 1/19-1-1	IL	Mali	G	-	W/P/R	Sorghum midge susceptible
V13	CGM 19/9-1-1	IL	Mali	G	-	W/B/R	Striga susceptible
V14	CGM 19/9-1-2	IL	Mali	G	-	W/P/R	-
V15	CK 60	IL	USA	K	-	W/P/R	Striga susceptible
V16	F2-20	IL	Burkina Faso	C	-	W/R/tan	Leaf anthracnose resistant, sorghum midge resistant, photoperiod sensitive, logging resistant
V17	Farkakofsi 781	LR	Burkina Faso	G	+	R/B/R	-
V18	Framida	IL	South Africa	KC	+	R/C/R	Striga resistant, photoperiod sensitive
V19	G 1296	LR	Burkina Faso	GC	-	R/R/R	Good for dyeing, grain mold resistant
V20	G 1414	LR	Burkina Faso	G	-	W/P/R	Photoperiod sensitive
V21	G 1636	LR	Burkina Faso	G	-	W/P/tan	photoperiod sensitive, soft grains (PSI >16)
V22	ICSV 1002	IL	Burkina Faso	C	-	W/P/tan	Leaf anthracnose resistant
V23	ICSV 1049	IL	Burkina Faso	C	-	W/P/tan	Sooty stripe resistant, photoperiod insensitive, post-flowering drought resistant, logging resistant
V24	ICSV 745	IL	India	C	-	W/P/tan	Sooty stripe susceptible, sorghum midge resistant
V25	IRAT 10	IL	Niger	C	-	W/B/R	Grain mold susceptible, drought resistant, photoperiod insensitive, logging resistant
V26	IRAT 174	IL	Burkina Faso	C	-	W/C/R	Photoperiod sensitive, logging resistant
V27	IRAT 202	IL	Senegal	C	+	W/R/tan	Pre-flowering drought resistant, photoperiod insensitive, logging susceptible
V28	IRAT 204	IL	Senegal	C	-	W/P/tan	Sooty stripe and anthracnose susceptible, post-flowering drought susceptible, photoperiod insensitive, logging susceptible
V29	IRAT 277	IL	Burkina Faso	C	-	W/R/tan	Leaf anthracnose resistant, photoperiod insensitive, soft grains (PSI >16)
V30	IRAT 9	IL	Cameroon	C	+	R/C/R	Grain mold resistant, photoperiod insensitive
V31	IS 15401	LR	Cameroon	GC	-	W/P/R	Striga resistant
V32	Kaapelga	LR	Burkina Faso	G	-	W/P/tan	photoperiod insensitive, post-flowering drought resistant, Hard grains (PSI< 10)
V33	Kapla-57	LR	Burkina Faso	G	+	R/P/R	Sorghum midge susceptible
V34	Kokologho	LR	Burkina Faso	C	+	W/B/R	Post-flowering drought resistant
V35	90L1235	IL	USA	GC	-	W/B/R	Sorghum midge resistant
V36	Magadji 1-509	LR	Burkina Faso	GC	-	R/B/R	Photoperiod insensitive
V37	Nafo-Natogué 775	LR	Burkina Faso	G	-	R/B/R	-
V38	Nazongala tan	IL	Burkina Faso	G	-	W/B/tan	Soft grains (PSI >16)
V39	Nongomsoba	LR	Burkina Faso	G	-	W/B/tan	Soft grains (PSI >16)
V40	S 29	LR	Burkina Faso	G	-	W/R/R	Striga susceptible
V41	Sariaso 10	IL	Burkina Faso	C	-	W/R/R	Post-flowering drought resistant, photoperiod insensitive
V42	Sariaso 11	LR	Burkina Faso	G	+	W/P/tan	Sooty stripe resistant, leaf anthracnose sensitive, striga resistant, post-flowering drought resistant, photoperiod sensitive
V43	Sariaso 12	LR	Burkina Faso	G	+	W/B/R	Photoperiod insensitive, post-flowering drought resistant
V44	Sariaso 14	IL	Burkina Faso	C	-	W/P/tan	Grain mold resistant, sorghum midge resistant, post-flowering drought resistant, photoperiod insensitive, thumbtacks sensitive, logging resistant
V45	Sariaso 9	LR	Burkina Faso	G	-	W/B/R	Sooty stripe resistant, photoperiod sensitive
V46	Segaolane	IL	Botswana	C	-	W/P/R	Pre-flowering drought resistant
V47	SRN 39	IL	Sudan	C	-	Y/P/tan	Striga resistant
V48	Tiamassie 289	LR	Burkina Faso	G	+	W/B/R	-
V49	Tx 7000	IL	USA	C	-	W/P/R	Post-flowering drought susceptible
V50	Zugilga	LR	Burkina Faso	G	+	R/B/R	-

[a]Abbreviations: C, *Caudatum*; G, *Guinea*; CG, *Guinea-Caudatum*; D, *Durra*; K, *Kafir*; KC, *Kafir-Caudatum*, R, red; W, white; Y, yellow; IL, Inbred line; LR, Landrace; PSI, particle size index. Grain with (+) or without (-) pigmented testa layer. /, not known.
[b]Gen. = genetic type.

Determination of enzyme activities

PAL activity was evaluated by measuring *trans*-cinnamic acid formation from L-phenylalanine as described previously (Dicko et al., 2005b; this thesis, **Chapter 4**). Commercial sodium *trans*-cinnamate was used as standard. The spectrophotometric assay for PPO was performed as described previously (Dicko et al., 2002a; this thesis, **Chapter 2**). 4-Hydroxyanisole (4HA) and 3,4-dihydroxyphenylpropionic acid (DHPPA) were used as substrates to determine the monophenolase and *o*-diphenolase activities of PPO, respectively. The enzyme extract (10 µL) was incubated with 150 µL 50 mM sodium acetate, pH 5.5, 10 µL 40% (v/v) DMF and 10 µL 50 mM 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) at 25°C for 5 min. The reaction was started by addition of 20 µL 100 mM of the phenolic substrate (prepared in 0.15 mM phosphoric acid). The reaction was monitored at 500 nm. Blanks, in which the enzyme extracts or substrates were replaced by buffer, were performed. One unit of PPO activity (U) is defined as the amount of enzyme producing 1 µmol of MBTH-*o*-quinone-adducts per min resulting from the oxidation of 4-HA or DHPPA. Prior to determination of POX activity, extracts from ungerminated and germinated sorghums were diluted 400 and 1000 fold, respectively in 50 mM Tris-HCl, pH 7.3, containing 0.5 M $CaCl_2$ and 1 mg/mL bovine serum albumin. POX activity was measured spectrophotometrically by monitoring the H_2O_2-dependent oxidation of ABTS, at 25°C (Dicko et al., 2002, this thesis, **Chapter 2**). The reaction mixture consisted of 10 µL of 200-fold diluted crude enzyme extract, 20 µL of 100 mM ABTS, 10 µL of 100 mM H_20_2 and 160 µL of 50 mM sodium acetate, pH 4.0. Blanks, in which the enzyme extract or substrates were replaced by buffer, were performed. The reaction was monitored at 405 nm. One unit of POX activity (U) is defined as the amount of enzyme releasing 1 µmol of ABTS radical/min under the assay conditions.

Statistical analysis

Statistical analyses were carried out as described previously (Dicko et al., 2002, 2005a, this thesis, **Chapters 2** and **3**). All spectrophotometric assays were carried out in 96-well microtiter plates (Nunclon, Denmark) using a multiwell plate reader (µQuant Bio-Tek Instrument, Inc) on line interfaced with a personal computer. The absorbances and slopes of absorbances per min (OD/min) were automatically recorded using KC junior software version 1.31.5 (Bio-Tek Instrument, INC, USA). All assays were carried out at least in duplicate. Mean values, standard deviations and standard errors are reported. Significant differences in mean performance for each composition among sorghum varieties were tested by the Student's t-test, $P < 0.05$ implies

significance. Pearson linear correlation coefficients were used to assess relationships among biochemical constituents.

Table 2. Agronomic properties of sorghum varieties

Type of stress	Plant/grain agronomic properties	Variety code
Biotic	sooty stripe resistant (n=3)	V23, V42, V45
	sooty stripe susceptible (n=3)	V3, V24, V28
	anthracnose resistant (n=4)	V11, V16, V22, V29
	anthracnose susceptible (n=3)	V7, V28, V42
	sorghum midge resistant (n=4)	V16, V24, V35, V44
	sorghum midge susceptible (n=2)	V12, V33
	striga resistant (n=4)	V18, V31, V42, V47
	striga susceptible (n=3)	V13, V15, V40
	grain mold resistant (n=3)	V19, V30, V44
	grain mold susceptible (n=2)	V3, V25
Abiotic	logging resistant (n=7)	V3, V10, V16, V23, V25, V26, V44
	logging susceptible (n=2)	V27, V28
	pre-flowering drought resistant (n=3)	V1, V27, V46
	pre-flowering drought susceptible (n=1)	V3
	Post-flowering drought resistant (n=8)	V3, V23, V32, V34, V41, V42, V43, V44
	Post-flowering drought susceptible (n=4)	V4, V11, V28, V49
	photoperiod insensitive (n=14)	V5, V7, V11, V23, V25, V27, V28, V29, V30, V32, V36, V41, V43, V44
	photoperiod sensitive (n=7)	V6, V18, V20, V21, V26, V42, V45

RESULTS AND DISCUSSION

The contents of phenolic compounds and the activities of related enzymes in varieties grouped according to their agronomic properties are given in **Table 3**. Difference in composition was determined using the Student *t*-test for paired comparison between varieties resistant and varieties susceptible to specific biotic or abiotic stresses (**Table 4**).

Correlations between phenolic compounds and related enzyme and resistance or susceptibility to biotic stress

In the present study, independent of grain germination, no significant difference in total phenolic compounds content between sorghum varieties susceptible and those resistant to biotic

stresses was found. However, leaves of sorghum resistant to fungi contained a higher content of total phenolics than leaves of susceptible ones upon pathogen challenge (Luthra et al., 1988). This suggests that total phenolic compounds content in sorghum grains, which are not challenged by pathogens, is not a good indicator for the resistance to biotic stress.

When focusing on individual classes of phenolic compounds, it can be seen that PAs levels were higher on average in varieties resistant to sooty stripe, sorghum midge, and grain mold than susceptible ones. In contrast, the resistance to anthracnose and striga was not correlated with the content of PAs. The high contents of PAs in varieties resistant to mold is in line with previous observations showing that high PAs containing sorghums are generally resistant to grain molding and weathering (Waniska et al., 2001). Sorghum midge resistance has also been found to be associated with PAs contents in another study (Sharma and Hariprasad, 2002).

The contents of 3-DAs are higher in all varieties resistant to the referred biotic stresses compared to those of the susceptible ones. This indicates a function as phytoalexins (Weiergang et al., 1996; Lo et al., 1999). For example, the content of 3-DAs, notably apigeninidin is an indicator of grain resistance to fungi such as *Colletrichum graminicola*, *Fusarium oxysporum*, *Gibberelle zeae*, and *Gliocladium roseum* (Schutt and Netzly, 1991) and sorghum resistance to anthracnose (Lo et al., 1999). Colored phenolic compounds, probably 3-DAs, have been suggested to be involved in sorghum resistance to striga (Arnaud et al., 1999). Recently, the importance of phenolic compounds as allelochemicals, involved in plant-parasite interactions, were indicated (Weir et al., 2004). Our results suggest that the content of 3-DAs in sorghum grain is not only an indicator of resistance to mold, but also to sooty stripe, anthracnose, sorghum midge and striga.

The content of flavan-4-ols (apiforol and luteoforol) is on average higher in varieties resistant to mold than in susceptible ones. This is in line with previous findings showing that flavan-4-ols content is an indicator to grains resistance to molds (Schutt and Netzly, 1991; Melake-Berhan et al., 1996). Moreover, the results presented here show that independent of germination, the flavan-4-ols content was on average higher in varieties resistant to sooty stripe, sorghum midge, leaf anthracnose, and striga, than in susceptible ones.

Table 3. Phenolic compounds and phenolic enzymes in groups of sorghum varieties* resistant and susceptible to stresses.

Type of stress		Total phenolics (%)[a]	PAs (%)[b]	3-DAs (%)[c]	Flavan-4-ols (%)	PAL (mU/mg)[d]	POX (U/mg)[e]	Mono-PPO (mU/mg)[f]	Dipheno-PPO (mU/mg)[g]
	Ungerminated grains								
biotic	sooty stripe resistant (n=3)	0.79	0.22	0.06	0.18	6.2	24.5	0.9	47.3
	sooty stripe susceptible (n=3)	0.78	nd	nd	nd	2.3	20.7	0.7	43.6
	anthracnose resistant (n=4)	0.73	nd	0.02	nd	6.0	34.9	0.9	42.9
	anthracnose susceptible (n=3)	0.76	0.09	nd	nd	4.7	36.2	1.0	47.7
	sorghum midge resistant (n=4)	0.74	0.45	0.05	0.16	6.3	30.8	0.8	45.5
	sorghum midge susceptible (n=2)	0.62	0.05	nd	nd	2.7	55.1	1.0	41.4
	striga resistant (n=4)	1.06	0.39	0.05	0.24	6.7	37.6	1.1	48.1
	striga susceptible (n=3)	0.70	0.13	0.04	nd	9.0	43.2	0.9	41.8
	grain mold resistant (n=4)	1.65	1.31	0.25	0.35	4.5	21.7	0.8	48.0
	grain mold susceptible (n=2)	0.77	0.05	nd	nd	4.0	19.2	0.9	41.0
abiotic	logging resistant (n=7)	0.68	0.06	0.03	nd	5.4	29.7	0.9	46.4
	logging susceptible (n=2)	1.08	0.53	nd	nd	2.2	15.5	0.9	46.8
	pre-flowering drought resistant (n=3)	0.80	0.24	0.04	nd	nd	40.2	1.0	45.2
	pre-flowering drought susceptible (n=1)	0.72	nd	nd	nd	2.3	17.4	0.7	39.3
	Post-flowering drought resistant (n=8)	0.71	0.26	0.04	0.17	4.9	22.6	0.9	46.4
	Post-flowering drought susceptible (n=4)	0.69	0.07	nd	nd	3.2	38.1	0.7	46.0
	photoperiod insensitive (n=14)	0.82	0.23	0.05	0.23	4.5	38.6	0.9	45.2
	photoperiod sensitive (n=7)	1.03	0.44	0.04	0.22	4.6	28.6	0.9	47.6
	Germinated grains								
biotic	sooty stripe resistant (n=3)	0.70	0.14	0.04	0.12	7.06	64.3	1.3	31.8
	sooty stripe susceptible (n=3)	0.59	nd	nd	nd	6.80	50.9	0.8	22.8
	anthracnose resistant (n=4)	0.72	nd	nd	nd	3.04	89.3	0.4	16.8
	anthracnose susceptible (n=3)	0.68	0.05	nd	nd	11.81	66.6	1.0	25.9
	sorghum midge resistant (n=4)	0.73	0.34	0.07	nd	8.54	85.0	1.1	34.7
	sorghum midge susceptible (n=2)	0.89	0.06	0.02	nd	8.37	96.3	0.9	29.0
	striga resistant (n=4)	1.00	0.29	0.04	0.17	7.49	87.9	1.6	28.3
	striga susceptible (n=3)	1.02	0.17	0.05	nd	12.38	97.6	1.0	16.7
	grain mold resistant (n=4)	1.59	1.02	0.14	0.32	8.86	82.0	1.6	27.0
	grain mold susceptible (n=2)	0.71	0.05	nd	nd	6.10	89.2	0.8	20.8
abiotic	logging resistant (n=7)	0.76	0.09	0.03	nd	5.33	92.3	0.8	22.1
	logging susceptible (n=2)	0.92	0.28	nd	nd	6.47	16.0	0.7	16.1
	pre-flowering drought resistant (n=3)	0.96	0.16	0.05	nd	7.59	54.2	1.0	22.6
	pre-flowering drought susceptible (n=1)	0.69	nd	nd	nd	9.86	79.9	0.8	24.4
	Post-flowering drought resistant (n=8)	0.78	0.24	0.05	0.15	10.65	58.7	1.4	35.6
	Post-flowering drought susceptible (n=4)	0.64	0.06	nd	nd	7.71	71.8	0.8	20.1
	photoperiod insensitive (n=14)	0.84	0.17	0.03	0.20	9.39	74.0	1.1	24.2
	photoperiod sensitive (n=7)	0.96	0.32	0.04	0.16	8.04	79.4	1.1	22.5

[a]Total phenolic compounds (w/w, gallic acid equivalents), [b]PAs = proanthocyanidins (w/w, procyanidin equivalents), [c]3-DAs = 3-deoxyanthocyanidins (w/w, apigeninidin equivalents), [d]PAL= phenylalanine ammonia lyase, [e]POX= peroxidase, [f]mono-PPO = monophenolase activity of polyphenol oxidase (PPO), [g]dipheno-PPO= diphenolase activity of PPO. *Data from previous study (Dicko et al., 2005a, 2005b; this thesis, **Chapters 3** and **4**).

This suggests that the flavan-4-ols content in sorghum grain is a good indicator for plant resistance to sooty stripe, sorghum midge, and striga. For leaf anthracnose, no correlation could be made with the grain content in flavan-4-ols.

Before germination, PAL activity was higher in varieties resistant to sooty stripe and sorghum midge than in susceptible ones. However, after germination there was no difference between them. PAL activity in germinated varieties resistant to anthracnose was higher than in susceptible ones. This supports that high PAL activity in sorghum is associated to the resistance to *Colletotrichum* species (Kamida et al., 2000).

POX and PPO activities before germination could not be linked to resistance or susceptibility to biotic stresses. Germination did not affect this trend for POX. After germination, the mono-PPO activity was increased in sooty stripe resistant varieties and decreased in anthracnose resistant varieties. These results support that PPO may confer resistance to some fungal species such as *Ramulispora sorghicola* (sooty stripe agent) (Luthra et al., 1988).

Correlations between levels of phenolic compounds and related enzymes and the resistance or susceptibility to abiotic stress

Varieties resistant to logging had significantly less PAs than susceptible ones, both before and after germination. From this it may be inferred that the PAs content of the grain is a marker for plant resistance to logging. Independent to grain germination, varieties resistant to both pre-flowering and post-flowering drought contained apparently more PAs, 3-DAs, and flavan-4-ols than susceptible ones. This points at the importance of polyphenols in drought resistance.

For other plants (*Mangifera indica*), the phenolic compounds content is influenced by their response to light (Tovar et al., 2002). However, no significant difference in content of total phenolic compounds, PAs, 3-DAs and flavan-4-ols was found between the grain of sorghum varieties resistant and susceptible to photoperiod.

POX activity in ungerminated grains was not significantly different between varieties resistant and susceptible to abiotic stresses. After germination, POX activity appeared to be on average higher in varieties resistant to logging than in susceptible ones. High activity of POX in varieties resistant to logging could be related to the role of POX in formation of physical polymeric barriers such as suberin and lignin (Cosgrove, 1997; Quiroga et al., 2000), which might confer the plant a high rigidity.

Table 4. Student *t*-test results indicating level of significance in composition of phenolic compounds and related enzymes for paired comparison between sorghum varieties resistant and susceptible to stresses.

Type of stress	Group of varieties	Total phenolics	PAs[a]	3-DAs[b]	Flavan -4-ols	PAL[c]	POX[d]	Mono-PPO[e]	Di-PPO[f]
	Ungerminated grains								
biotic	sooty stripe resistant (n=3)	NS	+	+	+	+	NS	NS	NS
	sooty stripe susceptible (n=3)	NS	-	-	-	-	NS	NS	NS
	anthracnose resistant (n=4)	NS	-	+	NS	NS	NS	NS	NS
	anthracnose susceptible (n=3)	NS	+	-	NS	NS	NS	NS	NS
	sorghum midge resistant (n=4)	NS	+	+	+	+	NS	NS	NS
	sorghum midge susceptible (n=2)	NS	-	-	-	-	NS	NS	NS
	striga resistant (n=4)	NS	NS	+	+	NS	NS	NS	NS
	striga susceptible (n=3)	NS	NS	-	-	NS	NS	NS	NS
	grain mold resistant (n=4)	NS	+	+	+	NS	NS	NS	NS
	grain mold susceptible (n=2)	NS	-	-	-	NS	NS	NS	NS
abiotic	logging resistant (n=7)	NS	-	+	NS	+	NS	NS	NS
	logging susceptible (n=2)	NS	+	-	NS	-	NS	NS	NS
	pre-flowering drought resistant (n=3)	NS	+	+	NS	-	NS	NS	NS
	pre-flowering drought susceptible (n=1)	NS	-	-	NS	+	NS	NS	NS
	Post-flowering drought resistant (n=8)	NS	+	+	+	NS	NS	NS	NS
	Post-flowering drought susceptible (n=4)	NS	-	-	-	NS	NS	NS	NS
	photoperiod insensitive (n=14)	NS	NS	NS	NS	NS	NS	NS	NS
	photoperiod sensitive (n=7)	NS	NS	NS	NS	NS	NS	NS	NS
	Germinated grains								
biotic	sooty stripe resistant (n=3)	NS	+	+	+	NS	NS	+	NS
	sooty stripe susceptible (n=3)	NS	-	-	-	NS	NS	-	NS
	anthracnose resistant (n=4)	NS	-	+	NS	+	NS	-	NS
	anthracnose susceptible (n=3)	NS	+	-	NS	-	NS	+	NS
	sorghum midge resistant (n=4)	NS	+	+	NS	NS	NS	NS	NS
	sorghum midge susceptible (n=2)	NS	-	-	NS	NS	NS	NS	NS
	striga resistant (n=4)	NS	NS	+	+	NS	NS	NS	NS
	striga susceptible (n=3)	NS	NS	-	-	NS	NS	NS	NS
	grain mold resistant (n=4)	NS	+	+	+	NS	NS	NS	NS
	grain mold susceptible (n=2)	NS	-	-	-	NS	NS	NS	NS
abiotic	logging resistant (n=7)	NS	-	+	NS	NS	+	NS	NS
	logging susceptible (n=2)	NS	+	-	NS	NS	-	NS	NS
	pre-flowering drought resistant (n=3)	NS	+	+	NS	NS	NS	NS	NS
	pre-flowering drought susceptible (n=1)	NS	-	-	NS	NS	NS	NS	NS
	Post-flowering drought resistant (n=8)	NS	+	+	+	NS	NS	+	+
	Post-flowering drought susceptible (n=4)	NS	-	-	-	NS	NS	-	-
	photoperiod insensitive (n=14)	NS	NS	NS	NS	NS	NS	NS	NS
	photoperiod sensitive (n=7)	NS	NS	NS	NS	NS	NS	NS	NS

[a]PAs = proanthocyanidins, [b]3-DAs = 3-deoxyanthocyanidins, [c]PAL = phenylalanine ammonia lyase, [d]POX = peroxidase, [e]mono-PPO = monophenolase activity of polyphenol oxidase (PPO), [f]dipheno-PPO = *o*-diphenolase activity of PPO. NS, not significant ($P>0.05$); (+), significantly high ($P<0.05$); (-), significantly low ($P<0.05$).

It has been observed that specific POX isoenzymes in leaves, notably the cationic ones are correlated with photoperiod sensitivity in sorghum and play a role in the plant adaptation (Pao and

Morgan, 1988). However, in the present study the POX activity of the grain could not be linked to the plant sensibility to photoperiod.

Before grain germination, PPO activities could not be linked to abiotic stresses. After germination, both the monophenolase and *o*-diphenolase activities of PPO were higher in post-flowering drought resistant varieties than in susceptible ones. This suggests a role of PPO in post-flowering drought resistance, in agreement with earlier findings (Mayer and Harel, 1991). Independent of grain germination, PPO activities could not be related to resistance to logging and photoperiod. PAL activity in the grain cannot be used as marker for the resistance to logging, drought and photoperiod variation as well.

Overall impact of phenolic compounds and related enzymes in stress resistance

Results in **Table 4** show that content of 3-DAs is a good marker for sorghum resistance to most of both the biotic and abiotic stresses because 3-DAs are positively correlated for the resistance to all stresses except for photoperiod sensitivity. The second marker for stress resistance is the content of PAs. Total phenolic compounds and the activities of related enzymes are not good markers for stress resistance in sorghum grains. For photoperiod sensitivity, none of the screened biochemical compounds could be used as marker of resistance in sorghum grain.

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REFERENCES

Arnaud, M. C.; Véronési, C.; Thalouarn, P. Physiology and histology of resistance to *Striga hermontica* in *Sorghum bicolor* var. Framida. *Aust. J. Plant Physiol*. **1999**, *26*, 63-70.

Audilakshmi, S.; Stenhouse, J. W.; Reddy, T. P.; Prasad, M. V. R. Grain mould resistance and associated characters of sorghum genotypes. *Euphytica* **1999**, *107*, 91–103.

Choudhary, S.; Bohra, S. P.; Swarnkar, P.L. Heat shock responses in sorghum. *J. Phytological. Res.* **1993,** *6*, 39-42.

Cosgrove D. J. Assembly and enlargement of the primary cell wall in plants. *Annu. Rev. Cell Dev. Biol.* **1997**, *13,* 171-201.

Cowan, M. M.. Plants products as antimicrobial agents. *Clin. Microbiol. Rev*. **1999,** *12*, 564-582.

Cui, Y.; Magill, J.; Frederiksen, R.; Magill, C. Chalcone synthase and phenylalanine ammonia-lyase mRNA levels following exposure of sorghum seedlings to three fungal pathogens. *Physiol. Mol. Plant Pathol*. **1996**, *49*, 187-199.

Dayan, F. E.; Kagan, I. A.; Rimando, A. M. Elucidation of the biosynthetic pathway of the allelochemical sorgoleone using retrobiosynthetic NMR analysis. *J. Biol. Chem*. **2003**, *278*, 28607-28611.

Deu, M. Gonzalez de Leon, D.; Glaszman, J.C.; Degremont, I.; Chantereau, J.; Lanaud, C.; Hamon, P. RFPL diversity in cultivated sorghum in relation to racial differentiation. *Theoret. Appl. Gen.* **1994**, *88*, 838-844.

Dicko, M. H.; Gruppen. H..; Traoré, A. S.; van Berkel, W. J. H.; Voragen, A. G. J. Evaluation of the effect of germination on phenolic compounds and antioxidant activities in sorghum varieties *J. Agric. Food Chem.* **2005**, *53***,** 2581-2588.

Dicko, M. H.; Gruppen. H..; Zouzouho, O. C.; Traoré, A. S.; van Berkel, W. J. H.; Voragen, A. G. J. Effects of germination on the activities of amylases and phenolic enzymes in sorghum varieties grouped according to end-use properties. **2005b**, submitted

Dicko, M. H.; Hilhorst. R.; Gruppen, H.; Traoré, A. S.; Laane, C.; van Berkel, W. J. H.; Voragen. A. G. J. Comparison of content in Phenolic compounds. polyphenol oxidases and Peroxidases in grains of fifty sorghum varieties from Burkina Faso. *J. Agric. Food Chem.* **2002**, *50*, 3780-3788.

Kamida, H. M.; Pascholati, S. F.; Bellato, C. M. Influence of *Saccharomyces cerevisiae* in the gene expression of phenylalanine ammonia-lyase in sorghum tissue protected against *Colletotrichum sublineolum*. *Summa Phytopathologica*. **2000**, *26*, 74-77.

Lo, S. C. C.; De Verdier, K.; Nicholson, R. L. Accumulation of 3-deoxyanthocyanidin phytoalexins and resistance to *Colletotrichum sublineolum* in sorghum. *Physiol. Mol. Plant Pathol*. **1999**, 55, 263-273.

Luthra, Y. P.; Ghandi, S. K.; Joshi, U. N.; Arora, S. K. Total phenols and their oxidative enzymes in sorghum leaves resistant and susceptible to *Ramulispora sorghicola* Harris. *Acta Phytopathol. Entomol. Hungarica* **1988**, *23*, 393-400.

Mayer , A. M.; Harel, E. Phenoloxidases and their significance in fruit and vegetables. *in Food Enzymology*. Vol I; P. F. Fox Ed.; Elsevier Science publishers, London, **1991**, 373-398.

Melake-Berhan, A.; Butler. L. G.; Ejeta. G.; Menkir, A. Grain mold resistance and polyphenol accumulation in sorghum. *J. Agric. Food Chem*. **1996**, *44*, 2428-2434.

Mohammadi, M.; Kazemi, H. Changes in peroxidase and polyphenol oxidase activities in susceptible and resistant wheat heads inoculated with *Fusarium graminearum* and induced resistance. *Plant Sci.* **2002**, *162*, 491-498.

Mohan, S.; Purushothaman, D.; Jayaraj, S.; Rangarajan, A. V. Phenylalanine ammonia lyase activity in the roots of *Sorghum bicolor* (L.) inoculated with *Azospirillum*. *Cur. Sci*. **1988**, *57*, 492-3.

Neya A.; Le Normand, M. Responses of sorghum genotypes to leaf anthracnose (*Collelotrichum graminicola*) under field conditions in Burkina Faso. *Crop Prot.* **1998**, *17*, 47-53.

Pao, C. I.; Morgan, P. W. Patterns of total peroxidase activity and isozyme complement during development of selected maturity genotypes of sorghum. *Crop Science*. **1988**, *28*, 531-535.

Schutt, C.; Netzly, D. Effect of apiforol and apigeninidin on growth of selected fungi. *J. Chem. Ecology.* **1991**, *17*, 2261-2266.

Sharma, H. C.; Hariprasad, K. V. Flowering events in sorghum in relation to expression of resistance to sorghum midge, *Stenodiplosis sorghicola***.** *Euphytica* **2002**, *127*, 411-419.

Sheen, J. C4 gene expression. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1999***, 50, 187–217.*

Stafford, Helen A. Changes in phenolic compounds and related enzymes in young plants of sorghum. *Phytochemistry*. **1969**, *8*, 743-52.

Tao, Y.; Hardy, A.; Drenth, J.; Henzell, R. G.; Franzmann, B. A, Jordan, D. R; Butler, D. G.; McIntyre, C. L. Identifications of two different mechanisms for sorghum midge resistance through QTL mapping. *Theor. Appl. Genet*. **2003**, *107*, 116-22.

Tao, Y.; Manners, J. M.; Ludlow, M.M.; Henzell, R. G. DNA polymorphism in grain sorghum (*Sorghum bicolor* L. Moench). *Theoret. Appl. Gen.* **1993**, *86*, 679-688.

Tenkouano, A. Current trends in regional integration of sorghum research in Western and Central Africa. International Sorghum and Millet Newsletter ; SICNA and ICRISAT, Eds.;. **1995**, *36*, 41-44.

Tovar, M. J.; Romero, M. P.; Girona, J.; Motilva, M. J. L-phenylalanine ammonia-lyase and concentration of phenolics in developing olive (*Olea europaea* L cv Arbequina) fruit grown under different irrigation regimes. *J. Sci. Food Agric.* **2002**, *82*, 892-898.

Trouche, G. ; Da, S. ; Pale, G. ; Sohoro, A.; Ouédraogo, O. ; Den Gosso, G. Evaluation participative de nouvelles variétés de sorgho. In Sélection participative; Hocdé, H.; Lançon, J.; Trouche, G. Eds.; CIRAD, Montpellier, **2001**, pp. 36-55.

Waniska, R. D.; Venkatesha, R. T.; Chandrashekar, A.; Krishnaveni, S.; Bejosano, F. P.; Jeoung, J.; Jayaraj, J.; Muthukrishnan, S.; Liang, G. H. Antifungal proteins and other mechanisms in the control of sorghum stalk rot and grain mold**.** *J. Agric. Food Chem*. **2001**, *49*, 4732-4742

Weiergang, I.; Hipskind, J. D.; Nicholson R. L. Synthesis of 3-deoxyanthocyanidin phytoalexins in sorghum occurs independent of light. *Physiol. Mol. Plant Pathol.* **1996**, *49*, 377-388.

Weir, T. L.; Park, S. W.; Vivanco, J. M. Biochemical and physiological mechanisms mediated by allelochemicals. *Curr. Opin. Plant Biol.* **2004**, *7*, 472-479.

Weston, L. A.; Nimbal, C. I.; Jeandet, P. Allelopathic potential of grain sorghum [*Sorghum bicolor* (L.) Moench] and related species, *in* Principles and Practices in Plant Ecology: Allelochemical Interactions.; Dakshini, K. M. M.; Foy, C. L. Eds.; CRC Press, Boca Raton, Florida. **1999**, pp. 467–477.

CHAPTER 6

Zymography of peroxidase, monophenolase and *o*-diphenolase activities of polyphenol oxidase

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ABSTRACT

A new procedure for the zymography of monophenolase and *o*-diphenolase activities of polyphenol oxidase (PPO), and peroxidase (POX) is proposed using a highly sensitive, chromogenic nucleophile 3-methyl-2-benzothiazolinone hydrazone (MBTH), which traps quinones. The procedure allowed the distinction between PPO isoenzymes from sorghum and mushroom in the same gel, as well as between monophenolase and *o*-diphenolase activities of PPO isoenzymes from crude extracts of mushroom. Three isoforms were detected with monophenolase activity, and at least seven isoforms were detected with *o*-diphenolase activity. The procedure also allows the identification of PPO isoforms exhibiting monophenolase activity from a crude extract. The sensitivity, speed and ability to discriminate between mono and *o*-diphenolase activities could make the newly developed procedure a universal and powerful method for the routine zymography of PPOs and POXs in biological materials. The assay also discriminates the activities of PPOs, POXs and laccases.

Keywords: zymography, polyphenol oxidase, peroxidase, mushroom, laccase, sorghum.

INTRODUCTION

Polyphenol oxidase [monophenol, *o*-diphenol: oxygen oxidoreductase: EC 1.14.18.1, PPO] is a binuclear copper-cluster containing monooxygenase ubiquitously present in biological systems (Martinez and Whitaker, 1995; Klabunde et al., 1998). PPO may be distinguished from the related oxidase laccase [*p*-diphenol:oxygen oxidoreductase, EC 1.10.3.2] by its substrate specificity and the type and number of catalytic coppers (Timothy et al., 2001). PPO catalyses the oxidation of *o*-diphenols (*o*-diphenolase or catecholase activity) to the corresponding *o*-quinones, by consumption of molecular oxygen. It may also catalyze the regioselective *ortho*-hydroxylation of monophenols to catechols and their subsequent oxidation to *o*-quinones (monophenolase, cresolase or hydroxylase activity). The resulting quinones may undergo non-enzymatic autopolymerization or covalent heterocondensation with proteins to produce colored compounds (Klabunde et al., 1998). Peroxidases (POXs) [donor: H_2O_2 oxido-reductase, EC 1.11.1.7] are heme-binding enzymes which mediate a highly H_2O_2-dependent one electron oxidation of aromatic compounds with a very broad substrate specificity (Dunford, 1999). Some POXs have oxidase activity in the absence of exogenous hydrogen peroxide.

Browning in plant materials is associated with the enzymatic oxidation of phenolic compounds. Therefore, much interest exists in the rapid quantitation and in-electrophoresis gel detection of oxidases in plant tissues and food materials. In addition, it is known that for instance not all PPO isoforms have hydroxylase activity (Martinez and Whitaker, 1995; Klabunde et al., 1998). Hence, the identification of isoforms from crude biological extracts, possessing the monophenolase activity could be of great importance. The interest in these isoforms is not only to elucidate their physiological function but also their application as biocatalysts in the synthesis of catechols from monophenolic substrates (Espin et al., 2001).

The zymography of PPO is routinely conducted by the visualization of the oxidation products (dopachrome or melanochrome) of L-3,4-dihydroxyphenylalanine (L-DOPA), or the reaction products of the released quinones with aromatic amines such as *p*-phenylenediamine and 4-amino-*N,N'*-diethylaniline sulphate (Janusz, 1994; Rescigno et al., 1997). Current procedures of PPO zymography are time consuming, require relatively high amounts of enzyme (1-6 µg) for the activity staining, and are suitable to monitor exclusively the *o*-diphenolase activity of PPO. The zymography of PPO from mushrooms has revealed four isoforms when L-DOPA was used as a substrate, two for catechin and chlorogenic acid, while only one was detected using tyrosine and *p*-

cresol (Janusz, 1994). These results show clearly the substrate specificity of PPO and, therefore, the importance of the choice of substrate for the determination of PPO in activity staining procedures. Recently, Hoopes and Dean (2001) developed a new method of zymography of laccase and POX using 1,8-diaminonaphthalene as substrate. This assay is essentially based on detecting enzyme activity after SDS-PAGE analysis and counterstaining with Coomassie brilliant blue.

In recent years, Espín and co-workers have developed a highly sensitive, reliable and accurate spectrophotometric method for the determination of the monophenolase and *o*-diphenolase activities of PPO from fruits and vegetables (Espin et al., 1995, 1997, 1998, 1999, 2001; Espin and Wichers, 1999). In this method, the chromogenic nucleophile 3-methyl-2-benzothiazolinone hydrazone (MBTH) traps *o*-quinone products originating from the oxidation of phenolic compounds by PPO. The stable MBTH-quinones are kept in solution by addition of 2% *N,N'*-dimethyl formamide (DMF), allowing the continuously monitoring of the reaction. The most suitable substrates for the monophenolase and *o*-diphenolase activities of PPO were found to be 4-hydroxyanisole (4HA) and 3,4-dihydroxyphenylpropionic acid (DHPPA), respectively (Espín et al., 1995, 1997, 1998).

Whereas PPO activity from ungerminated grains of sorghum [*Sorghum bicolor* (L.) Moench] could be quantified spectrophotometrically with the MBTH assay, the routinely used methods of PPO zymography failed to detect the enzyme in gel, after IEF. Therefore, we have developed a new procedure for the zymography of PPO and also POX, by making use of the chemistry of the spectrophotometric assay developed by Espin and co-workers (1995, 1997, 1998). Furthermore, it is demonstrated that this method can also be used to discriminate between PPO, laccase and POX activities.

MATERIALS AND METHODS

Materials and reagents

Purified mushroom *(Agaricus bisporus)* PPO (EC 1.14.18.1, grade II, lot N° T-7755), and catalase (EC 1.11.1.6, lot N° 119F3798) were products of Sigma. Horseradish peroxidase (HRP) (EC 1.11.1.7) was obtained from Sigma (grade II, lot N° 16H9522) and from Boehringer Mannheim (grade II, lot N°12486022-85). Crude extract of PPO from mushroom fruit bodies, prepared essentially as described by Espín and co-workers (1999), was a gift of Dr. Harry J. Wichers from Agrotechnological Research Institute (ATO-BV), Wageningen, The Netherlands. Ungerminated grains of *S. bicolor* (L.) Moench var. Cauga 108-15, were kindly provided by the Centre

International pour la Recherche Agronomique et de Développement at Ouagadougou, Burkina-Faso. Crude extract of sorghum PPO was prepared by mixing 250 mg of sorghum flour with 1.2 mL of 50 mM Tris-HCl buffer pH 7.3 containing 0.5 M $CaCl_2$ and 2% (w/v) insoluble polyvinylpyrrolidone (PVP), at 4°C for 1 hour. The homogenate was centrifuged at 14000g, at 4°C, for 45 min. The supernatant was used as crude extract of PPO. IEF gels (pH 3-9) were purchased from Amersham Pharmacia Biotech. 4-Hydroxyanisole (4HA) was from Aldrich. 3,4-Dihydroxyphenylpropionic acid (DHPPA) was from Acros Organics. Bovine serum albumin and 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) were from Sigma. All other chemicals were of analytical grade.

Spectrophotometric assay

The monophenolase and *o*-diphenolase activities of PPO were determined using 4HA and DHPPA as substrate, respectively, as described previously by Espín and co-workers (1995, 1998). The reactions were monitored with a spectrophotometer multiwell plate reader SLT340 ATTC (Labinstruments, Australia) on-line interfaced to a Macintosh Performa 450 computer. Data were analyzed with the software DelatSoftII version 4.1S (Biometallic, Inc.). One unit of enzyme activity (U) is defined as the amount of enzyme producing 1 µmol of MBTH-quinone adducts per min during the linear phase of the reaction. POX content was determined using a molar absorption coefficient of 100 mM^{-1} cm^{-1} at the Soret region (403 nm). Protein content was determined by the linearized method of Bradford (Zor and Selinder, 1996), using the ratio A_{620}/A_{450} versus protein concentration. Bovine serum albumin was used as standard.

Zymography

Aliquots of enzyme preparations were applied on pH 3-9 IEF gradient gels. The IEF was carried out with a PhastSystem unit (Amersham Pharmacia Biotech) according to the manufacture's instructions. For PPO detection, gels were incubated after IEF in 20 mL of 0.1 M sodium acetate pH 6.0 containing 10 mM MBTH and 10 mM of the phenolic substrate, at 25°C, for 3-5 min. 4HA and DHPPA were used as phenolic substrates for the detection of the monophenolase and *o*-diphenolase activities of PPO, respectively. To check for possible oxidation of 4HA and DHPPA by laccase, the PPO above was replaced by laccase, for 30 min and up to 12 hours of incubation. For the detection of hydrogen peroxide-independent POX oxidation, gels were incubated after IEF in 20 mL of 50 mM sodium citrate buffer pH 6.0 containing 10 mM MBTH, 10 mM of phenolic substrate, and 50

U/mL of catalase. For the detection of hydrogen peroxide-dependent POX oxidation, staining was as above with catalase replaced by 5 mM hydrogen peroxide. Note that the choice of the incubation pH was guided by the fact that in the absence of DMF, the MBTH is poorly soluble above pH 6. Citrate buffer was preferred for POX activity staining because, it is known to be a potential inhibitor of copper oxidases. DMF, previously used in the spectrophotometric assay (Espín et al., 1995, 1997, 1998), was omitted to allow red spots of the precipitate of the reaction products of MBTH with quinones of 4HA and DHPPA to appear. After incubation, the gels were washed for 3-5 min with 20 mL of 40 % DMF to reduce the eventual background color, twice (3-5 min) with 20 mL distilled water and dried at room temperature.

RESULTS AND DISCUSSION

The monophenolase and *o*-diphenolase activities of PPO from sorghum (p*I* 9) could be easily detected after IEF of crude extracts using 4HA and DHPPA as substrates, respectively (**Figures 1A** and **1B**). A short incubation time (3-5 min) was enough to detect the enzyme. Prolonged incubation did not increase the intensity of the bands, possibly because the PPO is inhibited by the quinoids (Rescigno et al., 1997). The bands were not water-extractable and no protein diffusion was observed. **Figure 1C** shows that the method allows to distinguish PPO isoenzymes from sorghum (p*I* 9) and mushroom (p*I* 4.3) in the same gel. **Figure 2** shows that the assay can distinguish the monophenolase and *o*-diphenolase activities of PPO isoenzymes from crude extract of mushroom. Indeed, three isoforms (a, b and c) were detected with monophenolase activity (**Figure 2A**) and at least seven isoforms (a, b, c, d, e, f and g) with *o*-diphenolase activity (**Figure 2B**). The low intensity of bands b, c, d, e, f, and g denotes the low abundance of theses isoforms in the crude extract of mushroom. Thus, this method allows identifying directly from a crude extract PPO isoforms exhibiting monophenolase activity. All detected mushroom PPO isoforms are anionic with a p*I* ranging from 4 to 5.5. The most abundant isoenzyme (a) having the lowest p*I* 4.3, corresponds to the commercially available PPO from mushroom (p*I* 4.1-4.3) (Espín et al., 1999).

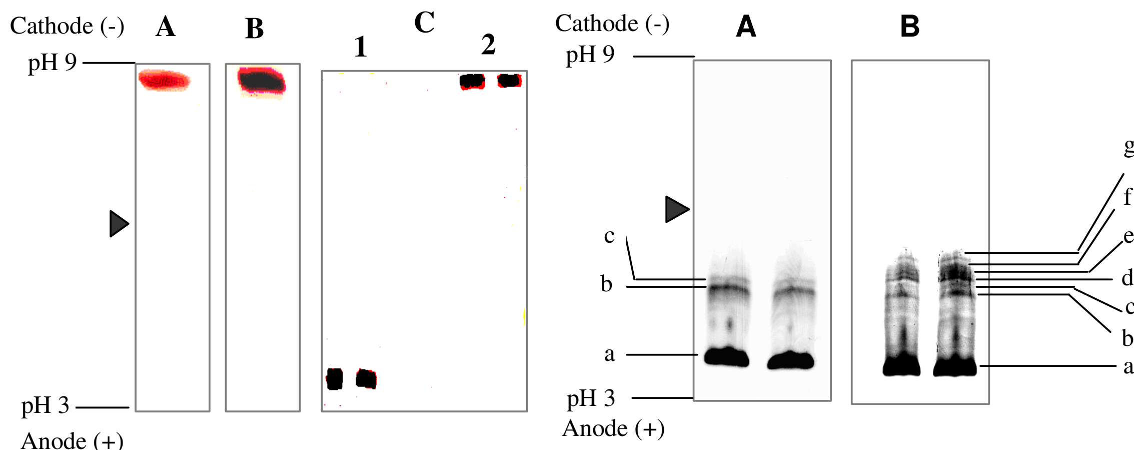

Figure 1. Zymogram of PPO. A and B, detection of the monophenolase and *o*-diphenolase activities of sorghum PPO, respectively. Note that after IEF, the gel was split into two parts, and the pieces were incubated separately for the detection of the monophenolase (A) and *o*-diphenolase (B) activities of sorghum, in the presence of MBTH. C, detection of the *o*-diphenolase activity (samples were applied in duplicate) of commercial mushroom (Lane C1) and sorghum (Lane C2) PPO isoenzymes in the same electrophoretic gel. Prior to IEF, the crude extract of PPO from sorghum (3.8 µg) and commercial mushroom (0.2 µg) were loaded (4 µL) on the gel using the PhastSystem sample applicator 6/4. The samples were applied in the middle of the gels (►).

Figure 2. Zymogram of the monophenolase (A) and *o*-diphenolase (B) activities of crude extract of mushroom. Prior to IEF, the crude extract of PPO from mushroom (1.8 µg) were loaded (4 µL) on the gel using the PhastSystem sample applicator 6/4. After IEF, the gel was split into two parts, and the pieces were incubated separately for the detection of the monophenolase (A) and o-diphenolase (B) activities, in the presence of MBTH. Two sample applications were performed for each detection. The samples were applied in the middle of the gels (►). Letters a-g indicate the detected PPO isoenzymes.

To assess the sensitivity of the method, commercial PPO was subjected to IEF at different concentrations. **Figure 3** shows a typical zymogram using either 4HA (**Figure 3A**) or DHPPA (**Figure 3B**) as substrate. The sensitivity limit of the assay for the monophenolase activity of commercial PPO is about 0.1 µg (**Figure 3A**). However, an amount of PPO as low as 0.05 µg could be detected for the *o*-diphenolase activity (**Figure 3B**). The higher sensitivity of the *o*-diphenolase activity is in agreement with the spectrophotometric assay revealing a specific activity 3.02 ± 0.11 U/mg and 0.62 ± 0.03 U/mg for the *o*-diphenolase and monophenolase activities of PPO, respectively. The rate of the oxidation of *o*-diphenols is higher than that of monophenols because the hydroxylation step is generally admitted to be rate limiting (Rodríguez-López et al., 1992; Martinez and Whitaker, 1995).

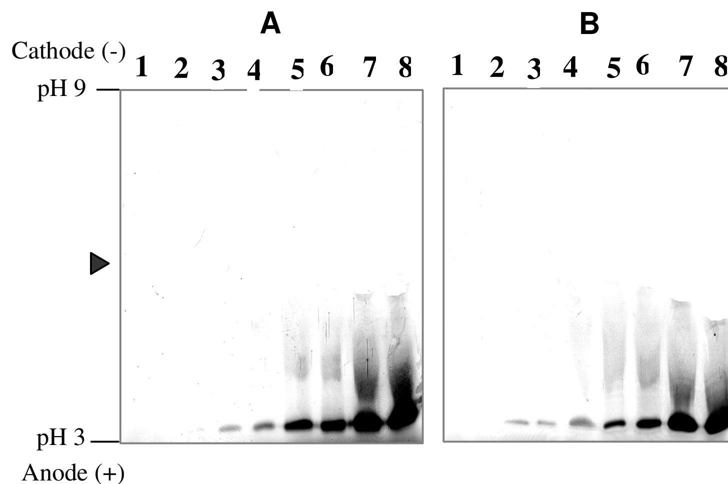

Figure 3. Zymogram of the monophenolase (A) and *o*-diphenolase (B) activities of commercial mushroom PPO. Dilutions of PPO were made in 50 mM Na-phosphate buffer pH 6.5 and aliquots of 1 µL of the enzyme solution were loaded on the gel using the PhastSystem sample applicator 8/1. Lane 1, bovine serum albumin (30 µg) used as the inert control. Lanes 2-8 represent the amounts of PPO of 0.05, 0.1, 0.25, 0.5, 1, 3, 6 µg, respectively, applied on the gel. After IEF, the gels were incubated separately for the enzymatic oxidation of 4HA (A) and DHPPA (B), in the presence of MBTH. Samples were applied in the middle of the gels (►).

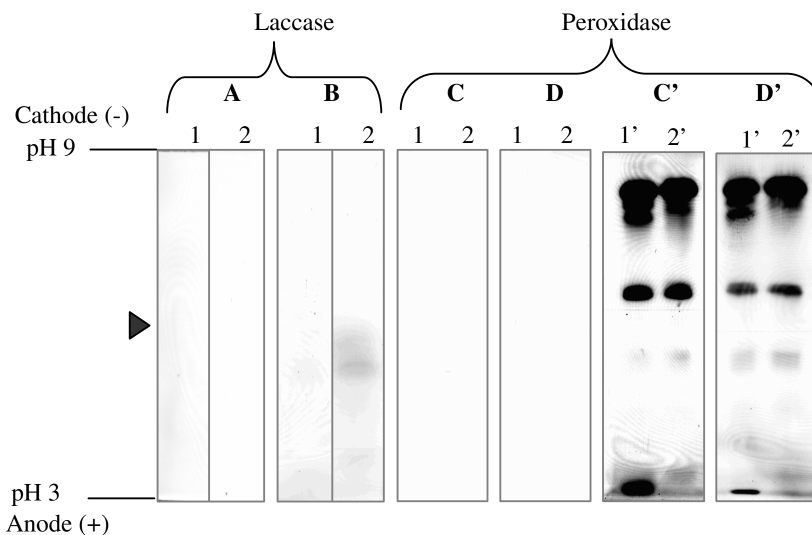

Figure 4. Zymogram of laccase (A and B) and peroxidase (C, D, C' and D') using 4HA and DHPPA as substrates. A and B, detection of laccase activity (1 µg was loaded in each well) in the presence of 4HA and DHPPA, respectively, after 30 min (lanes A1 and B1) and 12 hours (lanes A2 and B2) of incubation (in the presence of MBTH). C and D, detection of POX activity (10 ng were loaded in each well) using 4HA and DHPPA, respectively, as substrates in the presence of catalase (50 U/mL) and MBTH for 3-5 min. Lanes C1 and D1 contained HRP from Sigma and lanes C2 and D2 contained HRP from Boehringer. C' and D', are the same conditions like in C and D, respectively, except that catalase was replaced with 5 mM H_2O_2. Enzymes (1 µL) were loaded on the gels using the PhastSystem sample applicator 8/1. Samples were applied in the middle of the gels (►).

Figures 4A (lane 1) and **4B** (lane 1) show that both 4HA and DHPPA are not readily oxidized by laccase in conditions where they are oxidized by PPO. After prolonged incubation time (12 hours, at 25 °C) laccase slightly oxidized DHPPA (**Figure 4B**, lane 2, p*I* 5.2-5.4) without noticeable oxidation of 4HA. These results are in agreement with the fact that laccases are more specific for *p*-diphenols and aromatic amines and suggest that they do not interfere markedly with the detection of PPO.

Figures 4C and **4D** show that in the absence of hydrogen peroxide, POX is not able to oxidize neither 4HA nor DHPPA. However, in the presence of hydrogen peroxide POX activity was sensitively detected with the newly developed MBTH zymography method (**Figure 4C'** and **4D'**). Indeed, several cationic, neutral and anionic HRP isoenzymes could be clearly detected loading only 10 ng of protein (**Figure 4C'** and **4D'**). To determine the lowest limit of POX detection, various amounts of POX were analyzed by MBTH zymography (**Figures 5A** and **5B**). In this way, POX could be detected in the nanogram range using both 4HA and DHPPA as hydrogen donor. Thus, the zymography of POX is up to 50 times more sensitive than that of PPO using 4HA or DHPPA as substrate.

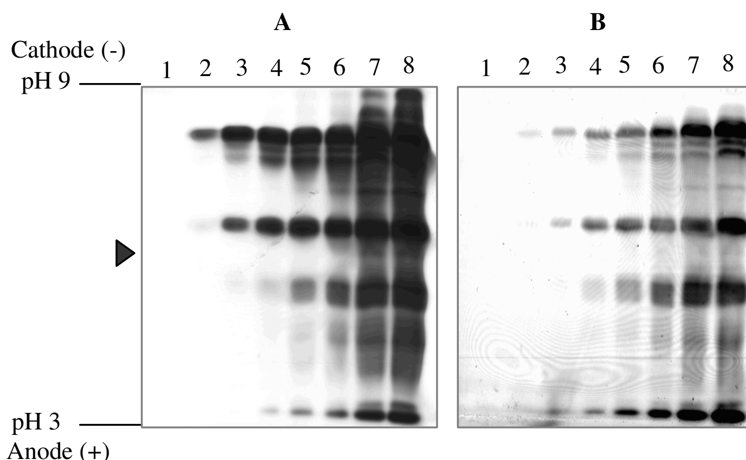

Figure 5. Zymogram of the H_20_2-dependent POX oxidation of 4HA (A) and DHPPA (B). Dilutions of POX (HRP from sigma) were performed in 50 mM Na-citrate buffer pH 6 and aliquots of 1 µL of the enzyme were loaded on the gel using the PhastSystem sample applicator 8/1. Lane 1, bovine serum albumin (30 µg) used as the inert control. Lanes 2-8 represent the amounts of POX of 1, 4, 8, 16, 30, 100 and 200 ng, respectively, applied on the gel. After IEF, the gels were incubated separately for the H_2O_2-dependent oxidation of 4HA (A) and DHPPA (B), in the presence of MBTH for 3-5 min. Samples were applied in the middle of the gels (►).

The detection limit of our method is lower than the current methods of PPO and POX zymography (Rescigno et al., 1997; Hoopes and Dean, 2001), which require 1-6 μg of enzyme for the activity staining in native polyacrylamide electrophoresis gels. The sensitivity, speed and ability to discriminate between mono and *o*-diphenolase activities could make the newly developed procedure an universal and powerful method for the routine zymography of PPO in biological materials. Aside from this, the MBTH zymography method can also be used for the specific and sensitive detection of POX isoforms.

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REFERENCES

Dunford, H. B. *Heme peroxidase*, John Willey.; Sons Inc.; New York. **1999.**

Espín, J. C.; Wichers, J. H. Slow-binding inhibition of mushroom (*Agaricus bisporus*) tyrosinase isoforms by tropolone. *J. Agric. Food Chem.* **1999**, *47*, 2638-2644.

Espín, J. C.; Morales, M.; García-Ruiz, P. A.; Tudela, J.; García-Cánovas, F. Improvement of a spectrophotometric method for determining the monophenolase and diphenolase activities of mushroom polyphenol oxidase. *J. Agric. Food Chem.* **1997**, *45*, 1084-1090.

Espín, J. C.; Morales, M.; Varón, R.; Tudela, J.; García-Cánovas, F. A continuous spectrophotometric method for determining the monophenolase and diphenolase activities of apple polyphenol oxidase. *Anal. Biochem.* **1995**, *231*, 237-246.

Espin, J. C.; Soler-Rivas, C.; Cantos, E.; Tomas-Barberan, F. A.; Wichers, H. J. Synthesis of the antioxidant hydroxytyrosol using tyrosinase as biocatalyst. *J. Agric. Food Chem.* **2001**, *49*, 1187-1193.

Espín, J. C.; Tudela, J.; Garcia-Cánovas, F. 4-hydroxyanisole and the most suitable monophenolic substrate for determining spectrophotometrically the monophenolase activity of polyphenol oxidase. *Anal. Biochem.* **1998**, *259*, 118-126.

Espín, J. C.; Van Leeuwen, J.; Wichers, H. J. Kinetic study of the activation process of a latent mushroom (*Agaricus bisporus*) tyrosinase by serine proteases. *J. Agric. Food Chem.* **1999**, *47*, 3509-3517.

Hoopes, J. T.; Dean, J. F. D. Staining electrophoretic gels for laccase and peroxidase activity using 1,8-diaminonaphthalene. *Anal. Biochem.* **2001**, *293*, 96-101.

Janusz, C. Substrate specificity.; inhibitors of polyphenol oxidase in aspect of darkening of fresh and frozen mushrooms (*Agaricus bisporus* (Lange) Sing.). *Acta Agrobotanica.* **1994**, *47*, 103-110.

Klabunde, T.; Eicken, C.; Sacchettini, J. C.; Krebs, B. Crystal structure of a plant catechol oxidase containing a dicopper center. *Nature Struct. Biol.* **1998**, *5*, 1084-1090.

Martinez, M. V.; Whitaker, J. R. The biochemistry and control of enzymatic browning. *Trends Food Sci. Technol.* **1995**, *6*, 195-200.

Rescigno, A.; Sollai, F.; Rinaldi.; A. C.; Soddu, G.; Sanjust, E. Polyphenol oxidase activity staining in polyacrylamide electrophoresis gels. *J. Biochem. Biophys. Methods* **1997**, *34*, 155-159.

Rodríguez-López, J. N.; Tudela, J.; Varón, R.; García-Carmona, F.; García-Cánovas, F. Analysis of a kinetic model for melanin biosynthesis pathway. *J. Biol. Chem.* **1992**, *267*, 3801-3810.

Timothy, E. M.; Quintanar, L.; Palmer, A. E.; Hasset, R.; Severance, S.; Kosman, D. J.; Solomon, E. I. Spectroscopy and reactivity of the type 1 copper site in Fet3p from S*accharomyces cerevisiae*: correlation of structure with reactivity in the multicopper oxidases. *J. Am. Chem. Soc.* **2001**, *123*, 5507-5517.

Zor, T.; Selinger, Z. Linearization of the Bradford protein assay increases its sensitivity: theoretical.; experimental studies. *Anal. Biochem.* **1996**, *236*, 302-308.

CHAPTER 7

Biochemical characterization of the major sorghum grain peroxidase

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(Submitted for publication)

ABSTRACT

The major cationic peroxidase (SPC4) in sorghum [Sorghum bicolor (L.) Moench] was purified to apparent homogeneity, and found to be a highly basic protein (p*I*~11), containing a type-b heme. MS analysis showed that SPC4 consists of two glycoforms with molecular masses of 34227 and 35629 Da. Chemical deglycosylation allowed to estimate sugar contents of 3.0% and 6.7% (w/w) in glycoform I and II, respectively, and a mass of the apoprotein of 33 246 Da. SPC4 has a relatively low sugar content compared to other peroxidases. High performance anion exchange chromatography (HPAEC) revealed the presence of mannose, xylose, fucose and *N*-acetylglucosamine as constituents of the polysaccharide chains. Combination of MS and HPAEC analyses showed that the proportions of glycoforms I and II are 35 and 65%, respectively. SPC4 is localized in the chromosome 1 of sorghum, and its primary structure has a high similarity with other cereal peroxidases. SPC4 was activated by calcium ions. Ca^{2+} binding increased the protein conformational stability by raising the melting temperature (T_m) from 67 to 82°C. SPC4 catalyzed the oxidation of a wide range of aromatic substrates, being catalytically more efficient with hydroxycinnamates than with tyrosine derivatives. SPC4 displayed indole-3-acetic acid oxidase activity suggesting a physiological role in auxin metabolism.

Keywords: sorghum, peroxidase, heme, glycoprotein, glycoform, isoenzyme, matrix-assisted laser-desorption ionization time-of-flight mass spectrometry.

INTRODUCTION

Plant secretory peroxidases [donor: H_20_2 oxidoreductase, EC 1.11.1.7, POXs] are class III peroxidases that contain a non-covalently bound Fe^{III}-protoporphyrin-IX. They catalyze the conversion of a large number of substrates, notably phenolic compounds for biosynthetic and catabolic functions. In general, they use hydrogen peroxide as electron acceptor (Dunford, 1999). Multigene families of POXs exist, and in the genomes of rice (*Oryzae sativa*) and thale cress (*Arabidopsis thaliania*) up to 138 and 73 of POX genes, respectively, were discovered (Duroux and Welinder, 2003; Passardi et al., 2004). The physiological functions of POXs are associated with defence mechanisms, auxin metabolism and the biosynthesis of cell-wall polymers such as lignin and suberin (Christensen et al., 1998; Dunford, 1999; Boerjan et al., 2003).

Most POXs are glycoproteins occurring in different glycoforms, which may contain different glycan chains (Christensen et al., 1998). For instance, barley POX (BP1) consists of two forms; one glycosylated at Asn300 (BP1a) and the other (BP1b) non-glycosylated (Johansson et al., 1992; Henriksen et al., 1998). The major glycan chain in BP1a represents 70% of the total carbohydrate content and has as structure Manα1-6(Xylα1-2)Manα1-4GlcNAcα1-4(Fucα1-3)GlcNAc (Johansson et al., 1992). Next to iron, Ca^{2+} is an important metal cofactor of POX. Ca^{2+} ions play both a structural and catalytic role in class-III POXs, and their removal decreased the enzyme activity and stability (van Huystee et al., 2004).

Cereal POXs *hitherto* characterized are from barley (Johansson et al., 1992), wheat (Hertig et al., 1991), rice (Chittor et al., 1997) and maize (Teichmann et al., 1997). All these enzymes are monomers with molecular masses ranging from 35-40 kDa. The crystal structure of BP1, with two domains and four disulfide bridges (C18-C99, C51-C56, C106-C301 and C186-C213) is highly similar to the structure of the archetypical horseradish peroxidase (Henriksen et al., 1998).

Relatively little is known about the structure and properties of POX from sorghum [*Sorghum bicolor* (L.) Moench]. Sorghum is the fifth most important cereal crop in the world after wheat, rice, maize, and barley. Properties of a crude sorghum POX preparation such as p*I* (9-10) and molecular mass (43 kDa) have been reported (Sae et al., 1971). However, until now no sorghum grain POX has been purified to homogeneity and characterized. When screening for POX activity in the seeds of fifty sorghum varieties originating from different parts of the world, the cationic POX was ubiquitously present in all varieties. It was also the most abundant isoenzyme in both ungerminated and germinated sorghum grains (Dicko et al., 2002, 2005; this thesis, **Chapters 2** and **4**). In other

cereals, the cationic isoenzymes are also the most abundant enzymes and account for more than 80% of total POX activity (Johansson et al., 1992; Converso and Fernandez, 1995).

In recent years, it has been shown that cationic POXs are more active with phenolic compounds than anionic POXs and laccases (Wallace and Fry, 1999). Thus, cationic POXs may be of interest for biocatalytic applications such as the production of useful polymers, the treatment of wastewater streams polluted with toxic aromatic compounds, and various other clinical and biotechnological applications (Regalado et al., 2004). Cationic POXs may also find interest in food biotechnology by modification of functional properties of food proteins and carbohydrates (Oudgenoeg et al., 2001; Boeriu et al., 2004). In this study, we have purified and characterized the cationic POX isoenzyme from sorghum grain.

MATERIALS AND METHODS

Chemicals

Horseradish peroxidase [HRP, EC 1.11.1.7] (grade II, lot N° 16H9522), *p*-coumaric acid, ferulic acids, L-tyrosine, and indole-3-acetic acid were from Sigma-Aldrich. Modified trypsin [EC 3.4.21.4] sequencing grade was from Roche. Electrophoresis gels (IEF, pH 3-9) were purchased from Amersham Biosciences. *N*-acetyl tyrosine, N-Acetyl tyrosine methyl ester and Gly-Tyr-Gly were from Bachem, Bubendorf, Switzerland. SDS-PAGE gradient gels (10-18%) were from Biorad. Immobilon-P transfer membrane was from Millipore. Maltodextrin MD05 standards were obtained from Spreda (Burghof, Switzerland). Trifluoromethanesulphonic acid was from Aldrich. Hydrogen peroxide was from Merck. All other chemicals were of analytical grade.

Enzyme purification

The grains of sorghum variety [*Sorghum bicolor* (L.) Moench var. Cauga 108-15] grown in 1998 were used (Dicko et al., 2002a, this thesis, **Chapter 2**). POX isoenzymes were extracted from flour as described previously (Dicko et al., 2002a, 2005b; this thesis, **Chapters 2** and **4**). Protein precipitation was performed with slow addition of acetone (-30°C) to the crude extract, followed by centrifugation (10 000 g, 30 min). The precipitate obtained between 40-80% (v/v) acetone was resuspended in the extraction buffer and dialyzed overnight against 20 mM Bis-Tris-Cl, pH 7.0, containing 1 mM $CaCl_2$ (starting buffer), at 4°C. Insoluble material was removed by centrifugation (15 000 g, 45 min, 4°C).

Subsequent chromatography steps were performed at room temperature (20-22°C). Protein eluates were monitored at wavelengths of 280 nm and 403 nm. Reinheitszahl (RZ) values (A_{403}/A_{280}) were calculated directly from the chromatograms (Christensen et al., 1998). The supernatant (150 mL) obtained after acetone precipitation and subsequent dialysis was loaded onto a Superdex 75-PG gel filtration column (65 x 15 cm) equilibrated with starting buffer. Proteins were eluted at a flow rate of 25 mL/min. Fractions containing POX activities were pooled and loaded onto a Resource-Q column (Source™ 15Q, 1 mL, Amersham Biosciences; flow rate 2 mL/min) equilibrated with starting buffer. After washing with 10 mL of starting buffer, elution was performed with a 10 mL linear gradient of 0 to 0.5 M NaCl in starting buffer. Fractions containing cationic POX isoenzymes were concentrated by ultrafiltration with a Y10 membrane (Amicon) and equilibrated in 50 mM sodium acetate, pH 6.0. The protein sample was applied onto a Mono-S cation-exchange column (HR 5/5, 5 x 50 mm, Amersham Biosciences; flow rate 1 mL/min), equilibrated in the same buffer. After washing with 10 mL of equilibration buffer, the POX isoenzymes were eluted with a gradient of 0 to 1 M NaCl in 50 mM sodium acetate, pH 6.0. The enzyme fractions were pooled and frozen in liquid nitrogen and stored at -80°C. To estimate the apparent molecular mass of the enzyme by molecular sieving, an analytical Superdex 75 column (HR 60 x 16 mm, Amersham Biosciences) was calibrated using blue dextran (V_o = 8.42 mL), bovine serum albumin (67 kDa, V_e = 10.71 mL), ovalbumin (43 kDa, V_e = 12.86 mL), chymotrypsin A (25 kDa, V_e = 13.79 mL), and ribonuclease A (13.7 kDa, V_e = 15.21 mL). The column was running in 50 mM sodium acetate pH 5.0, containing 155 mM NaCl. Reference proteins and the purified POX were loaded (100 µL) and eluted with the same buffer at 0.5 mL/min.

Primary structure analysis

Total protein was quantified by the linearized method of Bradford adapted to a microtiter plate (Dicko et al., 2002a, this thesis, **Chapter 2**) using BSA as standard. Protein concentration of pure enzyme was determined from the absorbance at the Soret region using a value of ε_{403} = 104 $mM^{-1}cm^{-1}$. SDS-PAGE was performed with 10-18% gradient gels. SDS-PAGE gels were calibrated using a low-molecular weight marker kit (Amersham Biosciences). Protein bands were stained with Coomassie brilliant blue R250. Isoelectrofocusing and zymography were performed as described previously (Dicko et al., 2002b; this thesis, **Chapter 6**).

Amino acid composition was performed on a Biochrom amino acid analyzer (Amersham Biosciences). Proteins were hydrolyzed in conditions allowing cysteine determination, essentially as

described previously (Christensen et al., 1998). Protein samples were hydrolyzed in 6 N HCl, 0.05 % (v/v) phenol, 0.1% 3,3'-dithiodipropanoic acid in nitrogen atmosphere, at 110°C, for 22h. Tryptophan content was estimated by a fluorimetric method (Pajot, 1976). Theoretical isoelectric point values were estimated using the software developed by Bjellqvist and co-workers (1993) available at http://expasy.ch/tools/#primary.

Prior to N-terminal microsequencing according to automated Edman degradation, the enzyme was separated by SDS-PAGE, using a 10-20% gradient gel and blotted onto poly(vinylidene difluoride) membrane according to Matsudaira (1987). The protein band was excised and N-terminal amino-acid sequencing of the polypeptide was carried out on an automated amino acid sequencer (Institute for Biomembranes Sequence Center, University of Utrecht, Utrecht, The Netherlands). In order to obtain the full sequence of the enzyme, the N-terminal sequence was used as input for tBLASTn searches (Altschul et al., 1997) within different databases. Sequence alignments of cereal POXs were performed using Clustal W (Thomson et al., 1994) available at the European Bioinformatic Institute (http://www.ebi.ac.uk/).

Determination of carbohydrate composition

The carbohydrate composition of SPC4 was determined by hydrolysis in 2M trifluoroacetic acid at 100ºC for 2h. After hydrolysis, the sample was dried, then dissolved in water, centrifuged (10 000g, 10 min), and analyzed by high performance anion exchange chromatography (HPAEC). The HPAEC system consisted of a Dionex Bio-LC GPM-II quaternary module (Dionex Corporation, Sunnyvale, CA, USA) system, equipped with an eluent degas (He) module, a CarboPac PA10 column (4 x 250 mm) with CarboPac PA100 guard column and a pulsed electrochemical detector (PED-2) in the pulsed amperometric detection (PAD) mode. A Spectra Physics AS3000 autosampler was used and chromatograms were recorded using PC1000 software. The effluent was monitored using the PED-2 detector (reference electrode Ag/AgCl) containing a gold electrode. Potentials of E1 0.1, E2 0.7 and E3 -0.1 were applied for duration times of T1 0.4 s, T2 0.2 s and T3 0.4 s. The gradient (1 mL/min) was obtained by mixing Millipore water, 0.1 M NaOH and 1 M sodium acetate in 0.1 M NaOH. After equilibration with Millipore water, 20 µl of the sample was injected and the elution program was 0→40 min, Millipore water isocratic, followed by regeneration: 40→70 min, 0→1 M sodium acetate; 70→75 min, 1 M sodium acetate; 75→76 min, Millipore water; 76→96 min, re-equilibration using Millipore water isocratic. The post-column eluent was 2.5 M NaOH eluted at a flow rate of 0.5 mL/min. Standard carbohydrates used were

D-glucose, D-galactose, D-mannose, D-ribose, D-xylose, L-fucose, L-arabinose, L-rhamnose, D-fructose, D-mannosamine, *N*-acetyl-D-mannosamine, D-glucosamine, *N*-acetyl-D-glucosamine, D-galactosamine and *N*-acetyl-D-galactosamine. Prior to HPAEC analysis, standards were subjected to trifluoroacetic acid hydrolysis using the same conditions as with the protein sample.

Chemical deglycosylation of the enzyme was performed by incubating the enzyme with a mixture of trifluoromethane sulfonic acid and anisole (Christensen et al., 1998) for 5h at room temperature. Horseradish peroxidase (HRP) was used as positive control. After deglycosylation, chemicals were removed by thoroughly washing the proteins with water using a Centricon-3 device (3 kDa molecular sieve, Millipore Corp, The Netherlands). The deglycosylated protein samples were kept at -20°C prior to mass spectrometry analysis.

Mass spectrometry

MALDI-TOF-MS was performed with a Voyager-DE-RP Biospectrometry Workstation elite reflectron time of flight mass spectrometer (PerSeptive Biosystems, Inc., Framingham, Manchester, England) with a delayed extraction MALDI ion source. Between 100 and 256 scans were averaged for each of the spectra shown. Samples were deposited in non-welled gold plates.

The mass of the heme cofactor of sorghum POX was analyzed by using 3,5-dihydroxybenzoic acid saturated in methanol/water (60/40) as matrix. MALDI-TOF-MS was performed using maltodextrins (MDO5) for calibration as external standard as described previously (Dicko et al., 2001).

Intact and deglycosylated protein samples (5 µL) were cleaned up using a ZipTip$_{C4}$ reverse phase microcolumn according to the manufacturer's instructions (Millipore Corp, The Netherlands) and mixed (1:1, v/v) with the MALDI-TOF-MS matrix. The matrix used for intact protein analysis was a freshly prepared solution of 10 mg/mL sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) in aqueous 30% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. Intact protein detection was performed in the linear mode. External calibrations under identical conditions were performed according to the manufacturer's description using bradykinin (1060.56 Da), ACTH-1-17 (2093.08 Da), insulin (5734.59 Da), ribonuclease A (13682.30 Da), apomyoglobin (16952.56 Da) and β-lactoglobulin B (18278.20 Da) as standards for the MALDI-TOF-MS analysis.

In-gel tryptic digestion was performed as described previously (Schevchenko et al, 1996). Prior to MALDI-TOF-MS analysis, the peptide samples were cleaned using a ZipTipC$_{18}$ (Millipore Corp, The Netherlands) reverse phase microcolumn. A freshly prepared α-cyano-4-hydroxy-*trans*-

cinnamic acid at 20 mg/mL in 50% aqueous acetonitrile containing 0.1% (v/v) trifluoroacetic acid was used as a matrix for peptide analysis. Peptide mass spectra were acquired in the reflector mode with delayed extraction. The above mentioned external calibrations for proteins were also used for peptides. Mass spectrometry database searching and prediction of tryptic cleavage sites were performed with the MS-fit and MS-digest programs, respectively (http://prospector.ucsf.edu). Restrictions for database searches were made according to Kristensen and co-workers (1999).

Spectral properties

Absorption spectra were recorded on a Hewlett-Packard 8453A diode array spectrophotometer at 25°C. The effect of sodium azide on the spectral properties of the enzyme was determined by incubating the native enzyme (4.4 µM) with 0-500 µM sodium azide in 50 mM sodium acetate pH 5.0. The heme cofactor of the enzyme was extracted with butanone adjusted to pH 2.0 with acetic acid. The UV-spectrum of the heme was recorded in the extraction solution. Fluorescence emission spectra were recorded on a Cary Eclipse fluorimeter (VARIAN, Australia). Ca^{2+} binding of the enzyme was studied at 25°C by monitoring the intrinsic tryptophan fluorescence of the enzyme (2.2 µM) in 10 mM sodium acetate pH 5.0 as a function of $CaCl_2$ concentration (0-10 mM). The excitation wavelength was 295 nm. The fluorescence titration data were analyzed as described by Leskovac (2003), assuming a single non-structural Ca^{2+} binding site.

Far-UV circular dichroism (CD) spectra of SPC4 (3.9 µM) were recorded in 10 mM sodium acetate pH 5.0, with a Jasco J-715 spectropolarimeter (Jasco Corp., Japan) at 20°C. A quartz cell with an optical path length of 1 mm was used. The CD intensity is expressed as molar ellipticity [θ], in deg cm $dmol^{-1}$. The secondary structure of the enzyme was analyzed as described by Venyaminor et al. (1993). The effect of Ca^{2+} binding on the protein secondary structure was determined by recording CD spectra in the presence of increasing concentrations of $CaCl_2$ (0 to 10 mM).

Thermal stability

Thermal stability was studied by incubating SPC4 in the absence or presence of 5 mM $CaCl_2$ at temperatures ranging from 40 to 95°C. Enzyme aliquots (0.27 µM) in 50 mM sodium acetate pH 5.0 were heated in a thermocycler (Amplitron II Thermolyne; temperature accuracy ± 0.1°C) at indicated temperatures for the times specified. The time needed for the temperature to reach equilibrium was less than 15 s. Following heating, samples were immediately cooled on ice and the residual enzyme activity was determined under standard assay conditions. For each

incubation temperature, control samples with or without Ca^{2+} were analyzed in parallel. The apparent first-order rate constants of enzyme inactivation (k_{inact}) were obtained from the slopes of $\log(A\text{-}A_{\infty} / A_{o}\text{-}A_{\infty})$ versus time plots where A_o is the initial enzyme activity, A is the activity after heating for time t, and A_{∞} the background activity at infinite time. Activation energies for heat inactivation were calculated from the slopes of Arrhenius plots. Heat-induced unfolding of SPC4 was studied by monitoring the intrinsic tryptophan fluorescence of the enzyme (2.2 µM) in 10 mM sodium acetate pH 5.0 in the absence or presence of 5 mM $CaCl_2$. The temperature of the continuously stirred protein solution, as measured with a digital sonde with a reading precision of ± 0.01 °C, was increased from 25 °C to 95°C at a speed of 0.5°C per min (Rodriguez et al., 2002). The fluorescence emission at 342 nm was measured at 0.5 min interval with excitation at 295 nm. To determine the temperature at midpoint transition (T_m), also referred to as melting temperature, the changes in fluorescence emission were analyzed according to a two-state mechanism of unfolding (van Mierlo et al., 1998). Data were fitted by non-linear, least-squares analysis using the general curve fit option of the Profit program (Quantum Soft, Zurich, Switzerland).

Enzyme activity

POX activity was measured spectrophotometrically by monitoring the H_2O_2-dependent oxidation of ABTS, at 25°C (Dicko et al., 2002a, this thesis, **Chapter 2**). Incubations were performed in 50 mM sodium acetate pH 4.0. The pH optimum of POX activity was determined with ABTS, ferulic acid and *N*-acetyl-L-tyrosine as substrates. Activity measurements were performed in 50 mM McIlvaine buffers at pH values between 2.5 and 9.0, using the standard assay. The effect of pH on enzyme stability was determined by preincubating the enzyme in various pH buffers (Dicko et al., 2001) and determining the residual activity with the standard assay. The effect of Ca^{2+} ions on POX activity was analyzed by measuring POX activity in the presence of varying (0–10 mM) concentrations of $CaCl_2$. Prior to the assay, the enzyme was desalted using a Y10 centricon (Amicon) and preincubated with known concentrations of $CaCl_2$ for 10 min, at 25°C. The reaction was then started by adding hydrogen peroxide. The concentration of the enzyme in the reaction medium was 10 nM.

The inhibitory effect of sodium azide on POX activity was determined by measuring the standard POX activity in the presence of 0 to 16 µM sodium azide. The concentration of the enzyme in the reaction medium was 10 nM.

Substrate specificity

Steady-state kinetics of SPC4 were performed by measuring the initial rate of enzyme activity in 50 mM sodium acetate pH 5.5, containing 1 mM $CaCl_2$, in the presence of 2.5 mM H_2O_2 and varying concentrations of hydrogen donor, at 25°C. The enzyme concentration was 10 nM and the reaction was started by the addition of H_2O_2. Spectral changes were recorded using a Hewlett-Packard 8453A diode array spectrophotometer. Blanks where the enzyme was replaced with buffer were prepared for each incubation to correct for autooxidation. Initial rates were determined as the slope of initial change in absorbance of substrate disappearance or product formation. Unless otherwise indicated, stock solutions of hydrogen donors were prepared in 10% ethanol at concentrations ranging from 1 to 25 mM. The concentration of ethanol in the assay mixture never exceeded 0.25% (v/v). Molar absorption coefficients of hydrogen donors at their maximum wavelengths were determined in 50 mM sodium acetate pH 5.5 (**Table 4**). The molar absorption coefficients of oxidation products were retrieved from literature (Oudgenoeg et al. 2001; Marco et al., 1999; Liu et al. 1999; Nielsen et al., 2001; Michon et al., 1997). The following substrates were used: ABTS, ferulic acid, *p*-coumaric acid, *N*-acetyl tyrosine, *N*-acetyl tyrosine methyl ester, tyrosine, catechol, and the tripeptide Gly-Tyr-Gly. For each substrate concentration, the enzymatic reaction rate was determined in duplicate. For the determination of indole-3-acetic acid (IAA) oxidase activity, IAA was used as substrate without addition of H_2O_2. Steady state kinetics were performed as indicated above. Blank reactions without enzyme served to correct for IAA autooxidation.

RESULTS AND DISCUSSION

Purification of major peroxidase from sorghum seed

At least four sorghum POX cationic isoenzymes, denoted SPC1, SPC2, SPC3 and SPC4, according to their order of elution, could be distinguished and separated by the Mono-S cation exchange chromatographic step (**Figure 1A**). SPC4 was by far the most abundant isoenzyme. Zymography (**Figure 2A**) showed that this enzyme has an experimental p*I* > 9. Three independent repetitions of all purification steps were performed to confirm the profile and abundance of isoenzymes within sorghum grain. The purification by three chromatographic steps resulted in a final enrichment of SPC4 by 105-fold, with an activity yield of 28% (**Table 1**). The purity of SPC4 was assessed by the single band obtained by SDS-PAGE (**Figure 2B**) and the high RZ value (4.0) of the enzyme. The purification of SPC4 is summarized in **Table 1**. The final specific activity of SPC4 for the H_2O_2-dependent oxidation of ABTS was 1071 U/mg. The purified enzyme was soluble in aqueous acetone, methanol and ethanol up to proportions of 40% (v/v) of organic solvent. The enzyme eluted from a Superdex G75 column in one symmetrical peak with an apparent mass of 32 kDa (**Figure 1B**), calculated using the molecular markers proteins as described in Materials and Methods. The molecular masses obtained by SDS-PAGE (38 kDa, **Figure 2B)** and MALDI-TOF-MS (34283-35631 Da, **Figure 3A**), show that SPC4 is a monomer.

Table 1. Purification of the major sorghum peroxidase.

Step	Total activity	Total protein	Specific activity	Yield
	U	mg	U mg^{-1}	%
Crude extract	10 710	1 050	10	100
Acetone fraction	7 497	407	18	70
Superdex 75	5 890	200	29	55
Resource-Q	4 820	12.7	379	45
Mono-S	2 998	2.8	1071	28

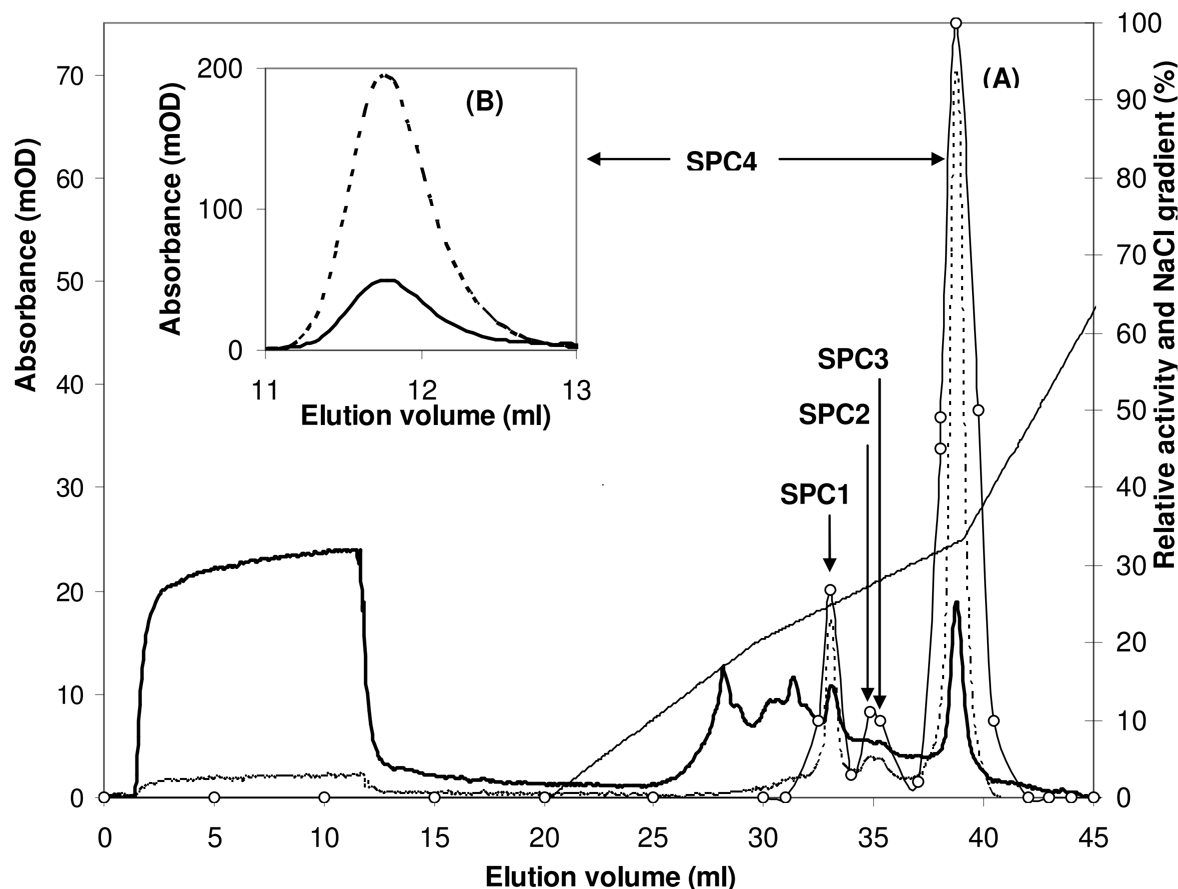

Figure 1. Purification of cationic isoforms of sorghum POX. **A**) Mono-S cation exchange chromatography: POX activity (o), absorbance at 280 nm (▬), absorbance at 403 nm (------), and 0-1M NaCl gradient (—). **B**) Elution profile of Mono S purified SPC4 on Superdex 75 PG.

Molecular characterization

Carbohydrate composition

MALDI-TOF-MS analysis revealed that SPC4 consists of two species with masses of 34 283 and 35 631 Da, respectively (**Figure 3A**). Chemical deglycosylation of the enzyme yielded a single protein peak with a mass of 33 449 Da (**Figure 3B**). This indicated that the heterogeneity of the enzyme is exclusively related to its glycan composition and that SPC4 has two glycoforms. For convenience, the species with a mass of 34 283 Da is further referred to as glycoform I and the species with a mass of 35 631 Da as glycoform II. The chemical deglycosylation was not complete because it leaves one unit of GlcNAc (203 Da) remaining on the polypeptide chain at each

attachment site (Edge et al., 1981). Thus, the molecular mass of fully deglycosylated SPC4 is at most 33 246 Da. The sugar contents estimated by MALDI-TOF-MS are 3.0% and 6.7% in glycoform I and II, respectively.

Carbohydrate analysis of SPC4 by HPAEC showed an average carbohydrate content of approximately 5.4% (**Table 2**). From the overall sugar content (HPAEC) and the estimated sugar contents of the individual glycoforms (MALDI-TOF-MS), the proportions of glycoforms I and II can be calculated to be 35 and 65%, respectively. HPAEC analysis showed that the main sugar constituents of the glycan chains are fucose, mannose, xylose and *N*-acetyl-glucosamine (**Table 2**).

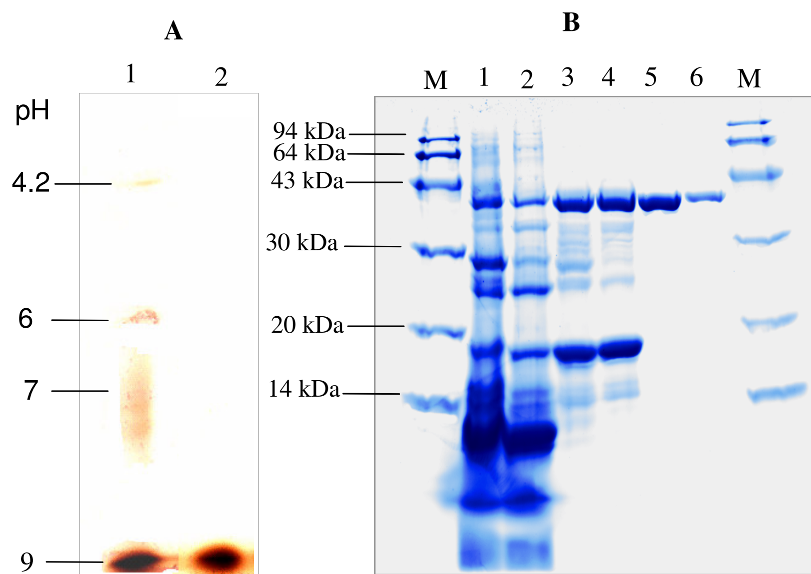

Figure 2. Zymogram and SDS-PAGE of sorghum peroxidase. **A**) Zymography: lane 1, crude extract and lane 2, purified SPC4. **B**) SDS-PAGE of purification steps of SPC4: lane M, marker proteins; lane 1, crude extract; lane 2, acetone precipitate; lane 3, preparative Superdex 75 fraction; lane 4, unbound Resource-Q fraction; lane 5, Mono-S fraction; lane 6, analytical Superdex 75 fraction.

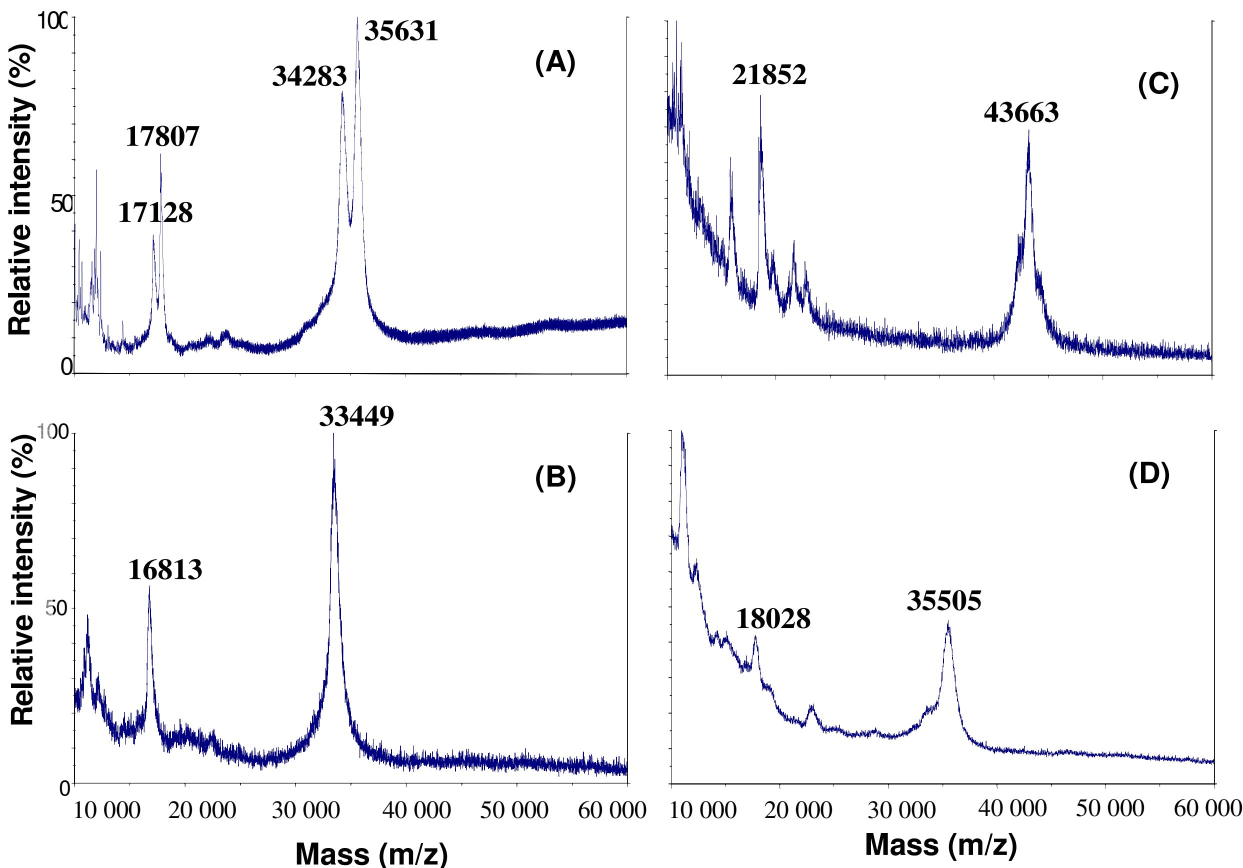

Figure 3. MALDI-TOF-MS analysis of native and deglycosylated forms of SPC4 and HRP. **A**) native SPC4, **B**) deglycosylated SPC4, **C**) native HRP, **D**) deglycosylated HRP.

MALDI-TOF-MS analysis of HRP as positive control showed masses of the native and deglycosylated form of 43663 Da and 35505 Da, respectively (**Figures 3C** and **3D**). Since HRP has eight glycan chains (Tams and Welinder, 1995), at least 8 GlcNAc residues will remain after chemical deglycosylation. Thus, the fully deglycosylated HRP would have a mass of 33 881 Da (35 505 – 203 x 8 Da) which is in good agreement with data obtained by electrospray ionization mass spectrometry (Green and Olivier, 1991), and also with the calculated mass based on the primary structure (**Table 2**). The mass of the sugar moiety in HRP is therefore 9 782 Da, corresponding to 22.4% (w/w). HPAEC analysis of the HRP C sugar composition revealed a carbohydrate content of 22.1% (w/w). The comparison of sugar composition between SPC4 and HRP C is illustrated in **Table 2**. The sugar content of SPC4 is much lower than observed with HRP (Tams and Welinder,

1995) as well as from other cationic peroxidases except for BP1, which also has a low sugar content (**Table 3**).

Table 2. Molecular mass and sugar composition of SPC4 and HRP

	Mass of intact protein (Da)	Mass of carbohydrate moiety (Da)		Proportion of carbohydrate (%, w/w)		Number of residues (mol/mol) determined by HPAEC			
	MS[a]	MS	HPAEC[b]	MS	HPAEC	Fucose	Mannose	Xylose	NGlc
SPC4[c]	I: 35631 II: 34283	I:1037 II: 2385	1903	I: 3.0 II: 6.7	5.4	1.4	5.6	1.7	2.7
HRP[d] (present study)	43663	9782	9689	22.4	22.1	9.5	26.8	8.0	14.2
HRP[e]	42200 to 44000	/	/	22 to 27	/	8	24	8	16

[a]MS = mass spectrometry analysis of the two glycoforms I and II; [b]HPAEC = high performance anion exchange chromatography analysis of both glycoforms; [c]SCP4 = sorghum cationic peroxidase (for HPAEC analysis, the average molecular mass and sugar composition of the two glycoforms was considered); [d]Horseradish peroxidase according to the present study ; [e]Horseradish peroxidase according to theoretical prediction (Tams and Welinder, 1995).

Amino acid composition and N-terminal sequence analysis

The amino acid composition of SPC4 together with those of other cationic POXs is given in **Table 3**. The average amino acid calculated mass of cationic POXs is 106.7 Da (**Table 4**), allowing to estimate 311 amino acid residues in SPC4. From this amino acid composition, a theoretical p*I* value of 11 was calculated, assuming that all 8 cysteines are involved in disulfide bridges (Dunford, 1999; Welinder, 1976; Henriksen et al., 1998). The low ratio of (Asx+Glx) to (Arg+Lys) of SPC4 and its p*I* indicate that in comparison to other cationic POXs, SCP4 is highly basic (**Table 3**).

Table 3. Amino acid composition of SPC4 and other cationic plant POXs

Amino acid	SPC4*		RP[a]	WP[b]	BP1[c]	CC[d]	HRPC[e]	PNC21[f]	SB1[g]	TP7[h]
Ala	31	(10.0)	50	39	22	37	23	27	29	32
Arg	23	(7.4)	15	12	30	21	21	19	22	17
Asp+Asn	35	(11.1)	32	38	34	31	48	35	35	39
Cys	8	(2.5)	8	9	8	9	8	8	9	8
Glu+Gln	12	(3.9)	13	15	26	21	20	22	27	14
Gly	29	(9.3)	22	24	25	26	17	28	26	24
His	5	(1.6)	5	4	4	5	3	5	4	3
Ile	8	(2.6)	14	11	11	13	13	13	12	15
Leu	29	(9.4)	32	31	30	28	35	25	30	21
Lys	14	(4.5)	7	10	6	4	6	12	8	10
Met	3	(1.0)	7	8	2	8	4	3	6	6
Phe	16	(5.2)	11	12	17	13	20	18	17	14
Pro	15	(4.8)	12	10	21	17	17	11	15	11
Ser	27	(8.7)	36	37	26	31	25	29	30	42
Thr	25	(8.1)	26	27	16	19	25	22	16	16
Trp	2	(0.6)	1	1	1	1	1	2	2	1
Tyr	6	(1.9)	6	4	4	6	5	4	9	4
Val	23	(7.4)	17	20	26	17	17	24	25	19
(Asx+Glx)/ (Arg+Lys)	1.27		2.05	2.41	1.67	2.08	2.52	1.84	2.07	1.96
Sum	311	(100)	314	312	309	307	308	307	322	296
Apoprotein Mw**	33 226		32 437	32 382	33 825	32 508	33 918	32 954	35 029	31 086
Carbohydrate proportion	3-6%		/***	/	0-3%	/	22-27%	12-19%	/	7%
Accession code[$]	P84516		O22440	Q05855	Q40069	Q43416	P00433	AAA32676	Q9SSZ9	POO434

[a]Rice (Chittoor et al. 1997), [b]Wheat (Hertig et al. 1999), [c]Barley (Johansson et al., 1992), [d]*Cenchrus ciliaris* (Ross, 1994), [e]Horseradish (Welinder, 1976), [f]Peanut (Buffard et al. 1990), [g]*Scutellaria baicalensis* (Morimoto et al. 1999), [h]Turnip (Mazza and Welinder, 1980).
*Results of SPC4 are presented in number of amino acid/protein and in mol % (mol/mol) in brackets.
**Calculated molecular weights using a software to compute p*I*/Mw/titration curve, available at http://expasy.ch/tools/#primary.
/***, sugar composition not given.
[$]Entries in Swiss-Prot/TrEMBL

```
Sorghum  1    MSRAPTLAAAAAVAAVVLICSSSTATAADGNA-RQPPLAPGLSFDFYKRSCPKAESIVRS
Barley   1    MARVPLL--AALVVAMAVLVASSLG---PRASAAEPPVAPGLSFDFYWQTCPRAESIVRE
Rice     1    MASATTL--VMLVAAVACLCAPAAAGDGNADATRQPPVVSGLSFDFYRKSCPKAESVVRK
Wheat    1    MARAPLL--AALVV--AVVVASSLG---HGASAAEPPVARGLSFDFYRRTCPRAESIVRG
Maize    1    MARSSGS--RPVALVLLALCAAALS---SATVTVNEPIANGLSWSFYDVSCPSVEGIVRW

Sorghum  60   FVQDAVRRDVGLAAGLLRLHFHDCFVQGCDASVLLDGSATGPGEQQAPPNLTLRPTAFKA
Barley   56   FVQEAVRKDIGLAAGLLRLHFHDCFVQGCDASVLLDGSATGPGEQQAPPNLTLRPSAFKA
Rice     59   FVRDAVRKDIGLAAGLLRLHFHDCFVQGCDASVLLDGSATGPGERQAPPNLTLRPSAFKA
Wheat    54   FVQDAVRKDIGLAAGLLRLHFHDCFVQGCDASVLLDGSATGPGEQQAPPNLTLRPSAFKA
Maize    56   HVAEALRRDIGIAAGLIRIFFHDCFPQGCDASVLLSGSNS---EQIEVPNQTLRPEALKL
                               *  **                           *

Sorghum  120  INDIHDRLHKECGGTVVSCSDVLALAARDSVVVSGGPSYKVPLGRRDSASFATQQDVLSG
Barley   116  VNDIRDRLERECRGAVVSCSDILALAARDSVVVSGGPDYRVPLGRRDSRSFASTQDVLSD
Rice     119  VNDIRDRLEKACGASVVSCSDILALAARDSVVASGGPEYKVPLGRRDSAEFASQQDVLSG
Wheat    114  VNDIRDRLERECRGAVVSCADILALAARDSVVVSGGPDYRVPLGRRDSRRFATRQDVLSD
Maize    113  IDDIRAAVHAVCG-PTVSCADITTLATRDAVVASGGPFFEVPLGRRDGLAPASS-DLVGT
                                                       *

Sorghum  180  LPPPTAAVPALLAVLSKINLDATDLVALSGGHTI--------------------------
Barley   176  LPGPSSNVQSLLALLGRLGLDATDLVTISGGHTIGLAHCSSFEDRLFPRPDP-TISPTFL
Rice     179  LPPPTAAVPALLDALAKIKLDATDLVALSGGHTVGLAHCSSFEGRLFPRRDP-AMNATFA
Wheat    174  LPAPSSNVPSLLALLRPLGLDATDLVTISGGHTIGQAHCSSFEDRLFPRPDP-TINPPFL
Maize    171  LPAPFFDVPTLIESFKNRSLDKADLVALSGAHTVGRGHCVSFSDRLPPNADDGTMDPAFR
                                             *
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Figure 4. Multiple sequence alignment of sorghum POX (P84516) with other cereal POXs. The N-terminal fragment of SPC4 is aligned with barley BP1 (gi:167080), rice Prx23 (gi:55700912), wheat WSP1 (gi:22001284), and maize POX (gil:2224845). The highly conserved catalytic residues among all class III POXs are marked with asterisks. The N-terminal sequence of SPC4 obtained by Edman sequencing is underlined. The signal peptides of SPC4 and BP1 are shown in the box.

Like BP1 (Johansson et al., 1992), the N-terminal sequence of SPC4 is not blocked by pyroglutamate, in contrast to most other POXs (Welinder, 1976; Mazza and Welinder, 1980). The first 20 amino acid residues are shown in **Figure 4**. The tBLASTn search at the Gramene website (www.gramene.org) indicated that the SPC4 gene is localized in the sorghum chromosome 1. At the Institute for Genomic Research (www.tigr.org), the best match with 100% identity was found with gene indice TC102191 (213 amino acids). MALDI-TOF-MS analysis (**Figure 5**) showed that six peptides, including the N-terminal sequence of SPC4 matched with the predicted tryptic peptides of TC102191, indicating that TC102191 codes for the N-terminal part of the sequence of SPC4. SPC4 has a signal peptide of 31 amino acids (**Figure 4**). Since the expected full length of SPC4 is about 311 amino-acid residues, the C-terminal sequence of about 129 amino-acids is unknown. With the currently ongoing sorghum genome project (http://fungen.botany.uga.edu), the sequence of this

fragment will be available soon. The sequence of the N-terminal part of SPC4 was analyzed by searching for specific POX motifs (rpsblast at NCBI: www.ncbi.nlm.nih.gov), and protein families (Pfam at Sanger Institute: www.sanger.ac.uk). SPC4 consists of two domains and has an N-terminal extension of one and eight residues, compared to BP1 (Johansson et al., 1992) and HRP-C (Welinder, 1976), respectively. The key catalytic residues (Arg46, Phe49, His50, Asn78, Pro150 and His180) and cysteines involved in intramolecular disulfide bridges (Cys19-Cys100; Cys52-Cys57; Cys107) are all conserved (**Figure 4**). With the N-terminal fragment of SPC4 the only putative glycosylation site found is Asn78 (active site residue), which is unlikely glycosylated among plant POXs (Johansson et al., 1992). Thus, like found for most POXs, the glycosylation sites of SPC4 are localized in the C-terminus part of the enzyme. The sorghum peroxidase sequence data reported in this paper will appear in the Swiss-Prot and TrEMBL knowledgebase under the accession number P84516.

A tBLASTn search against the non-redundant database at NCBI (www.ncbi.nlm.nih.gov) indicated that SPC4 is most closely related to cereal POXs (**Figure 4**). The N-terminal fragment of SPC4 has a high sequence identity with barley BP1 (85%), rice Prx23 (90%), wheat WSP1 (82%) and maize (58%), indicative for a common ancestor (Paterson et al., 2003).

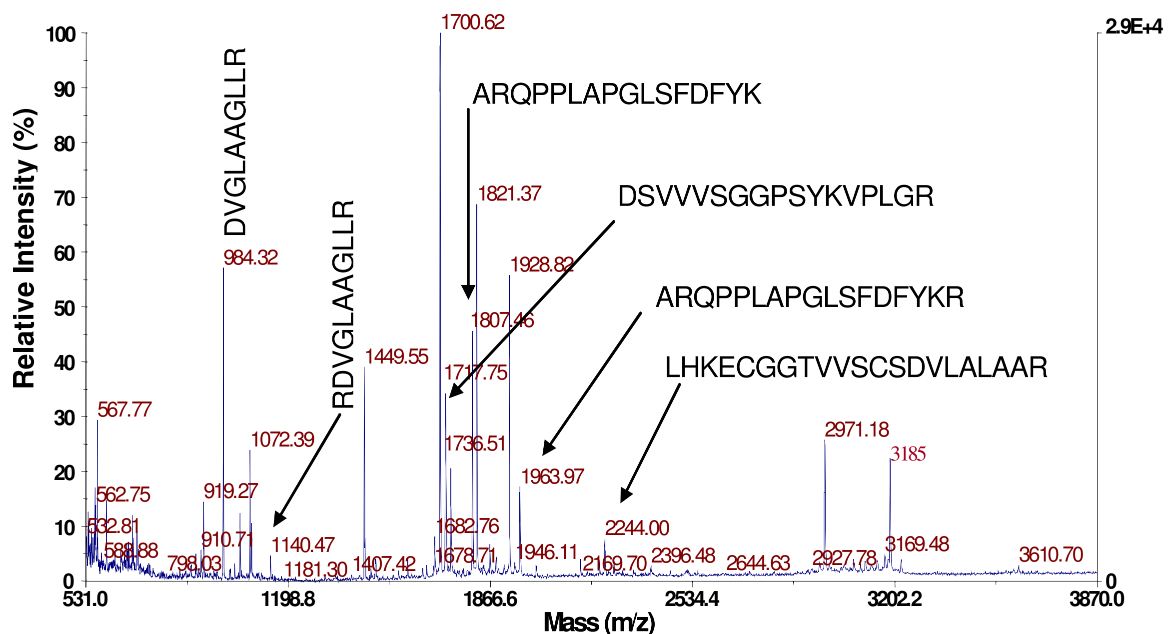

Figure 5. MALDI-TOF-MS peptide mass fingerprint of SPC4.

Spectral properties

The absorption spectrum of native SPC4 showed characteristics typical of high-spin ferric heme proteins, with a maximum in the Soret region at 403 nm and charge transfer band at 497 nm (**Figure 6**). With the spectrum of the heme, a β-band at 532 and another charge transfer band at 637 nm were observed. This spectrum is characteristic for a Fe^{III}-protoporphyrin-IX cluster. The molar absorption coefficient of SPC4 at 403 nm was determined to be approximately 104 $mM^{-1}cm^{-1}$.

Figure 7 shows the mass spectral analysis of the heme cofactor of SPC4. The mass of 616 Da corresponds to the Fe^{III}-protoporphyrin-IX cluster, confirming that SPC4 contains a type-b heme. The peak with a mass of 563 Da is ascribed to the partial loss of iron by the protoporphyrin-IX cluster. The MALDI-TOF-MS spectrum also shows an intense peak with a mass of 650, which is assigned to a heme-H_2O_2 adduct. Thus, SPC4 is a type-b heme-containing POX, which shares similar molecular properties with cereal POXs (Johansson et al., 1992; Converso and Fernandez, 1995; Dunford 1999).

Far UV-circular dichroism spectroscopy showed that SPC4 contained 42 ± 6% α-helix, 35 ± 7% β-sheet and 24 ± 7 % β-turns. This secondary structure is similar to that of other plant peroxidases (Melo et al., 1997).

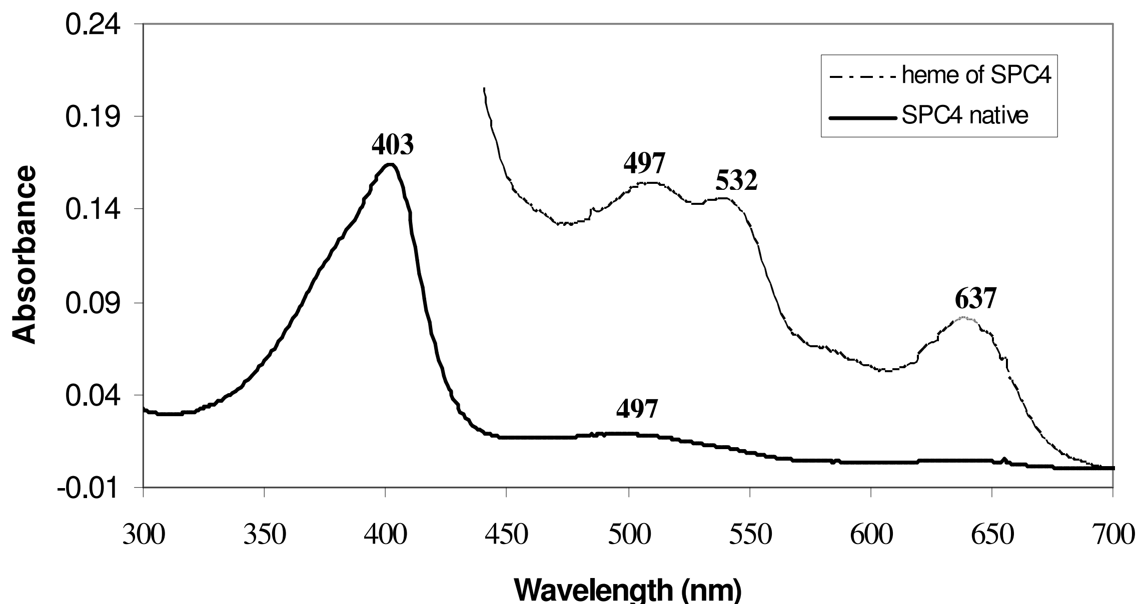

Figure 6. UV-visible spectra of SPC4 and extracted heme. The spectrum of native enzyme was recorded in 50 mM sodium acetate pH 5. The spectrum of heme was recorded in acidified butanone at pH 2.

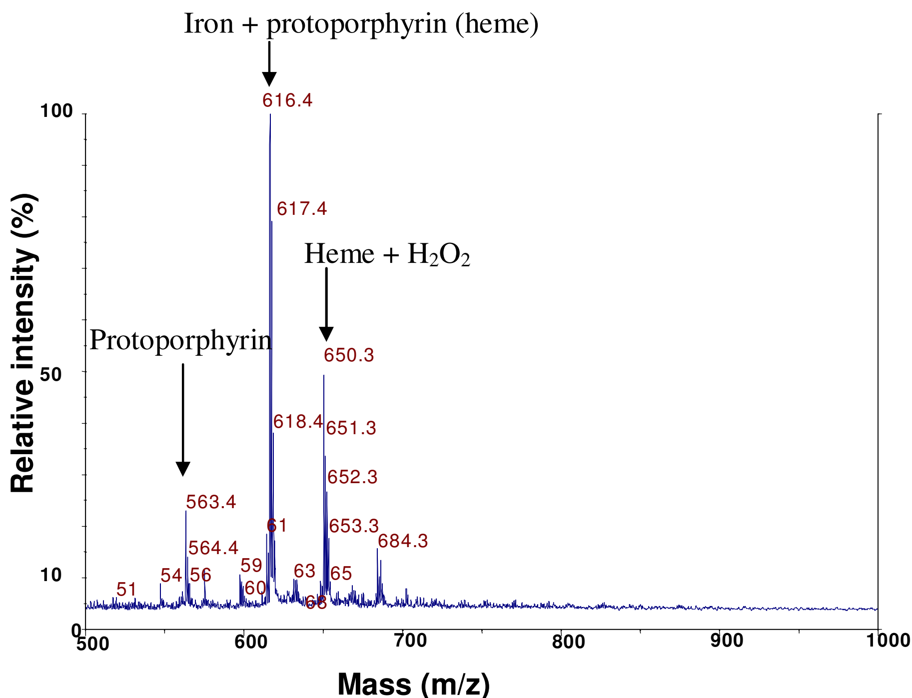

Figure 7. MALDI-TOF-MS analysis of SPC4 heme

Catalytic properties

SPC4 was stable between pH 3 and pH 7 for 2h at 25ºC. The enzyme showed optimal activity with ABTS, ferulic acid and *N*-acetyl-L-tyrosine at pH 3.8, 5.5 and 6.5, respectively. These different pH optima are in line with reported properties of other peroxidases (Melo et al., 1997). Nevertheless, SPC4 remarkably differs from BP1 (Rasmussen et al., 1998) in being active with aromatic compounds above pH 5. The enzyme was inactivated by sodium azide. This compound is a common inhibitor of POXs because it reacts with the heme group (Dunford, 1999).

Stafford and Brown (1976) reported an oxidative dimerization of ferulic acid by sorghum grain extracts. Furthermore, using a crude extract from sorghum variety NK300, a high POX activity on ferulic acid and no activities on tyrosine and other phenolics was found (Glennie, 1981). Here we found that purified SPC4 has a high preference for hydroxycinnamates, including ferulic acid and *p*-coumaric acid, which are among the most abundant phenolic compounds in sorghum (Awika, 2004). Kinetic studies performed at pH 5.5 showed that the catalytic efficiency of SPC4 with phenolic compounds decreased in the following order: ferulic acid > *p*-coumaric acid > *N*-acetyl-tyrosine methyl ester > *N*-acetyl-tyrosine> tyrosine > catechol > Gly-Tyr-Gly (**Table 4**).

Table 4. Substrate specificity of sorghum POX

Substrate	Substrate λ_{max}	Substrate molar absorption coefficient	Product λ_{max}	Apparent V_{max}/K_m
	nm	$mM^{-1}\ cm^{-1}$	nm	$M^{-1}\ s^{-1}$
ABTS	317	34	414#	1.16
ferulic acid	310*	14.9	318[a]	0.92
p-coumaric acid	287*	19.7	308[b]	0.23
indole-3-acetic acid	280*	5	261[c]	0.08
N-acetyl tyrosine methyl ester	275	1.4	318[a]#	0.07
N-Acetyl tyrosine	275	1.4	293[d]#	0.05
tyrosine	276	2.7	318[a]#	0.03
catechol	276	2.3	398[e]#	0.01
Gly-Tyr-Gly	275	1.3	318[a]#	0.01

The substrates are ranked by order of preference. [a]Oudgenoeg and co-workers (2001), [b]Nielsen and co-workers (2001) ; [c]Marco and co-workers (1999);[d]Michon and co-workers (1997); [e]Liu and co-workers (1999); The reaction was followed by *substrate disappearance or #product formation.

The high reactivity with hydroxycinnamic acid derivatives suggests that the enzyme is involved in the cross-linking of the plant cell wall. On the other hand, the low catalytic efficiency of SPC4 with tyrosine and tyrosine-containing peptides suggests that the enzyme probably is not involved in protein modification via tyrosine cross-linking. The low activity of BP1 with aromatic substrates compared to HRP-C has been justified by the presence of Ser152 and Ser153 at the substrate binding region in BP1 instead of Phe142 and Phe143 in HRP (Johansson et al., 1992). Recently, analysis of the crystal structure of the HRP C-ferulic acid complex confirmed the involvement of these residues in substrate binding (Henriksen et al., 1999). In case of SPC4, the corresponding residues are Thr153 and Ala154. These amino acid replacement may justify the low activity of SPC4 on some phenolic compounds.

SPC4 also displayed IAA (auxin) activity. This oxidase activity is not a property of all plant POX isoenzymes (Marco et al., 1999), suggesting that SPC4 plays a role in auxin regulation. Some POXs regulate the level of this plant hormone either by directly degrading auxin or by oxidizing endogenous flavonoids, which are inhibitors of auxin transport (Mathesius, 2001). The activity of

SPC4 on IAA might be related to the presence of His48 (His40 in HRP) in the distal domain, near the heme, which is believed to play a role in auxin catabolism based on sequence similarity with auxin binding proteins (Savitsky et al., 1999).

The activity of SPC4 was stimulated in the presence of $CaCl_2$. The maximum increase of activity of the purified enzyme was two-fold with an apparent semi-maximal activation at 0.7 mM $CaCl_2$. A similar, but somewhat more stronger activation, was observed for BP1 (Rasmussen et al., 1998). The Ca^{2+} activation of POX is of particular interest because not all POXs, including HRP are activated by Ca^{2+} (Converso and Rodriguez, 1996). Binding of Ca^{2+} decreased the intrinsic tryptophan fluorescence intensity of SPC4. From the binding curve, a dissociation constant for the SPC4-Ca^{2+} ion complex, K_d = 2.4 ± 0.3 mM, was estimated. The affinity of SPC4 for Ca^{2+} was higher than that of BP1 (K_d = 4 mM; Rasmussen et al., 1998). The binding of Ca^{2+} has been proposed to change the electronic properties of the heme iron or the topology of the heme vicinity and might improve substrate binding (Converso and Fernandez, 1996; Rasmussen et al., 1998; Henriksen et al., 1998, van Huystee et al., 2004). In the case of SPC4, such structural perturbations must be small because circular dichroism analysis revealed that Ca^{2+} binding does not change the secondary structure of the enzyme (not shown).

Thermal stability

In the absence of added $CaCl_2$, the enzyme readily lost activity when incubated at temperatures above 55°C (**Figure 8A**). However, in the presence of added Ca^{2+} ions, the enzyme kept its full activity up to 65°C for 90 min incubation (**Figure 8B**). Arrhenius plots (**Figure 8C**) of the thermoinactivation data revealed straight lines and showed that Ca^{2+} binding increased the activation energy of heat inactivation of SPC4 from 157 ± 12 kJ mol^{-1} to 170 ± 14 kJ mol^{-1}. The increased stability of SPC4 in the presence of Ca^{2+} ions was confirmed by fluorescence experiments. Upon heating, both in the absence and presence of Ca^{2+} ions, a strong increase in protein tryptophan fluorescence was observed (**Figures 9A** and **9B**). Independent of the presence of Ca^{2+} ions, and treating the data according to van Mierlo and co-workers (1998), SPC4 followed a simple two-state mechanism of heat-induced unfolding. This is in agreement with other plants POXs (Rodriguez et al., 2002).

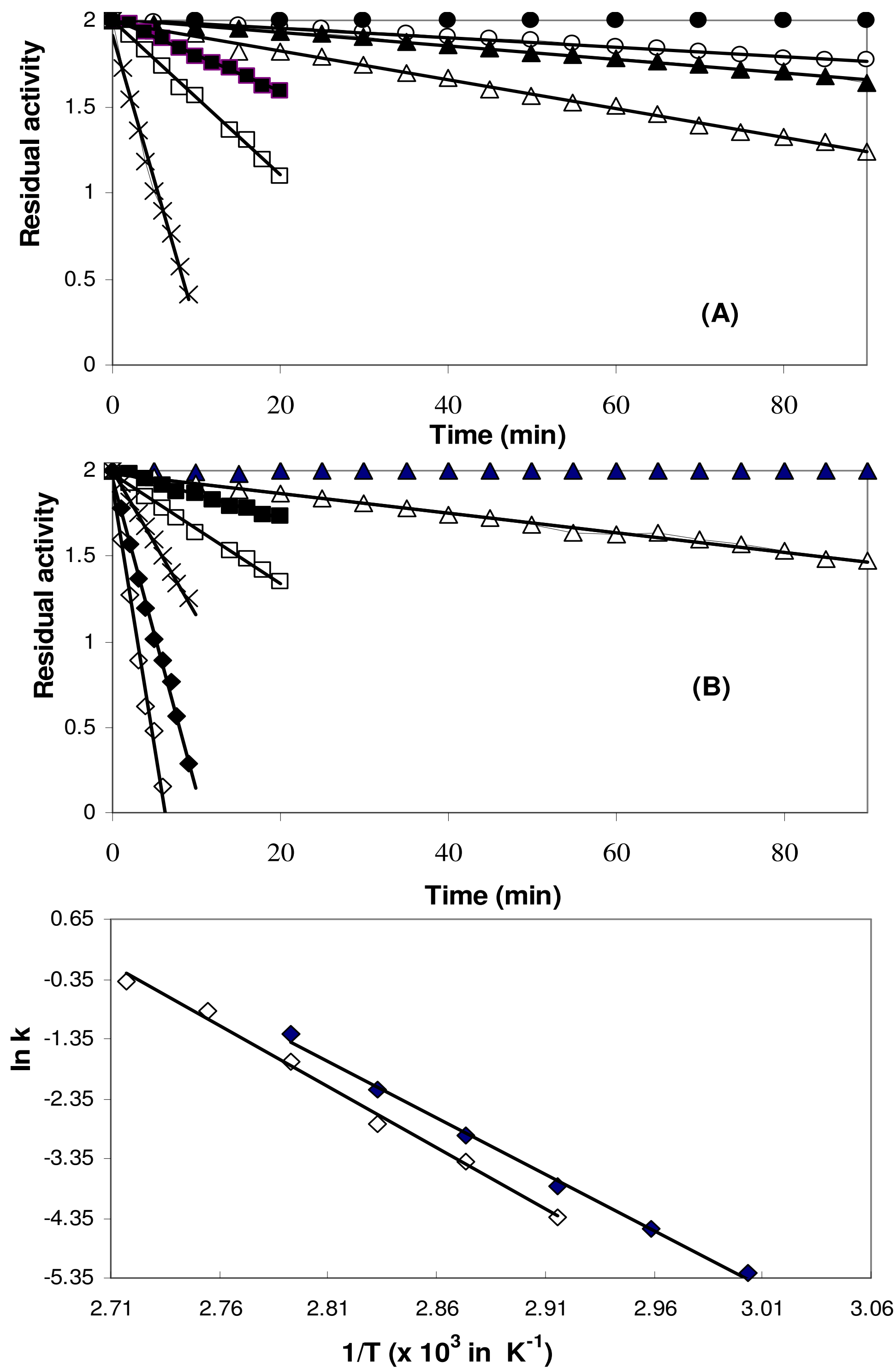

Figure 8. Thermoinactivation of SPC4. The enzyme (270 nM) was incubated at different temperatures in 50 mM sodium acetate pH 5, either in the absence (**A**) or presence (**B**) of 5 mM $CaCl_2$: 55ºC (●), 60ºC (○), 65ºC (▲), 70ºC (Δ), 75ºC (■), 80ºC (□), 85ºC (**X**); 90ºC (◆), 95ºC (◇). **C**) Arrhenius plot for heat inactivation of SPC4 in the absence (■) or presence ( ) of calcium. Vertical bars indicate the average standard error of each experiment.

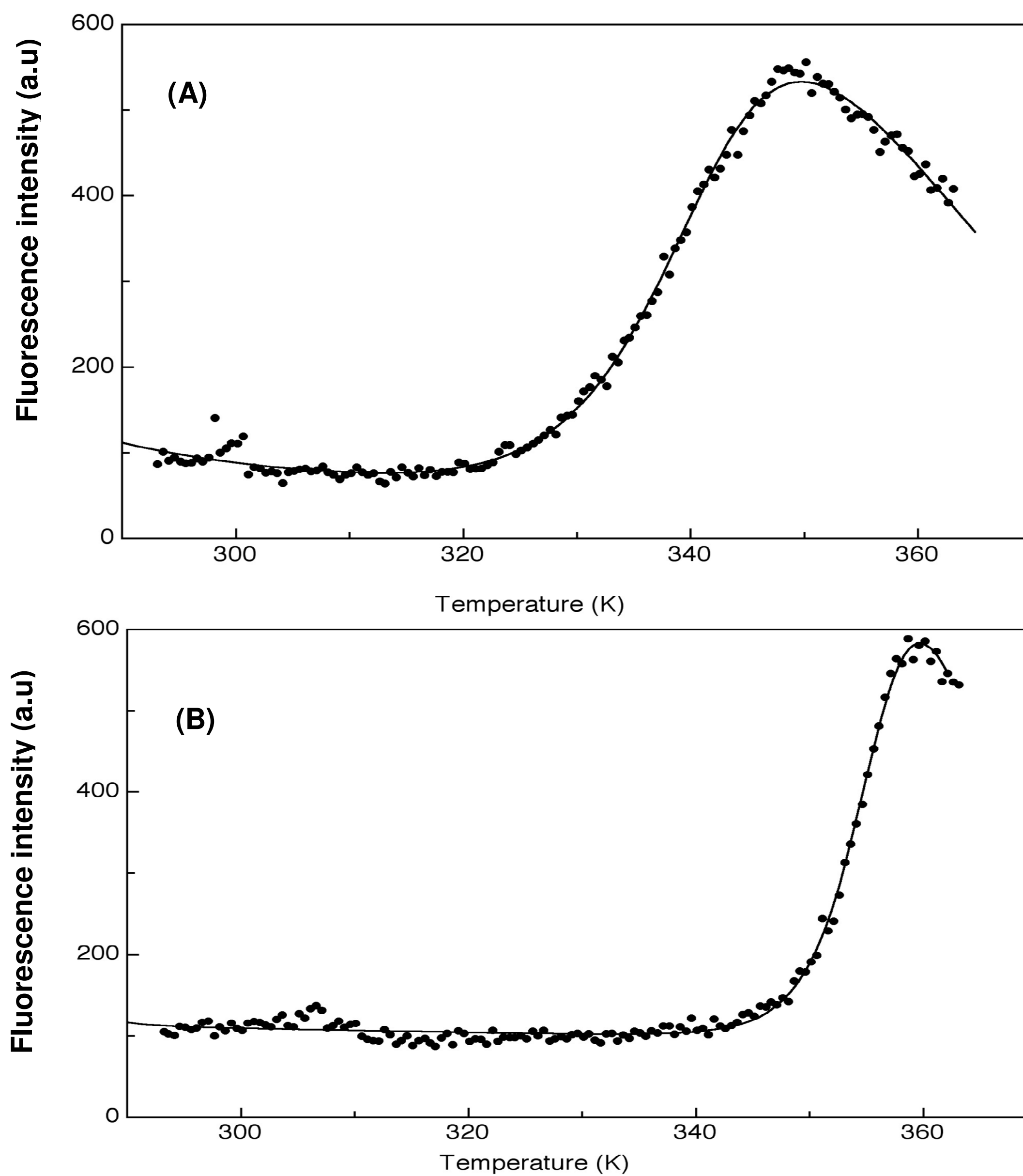

Figure 9. Thermal unfolding of SPC4 as followed by intrinsic tryptophan fluorescence. The enzyme (2.22 µM) was heated in 10 mM sodium acetate pH 5, either in the absence (A) or presence (B) of 5 mM $CaCl_2$ at a rate of 0.5ºC/min. The excitation wavelength was 295 nm. The emission at 342 nm was monitored at 0.5 min intervals. Solid lines are the best fit of the two states unfolding equation (van Mierlo et al., 1998).

Thermal unfolding of SPC4 induced not only an increase of fluorescence intensity but also a bathochromic shift of the fluorescence maximum from 338 nm to 348 nm (not shown). Tm values of 67°C and 82°C for the free and metal ion bound form, respectively, were found. In the absence of Ca^{2+}, the melting temperature of SPC4 was between that of HRP C (T_m = 60°C) and the African palm tree POX (T_m = 74°C) (Rodriguez et al., 2002). In the presence of Ca^{2+} the T_m of SPC4 is near that of soybean POX, which is reputed to be the most stable POX with a T_m of 90°C in the presence of calcium (McEldoon and Dordick, 1996). Thus, SPC4 may be considered as one of the most thermostable cationic POXs.

In conclusion, the major isoenzyme in sorghum grain (SPC4) was shown to be a cationic POX having two glycoforms with unusual basic character and a high heat stability in the presence of calcium. The enzyme has a relatively low carbohydrate content. It shares similar molecular properties with other cereal peroxidases and may develop as an alternative peroxidase for biochemical and clinical assays, and biocatalysis.

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REFERENCES

Altschul, S. F.; Madden, T. L.; Schäffer, A. A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D. J. "Gapped BLAST andPSI-BLAST: a new generation of protein database search programs". *Nucleic Acids Res.* **1997**, *25*, 3389-3402.

Awika, J. M.; Rooney, L. W. Sorghum phytochemicals and their potential impact on human health. *Phytochemistry* **2004**, *65*, 1199-1221.

Bjellqvist, B.; Hughes, G.J.; Pasquali, Ch.; Paquet, N.; Ravier, F.; Sanchez, J.-Ch.; Frutiger, S.; Hochstrasser, D. F. The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. *Electrophoresis* **1993**, *14*, 1023-1031.

Boeriu, C. G.; Oudgenoeg, G.; Spekking, W. T. J.; Berendsen, L. B. J. M.; Vancon, L.; Boumans, H.; Gruppen, H.; van Berkel, W. J. H.; Laane, C.; Voragen, A. G. J. Horseradish peroxidase-catalyzed cross-inking of feruloylated arabinoxylans with β-casein. *J. Agric. Food Chem*. **2004**, 52, 6633-6639.

Boerjan, W.; Ralph, J.; Baucher, M. Lignin biosynthesis. *Annu. Rev. Plant Biol.* **2003**, *54*, 519–546.

Buffard, D.; Breda, C.; van Huystee, R. B.; Asemota, O.; Pierre, M.; Ha, D. B.; Esnault, R. Molecular cloning of complementary DNAs encoding two cationic peroxidases from cultivated peanut cells. *Proc. Natl. Acad. Sci.* U.S.A. **1990**, *87*, 8874-8878.

Chittoor, J. M.; Leach, J. E.; White, F. F. Differential induction of a peroxidase gene family during infection of rice by *Xanthomonas oryzae* pv. *oryzae*. *Mol. Plant Microbe Interact.* **1997**, *10*, 861-871.

Christensen, J. H.; Bauw, G.; Welinder, K. G.; Montagu, M. V.; Boerjan, W. Purification and characterization of peroxidases correlated with lignification in poplar xylem. *Plant Physiol.* **1998**, *118*, 125-135.

Converso, D. A.; Fernández, M. E. Ca^{2+} activation of wheat peroxidase: a possible physiological mechanism of control. *Arch. Biochem. Biophys.* **1996**, *333*, 59-65.

Dicko, M. H.; Gruppen. H.; Traoré, A. S.; Zouzouho, O. C.; van Berkel, W. J. H. and Voragen, A. G. J. Effects of germination on the activities of amylases and phenolic enzymes in sorghum varieties. **2005**, submitted

Dicko, M. H.; Hilhorst R.; Gruppen, H.; Traoré, A. S.; Laane, C.; van Berkel, W. J. H.; Voragen, A. G. J. Comparison of content in phenolic compounds, polyphenol oxidases and peroxidases in fifty sorghum varieties from Burkina Faso. *J. Agric. Food Chem*. **2002a**, *50*, 3780-3788.

Dicko, M. H.; Hilhorst, R.; Gruppen, H.; Laane, C.; van Berkel, W. J. H.; Voragen, A. G. J. Zymography of the monophenolase and *o*-diphenolase activities of polyphenol oxidase. *Anal. Biochem.* **2002b**, *360*, 336-339.

Dicko, M. H.; Searle-van Leeuwen, Hilhorst, R.; Traoré, A. S.; Beldman, G. Polysaccharide hydrolases from leaves of *Boscia senegalensis:* properties of the endo-(1→3)-β-D-glucanase. *Appl. Biochem. Biotechnol.* **2001**, *94*, 225-241.

Dunford, H. B. *Heme peroxidases*. John Wiley.; Sons, Inc. **1999**.

Duroux, L.; Welinder, K. G. The peroxidase gene family in plants: a phylogenetic overview. *J. Mol. Evol.* **2003**, *57*, 397-407.

Edge, A. S. B.; Faltynek, C. R.; Hof, L.; Reichert, L. E.; Weber, P. Deglycosylation of glycoproteins by trifluoromethanesulfonic acid. *Anal. Biochem.* **1981**, *118*, 131-137.

Glennie, C. W. Preharvest changes in polyphenols, peroxidase, and polyphenol oxidase in Sorghum grain. *J. Agric. Food Chem*. **1981,** *29*, 33-36.

Green, B. N.; Olivier, R. W. A. The study of intact proteins and glycoproteins by electrospray mass spectrometry. Biochem. Soc. Trans. **1991**, *19*, 929-934.

Henriksen, A.; Welinder, K. G.; Gajhede, M. Structure of barley grain peroxidase refined at 1.9-Å resolution: a plant peroxidase reversibly inactivated at neutral pH. *J. Biol. Chem.* **1998**, *273*, 2241-2248.

Henriksen, A.; Smith, A. T.; Gajhede, M. The Structures of the horseradish peroxidase C-ferulic acid complex and the ternary complex with cyanide suggest how peroxidases oxidize small phenolic substrates. *J. Biol. Chem*. **1999**, *274*, 35005-35011.

Hertig, C.; Rebmann, G.; Bull, J.; Mauch, F.; Dudler, R. Sequence and tissue-specific expression of a putative peroxidase gene from wheat (*Triticum aestivum* L.). *Plant Mol. Biol*. **1991**, *16*, 171-174.

Johansson, S. K.; Rasmussen, J. E.; Harthill, J. E.; Welinder, K. G. cDNA, amino acid and carbohydrate sequence of barley seed-specific peroxidase BP1. *Plant Mol. Biol*. **1992**, *18*, 1151-1161

Kristensen, B. K.; Bloch, H.; Rasmussen, S. K. Barley coleoptyl peroxidases. Purification, molecular cloning, and induction by pathogens. *Plant Physiol.* **1999**, *120*, 501-512.

Leskovac, V. Comprehensive enzyme kinetics; Leskovac, V. Ed.; Kluwer Academic/Plenum Publishers. New York, Boston, Dordrecht, London, Moscow, **2003**.

Liu, W.; Fang, J.; Zhu, W. M.; Gao, P. J. Isolation, purification and properties of the peroxidase from the hull of Glycine max var HH2. *J. Sci. Food Agric*. **1999,** *79*, 779-785.

Marco, A.; Guzzardi, P.; Jamet, E. Isolation of tobacco isoperoxidases accumulated in cell-suspension medium and characterization of activities related to cell wall metabolism. *Plant Physiol*. **1999**, *120*, 371-381.

Mathesius, U. Flavonoids induced in cells undergoing nodule organogenesis in white clover are regulators of auxin breakdown by peroxidase. *J. Exp. Bot*. **2001**, 52, 419-426.

Matsudaira, P. Sequence from picomole quantities of proteins electroblotted onto polyvinylene difluoride membranes. *J. Biol. Chem.* **1987**, *262*, 10035-10038.

Mazza, G.; Welinder, K. G. Covalent structure of turnip peroxidase 7. Cyanogen bromide fragments, complete structure and comparison to horseradish peroxidase C. *Eur. J. Biochem*. **1980**, *108*, 481-489.

McEldoon, J. P.; Dordick, J. S. Unusual thermal stability of soybean peroxidases. *Biotechnol. Prog*. **1996**, *12*, 555-558.

Melo, N. S.; Larsen, E.; Welinder, K. G.; Fevereiro, P. S. Characterization of two major cationic peroxidases from cell suspension cultures of *Vaccinium myrtillus*, *Plant Sci.* **1997**, *122*, 1-10.

Michon, T.; Chenu, M.; Kellershon, N.; Desmadril, M.; Gueguen, J. Horseradish peroxidase oxidation of tyrosine-containing peptides and their subsequent polymerization: a kinetic study. *Biochemistry* **1997**, *36*, 8504-8513.

Morimoto, S.; Tateishi, N.; Inuyama, M.; Taura, F.; Tanaka, H.; Shoyama, Y. Identification and molecular characterization of novel peroxidase with structural protein-like properties. *J. Biol. Chem.* **1999**, *274*, 26192-26198.

Nielsen, K. L.; Indiani, C.; Henriksen, A.; Feis, A.; Becucci, M.; Gajhede; M.; Smulevich, G.; Welinder, K. G. Differential activity and structure of highly similar peroxidases. Spectroscopic, crystallographic, and enzymatic analyses of lignifying *Arabidopsis thaliana* peroxidase A2 and horseradish peroxidase A2. *Biochemistry* **2001**, *40*, 11013-11021.

Oudgenoeg, G.; Hilhorst. R.; Piersma. S. R.; Boeriu. C. G.; Gruppen. H.; Hessing. M.; Voragen. A. G. J.; Laane C. Peroxidase-mediated cross-linking of a tyrosine-containing peptide with ferulic acid. *J. Agric. Food Chem*. **2001**, *49*, 2503-2510.

Pajot, P. Fluorescence of proteins in 6M guanidine hydrochloride: a method for quantitative determination of tryptophan. *Eur. J. Biochem.* **1976,** *63*, 263-269.

Passardi, F.; Longet, D.; Penel, C.; Dunand, C. The class III peroxidase multigenic family in rice and its evolution in land plants. *Phytochemistry* **2004**, *65*, 1879-1893.

Paterson, A. H.; Bowers, J. E.; Peterson, D. G.; Estill, J. C.; Chapman, B. Structure evolution of cereal genomes. *Curr. Opin. Gen. Dev.* **2003**, *13*, 644-650.

Rasmussen, C. B.; Hiner, A. N. P.; Smith, A. T.; Welinder, K. G. Effect of calcium, other ions, and pH on reactions of barley peroxidase with hydrogen peroxidase and fluoride: control of activity through conformational change. *J. Biol. Chem.* **1998**, *273*, 2232-2240.

Regalado, C.; Garcia-Almendárez, B. E.; Duarte-Vázquez, M. A. Biotechnological applications of peroxidases. *Phytochemistry Rev*. **2004**, *3*, 243-256.

Rodríguez, A.; Pina, D. G.; Yélamos, B.; Léon, J. J. C.; Zhadan, G. G.; Villar, E.; Gavilanes, F.; Roij, M. G.; Sakharov, I. Y.;. Shnyrov, V. L. Thermal stability of peroxidase from the African oil palm tree *Elaeis guineensis*. *Eur. J. Biochem.* **2002***, 269*, 2584-2590.

Ross, A. H. Investigation of peroxidase genes and genetic transformation in buffel grass. PhD thesis, University of Queensland, Queensland 4072, Australia, **1994**.

Sae, S. W.; Kadoum, A. M.; Cunningham, B. A. Purification and some properties of sorghum grain esterase and peroxidase. *Phytochemistry*. **1971**, *10*, 1-10.

Savitsky, Pavel A.; Gazaryan, Irina G.; Tishkov, Vladimir I.; Lagrimini, L. Mark, Ruzgas, Tautgirdas, Gorton, Lo. Oxidation of indole-3-acetic acid by dioxygen catalyzed by plant peroxidases: specificity for the enzyme structure. *Biochem. J*. **1999**, *340*, 579-583.

Schevchenko, A.; Wilm, M.; Vorm, O.; Mann, M. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal. Chem.* **1996**, *68*, 850-858.

Stafford, H. A.; Brown, M. A. Oxidative dimerization of ferulic acid by extracts from sorghum. *Phytochemistry* **1976**, *15*, 465-469.

Tams, J. W.; Welinder, K. G. Mild chemical deglycosylation of horseradish peroxidase yield a fully active, homogeneous enzyme. *Anal. Biochem.* **1995**, *228*, 48-55.

Tams, W. J.; Vind, J.; Welinder, K. G. Adapting protein solubility by glycosylation. N-glycosylation mutants of *Coprinus cinereus* peroxidase in salts and organic solutions. *Biochem. Biophys. Acta*. **1999**, *1432*, 214-221.

Teichmann, T.; Guan, C.; Kristoffersen, P.; Muster, G.; Tietz, O.; Palme, K. Cloning and biochemical characterization of an anionic peroxidase *from Zea mays*. *Eur. J. Biochem*. **1997**, *247*, 826-832.

Thompson, J. D.; Higgins, D. G.; Gibson, T. J. CLUSTAL W. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **1994**, *22*, 4673-4680.

van Huystee, R. B.; Roig, M. G.; Shnyrov, V. L.; Sakharov, I. Y. Peroxidase stability related to its calcium and glycans. *Phytochem. Rev*. **2004**, *3*, 19-28.

van Mierlo, C. P. M.; van Dongen, W. M. A. M.; Vergeldt, F.; van Berkel, W. J. H.; Steensma, E. The equilibrium unfolding of *Azotobacter vinelandii* apoflavodoxin II occurs via a relatively stable folding intermediate. *Prot. Sci*. **1998**, *7*, 2331-2344.

Venyaminor, S. Y.; Baikalov, I. A.; Wu, C. S. C.; Shen, Z. M.; Yang, Y. T. Circular dichroism analysis of denatured proteins: inclusion of denatured proteins in the reference set. *Anal. Biochem*. **1993**, *214*, 17-24.

Wallace, G.; Fry, S. C. Action of diverse peroxidases and laccases on six cell wall-related phenolic compounds. *Phytochemistry*. **1999**, *52*, 769-773.

Welinder, K. G. Covalent structure of the glycoprotein horseradish peroxidase [EC 1.11.1.7]. *FEBS Lett.* **1976**, *72*, 19-23.

CHAPTER 8

General discussion

Inter-relations between biochemical markers and food quality

As illustrated by the presence of several thousands of sorghum germplasms, sorghum is genetically very diverse. Therefore, it was a challenge to select a representative number of sorghum varieties cultivated in Burkina Faso to screen for biochemical markers, which can serve as determinants for sorghum grain quality in food processing. The varieties were chosen according to their agronomic characteristics (resistance or susceptibility to biotic and abiotic stresses) and food end-use properties: "tô", thin porridge, couscous and "dolo". In this study, biochemical markers for food-quality, such as phenolic compounds and enzymes involved in their biosynthesis (phenylalanine ammonia lyase) and their oxidation (polyphenol oxidases and peroxidases), and the reserve polysaccharide starch and its hydrolytic enzymes (amylases) were investigated. **Figure 1** shows the inter-relations between the biochemical markers screened. Since germination influences the biochemical composition of the grain, the effect of germination on these markers was also assessed.

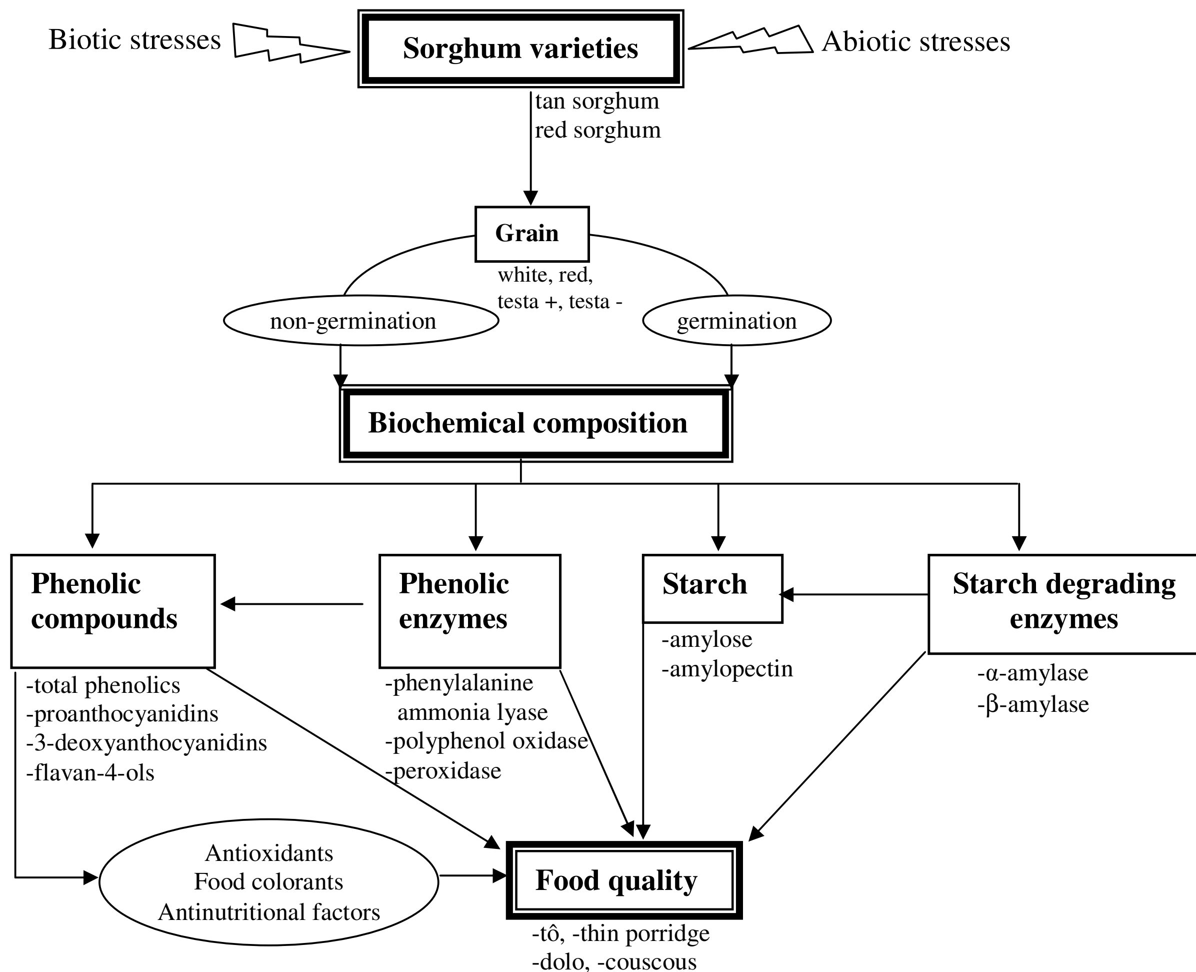

Figure 1. Relationships between biochemical markers relevant for food quality

Biotic and abiotic stresses

The (bio)chemical markers of sorghum studied in this thesis are not only important for food quality but are also determinants for the grain and even the whole plant resistance to several biotic and abiotic stresses (Weiergang et al., 1996; Lo et al., 1999; Parr et al., 2000; Tomas-Barberan and Espin, 2001). It was, therefore, of interest to compare the levels of these compounds in groups of varieties either resistant or susceptible to known stresses that affect sorghum production in Burkina Faso (Neya et al., 1998; Trouche et al., 2000; 200; Sharma et al., 1999;). Such a comparison has not been done before for sorghum and other cereals. In **Chapter 5**, we found that the contents of phenolic compounds and activities of related enzymes before and after sorghum grain germination are significantly different between varieties resistant and susceptible to biotic (sooty stripe, sorghum midge, leaf anthracnose, striga, and grain molds) and abiotic (logging, drought resistance and photo-period sensitivity) stresses. The total phenolic compounds content could not be linked to any stress. However, independent of germination, sorghum varieties resistant to biotic and abiotic stresses have on average higher contents in PAs, 3-DAs and flavan-4-ols than varieties susceptible to the stresses mentioned. PAL activities in ungerminated grain are higher in varieties resistant to sooty stripe, sorghum midge and logging than in susceptible ones. In germinated grain, POX is higher in varieties resistant to logging than in susceptible ones. PPO activities in germinated sorghum are higher in drought resistant varieties than in susceptible ones. These results show that contents of phenolic compounds and activities of phenolic enzymes are candidate markers for resistance and susceptibility of grain or plant to stress.

Relevance of phenolic compounds for food quality

Among phenolic compounds of particular interest in sorghum are proanthocyanidins (PAs), 3-deoxyanthocyanidins (3-DAs) and flavan-4-ols. This is because of their importance in human nutrition and because of their agronomic properties in grain preservation. For instance, the content of PAs is important in assessing the food grade quality of sorghum because of their possible antinutritional effect linked to the inhibition of hydrolytic enzymes (Butler, 1992). While most plants, including other cereals, are lacking 3-deoxyanthocyanidins, e.g. apigeninidins and luteolinidins, sorghum is unique in containing a relatively high level of these compounds (Awika et al., 2004). Reports on the levels of phenolic compounds in sorghum varieties are suffering from the limited number of varieties screened (Subramanian, et al., 1992; FAO, 1995; Iwuoha and Aina, 1999; Bvochora et al., 1999). The studies described in this thesis were conducted with a representative number of fifty sorghum varieties. Flavan-4-ols, present in only rare sorghum

varieties (Audilakshmi et al., 1999), are of particular interest for grain storage (Melake-Berhan et al., 1996) and for treating various cancers (Ferreira and Slade, 2002). **Chapters 2** and **3** describe for the first time the comparison of the content of total phenolics, PAs, 3-DAs and flavan-4-ols in a large number of sorghum varieties. They reveal that the contents of these compounds among sorghum varieties from Burkina Faso vary more than in other sorghums for instance from Nigeria, India and Zimbabwe. The majority of sorghum varieties from Burkina Faso do not contain high amounts of PAs, which is an advantage for weaning foods preparation.

Despite their importance for nutrition, the effect of germination on PAs, 3-DAs, and flavan-4-ols in sorghum varieties has been scarcely studied. Previous reports on 2-4 varieties indicated that the analyzable content of phenolic compounds may increase (Nwanguma and Eze, 1996) or decrease upon germination (Iwuoha and Aina, 1997). In **Chapter 3** it is shown that, on average germination does not affect the total phenolic compounds content of the fifty selected varieties. This could be related to the fact during germination, both enzymes involved in phenolic compounds biosynthesis as well as enzymes involved in their degradation are activated (**Chapter 4**). A clear decrease in PA, 3-DA and flavan-4-ol contents was observed. The observation that the means of PAs, 3-DAs and flavan-4-ols decrease upon germination while the mean of total phenolic compounds is more constant is an overall observation for the fifty screened varieties. However, considering a single variety we observed different fluctuations. The assays used do not reveal all the complexity of the chemistry that occurs in the plant tissue during germination, but showed the chemical diversity in terms of classes of chemical compounds. In addition, sorghum contains several other important groups of phenolic compounds such as hydroxybenzoic acids and hydroxycinnamic acids (for instance ferulic and *p*-coumaric acids are abundant in sorghum) and other flavonoids which were not individually quantified to assess the effect of germination on their levels. The decrease in extractable PAs and DAs upon germination could be due to leaching of water-soluble PAs and DAs, which are located in the pericarp and testa (Beta et al., 1999; Waniska et al., 2000) or due to formation of insoluble complexes between PAs and proteins (Riedl and Hagerman et al., 2001).

Nowadays, phenolic compounds in food are receiving much attention because their antioxidant activities are proven to be of beneficial effect on human health (Moosmann and Belh, 1999; Santos-Buelga and Scalbert, 2000; Parr and Bolwell, 2000; Yao et al., 2004). Recently, Awika and co-workers (2003) reported methods to determine antioxidant activities of sorghum grains. In **Chapter 3**, it is shown that independent of germination, there are large inter-varietal differences in antioxidant activities among sorghum varieties. Phenolic compounds and antioxidant activities were

well correlated before germination, but germination lowered this correlation. This study revealed that although white sorghum varieties have lower antioxidant activities than red varieties, these activities are comparable to the most important sources of natural antioxidants such as *Vaccinium* species, e.g. blackberries (Pellegrini et al., 2003). Moreover, the red sorghum varieties, e.g. V17, V19, V30, V37 and V50 are among the most important potential sources of natural antioxidants. Since sorghum is a staple food in Africa, it can be tentatively inferred that it is the main potential source of natural antioxidants for people relying on it as a main source of energy. Cardiovascular disease (CVD) is one of the most important diseases in Western countries. Epidemiological studies suggest that the consumption of whole cereal grains, including sorghum lowers the mortality from CVD, linked probably to their antioxidant properties (Kushi et al., 1999; Anderson et al., 2003; Awika and Rooney, 2004). This implies that several sorghum varieties characterized in the present study could be candidate to be processed into food not only for Africa, but also for other countries. For sorghum varieties displaying high levels of antioxidant activities, it would be interesting to perform a further analysis of the nature of phenolic compounds contained in these varieties and to study their antioxidant or pro-oxidant properties. A thorough analysis of the phenolic composition of these selected varieties may allow discovering new flavonoids displaying anti-HIV-1 activities (Li et al., 1998; Kitamura et al., 1998; Yao et al., 2004). The same analysis could allow to find lignans and other bioactive phenolic compounds in sorghum grains.

Phenolic enzymes

Enzymes involved in the biosynthesis and oxidation of phenolic compounds have been shown to be determinants for the quality of plant-derived foods (Tomas-Barberan and Espin, 2001). In **Chapter 4**, the presence of PAL activity in sorghum grain and its activation upon germination was assessed. Although PAL is indirectly involved in the synthesis of almost all phenolic compounds, its activity was not correlated with the contents in phenolic compounds in both ungerminated and germinated varieties. This lack of correlation may be due to the presence of phenolic oxidizing enzymes in the grain.

A few decades ago, it was found that sorghum grains contain polyphenol oxidase (PPO) activity (Stafford and Dresler, 1972). However, the inter-varietal difference of PPO activity and the effect of germination on the occurrence of PPO isoenzymes in sorghum varieties are unknown. In **Chapter 2** and **4**, it is shown that sorghum varieties display different monophenolase and *o*-diphenolase activities. Like most other plant PPOs (Martinez and Whitaker, 1995), the sorghum

PPOs are more active with *o*-diphenols than with monophenols. Germination decreases the *o*-diphenolase activity and slightly increases the monophenolase activity.

When we started our research, current methods to detect PPO by zymography were time consuming and suffered from lack of sensitivity (Rescigno et al., 1997; Hoopes and Dean, 2001). In **Chapter 6** we present a fast and sensitive new method of PPO zymography. Interestingly, this method allows to discriminate between the monophenolase and *o*-diphenolase activities of PPO, as well as between these activities and the activities of laccase and POX. The method is particularly useful for the high sensitive detection of POX isoenzymes in the presence of hydrogen peroxide (**Chapter 4**). The zymography of PPO revealed that germination does not induce new PPO isoenzymes in sorghum grain (**Chapter 4**). As reported for wheat (Hatcher and Kruger, 1993), the decrease of *o*-diphenolase activity of PPO might be related to its localization in the pericarp, where it can leach out during germination or form complexes with phenolic compounds. Since both activities of PPO can be kinetically controlled for the synthesis of *o*-diphenols or *o*-quinones, sorghum varieties possessing these activities may find interesting applications in the food and chemical industries (Dubey et al., 1998; Espin et al., 2001).

POX activities in sorghum varieties and the occurrence of isoenzymes have been reported (Ollitrault et al., 1989; Bvochora et al., 1999). However, the effect of germination on these enzymes was not addressed. The studies in **Chapters 2** and **4** revealed that there is a strong inter-varietal difference of POX activity among sorghum varieties, before and after germination. Germination increased POX activity due to the activation of already present isoenzymes and/or due to *de novo* synthesis of POX isoenzymes. Interestingly, cationic POX isoenzymes with p*I* ≥9 are ubiquitously present in both ungerminated and germinated varieties, and as found for other cereals (wheat, barley and maize). However, an anionic isoenzyme (p*I* 3.1) is expressed only in germinated grain. This germination-associated isoenzyme may play a role in cell wall synthesis and protect the seed against pathogens (Passardi et al., 2004). Purification and characterization of this anionic isoenzyme will give more insight in its specificity for phenolic compounds that are involved in plant growth.

Cationic POXs are generally more active on phenolic compounds than laccases and anionic POXs. Therefore, they have better promise as biocatalysts (Wallace and Fry, 1999). In **Chapters 2** and **4** we found that the cationic POX is the most abundant in sorghum, and it is ubiquitously present in all varieties, in both ungerminated and germinated grains. Therefore, the characterization of this isoenzyme was a challenge. In other cereals such as barley, wheat and maize, the cationic isoenzymes were also the most abundant, accounting for 80-90% of total POX activity (Johansson et al., 1992; Converso and Fernandez, 1995; Billau et al., 1999). Except for sorghum, the major

peroxidases of all these cereals have been purified, sequenced and biochemically characterized. In **Chapter 7**, it is shown that sorghum contains several isoenzymes that can be purified. The major sorghum cationic POX (SPC4) was purified and characterized for the first time. Mass spectral analysis showed that SPC4 consists of two glycoforms. Chemical deglycosylation allowed to estimate carbohydrate contents of 3% and 6.7% (w/w) in glycoform I and II, respectively, and a mass of the apoprotein of 33 246 Da. The glycoform II was the major (65%) form of the enzyme. Through N-terminal sequencing, MALDI-TOF-MS peptide fingerprinting and analyzing the currently released sorghum expressed sequence tags (ESTs) it was possible to identify the amino-acid sequence of the first 213 residues of SPC4 (TC102191). With the currently ongoing sorghum genome sequencing project, the full length sequence of SPC4 is expected to be obtained. Sequence analysis and spectroscopic studies showed that, apart from an extremely high isoelectric point (p*I* 11), SPC4 has many properties in common with other cereal peroxidases (maize, barley, wheat and rice). The enzyme is soluble in organic solvents and Ca^{2+} binding increases its denaturation temperature (T_m) from 67 to 82°C. Interestingly, the thermal stability of the enzyme is between that of HRP (Rodriguez et al., 2002) and soybean POX (Amisha and Behere, 2002). SPC4 showed higher activity with hydroxycinnamates than with tyrosine derivatives. Therefore, SPC4 may be applied in food biotechnology for the modification of carbohydrate-containing hydroxycinnamates (Oudgenoeg et al., 2001, 2002; Boeriu et al., 2004; Regalado et al., 2004). Furthermore, SPC4 displayed indole-3-acetic acid oxidase activity. These reactions are useful for (bio)chemical, clinical and biocatalytic applications (Adam et al., 1999; Passardi et al., 2004).

Starch and starch degrading enzymes

Starch is the principal source of calorific energy in the developing world because cereals are the staple food in those countries. The screening of starch, amylose and amylopectin in 50 sorghum varieties before and after germination showed that there is an inter-varietal difference of content in these compounds (**Chapter 4**). Although, some varieties contained relatively little amylose, there is no waxy sorghum, e.g. containing only amylopectin (FAO, 1995) among cultivated sorghums in Burkina Faso. This is probably because cultivated varieties were primarily selected and bred for tô, for which a high amylose content is required (Bello et al., 1999; Trouche et al., 2000). So far, comparison of α-amylase and β-amylase activities were performed in sorghum varieties from other parts of the world but not for those from Burkina Faso. In addition, comparison of the effect of germination on α-amylase and β-amylase activities in sorghum varieties is rare, because most studies focused only on germinated grains (Dufour et al., 1992; Beta et al., 1999). Contrary to

previous results stating that α-amylase and β-amylase activities were not detected in ungerminated sorghum varieties (Ahmed et al., 1996), it is shown in **Chapter 4** that these enzymes are present in all sorghum varieties. The results of Ahmed and co-workers have been biased because of the low number (three) of varieties screened, and also because of the used ferricyanide assay, which is less specific for β-amylase than the assays we used in the present study (McCleary BV and Codd, 1989). While β-amylase activity did not show an overall increase after germination, α-amylase activity increased up to 20 fold in some varieties. The main reason for the difference of the effect of germination between α-amylase and β-amylase may be due to the fact that β-amylase is not *de novo* synthesized during germination (Ziegler, 1999). Using a statistically significant number of samples, it is shown that although α-amylase and β-amylase have a common substrate, e.g. starch, their activities are not correlated in sorghum grain both before and after germination. The clear polymorphism of β-amylase and α-amylase activities in sorghum varieties may give direction for the selection of sorghum varieties containing these enzymes for specific food utilization.

Recommended varieties as potential source of bioactive components

The investigation of several constituents within sorghum varieties has shown their wide (bio)chemical diversity. This large (bio)chemical diversity could be rationally exploited. Among the phenolic compounds and enzymes studied, some are of particular interest as bioactive constituents or biocatalysts. Therefore, it is useful to recommend some varieties as sources of specific molecules. For instance, varieties containing a high level of total phenolic compounds could be novel sources of antioxidants (**Chapter 3**). Varieties containing high levels of PAs could be candidate to isolate these compounds because they are currently gaining attention as antibacterial and anti-HIV1 agents (Chan and Kim, 1998). Varieties containing high levels of 3-DAs will be of interest for the isolation of apigeninidins and luteolinidins which are currently commercialized as food colorants and various other industrial utilizations (Morazzoni and Magistretti, 1990; Coultate, 1996). Based on the literature, a recommendation of some sorghum varieties as sources of specific biochemical components is given in **Table 1**.

Table 1. Recommended varieties as potential source of biochemical constituents

Recommended five varieties	Germination requirement	(Bio)chemical component	Utilizations	References
V17, V19 V30, V37, V50	no	Total phenolic compounds	antioxidant, food additive, antimicrobial	1, 2, 3
V6, V18, V19, V30, V37	no	Proanthocyanidins	antioxidant, antimicrobial and antiviral agent	4, 5, 6
V17, V18, V19, V30, V37	no	3-deoxyanthocyanidins	food colorant	7, 8
V17, V18, V19, V30, V37	no	Flavan-4-ols	anti-cancer agent	9, 10
V31, V35, V40, V41, V42 V10, V44, V46, V47, V50	no	Polyphenol oxidase	synthesis of *o*-diphenols and *o*-quinones	11, 12, 13
V5, V15, V16, V31, V38	yes	Peroxidase	synthesis of various organic compounds, functional modification of carbohydrates and proteins	14, 15, 16, 17, 18
V5, V6, V7, V14, V27	no	Starch	stabilizing agent in pharmaceutical and food industries	19

[1]Awika et al. (2003); [2]Pellegrini et al. (2003); [3]Tomas-Barberan and Espín (2001); [4]Scalbert (1991); [5]Chan and Kim (1998); [6]Lu et al. (2004); [7]Awika and Rooney (2004), [8]Kouda-Bonafos et al. (1996); [9]Ferreira and Slade (2002); [10]Okuda et al. (1991); [11]Dubey et al., 1998; [12]Espin et al. (2001); [13]Lamarino et al. (2003); [14]Guerra and Ferraz (2003); [15]Adam et al. (1999); [16, 17]Oudgenoeg et al. (2001, 2002); [18]Boeriu et al. (2004); [19]Whistler et al. (1984).

Recommended varieties for food processing

As shown in **Figure 1**, the relationships between the screened biochemical markers may give directions for the selection of sorghum varieties for specific foods.

In tô preparation, the formation of a thick paste linked to high amylose content is necessary. Low α-amylase activity found in varieties good for tô is beneficial to obtain a relatively stick porridge. In analogy with what was found in wheat dough (Hilhorst et al., 1999), POX may mediate the thickness of sorghum flour during tô preparation by cross-linking carbohydrates containing endogenous hydroxycinnamate derivatives.

On the other hand, sorghum varieties with low viscosity are desired in the formulation of weaning foods with high energy density (WHO, 1998). In that case low amylose content and high α-amylase activities are determinant. For infant porridges, low PAs containing varieties, and low PPO and POX activities may be more convenient by avoiding enzyme-mediated oxidation of endogenous phenolic compounds into colored products. Interestingly, food-based sorghum varieties

rich in PAs can be suggested to obese people and diabetic patients by analogy with the 50% weight loss observed with animals (rabbits, pigs, etc.) fed with sorghums containing high levels of PAs (Ambula et al, 2001). This is supported by the observation that in certain cultures in Africa, people prefer to consume PAs containing sorghums because they have a longer "staying power" in the stomach (Awika and Rooney, 2004). The low digestibility of high PAs containing sorghums through the inhibition of hydrolytic enzymes, together with their high antioxidant activities may be interesting from a nutritional standpoint for obese persons.

For dolo and industrial beer preparations, high α-amylase and β-amylase activities are desired (Dufour et al., 1992; Taylor and Robbins, 1993). In industrial brewing, a specific interest exists in high β-amylase-containing sorghum varieties because this enzyme is usually deficient in sorghum (Dufour et al., 1992; Taylor and Robbins, 1993; Verbruggen et al., 1993, 1996). In **Chapter 4,** it is shown that some sorghum varieties contain β-amylase activities comparable of that of barley (Beta et al., 1995). These varieties can be suggested for industrial brewing. A constraint in the utilization of sorghum for industrial brewing is the high starch gelatinization temperature (Okafor and Aniche, 1987). Since amylose has a higher gelatinization temperature than amylopectin (Whistler et al., 1984), sorghum with low amylose content could be targeted for industrial brewing. Low POX and PPO activities to avoid beer darkness and haze occurrence by the oxidation of endogenous phenolic compounds are other criteria for selection of sorghum for industrial brewing (Clarkson et al., 1992).

For couscous preparation, the formation of a gel, mediated by POX via the cross-linking of macromolecules, is not desired. In addition, low α-amylase activity is required to avoid starch dextrinization during the process.

In Burkina Faso, bakers do not use composite sorghum/wheat flour. However, acceptable bread can be produced with 30-50% sorghum substitution for wheat (Anglani, 1998; Carson et al., 2000). Addition of sorghum flour possessing high POX activities (Hilhorst et al., 1999) in combination with suitable α-amylase activity (Baik et al., 2003) could lead to the cross-linking of sorghum glucuronoarabinoxylans. These polysaccharides are known to contain ferulic acid (Verbruggen et al., 1993; 1996) and may make addition of a higher percentage of sorghum flour acceptable, in analogy with the action of POX in wheat bread (Hilhorst et al., 1999). For bread making, it is important to have sorghum lines with low amylose contents (Lee et al., 2001; Martin et al., 2004). These criteria may give directions for selecting sorghum varieties of the present study for bread making.

The (bio)chemical characteristics screened are suggested to serve as determinants for sorghum utilization for various foods. The recommended varieties having important (bio)chemical

constituents as quality-grade markers for the preparation for foods are shown in **Table 2.** It is important to stress that the suitability of sorghum varieties for food and beverages is also dependent on the chemical and physical properties of kernels and process conditions. The recommendations made in **Table 2** need further investigations on technological levels and consumer acceptance for confirmations.

Table 2. Summary of apparent (bio)chemical properties of groups of sorghum varieties according to known properties and recommended applications

Utilization	Apparent (bio)chemical determinant								Recommended varieties
	Grain color	Germi-nation	Starch content	Amylose content	α-amylase and β-amylase	Phenolics content	[a]POX	[b]PPO	
Tô	white	no	high	high	low	medium	high	low	V1, V4, V5, V6, V8, V9, V10, V11, V12, V15, V20, V21, V22, V25, V26, V32, V34, V38, V40, V43, V45, V46, V47, V48, V49
Dolo	red	yes	high	high	high	high	high	low	V17, V18, V19, V30, V33, V36, V37, V42, V50
Couscous	white	no	medium	low	medium	low	medium	low	V3, V7, V14, V16, V27, V29, V31, V35
Infant porridge	white	yes	high	low	high	low	low	low	V2, V13, V23, V24, V28, V39, V41, V44
Industrial beer	white	yes	high	low	high	high	low	low	V40, V41, V45 V47, V48
Bread	white	no	high	high	high	high	high	low	V5, V7, V16, V31, V38

In conclusion, sorghum is a tropical cereal that nature gave us with the highest environment tolerance and the greatest possible (bio)chemical diversity. The findings in this thesis show that it is possible to use state-of-the-art (bio)chemical knowledge to give directions for selecting the most suitable sorghum varieties for specific food utilization. Research on endogenous bioactive components such as phenolic compounds and industrial enzymes, is essential to unleash sorghum's capacity to be the cornerstone of food security in Africa as well as in many developing countries.

REFERENCES

Adam, W.; Lazarus, M., Saha-Moller, C. R.; Weichold, O.; Hoch, U.; Haring, D.; Schreider, P. **1999**, *63*, 73-107.
Anglani, C. *Plant Foods Hum. Nutr*. **1998**, *52*, 85-89.
Ahmed, S. B.; Mahgoub, S. A.; Babiker, B. E. *Food Chemistry*. **1996**, *56*, 159-162.
Ambula, M. K.; Oguho, G. W.; Tuitoek, J. K. *Asian-Austr. J. Ani. Sci.* **2001.**14, 1276–1281.
Amisha, J. K.; Behere, D. V. *Biochemistry* **2002**, *41*, 9034-9042.
Anderson, J. W. *Proc. Nutr. Soc*. **2003**, *62*, 135–142.

Anon, K. B. Measurement of diastatic activity. In *Recommended Methods of Analysis of Barley, Malt and Adjuncts.* Adopted by the Council of the Institute of Brewing, **1971**, pp. 163-165.
Awika, J. M., Rooney, L. W., Wu, X., Prior, R. L., Cisneros-Zevallos, L. *J. Agric. Food Chem*. **2003**; 51, 6657-6662.
Awika, J. M.; Rooney, L. W. *Phytochemistry*. **2004**, *65*, 1199-1221.
Audilakshmi, S.; Stenhouse, J. W.; Reddy, T. P.; Prasad, M. V. R. Grain mould resistance and associated characters of sorghum genotypes. *Euphytica*, **1999**, *107*, 91-103.
Baik, B. K.; Czuchajowska, Z.; Pomeranz, Y. *J. Cereal. Sci*. **1994,** *19*, 291-296.
Baik, B.-K.; Park, C. S.; Paszczynska, B.; Konzak, C. F. *Cereal Chem*. **2003**, *80*, 627-633.
Bello AB, Rooney LD, Waniska RD. *Cereal Chem.* **1990**, *67*, 20-25.
Beta, T.; Rooney, L. W.; Waniska, R. D. *Cereal Chem*. **1995**, *72*, 533-538.
Beta. T.; Rooney. L. W.; Marovatsanga. L. T.; Taylor. J. R. N. *J. Sci. Food Agric*. **1999**, *79*, 1003-1010.
Billau, C., Louarme, L.; Nicolas, J. *J. Food Biochem*. **1999**, *23*, 145-172.
Boeriu, C. G.; Oudgenoeg, G.; Spekking, W. T. J.; Berendsen, L. B. J. M.; Vancon, L.; Boumans, H.; Gruppen, H.; van Berkel, W. J. H.; Laane, C.; Voragen, A. G. J. *J. Agric. Food Chem*. **2004**, *52*, 6633-6639.
Butler. L. G. Plant polyphenols: synthesis, properties and significance; Hemingway. R. W.; Laks. P. E. Eds.; Plenum press. New York. **1992**, pp. 693-698.
Bvochora, J. M., Reed, J. D., Read, J. S.; Zvauya, R. *Process Biochem*. **1999**, *35*, 21-25.
Carson, L.; Setser, C.; Sun, X. S. *Int. J. Food Sci. Technol.* **2000**, *35*, 465-471.
Chan, D. C.; Kim, P. S. *Cell* **1998**, *3*, 681-684.
Christensen, J. H.; Bauw, G.; Welinder, K. G.; Montagu, M. V. ; Boerjan, W. *Plant Physiol*. **1998**, *118*, 125-135.
Clarkson, S. P.; Large, P. J.; Bamforth, C. W. *J. Inst. Brew*. **1992**, *98*, 111-115.
Coultate. T. Food: The chemistry of its compounds. Edition Lavoisier, 3rd Edition ; Paris. **1996**.
Dubey, S.; Singh, D.; Misra, R. A. *Enz. Microb. Technol*. **1998**, *23*, 432-437.
Dufour, J. P.; Melotte, L.; Sebrenik, S. *ASBC Journal*. **1992**, *50*, 110-119.
Dunford, H. B. Heme peroxidases. John Wiley & Sons, Inc. **1999**, 509p.
Espin, J. C.; Soler-Rivas, C.; Cantos, E.; Tomas-Barberan, F. A.; Wichers, H. J. *J. Agric. Food Chem*. **2001**, *149*, 1187-1193.
Ferreira, D.; Slade, D. *Nat. Prod. Rep.* **2002**, *19*, 517-541.
Food and Agricultural Organization of the United Nations (FAO). FAO Food and Nutrition Series, No. 27. ISBN 92-5-103381-1. 1995. http://www.fao.org/DOCREP/T0818e/T0818E00.htm#Contents
Guerra, A.; Ferraz, A. *In* Wastewater treatment using enzymes. Research Signpost, Trivandrum, India. **2003**, pp. 73-91.
Hatcher, D. W.; Kruger, J. E. *Cereal Chem.* **1993**, *70*, 51-55.
Hilhorst, R., Dunnewind, B., Orsel, R., Stegeman, P., Vliet, T. van, Gruppen, H. and Schols, H. *J. Food Sci*. **1999**, *64*, 808-813.
Hoopes, J. T.; Dean, J. F. D. *Anal. Biochem*. **2001**, *293*, 96-101.
Iwuoha, C. I.; Aina, J. O. *Food Chem*. **1997**, *58*, 289-295.
Kitamura, K.; Honda, M.; Yoshizaki, H.; Yamamoto, S.; Nakane, H.; Fukushima, M.; Ono, K.; Tokunaga, T**.** *Antiviral Res*. **1998**, *37*, 131-140.
Kouda-Bonafos, M.; Nacro, M.; Ancian, B. *Magn. Reson. Chem*. **1996**, *34*, 389-394.
Kushi, L. H.; Meyer, K. A.; Jacobs, D. R. *Amer. J. Clin. Nutr*. **1999**, *70*, 451S–458S.
Lamarino, G.; Rao, M. A.; Nocerino, G.; Gianfreda, L. *Polyph. Actual.* **2003**, *23*, 20-22.
Li, B. Q.; Fu, T.; Yan, Y. D.; Mikovits, J. A.; Ruscetti, F. W.; Wang, J. M. Biochem. *Biophys. Res. Commun.* **1998**, *276*, 534-538.
Lee, Mee-Ryung; Swanson, Barry, G.; Baik, B. K. *Cereal Chem*. **2001**, *78*, 701-706.
Lo, S. C. C.; De Verdier, K.; Nicholson, R. L. *Physiol. Mol. Plant Pathol*. **1999**, *55*, 263-273.
Lu, L.; Liu, S.; Jiang, S.; Wu, S. *Acta Pharma.* **2004**, *25*, 213-218.
Martin, J. M.; Talbert, L. E.; Habernicht, D. K.; Lanning, S. P.; Sherman, J. D.; Carlson, G.; Giroux, M. J. *Cereal Chem.* **2004**, *81*, 188-193.
Martinez, M. V.; Whitaker, J. R. *Trends Food Sci. Technol*. **1995**, *6*, 195-200.
McCleary, B. V.; Sheehan, H. *J Cereal Sci.* **1989**, *6*, 237-251.
McCleary BV, Codd R. *J. Cereal Sci*. **1989**, *9*, 17-33.
Melake-Berhan, A.; Butler. L. G.; Ejeta. G.; Menkir, A. *J. Agric. Food Chem*. **1996**, *44*, 2428-2434.
Moosmann. B.; Behl, C. *Proc. Natl. Acad. Sci. U SA*. **1999**, *96*, 8867-8872.
Morazzoni. P. ; Magistretti. M. J. *Fitoterapia*. **1990***, 61*, 13-20.
Neya A.; Le Normand, M. *Crop Protec*. **1998**. *17*, 47-53.
Nwanguma, B. C.; Eze, M. O. *J. Inst. Brew*. **1995**, *101*, 275-276.
Nwanguma, B. C.; Eze, M. O. *J. Sci. Food Agric.* **1996**, *70*, 162-166.
Okafor, N.; Aniche, G. N. *J. Food Sci. Tech*. **1987**, *24*, 131-134.

Okuda, T.; Yoshida, T.; Hatano, T., Economic and Medicinal Plant Research, Vol 5; Wagner, H.; Farnsworth, N. R. Eds.; Academic Press, London, **1991**, 129-165.
Ollitrault, P.; Arnaud, M.; Chantereau, J. *Agron. Trop*. **1989**, *44*, 211-222.
Oudgenoeg, G., Dirksen, E., Ingemann, S., Hilhorst, R., Gruppen, H., Boeriu, C. G., Piersma, S. R., van Berkel, W. J., Laane, C.; Voragen, A. G. J. *J. Biol. Chem*. **2002**, *277,* 21332-213340.
Oudgenoeg. G.; Hilhorst. R.; Piersma. S. R.; Boeriu. C. G.; Gruppen. H.; Hessing. M.; Voragen. A. G. J.; Laane C. *J. Agric. Food Chem*. **2001**, *49*, 2503-2510.
Parr, A. J.; Bolwell, G. P. *J. Sci. Food Agric*. **2000**, *80*, 985-1012.
Passardi, F.; Penel, C.; Dunand, C. *Trends Plant Sci*. **2004**, *9*, 534-539.
Pellegrini, N.; Serafini, M.; Colombi, B., Del Rio, D.; Salvatore, S.; Bianchi, M.; Brighenti, F. *J. Nutr*. **2003**, *133*, 2812-2819.
Regalado, C.; Garcia-Almendárez, B. E.; Duarte-Vázquez, M. A. *Phytochemistry Rev*. **2004**, *3*, 243-256.
Rescigno, A., Sollai, F., Rinaldi., A. C., Soddu, G., and Sanjust, E. *J. Biochem. Biophys. Methods* **1997**, *34*, 155-159.
Riedl, K. M.; Hagerman, A. E. *J. Agric. Food Chem.* **2001**, *49,* 4917-4923.
Rodríguez, A.; Pina, D. G.; Yélamos, B.; Léon, J. J. C.; Zhadan, G. G.; Villar, E.; Gavilanes, F.; Roij, M. G.; Sakharov, I. Y.; Shnyrov, V. L. *Eur. J. Biochem*. **2002**, *269*, 2584-2590.
Santos-Buelga, C.; Scalbert, A. *J. Sci. Food Agric*. **2000**, *80*, 1094-1117.
Scalbert, A. *Phytochemistry* **1991**, *30*, 3875-3883.
Sharma, H. C.; Mukuru S. Z.; Hari-Prasad, K. V.; Manyasa, E.; Pande, S. *Crop Prot*. **1999**, *18*, 29-37.
Stafford, H. A.; Dresler, S. *Plant Physiol.* **1972**, *49*, 590-595.
Subramanian, V., Murty, D. S., Rao, N. S. and Jambunatan, R. *J. Sci. Food Agric*. **1992**, *58*, 35-40.
Taylor, J. R. N.; Dewar, J. Advances in Food and Nutrition Research, Vol. 43; S.L. Taylor, Ed.; Academic Press, San Diego, USA, **2001**.
Taylor, J. R. N.; Robbins, D.J. *J. Ins. Brew*. **1993**, *99*, 413-416.
Tomas-Barberan, F.; Espín. J. C. *J. Sci. Food Agric*. **2001**, *81*, 853-876.
Trouche, G.; Fliedel, G.; Chantereau J.; Barro, C. *Agric. Dev.* **2000**, *23*, *94*-107
Trouche, G.; Da, S. ; Pale, G.; Sohoro, A., Ouédraogo, O.; Den-Gosso, G. In Sélection participative. Hocdé, H., Lançon, J., Trouche, G. ed.; CIRAD, Montpellier. **2001**. pp. 36-55.
Verbruggen, M. A.; Beldman, G.; Voragen, A. G. J.; Hollemans, M. *J. Cereal Sci*. **1993**, *17*, 71-82.
Verbruggen, M. *Glucuroarabinoxylans from sorghum grain.* PhD thesis. Wageningen University, The Netherlands, **1996**.
Weiergang, I.; Hipskind, J. D.; Nicholson R. L. *Physiol. Mol. Plant Pathol*. **1996**, *49*, 377-388.
Wallace, G.; Fry, S. C. *Phytochemistry* **1999**, *52*, 769-773.
Weir, T. L.; Park, S. W.; Vivanco, J. M. *Curr. Opin. Plant Biol*. **2004**, *7*, 472-479.
Whistler, R. L.; BeMiller, J. N.; Paschall, E. F. Starch chemistry and technology, 2nd Edition. Academic Press, Inc. Orlando, San Diego, New York, London, Toronto, Montreal, Sydney, Tokyo, **1984**.
World Health Organization (WHO) Complementary feeding of young children in developing countries: A review of current scientific knowledge. UNICEF/University of California-Davis/WHO/ORSTOM, Geneva: WHO/NUT/98.1. 1998.
Yao, L. H.; Jiang, Y. M.; Shi, J.; Tomas-Barbéran, F. A.; Datta, N.; Singanusong, R.; Chen, S. S. *Plant Foods Hum. Nutr*. **2004**, *59*, 113-122.
Ziegler, P. *J. Cereal Sci.* **1999**, *29*, 195-204.

SUMMARY

Sorghum is a staple food grain in many semi-arid and tropic areas of the world, notably in Sub-Saharan Africa because of its good adaptation to hard environments and its yield of production. It is therefore important to select varieties meeting specific agricultural and food requirements from this great biodiversity to insure food security.

The major objective of this thesis is to screen for biochemical components in fifty sorghum varieties cultivated in Burkina Faso to identify the best sorghum variety to be used as source of bioactive component or for specific local foods, e.g. “tô”, thin porridges for infants, granulated foods “couscous”, and local beers “dolo”. The choice of screened biochemical components was based on their role in food processing and their importance for nutritional quality of end-products. The main screened biochemical constituents are: i) starch and starch degrading enzymes such as α-amylases and β-amylases; ii) phenolic compounds and phenolic synthesizing (phenylalanine ammonia lyase) and oxidizing (polyphenol oxidase and peroxidase) enzymes. Since ungerminated and germinated sorghum kernels are differently used according to the processed food, the effects of germination on the biochemical constituents were assessed.

The mean values of starch, amylose and amylopectin in sorghum varieties were 63%, 13.4% and 49.6%, respectively. Germination induced a reduction of starch, amylose and amylopectin content by 5%, 14% and 3%, respectively. The proportion of amylose and amylopectin in sorghum starch was on average 21.2% and 79.8%, respectively. While α-amylase activity increased by 2-20 fold, β-amylase activity did not uniformly increase after germination. Some phenolic compounds are beneficial in foods because of their antioxidant activities, whereas others such as proanthocyanidins (PAs) may impair nutritional qualities of grain. Most of the screened varieties are low PAs containing sorghums (≤ 0.25%, w/w) whereas only a few are high PAs containing sorghums (≥ 0.75%, w/w). Germination generally reduces the level of PAs. Thus, the majority of the cultivated sorghum varieties in Burkina Faso are PAs free sorghums. Other important phenolic compounds in sorghum are 3-deoxyanthocyanidins (3-DAs) and flavan-4-ols because of their involvement in grain resistance to molds and their utilization as natural food colorant. The present study revealed that some varieties had relatively high content in 3-DAs (> 0.4%, w/w), both before and after germination. Remarkably, only a minor fraction of the varieties contained flavan-4-ols. In general, germination decreased the content of 3-DAs and flavan-4-ols in sorghum varieties.

Independent of grain germination, all sorghum varieties displayed antioxidants activities due essentially to their phenolic contents. Germination did not strongly affect the content in total phenolic compounds and total antioxidant activities. Phenolic compounds and antioxidant activities were more positively correlated in ungerminated grains than germinated grains. Red sorghum varieties, notably those with pigmented testa layers had higher antioxidant activities (Trolox equivalents) than white varieties, and these activities are higher than currently reported for most natural phenolic antioxidants from plant foods.

Activity of the key enzyme in phenolics biosynthesis (phenylalanine ammonia lyase, PAL), was detected only in half of the varieties, before germination. However, germination activated PAL in all of them. Independent of germination, peroxidase (POX) activity was several fold higher than the monophenolase and *o*-diphenolase activities of polyphenol oxidase (PPO). Germination did not affect the monophenolase activity of PPO but induced a decrease of the *o*-diphenolase activity of PPO. In contrast, germination stimulated the POX activity by 1.2 to 10 fold. This indicates that POX might be more involved in the oxidation of endogenous phenolic compounds than PPO.

In the course of the study of POX and PPO expression in sorghum, a new method of in-gel detection of both POX and PPO after isoelectrofocusing was developed. The method allowed the distinction between the monophenolase and *o*-diphenolase activities of PPO isoenzymes. The newly

developed method revealed that PPO is not polymorph in sorghum varieties. For POX, a high polymorphism in isoenzyme pattern was observed. During germination, POX isoenzymes were *de novo* synthesized, allowing to detect at least twelve isoenzymes. While cationic POX isoenzymes with p*I* ≥9 are ubiquitously present in both ungerminated and germinated varieties, an anionic isoenzyme (p*I* 3.1) was expressed exclusively in germinated grain.

The major sorghum POX isoenzyme (SPC4) representing more than 80% of POX activity was purified to apparent homogeneity, and found to be a highly basic protein (p*I* ~ 11). MALDI-TOF-MS analysis showed that the enzyme consisted of two glycoforms with molecular masses of 34227 and 35629 Da, respectively. Chemical deglycosylation and HPAEC analysis allowed to estimate sugar contents of 3% and 6.7% (w/w) for glycoforms I and II, respectively, and a mass of the apoprotein of 33 246 Da. The proportions of glycoforms I and II are 35 and 65%, respectively. N-terminal sequencing and MALDI-TOF-MS peptide fingerprinting were performed to determine the primary structure of SPC4. Analyzing the currently released sorghum ESTs (expressed sequence tags) allowed to identify the first 213 amino acids (TC102191). The enzyme is localized in the chromosome 1 of sorghum. Spectroscopic studies, amino acid composition and N-terminal sequence analysis indicated that SPC4 is most related to other cereal peroxidases (barley, rice, wheat and maize). Inactivation and protein unfolding studies showed that the enzyme has an unusual thermal stability in the presence of Ca^{2+}. The substrate specificity of SPC4 suggests its involvement in the cross-linking of the plant cell wall. Moreover, the enzyme had indole-3-acetic acid oxidase activity suggesting a role in auxin regulation.

Agronomic characteristics of grains (presence of absence of pigmented testa layer and color of the glumes) and plants (red or tan) could be linked to grain contents in phenolic compounds and phenolic related enzymes (PAL, PPO and POX). Contents in phenolic compounds and related enzymes were also compared between varieties resistant and susceptible to biotic (sooty stripe, sorghum midge, leaf anthracnose, striga, and grain molds) and abiotic (logging, drought resistance and photo-period sensitivity) stresses. From this it was deduced that 3-deoxyanthocyanidins and proanthocyanidins are potential markers of sorghum stress.

The screened biochemical parameters could be linked to the preferences of varieties for specific local foods. For instance, among varieties used for “tô”, “dolo”, couscous and thin porridge preparation, the “dolo” varieties had the highest average content and diversity in phenolics as well as the highest antioxidant activities. Varieties used for “dolo” had relatively higher α-amylase activities than varieties poor for “dolo”. “Dolo” varieties had a high content in PPO and a low POX activity. Varieties good for “tô” had the highest contents of total starch and amylose, before and after germination and, interestingly, showed the lowest α-amylase specific activity. In general, “tô” varieties have low phenolic compounds content and a medium POX activity. Varieties good for infant porridges preparation have low amylose content and high α-amylase activity after germination. Varieties good for couscous have a characteristic low POX content. In conclusion, the screened biochemical markers are determinants for the utilization of sorghum grains for human nutrition.

SAMENVATTING

Sorghum is de voornaamste graansoort in vele half-droge en tropische gebieden van de wereld, met name in de Sub-Sahara van Afrika, vanwege zijn klimatologisch aanpassingsvermogen en zijn productie-opbrengst. Het is daarom belangrijk om uit de grote diversiteit aan sorghum soorten de juiste variëteiten te selecteren die voldoen aan de specifieke vereisten om mensen van voldoende veilig voedsel te voorzien.

Het hoofddoel van dit proefschrift was om een aantal biochemische bestanddelen van vijftig verschillende, in Burkina Faso gekweekte, sorghumvariëteiten te onderzoeken om zo de beste sorghumsoort te selecteren als bron voor voedingsingrediënt of voor specifieke lokale gerechten zoals "tô", dunne sorghumpap voor zuigelingen, gemalen voedsel "couscous" en plaatselijke bieren "dolo". De keuze van de te onderzoeken biochemische bestanddelen was gebaseerd op hun rol in de voedselverwerking en hun belang voor de voedingswaarde van de eindproducten. De belangrijkste biochemische bestanddelen zijn: i) zetmeel en zetmeel afbrekende enzymen zoals α-amylases en β-amylases; ii) fenolische verbindingen en iii) enzymen die fenolen synthetiseren (fenylalanine ammonia lyase) dan wel oxideren (polyfenol oxidase en peroxidase). Omdat niet-ontkiemde en ontkiemde sorghum pitten anders gebruikt worden voor de voedselbereiding, werden de effecten van ontkieming op de biochemische bestanddelen bestudeerd.

De gemiddelde gehaltes aan zetmeel, amylose en amylopectine in de sorghum variëteiten waren respectievelijk 63%, 13,4% en 49,6%. Ontkieming veroorzaakte een verlaging van de zetmeel, amylose en amylopectine gehaltes met respectievelijk 5%, 14% en 3%. Het amylose en amylopectine gehalte in sorghum zetmeel was gemiddeld 21,2% en 79,8%. Na ontkieming nam de gemiddelde α-amylase activiteit met een factor 2 tot 20 toe. De β-amylase activiteit steeg echter niet uniform en nam zelfs af in sommige variëteiten.

Sommige fenolische verbindingen uit sorghum zijn nuttig vanwege hun antioxidante eigenschappen, terwijl andere zoals proanthocyanidines (PAs) de voedingswaarde van de granen kunnen benadelen. De meeste sorghumvariëteiten bleken lage concentraties PAs (≤ 0,25%, w/w) te bevatten, terwijl slechts een klein deel een hoge concentratie (≥0,75%, w/w) bevat. Ontkieming vermindert het gehalte aan PAs en de meeste gecultiveerde sorghumvariëteiten in Burkina Faso zijn daarom PAs-vrij. Andere belangrijke fenolische componenten in sorghum zijn 3 deoxyanthocyanidines (3-DAs) en flavan-4-ols vanwege hun weerstand tegen schimmels en hun gebruik als natuurlijke voedselkleurstof. De huidige studie liet zien dat sommige variëteiten een relatief hoog gehalte aan 3-DAs bevatte (> 0,4%, w/w), zowel voor als na ontkieming. Opvallend genoeg, bevatte slechts een klein deel van de variëteiten flavan-4-ols. In het algemeen veroorzaakte ontkieming een verlaging van het gehalte aan 3-DAs en flavan-4-ols.

Door de aanwezigheid van fenolische verbindingen, vertoonden alle sorghum variëteiten antioxidant activiteit. Ontkieming beïnvloedde het totale gehalte aan fenolische verbindingen en anti-oxidanten niet in sterke mate. Rode sorghum variëteiten, vooral die met gekleurde testa lagen, hadden hogere antioxidant activiteiten (Trolox equivalenten) dan de witte variëteiten. Deze activiteiten zijn hoger dan van andere natuurlijke fenolische antioxidanten.

De activiteit van het sleutelenzym in de biosynthese van fenolen (fenylalanine ammoniak lyase, PAL), was voor ontkieming slechts in de helft van de variëteiten aanwezig. Ontkieming zorgde echter voor een activering van PAL alle variëteiten. Zowel voor als na ontkieming was de activiteit van peroxidase (POX) vele malen hoger dan de monofenolase en *o*-difenolase activiteit van polyfenol oxidase (PPO). Ontkieming had geen invloed op de monofenolase activiteit van PPO, maar veroorzaakte een afname van de *o*-difenolase activiteit. Door ontkieming werd echter de POX activiteit in alle variëteiten 1,2 tot 10 maal verhoogd. Dit wijst er op dat POX betrokken zou kunnen zijn bij de oxidatie van endogene fenolische verbindingen.

Tijdens de studie van POX en PPO expressie in sorghum, werd er een nieuwe methode ontwikkeld voor de in-gel detectie van zowel POX als PPO activiteit na iso-electrofocusering. Door deze methode kan er in de gel al onderscheid gemaakt worden tussen de monofenolase en *o*-difenolase activiteiten van de verschillende iso-enzymen van PPO. Met deze nieuwe methode werd aangetoond dat sorhum PPO niet polymorf is. In de iso-enzym patronen van POX werd een hoog polymorfisme waargenomen. Tijdens de ontkieming worden er diverse POX iso-enzymen *de novo* gesynthetiseerd. In totaal konden er aldus in sorghum tenminste twaalf POX iso-enzymen worden aangetoond. Kationische POX iso-enzymen met een p*I* ≥9 werden zowel voor als na ontkieming in hoge concentratie aangetroffen. Een anionisch iso-enzym met p*I* 3,1 komt alleen in ontkiemde graankorrels tot expressie.

Het iso-enzym (SPC4) dat in sorghum verantwoordelijk is voor meer dan 80% van de POX activiteit, werd gezuiverd en bleek een zeer basisch eiwit (p*I* ~ 11) te zijn. MALDI-TOF-MS analyse toonde aan dat het enzym in twee glycovormen aanwezig was met molecuulgewichten van respectievelijk 34227 en 35629 Da. Met behulp van chemische deglycosylering en HPAEC analyse kon het suikergehalte bepaald worden op 3% en 6,7% (w/w) voor respectievelijk glycovorm I en II. De massa van het apo-eiwit is 33 246 Da.

Door middel van de bepaling van de *N*-terminale sequentie, MALDI-TOF-MS peptide fingerprinting en analyse van beschikbare sorghum ESTs (Expressed Sequence Tags) was het mogelijk om de volgorde van de eerste 213 aminozuren (TC102191) van SPC4 te identificeren. Spectroscopische studies, aminozuur samenstelling en N-terminale sequentie analyse toonden aan dat SPC4 nauw verwant is aan andere graanperoxidases (uit gerst, rijst, tarwe en maïs). Inactiverings- en eiwitontvouwings studies lieten zien dat het enzym een ongewone thermostabiliteit bezit in aanwezigheid van Ca^{2+}. De substraatspecificiteit van het enzym duidt op een betrokkenheid in de cross-linking van de plantencelwand. Verder heeft SPC4 indool-3-acetaat oxidase activiteit wat een rol in de regulatie van auxine suggereert.

Agronomische kenmerken van graankorrels (aan- of afwezigheid van gekleurde testa lagen en kleur van het vlies) en planten (rood- of taan-kleurig) konden gerelateerd worden aan de sorhum gehaltes aan fenolische verbindingen en fenolen gerelateerde enzymen (PAL, PPO en POX). Deze gehaltes werden ook vergeleken met de resistentie van de variëteiten en de gevoeligheid voor biotische (“Sooty stripe”(langwerpige bruine vlekken met een gele kern), sorghum-insekt, bladgrijskleuring, Striga (parasietplant) en graanschimmels) en abiotische stress (wortelhechting in de grond, droogte resistentie en gevoeligheid voor licht/donker periodes). Hieruit kwam naar voren dat deoxyanthocyanidines en proanthocyanidines potentiële markers zijn voor sorghum stress.

De onderzochte biochemische parameters zouden gekoppeld kunnen worden aan de voorkeur voor variëteiten om specifieke lokale producten te bereiden. Bijvoorbeeld, onder de variëteiten die gebruikt worden voor de bereiding van “tô”, “dolo”, couscous en dunne pap, had de “dolo” variëteit zowel het hoogste gehalte en diversiteit aan fenolen en de hoogste antioxidant activiteit. De variëteiten die gebruikt worden voor “dolo” hadden relatief meer α-amylase activiteit dan de variëteiten die ongeschikt zijn voor de bereiding van “dolo”. “Dolo” variëteiten hadden ook een hoger gehalte aan PPO en een lage POX activiteit. Variëteiten geschikt voor de bereiding van “tô” hadden het hoogste gehalte aan zetmeel en amylose, zowel voor als na ontkieming, maar vertoonden verassend genoeg de laagste α-amylase activiteit. In het algemeen hebben “tô” variëteiten een laag gehalte aan fenolische verbindingen en een middelmatige POX activiteit. Variëteiten geschikt voor de bereiding van zuigelingenpap hebben, na ontkieming, een laag gehalte aan amylose en een hoge α-amylase activiteit. Variëteiten geschikt voor couscous hebben een karakteristiek laag POX gehalte. Samenvattend kan geconcludeerd worden dat de onderzochte biochemische eigenschappen een goede leidraad vormen voor het gebruik van sorghum in humane voeding.

RESUME

Le sorgho est un aliment de base dans beaucoup de zones semi-arides et tropiques du monde, plus particulièrement en Afrique Sub-Saharienne à cause de sa bonne adaptation aux environnements hostiles et son bon rendement. Il est donc important de sélectionner des variétés possédant des caractéristiques agronomiques et des propriétés nutritionnelles désirées au sein de cette biodiversité, afin d'assurer la sécurité alimentaire. L'objectif majeur de cette thèse sera de faire le screening des composés biochimiques dans cinquante variétés de sorgho cultivées au Burkina Faso pour identifier les meilleures variétés à être utilisées comme source de molécules bioactives ou pour la préparation de nourritures locales spécifiques, *ex :* "tô", bouillies infantiles, couscous, et la bière locale "dolo". Le choix des composants biochimiques étudiés est basé sur leur rôle dans la transformation alimentaire et leur importance dans la valeur nutritive du produit final. Le screening a porté sur les principaux constituants biochimiques suivants : i) l'amidon et les enzymes le dégradent telles que les α-amylases et les β-amylases; ii) les composés phénoliques, les enzymes les synthétisant (phenylalanine ammonia lyase) ou les oxydant (polyphenol oxidase et peroxydase). Puisque les grains non germés et germés sont utilisés pour différents besoins alimentaires, les effets de la germination sur les constituants biochimiques ont été évalués. Les valeurs moyennes de l'amidon, l'amylose et l'amylopectine dans les variétés de sorgho étaient respectivement de 63%, 13,4% et 49,6%. La germination a provoqué une réduction de l'amidon, l'amylose et l'amylopectin, respectivement de 5%, 14% et 3%. La proportion de l'amylose et l'amylopectin dans l'amidon de sorgho était en moyenne, respectivement de 21,2% et 79,8%. Pendant que les activités des α-amylases ont augmenté dans toutes les variétés de 2-20 fois après germination, les activités des β-amylases n'ont pas augmenté dans toutes les variétés. Certains composés phénoliques présents dans les grains sont bénéfiques à cause de leurs activités antioxydantes, tandis que d'autres tels que les proanthocyanidines (PAs) peuvent altérer les qualités nutritives des grains. Heureusement, parmi les variétés étudiées 82% ont des teneurs faibles en PAs (<0,25%, w/w), 8% ont des teneurs moyennes (0.25-0.75 %, le w/w) et seulement 10% ont des teneurs élevées en PAs (>0,75%, w/w). De ce fait, la majorité des variétés de sorgho cultivées au Burkina Faso sont sans PAs. La tendance générale dans les variétés de sorgho est que la germination réduit la teneur des PAs. Les autres groupes de composés phénoliques étudiés sont les 3-deoxyanthocyanidines (3-DAs) et les flavane-4-ols à cause de leurs impacts dans la résistance des grains aux moisissures et leurs utilisations comme colorants alimentaires naturels. La présente étude a révélé que quelques variétés de sorghos ont des teneurs relativement élevées en 3-DAs (> 0,4%, w/w), avant et après la germination. Remarquablement, seulement 28% des variétés contenaient les flavan-4-ols. En général, la germination a diminué les teneurs en 3-DAs et les flavan-4-ols dans les variétés de sorgho. Indépendant de la germination, toutes les variétés de sorgho ont affiché des activités antioxydantes essentiellement dues à leurs composés phénoliques. La germination n'a ni fortement affecté le taux des composés phénoliques ni réduit les activités antioxydantes totales. Les composés phénoliques et les activités antioxydantes sont plus positivement corrélés dans les grains non germés que dans les grains germés. Les variétés rouges de sorgho, notamment celles avec des couches de testa pigmentés avaient plus d'activités antioxydantes (30-80 µmol d'équivalents Trolox/g) que les variétés blanches (16-62 µmol d'équivalent de Trolox/g), et ces activités sont plus élevées que celles actuellement rapportées pour la plupart des antioxydants phénoliques naturels trouvés dans les plantes alimentaires. L'activité de l'enzyme clé dans la biosynthèse des composés phénoliques (phenylalanine ammonia lyase, PAL), a été seulement détectée dans 50% des variétés avant la germination, mais la germination a activé la PAL dans toutes les variétés. Indépendant de la germination, les activités des peroxydases (POXs) étaient plusieurs fois plus élevées que les activités monophénolasiques et *o*-diphénolasiques des polyphenols oxydases (PPO). La germination n'a pas affecté l'activité monophenolase des PPOs mais a induit une diminution de l'activité *o*-diphénolasique. Cependant, l'activité des POXs a

augmenté de 1,2 à 10 fois dans toutes les variétés, après germination. Ceci montre que les POXs pourraient être plus impliquées dans l'oxydation des composés phénoliques endogènes que les PPOs.

Au cours de l'étude d'expression des POXs et PPOs dans le sorgho, une nouvelle méthode de détection « dans-le-gel » à la fois des POXs et PPOs après l'électrophorèse sur gel de polyacrylamide natif a été développée. La méthode a permis la distinction entre les isoenzymes des PPOs du sorgho et celles du champignon dans un même gel, de même qu'entre les activités monophenolase et *o*-diphénolase des isoenzymes des PPOs. L'application de la méthode récemment développée a permis de montrer que les PPOs ne sont pas polymorphes dans les variétés de sorgho. Pour les POXs, un haut polymorphisme des isoenzymes a été observé dans les variétés. Pendant la germination, les isoenzymes des POXs étaient synthétisées *de novo*, permettant ainsi de détecter au moins douze isoenzymes. Pendant que les isoenzymes cationiques des POXs avec un $pI \geq 9$ étaient ubiquitaires à la fois dans les variétés non germées et germées, une isoenzyme anionique (pI 3,1) a été seulement exprimée dans le grain germé. L'isoenzyme majeure parmi les POXs des grains de sorgho (SPC4), représentant plus de 80% d'activités des POXs a été purifiée à l'homogénéité, et a s'est révélée être une protéine extrêmement basique ($pI \approx 11$). L'analyse par MALDI-TOF-MS a montré que l'enzyme avait deux glycoformes avec les masses moléculaires respectivement de 34227 et 35629 Da. La déglycosylation chimique et l'analyse par HPAEC ont permis d'estimer des teneurs en carbohydrates de 3% et 6,7% (p/p), respectivement dans les glycoformes I et II, et une masse de l'apoprotéine de 33 246 Da. Le séquençage N-terminal et l'empreinte digitale des peptides par MALDI-TOF-MS ont été réalisés pour déterminer la structure primaire de SPC4. L'analyse des étiquettes des séquences exprimées (ESTs) de sorgho actuellement disponibles dans les banques d'ADN a permis d'identifier les premiers 213 acides aminés (TC102191) de la protéine. Les études spectroscopiques, la composition en acide aminés et l'analyse de la séquence N-terminale indique que SPC4 est apparentée aux peroxydases des céréales (orge, riz, blé et maïs). L'enzyme est située sur le chromosome 1 du sorgho. L'étude des propriétés catalytiques de la protéine et le dépliement de sa conformation tertiaire ont montré que l'enzyme a une stabilité thermique insolite en présence de Ca^{2+}. L'enzyme a aussi une activité d'indole-3-acétique acide oxydase, suggérant un rôle physiologique dans la régulation de l'auxin. La spécificité de l'enzyme pour le substrat suggère son implication dans le crosslinking des carbohydrates contenant des acides hydroxycinnamiques au lieu de la modification des protéines. Les caractéristiques agronomiques des grains (la présence d'absence de couche de testa et la couleur des glumes) et des plantes (rouge ou tan) pourraient être correlées aux contenus des grains en composés phénoliques et les enzymes phénoliques (PAL, POX, et PPO). Les teneurs en composés phénoliques et les enzymes apparentées ont été aussi comparées entre les variétés résistantes et susceptibles aux stresses biotiques (la bande de suie, le moucheron du sorgho, l'anthracnose des feuilles, le striga, et les moisissures des grains) et abiotiques (la verse, la résistance à la sécheresse et la sensibilité à la photopériode). En moyenne, les variétés résistantes aux stresses ont des teneurs élevées et une plus grande diversité en composés phénoliques. Elles ont aussi des activités en enzymes oxydatives plus importantes que les variétés susceptibles. Les paramètres biochimiques dont le screening a été effectué pourraient être corrélés avec les préférences des variétés pour les nourritures locales. Par exemple, parmi les variétés utilisées pour le “tô”, le “dolo”, le couscous et les bouillies, les variétés bonnes pour le “dolo” avaient, en moyennes les teneurs les plus élevées et la plus grande diversité en composés phénoliques. Elles ont aussi les plus grandes activités antioxydantes. Les variétés bonnes pour le “dolo” avaient plus d'activités α-amylasiques que les variétés moins bonnes pour le “dolo”. Les variétés bonnes pour le “dolo” avaient des activités élevées en PPOs et de faibles activités en POXs. Les variétés bonnes pour le “tô” avaient les teneurs les plus élevées en amidon et amylose, avant et après la germination. De manière intéressante, elles ont les activités α-amylasiques les plus faibles. En général, les variétés bonnes pour le “tô” ont de faibles teneurs en composés phénoliques et une

activité élevée en POXs. Les variétés bonnes pour la préparation des bouillies infantiles ont des teneurs en amylose faibles, et des activités α-amylasiques élevées, après la germination. Les variétés bonnes pour le couscous ont des activités faibles en POXs. En conclusion, les paramètres biochimiques dont le screening a été effectué s’avèrent déterminants pour l'utilisation des grains de sorgho en nutrition humaine.

List of abbreviations

ABTS : 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
BLAST: basic local alignment search tool
BP1: barley peroxidase isoenzyme-1
CD: circular dichroism
DAB: 3,3'-diaminobenzidine
3-DAs: 3-deoxyanthocyanidins
DHPPA: 3,4-dihydroxyphenylpropionic acid
Di-PPO: *o-diphenolase* activity of polyphenol oxidase
DMF: *N,N'*-dimethylformamide
DP: degree of polymerization
FAC: ferric ammonium citrate
FAO: Food and Agricultural Organization of the United Nations
FPLC: fast protein liquid chromatography
4-HA: 4-hydroxyanisole
HPAEC: high performance anion exchange chromatography
HRP: horseradish peroxidase
IAA: 3-indole- acetic acid
ICRISAT: international crop research institute for the semi-arid tropics
IEF: isoelectrofocusing
MALDI-TOF-MS: matrix assisted laser desorption/ionization-time of flight mass spectrometry
MBTH: 3-methyl-2-benzothiazolinone hydrazone hydrochloride
Mono-PPO: monophenolase activity of polyphenol oxidase
NSP: non-starch polysaccharides
PAD: pulsed amperometric detection
PAs: proanthocyanidins
PC: phenolic compounds
PMA: phosphomolybdenum assay
PNPG5: *p*-Nitrophenyl maltopentaoside
POX: peroxidase
PPO: polyphenol oxidase
PVP: polyvinylpyrrolidone
TAE: Trolox antioxidant equivalent
TFA: trifluoroacetic acid
TFMS: trifluoromethanesulphonic acid
VCAE: vitamin C antioxidant equivalent

List of Publications

Dicko, M. H., Searle-van Leeuwen, M. J. F., Beldman, G., Ouédraogo, O. G., Hilhorst, R. and Traoré, A. S. (**1999**). Purification and characterization of β-amylase from *Curculigo pilosa. Applied Microbiology and Biotechnology* (Sringer). **52**, 802-805.

Dicko, M. H., Searle-van Leeuwen, M. J. F., Hilhorst, R., and Traoré, A. S. (**2000**). Extraction, partial purification and characterization of β-amylase from *Gladiolus klattianus. Bioresource Technology* (Elsevier). **73**, 183-185.

Dicko, M. H., Searle-van Leeuwen, M. J. F., Hilhorst, R., Traoré, A. S. and Beldman, G. (**2001**) Polysaccharide hydrolases from leaves of *Boscia senegalensis:* properties of endo-(1→3)-β-D-glucanase. *Applied Biochemistry and Biotechnology* (Humanapress, USA). **94,** 225-241.

Dicko, M. H., Hilhorst, R., Gruppen, H., Traoré, A. S., Laane, C., van Berkel, W. J. H. and Voragen, A. G. J. (**2002**) Comparison of content in Phenolic compounds, polyphenol oxidases and Peroxidases in grains of fifty sorghum varieties from Burkina Faso*. Journal of Agricultural and Food Chemistry* (ACS, USA). **50**, 3780-3788.

Dicko, M. H., Hilhorst, R., Gruppen, H., Laane, C., van Berkel, W. J. H. and Voragen, A. G. J. (**2002**) Zymography of monophenolase and *o*-diphenolase activities of Polyphenol oxidase. *Analytical Biochemistry* (Academic Press, USA). **360**, 336-339.

Karou, D., **Dicko, M. H**., Sanon, S., Simporé, J., Traoré, A. S. (**2003**) Antimalarial activity of *Sida acuta* Burm. f. *(Malvaceae)* and *Pterocarpus erinaceus* Poir. *(Fabaceae). Journal of Ethnopharmacology* (Elsevier). **89**, 291-294.

Doka, O., Bicanic, D. D., **Dicko, M. H.** and Slingerland, M. A. (**2004**) Photoacoustic approach to direct determination of the total phenolic content in red sorghum flours. *Journal of Agricultural and Food Chemistry* (ACS, USA). **52**, 2133-2136.

Dicko, M. H., Gruppen, H., Traoré, A. S.; van Berkel, W. J. H. and Voragen, A. G. J. (**2005)** Evaluation of the effect of germination on phenolic compounds and antioxidant activities in sorghum varieties. *Journal of Agricultural and Food Chemistry* (ACS, USA). **53**, 2581-2588.

Dicko, M. H., Gruppen, H., Zouzouho, O. C.; Traoré, A. S.; van Berkel, W. J. H. and VORAGEN, A. G. J. (**2005**) Effects of germination on the activities of amylases and phenolic enzymes in sorghum varieties grouped according to food end-use properties. Submitted

Dicko, M. H., Gruppen, H., Voragen, A. G. J. and van Berkel, W. J. H. (**2005**) Biochemical characterization of the major cationic sorghum peroxidase. Submitted

Dicko, M. H., Gruppen, H., Barro, C., van Berkel, W. J. H. and Voragen, A. G. J. (**2005**) Impact of phenolics and related enzymes in sorghum varieties for the resistance and susceptibility to biotic and abiotic stresses. Submitted

Karou, D., **Dicko, M. H**., Simporé, J., and Traoré, A. S. (**2005**) Antimicrobial and antioxidant activities of phenolic compounds from four medicinal plants of Burkina Faso. *African Journal of Biotechnology. In press*

Hamidou, F., **Dicko, M. H**., Zombré, G., Traoré, A. S., and Guinko, S. (**2005**) Physiological and agro-morphological traits of two varieties of cowpea resistant and sensitive to water deficit. *Cahiers Agricultures* (Eurotext, France). *In press*

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Curriculum vitae

Mamoudou Hama Dicko was born on December 31st, 1969 in Dori, Burkina Faso, from the royal family of Dori. In 1994, after a remarkable education at the University of Ouagadougou (Burkina Faso) where he was the leader of his promotions in all academic years, he obtained the diploma of "Maîtrise" (*equiv.* Bsc) in Biochemistry with the options of organic chemistry and natural substances. In 1996, he obtained his DEA (*equiv.* Msc) in Biochemistry (Food Enzymology), at the University of Ouagadougou. In 1997, he was awarded the Jan Tinbergen Fellowship Program via the NUFFIC (The Netherlands) as one of the best students of the University of Ouagadougou to come to Wageningen University for eight months. That allowed him to be acquainted with research at the Laboratory of Food Chemistry. His was welcomed at Wageningen by Prof. Frans Rombouts (former head of the laboratory of Food Microbiology), and Prof. Fons Voragen (Head of the Laboratory of Food Chemistry) who specifically allowed him to conduct research in his laboratory. During his stay, he has benefited the close supervision of Mrs Marjo Searle-van Leeuwen (Food chemistry), Dr. Gerrit Beldman (Food chemistry), and Dr. Riet Hilhorst (Biochemistry), for the purification and characterization of polysaccharide degrading enzymes. In 1999, he defended his thesis of "Doctorat de 3ième cycle" (former French first doctorate degree thesis) with the title: "Purification and physico-chemical properties of polysaccharide degrading enzymes (amylases and 1,3-β-D-glucan endohydrolase) from *Curculigo pilosa*, *Gladiolus klattianus* and *Boscia senegalensis*". The promotor of this thesis was Prof. Alfred S. Traoré, director of Centre de Recherche en Sciences Biologiques Alimentaires et Nutritionnelles (CRSBAN), and former Chancellor/President of the University of Ouagadougou. In 2000, he was welcomed at Wageningen University for a period of one year under the supervision of Dr. Riet Hilhorst (Biochemistry), Dr. Harry Gruppen (Food chemistry), Prof. Colja Laane (Biochemistry), and Prof. Fons Voragen (Food chemistry). In January 2001, he got a permanent position at the University of Ouagadougou as Assistant Professor of Biochemistry and Food Enzymology. In January 2001, he was officially enrolled as a PhD student at Wageningen University, between the Laboratories of Biochemistry and Food Chemistry, under the supervision of Dr. Willem J. H. van Berkel, Prof. Harry Gruppen, and Prof. Fons Voragen. The outcome of this research is summarized in this thesis. Since July 2002 he is the permanent secretary of the West African Biotechnology Network, created by the Association of African Universities. In July 2003, he was appointed senior lecturer of Biochemistry in Africa by the African and Malagasy Council for Higher Education (CAMES) with the highest rate (score A at the unanimity of the jury).

Training and supervision completed during the PhD research

Activities	Place	Period	Time, credit points
Theoretical and practical course on NMR	Université de Ouagadougou	2000	2 weeks, 2
Certificate of mass spectrometry in Biology	University of Amsterdam, Biocentrum	2000	1 week, 1
Certificate of English refresher course	Wageningen University	2000	1 week, 1
Certificate of fluency in English	Wageningen University	2001	1 week, 1
Certificate of Reaction Kinetics in Food Science	Wageningen University (VLAG)	2001	1 week, 1
Certificate of Protein engineering in agro-food biotechnology	Wageningen University (VLAG)	2001	1 week, 1
Certificate of laboratory quality management ISO-CEI 17025	Ouagadougou, Heinis International (France), PTB (Germany)	2003	1 week, 1
Workshop on standardization of food analysis methods for the zone UEMOA	Ouagadougou, UEMOA	2003	4 weeks, 4
Workshop on maintenance of lab equipments	Université de Ouagadougou	2003	1 week, 1
Certificate of ITC, Nutrition and BIT	Swedish Agricultural University. Uppsala	2004	5 weeks, 5
Certificate of Bioinformatics	Pasteur Institute (Senegal) and WT Sanger Institute (England)	2004	1 week, 1
Attend ITC meeting for creating Virtual Training Studio modules for students	Sri-Lanka	2004	1 week, 1
Attend Food Africa workshop	Yaoundé Cameroon	2003	1 week, 1
Attend the course HIV biology and prevention	Bobo-Dioulasso	2003	1 week, 1
Attend Netherlands Biotechnology workshop	Ede-Wageningen	2000	3 days, 0.6
Attend Netherlands Protein Society workshops	Luntheren	2000, 2001, 2004	6 days, 1.2
Organization Food Science and Nutrition workshop, MHO	Université de Ouagadougou	2003	3 months
Supervision 3 master students (1 DESS-IAA, 2 DEA)	Université de Ouagadougou	2002-2005	6 months
Supervision 2 doctoral degree students (1 plant physiology, 1 Biochemistry)	Université de Ouagadougou	2002-2004	6 months
Secretariat west African Biotechnology Network	Université de Ouagadougou	2002-2005	9 months
Senior lecturer (CAMES) of Biochemistry and Food Enzymology	Université de Ouagadougou	2001-2005	15 months