Hinc patriam sustinet
Instituto Superior de Agronomia
Universidade Técnica de Lisboa

Myrosinase Stability in Broccoli (*Brassica oleracea* var. *Italica*)

Influence of Temperature and Water Activity

João Diogo Cristóvão Pinto de Castelo-Branco

Dissertação para obtenção do Grau de Mestre em

Engenharia Alimentar

Orientador: Professora Doutora Margarida Gomes Moldão Martins

Lisboa, 2010

Acknowledgements

The time I spent in WUR and Wageningen, specially in PDQ group and with the people from all around the world I met here, was great. The interaction with people was really good for new ideas, know other cultures and healthy discussions.

I learn a lot about broccoli (sometimes too much), but I still like broccoli, and I have to say that my samples taste quite good. I want to thank Teresa for her attention, help and ideas discussion, Matthijs Dekker and Ruud Verkerk for accepting me in this project, for the scientific suggestions and discussions on the meetings, but also for the friendliness and good mood.
I also want to thank Charlotte, Xandra and Harry for their helpfulness every time I needed.
Finally, thanks to my family for giving me this opportunity, and to all my friends for the support.

During the time that I stayed in Wageningen, the most important thing I learn was that a worldwide community, with so big differences, can live in harmony.

Wageningen, 2010

Abstract

Epidemiological studies have shown that a diet rich in *Brassica* vegetables is associated with a decreasing risk of incidence of some types of cancer. These health promoting effects are most likely due to the breakdown products of glucosinolates formed by the action of myrosinase which catalyzes the hydrolysis of glucosinolates, found in *Brassica* plants.

Industrial processing of food can affect the content, composition, activity and bioavailability of glucosinolates breakdown products, due to cell lysis or glucosinolates and myrosinase degradation. The aim of this thesis is to investigate the combined effect of moisture content (water activity) and temperature on myrosinase stability.

The study was conducted in batches of freeze dried broccoli with different moisture contents. These batches of broccoli were then heated at different time/temperature combinations and myrosinase activity was analysed, the obtained results from these measurements were kinetically modeled.

Myrosinase was shown to be much less thermo stable in higher moisture content samples than in the driest samples.

Thermal stability (30-80 ºC) of myrosinase was described by a first order kinetic model.

Besides showing higher myrosinase thermal stability, driest samples show lower activation energies, *i.e*, lower increase of degradation rate when temperature increases.

Keywords : Myrosinase, broccoli, temperature, a_w, stability, kinetics

Resumo

Estudos epidemiológicos têm demonstrado que uma dieta rica em Brassicas está associada a uma diminuição do risco de incidência de alguns tipos de cancro. Estes efeitos de promotores de saúde devem-se, provavelmente, aos produtos de degradação de glucosinolatos formados pela acção de mirosinase que catalisa a hidrólise de glucosinolatos, encontrados em plantas da família *Brassicaceae*.

O processamento industrial pode afectar o conteúdo, a composição, actividade e biodisponibilidade de produtos de degradação de glucosinolatos, devido à lise celular ou degradação de glucosinolatos e mirosinase. Esta tese visa investigar o efeito combinado do teor de humidade (a_w) e temperatura na estabilidade da enzima mirosinase.

O estudo foi realizado em lotes de brócolos liofilizados com diferentes teores de humidade. Esses lotes foram tratados termicamente com diferentes combinações de tempo/temperatura, a actividade da mirosinase analisada, sendo os resultados obtidos modelados.

A mirosinase mostrou ser muito menos termo estável em amostras de maior humidade do que em amostras mais secas.

A estabilidade térmica (30-80 ºC) da mirosinase foi descrita por um modelo cinético de primeira ordem.

Além da mirosinase apresentar maior estabilidade térmica, as amostras mais secas apresentam menores energias de activação, isto é, menor incremento da taxa de degradação com aumento da temperatura.

Palavras-chave: Mirosinase; brócolos; temperatura; a_w; estabilidade; cinética

Resumo alargado

Já nas sociedades antigas algumas plantas foram utilizadas devido a propriedades estimulantes, curativas ou medicinais. Algumas plantas da família Brassicaceae foram, inicialmente, pelas suas propriedades medicinais e, posteriormente, com fins culinários e nutritivos. A consciencialização dos consumidores, nutricionistas, cientistas e indústria alimentar para os efeitos na saúde relacionados com os alimentos tem aumentado nas últimas décadas. Há uma quantidade crescente de evidências de que a fruta e os vegetais contêm grandes quantidades de compostos de benéficos para a saúde. Estudos epidemiológicos têm demonstrado que uma dieta rica em vegetais está associada com uma diminuição do risco de incidência de alguns tipos de cancro, como pulmão, bexiga, cólon e recto. Estes efeitos de promotores de saúde devem-se, provavelmente, aos produtos de degradação de glucosinolatos formados pela acção de mirosinase, após a lesão dos tecidos da planta, que catalisa a hidrólise de glucosinolatos, encontrados em plantas da família *Brassicaceae*.

Através de processamentos industriais de vegetais, tais como o branqueamento, enlatamento, congelamento ou secagem, bem como processamento doméstico, espera-se que o conteúdo, a composição, a actividade antioxidante e a biodisponibilidade dos antioxidantes seja afectado. Além disso, operações como cortar e fatiar podem induzir um rápido decréscimo de várias substâncias fitoquímicas que estão presentes naturalmente nas plantas, como resultado do rompimento celular que permite que o contacto entre substratos e enzimas. Geralmente, as concentrações e actividades de fitoquímicos em vegetais processados são inferiores aos dos vegetais não processados

O objectivo desta tese é investigar o efeito combinado do teor de humidade (actividade de água) e da temperatura na estabilidade da mirosinase, uma vez que não existe literatura disponível sobre este assunto.

O estudo foi realizado em lotes de brócolos liofilizado com diferentes teores de humidade. Esses lotes de brócolos foram tratados termicamente em diferentes combinações de tempo e temperatura, a actividade da mirosinase foi analisada, e os resultados obtidos a partir dessas medições foram modelados cineticamente.

A actividade da mirosinase após liofilização dos brócolos mostrou ser menor nas amostras de humidade intermédia (62,1%) e maior em amostras mais secas (26,1 e 13,4%) e em amostras de maior teor de humidade (82,5% e 90,1% (brócolos não liofilizados)).

A mirosinase mostrou-se muito menos termo estável em amostras de maior teor de humidade (82,5 e 62,1%) do que nas amostras mais secas (26,1 e 13,4%). A mirosinase das amostras de

teor de humidade de 13,4% mostrou ser a mais termo estável entre todas as amostras de diferentes teores de humidade, enquanto a mirosinase das amostras com 62,1% de humidade foi a mais termo lábil. Para obter uma redução de 75% na actividade da mirosinase, 30 minutos a 80 º C foram necessários para a amostra mais seca (13,4%), 10 minutos a 60 º C para as amostras de 26,1% humidade, e 5 e 15 minutos a 40 º C, respectivamente, para 62,1 e 82,5% de humidade.

A estabilidade térmica (30-80 º C) da mirosinase foi descrita por um modelo cinético de primeira ordem.

As taxas de inactivação ou degradação enzimática foram determinadas a 50 º C. Os resultados obtidos foram: 8,67 ± 3,92 ($\times 10^{-1}$), 1,36 ± 0,51, 1,90 ± 0,29 ($\times 10^{-2}$), 2,17 ± 0,67 ($\times 10^{-3}$) min^{-1}, respectivamente, para as amostras de 82,5, 62,1, 26,1 e 13,4% de humidade. As energia de activação de inactivação da mirosinase obtidas foram de 165 ± 22, 160 ± 20, 138 ± 18 e 82 ± 16 kJ /mol, por decrescente de teores de humidade.

Os valores mais baixos de energia de activação das amostras mais secas demonstram, que para além da mirosinase apresentar maior termo estabilidade, as amostras mais secas sofrem um menor incremento da taxa de degradação com o aumento de temperatura, isto é, a diferença nas taxas de inactivação entre as amostras de menor teor de humidade e as de maior teor de humidade aumentam com acréscimos de temperatura.

Index

1. Introduction

Since the introduction of agriculture on ancient societies, and even before, people noticed, although empirically, that plants have a lot of properties, as stimulant, curative or medicinal. Some of these plants were initially used for medical purposes, and only later began to be cooked, situation that is still present nowadays. The Indians, Egyptians, Chinese, and Sumerians are just a few civilizations that have provided evidence suggesting that foods can be effectively used as medicine to treat and prevent disease. Hippocrates of Cos (*ca*. 460 BC – *ca.* 370 BC), considered the Father of Medicine, said "Let food be thy medicine" (Milner, 1999).

The awareness of consumers, nutritionists, scientists and food industry to health related effects of foods has increased in recent decades.
It has been shown that the inclusion of fruits and vegetables in daily diet reduces the risk of cardiovascular diseases and other degenerative diseases in humans, including cancer, which is nowadays the group of diseases that cause most human lives and economical losses globally (American Institute for Cancer Research, 2010).
In consequence, scientific research is being conducted to determine the causes and ensure the bioavailability of protective properties in fruits and vegetables (Mithen *et al.*, 2000).
The beneficial effects have been partly attributed to the compounds which possess antioxidant activity. The major antioxidants of fruits and vegetables are vitamins and glucosinolates (Mithen *et al.*, 2000; Podsedek, 2005).
This last group of compounds is a group of plant secondary metabolites found exclusively in dicotyledonous plants (Oerlemans *et al.*, 2006). The highest concentrations are found in the Brassicaceae family, which comprises many consumed vegetable species, such as cauliflower, cabbage, Brussels sprouts or broccoli.
Epidemiological studies have shown that a diet rich in *Brassica* vegetables is associated with a decreasing risk of incidence of some types of cancer, such as lung, bladder, colon and rectum cancer (Jones *et al.*, 2006; Mithen *et al.*, 2000).
Research suggests that glucosinolates themselves are not directly bioactive, but their breakdown products, as isothiocyanates are largely responsible for the anticarcinogenic properties of these vegetables (Mithen *et al.*, 2000; Rungapamestry *et al.*, 2006). These breakdown products are formed by the action of myrosinase, which catalyzes the hydrolysis of glucosinolates, and is found in all glucosinolates containing plants, especially Cruciferae.

Vegetable industrial processing such as blanching, canning, freezing or drying, as well as domestic cooking, is expected to affect the content, composition, antioxidant activity and bioavailability of antioxidants. In addition, operations such as cutting and slicing may induce a rapid enzymatic depletion of several naturally occurring phytochemicals as a result of cellular disruption which allows contacts of substrates and enzymes. Generally, the phytochemical concentrations and activities in processed vegetables are lower than those of the corresponding raw samples (Podsedek, 2005).
Therefore, in order to reach an optimal level of breakdown products and increase their bioavailability, it is important to know how both myrosinase and glucosinolates behave along the food production and supply chain, and search procedures to control it (Ludikhuyze *et al.*, 1999).

Many researches focused on the influence of the temperature during the thermal treatment on the glucosinolates content, myrosinase activity and breakdown products formation, but no literature is available about the combined effect of water activity and temperature on these compounds.
The objective of this study was to investigate and kinetically model the influence of temperature and water activity on myrosinase activity in broccoli.
A batch of broccoli was freeze dried in order to obtain batches of broccoli with different moisture content. These batches of broccoli were then heated at different time/temperature combinations and analysed for myrosinase activity, the obtained results from these measurements were kinetically modelled.

2.Theoretical Background

2.1 Introduction

Broccoli (*Brassica oleracea* L. *cv. Italica*) is considered to be a healthy vegetable. It naturally is a significant source of nutritional antioxidants, such as vitamins and carotenoids, as well as biologically active dietary components, such as the flavonol glycosides, hydroxycinnamic acids and sulphur-containing compounds, such as the glucosinolates (Hamauzu *et al.*, 2004).
This last group of compounds, glucosinolates, co-exist with myrosinase, which is present in all glucosinolate-containing plants, especially in Brassicaceae plants (Ludikhuyze *et al* (1999), which comprises mustard, cauliflower, cabbages, kales or broccoli.
Myrosinase and glucosinolates are present in different cell structure, meaning that a physical separation exists in intact cells (Kissen *et al.*, 2009; Fahey *et al.*, 2001). When cell disruption occurs and water is available, myrosinase hydrolyzes glucosinolates producing a variety of breakdown products, dependent on environmental and food matrix conditions, including isothiocyanates. Some of breakdown products are described as having antinutritional properties for herbivores, but there is strong evidence that they are the main source of anticarcinogenic activity in Brassica vegetables, for humans.
The breakdown products are also, in part, responsible for the characteristic flavor and aroma of most Brassica plants. Thus, when present in high concentrations the can contribute to undesirable strong taste, biterness or pungency, which is of extreme importance for the consumer acceptance (Fenwick *et al.*, 1983).

2.2 Structure and Conformation of Myrosinase

Myrosinase is the trivial name for the plant endogenous enzyme thioglucoside glucohydrolase (E.C. 3.2.1.147), which is responsible for the hydrolysis of glucosinolates, and is thought to be involved in plant defense against herbivores together with glucosinolates, forming the glucosinolate-myrosinase defensive system (Bones & Rossiter, 1996).

The myrosinase is a glucopeptide containing a large number of carbohydrates, particularly mannose residues various thiol groups, disulfide and salt bridges and in the interface of the myrosinase subunits a zinc atom(Björkman & Janson, 1972). Myrosinases have traditionally been reported to be disulfide-linked dimers of 75 kDa subunits (Bones & Slupphaug, 1989)although immunological studies have shown that they can form complexes of higher molecular weight (MW) with myrosinase binding proteins and myrosinase associated proteins(Rask *et al.*, 2000). Myrosinases in the *Brassicas* can form complexes of different molecular weight (500–600 kDa, 270–350 kDa and 140–200 kDa) (Bellostas *et al.*, 2008).
Certain myrosinases also exist in complexes with other proteins where the oligomerization state of myrosinase is not clear. Various degrees of glycosylation account for at least part of the charge heterogeneity seen in this enzyme (Höglund *et al.*, 1990)
Myrosinase is most accurately classified as a class of functionally enzymes. Fourteen isoenzymes were indentified in extracts from seeds of *Sinapsis alba*. In *Brassica napus* L., it was shown that enzymatic activity of two isoenzymes differed depending on the substrate glucosinolate (James & Rossiter, 1991). Structural analysis by X-ray has shown that *Sinapis*

alba L. myrosinase is a dimer linked by a zinc atom and has a characteristic (α/β)8-barrel structure. A 3D structure of myrosinase from *Sinapsis alba* seeds is shown in Figure 2.1.

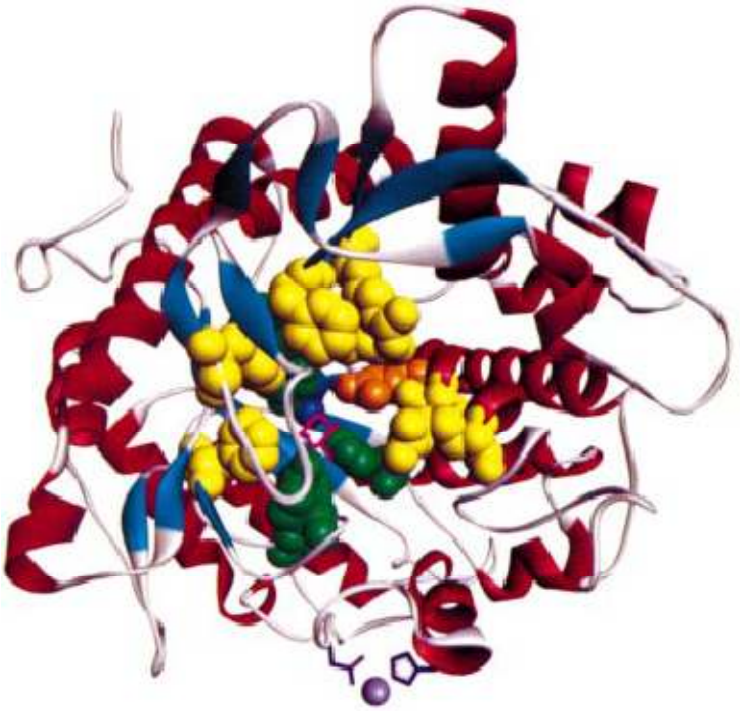


Fig 2.1 Schematic drawing of a myrosinase subunit showing the (β/α)8-barrel structure. The position of a substrate in the active site is indicated by the inhibitor 2-deoxy-2-fluoroglucotropaelin molecule in magenta covalently bound to the nucleophile (center). The view is from the substrate entrance part of the enzyme. N-terminus is at the bottom left and the C-terminus at the bottom center (Rask *et al*., 2000).

2.3 The myrosinase-glucosinolate system: Distribution and Localization of Myrosinase

The exact localization and distribution of the compounds of myrosinase-glucosinolates in plant cells is still unclear and not consensual among the scientific community, and therefore it is subject of study for researchers.

In early studies mainly based on morphological criteria, myrosinase was suggested to be localized in the interior of the myrosin grains (Rest & Vaughan, 1972; Werker & Vaughan, 1974, 1976), while others suggested a cytoplasmic distribution, sometimes in association with membranes (Maheshwari *et al.*, 1981). Studies in immunolocalization of myrosinase in sections of *Brassica napus* embryos showed that the enzyme was present in only a small number of cells. It was also found that the enzyme is either uniformly distributed in the cytoplasm or organized as particulate bodies(Höglund *et al.*, 1991).

In *Brassica* napus seeds, myrosinase was found solely in embryonic cells with a uniform distribution and no preference for either the cotyledons or the axis. The intracellular localization of the enzyme is likely to have a more pronounced accumulation around the vacuoles/ myrosin grains during this period. Approximately 2 to 5% of the cells in the mature seed appeared to contain myrosinase, and it is suggested that during the later stages of seed development, cells continue to accumulate myrosinase (Höglund *et al.*, 1991).

The captivity of myrosinase in only a few cells could indicate that a complex regulatory mechanism rules the expression of the myrosinase genes (Höglund *et al.*, 1991).

Based on these previous studies, it is assumed that degradation of glucosinolates by myrosinase occurs only when there is tissue damaging or disruption , and three major spatial distribution scenarios are possible:
1. Myrosinase and glucosinolates are localized in the same subcellular compartment of the same cells but in 'inactive' forms;
2. Localization in different subcellular compartments of the same cell;
3. Localization in different cells, either in the same or in different subcellular compartments (Kissen *et al.*, 2009).

Matile (1980) concluded that the stability of glucosinolates in the intact root tissues of horseradish *(Armoracia rusticana)* appeared to be due to the location of glucosinolates and myrosinase in distinct subcellular compartments of the same cell, and later was reported that myrosinase is a cytosolic enzyme which tends to bind to membranes (Lüthy & Matile, 1984), giving support to the second scenario described. Figure 2.2 shows a proposed model by Lüthy & Matile (1984) for compartmentation of glucosinolates, ascorbic acid and myrosinase.
Bones *et al*. (1991) concluded that a localization of glucosinolates and myrosinase in different vacuoles in the same cell is not likely, which contradicts the second hypothesis. The vacuoles of a myrosin cell do undergo considerable changes after sowing of which the fissions and fusions of the vacuoles in the cells are the most remarkable.
A more likely system would include compartmentation of glucosinolates in vacuoles of some cells and myrosinases in vacuoles of myrosin cells. The destruction of subcellular compartmentation by mechanical disruption or by micro- or macro-organisms feeding on the plants would cause the necessary contact between enzyme and substrate.

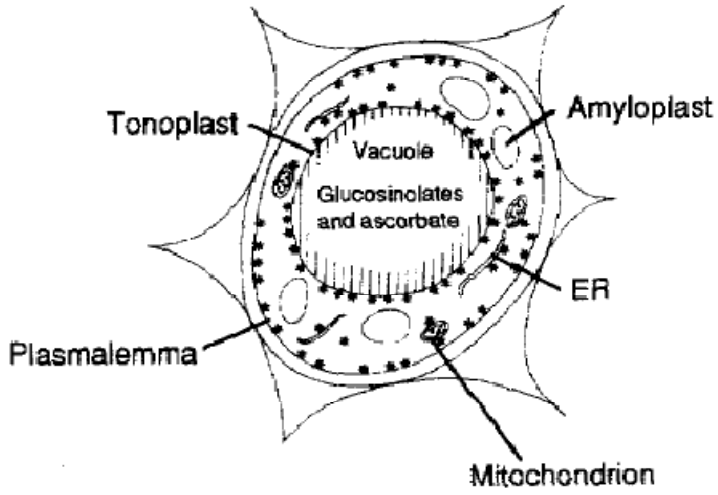


Figure 2.2 – Proposed compartmentation of glucosinolates, arcorbic acid and myrosinase. (•)Indicate suggested localization of myrosinase (Lüthy & Matile, 1984).

Kelly *et al.* (1998) showed that with exception of myrosin cells, glucosinolates are distributed in all cells of *Brassica* juncea cotyledons. One cell type had particular high sulphur content, hence referred to as S-cells, and myrosinase hydrolysis of the fluid collected from those cells indicated the presence of glucosinolates. S-cells are positioned in groups between the endodermis and the phloem cells of each vascular bundle (Koroleva *et al*. 2000) and are close

to or in direct contact with myrosin cells (Bones *et al*. 1991; Andreasson *et al*. 2001; Husebye *et al*. 2002).
A localization of glucosinolates in vacuoles of horseradish (*A. rusticana*) and oilseed rape (*B. napus*) has been reported (Matile 1980; Helmlinger *et al*. 1983; Yiu *et al*. 1984).

In this way, the third scenario hypothesized for spatial distribution of myrosinase-glucosinolates is the one that better fits the available data: localization of myrosinase and glucosinolates in different cells, either in the same or in different subcellular compartments (Figure 2.3). The observations indicate a location of myrosinase enzymes and glucosinolates in different but adjacent cells in the flower stalk of *Arabidopsis thaliana* (Bridges *et al*., 2002).
The position of myrosin cells together with S cells outside the phloem sieve elements is appropriate for the protection of the phloem and its content against microbes and insects. During insect attachment, myrosinase and glucosinolate mix, results in the release of toxic hydrolysis products for insects. The formation of toxic products for insects, after the hydrolysis of glucosinolates has led to a proposal that the myrosinase-glucosinolate system might have a function as a nonspecific defense system in the plant (Luthy & Matile, 1984). This proposal is in agreement with the findings from Höglund *et al.* (1991), where expression of myrosinase was found in guard cells and in xylem, which are both main locations for pathogen invasion.
Additionally, the location of myrosinase in phloem and guard cells can indicate a role in communication, internally by hydrolysis of glucosinolates and transport in phloem, and externally by distribution of volatile degradation products from guard cells. The fact that myrosinase activity is especially high in developing tissue may also suggest that myrosinase is involved in the control of plant growth and development (Kissen *et al.*, 2009).

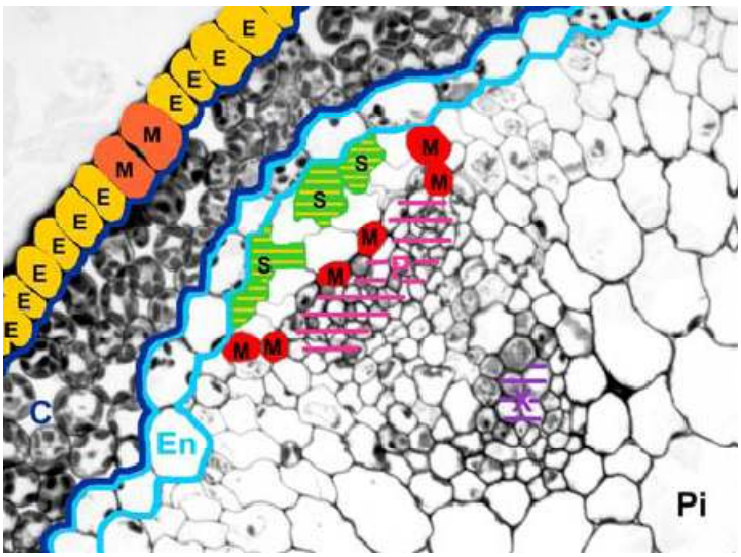


Figure 2.3 - Schematic representation of the spatial distribution of known components of the glucosinolate-myrosinase system superposed on a portion of an *A. thaliana* flower stalk transverse section. Cell types are labelled in the figure: green (‘‘S’’) represents S-cells containing glucosinolates; red and orange (‘‘M’’) are myrosinase-expressing phloem cells and guard cells respectively; yellow (‘‘E’’) represents epidermal cells expressing ESP; stripes in S-cells indicate cellular colocalization of glucosinolates and ESP For orientation purposes some tissues are indicated and marked by coloured lines: C: cortex (dark blue); En: endodermis (light blue); P: phloem (pink); X: xylem (purple); Pi: pith. (Kissen *et al.*, 2009)

From the data available, glucosinolates, myrosinases, myrosinase binding-proteins and myrosinase associated proteins seem to be present in vacuoles. Myrosinases are expressed in

myrosin cells that are different, but in close proximity of the cells accumulating glucosinolates - S cells. The possibility that minor amounts of myrosinase may be present in other compartments is still open (Kissen *et al.*, 2009). Since glycosylation may be a requisite for a vacuolar localization due to the hydrophilic properties added with the carbohydrates, this could indicate that myrosinase also can be localized in other places than the vacuoles of myrosin cells (Thangstad *et al*, 1991; Bones & Rossiter, 1996)

Since the majority of the localization and distribution studies of myrosinase were conducted in *A. thaliana, B. napus* and *A. rusticana* care must be taken when generalizations are made. Considering all the known components of the hydrolysis part (glucosinolates, myrosinases, myrosinase binding-proteins, myrosinase associated proteins, epithiospecifer protein) of the glucosinolate- myrosinase system, it is quite clear from the available data that the system is complex (Bones & Rossiter, 1996). The myrosin cells tissular distribution may vary between the same organs of different species and between organs of the same plant. Definitely, a complete scenario of the system is not yet available for any of the studied plants and the system also shows differences within each plant at organ and tissue level.
Although, it looks quite clear that hydrolysis of glucosinolates only occurs in damaged or disrupted cells by mechanical action or by macro and microrganisms, a fact of extreme importance for breeders and food technologists.

2.4 Formation of breakdown products

Glucosinolates are β-thioglycoside N-hydroxysulphates linked with a side chain R and a sulphur β-D-glucopyranose moiety (Fahey *et al*., 2001). The side chain R determines whether the GLs is aliphatic (e.g. glucoraphanin, sinigrin), arylic (e.g. glucotropaeolin) or indolic (e.g. glucobrassicin). Myrosinase hydrolysis of glucoraphanin, the glucosinolate most abundant in broccoli, can result in sulforaphane and sulforaphane nitrile.
Cell damage or disruption in Brassicaceae brings myrosinase and glucosinolates in contact and hydrolysis of glucosinolates occurs. The myrosinase activity results in the release of the glucose moiety of glucosinolates to form unstable intermediates – aglycones (thiohydroximate-O-sulfonates) (Mithen *et al*., 2000), which are spontaneously rearranged, with release of sulphate, frequently via a Lossen rearrangement (conversion of a hydroxamic acid to an isocyanate), to produce several products.
The products of glucosinolate hydrolysis include isothiocyanates, nitriles, thiocyanates, indoles and oxazolidinethiones. The chemistry of the formed product is dependent on several factors, such as pH, substrate, side-chain nature of glucosinolates, presence of proteins such as epithiospecifier protein, ascorbic acid or ferrous and hydrogen ions availability (Bones & Rossiter, 1996; Kleinwächter & Selmar, 2004). Figure 2.4 shows the formation of different glucosinolates breakdown products.
In the presence of ascorbic acid, sinigrin (2-propenyl glucosinolate) is degraded most rapidly and the indole glucosinolates glucobrassicin (indol-3-ylmethyl glucosinolate) and neoglucobrassicin (1-methoxyindol-3-ylmethyl glucosinolate) are degraded at the lowest rates. The presence of ferrous ions may result in the production of nitriles even when pH conditions favor isothiocyanate generation (Bones & Rossiter, 1996).

Isothiocyanates are usually produced at neutral pH while nitrile production occurs at lower pH. Indole-glucosinolates, like glucobrassicin undergo enzyme hydrolysis resulting in 3-indolemethanol, 3-indoleacetonitrile and 3,3'- diindolylmethane (Labague et al, 1991).

Isothiocyanates with a hydroxy group in the 2 position spontaneously cyclise to give oxazolidine- 2-thiones, as goitrin, derived from 2-hydroxy-but-3-enylglucosinolate (Bones & Rossiter, 1996).
Only three naturally occurring glucosinolates appear to undergo enzymatic degradation to thiocyanates. These are allyl-, benzyl- and 4 (methylthio)butylglucosinolates. The proposed mechanism for thiocyanate formation is a combined theory where an isomerase causes Z-E isomerisation of the aglycone, where only glucosinolates with stable cations are able to undergo E-aglycone rearrangement to the thiocyanate (Hasapis & MacLeod 1982). Thiocyanates are produced from the degradation of glucosinolates containing an indole or substituted indole side chain (Rosa *et al.*, 1997).
Epithioalkanes are produced from the hydrolysis of alkenyl glucosinolates when myrosinase co-occurs with epithiospecifier protein (Tookey, 1973).

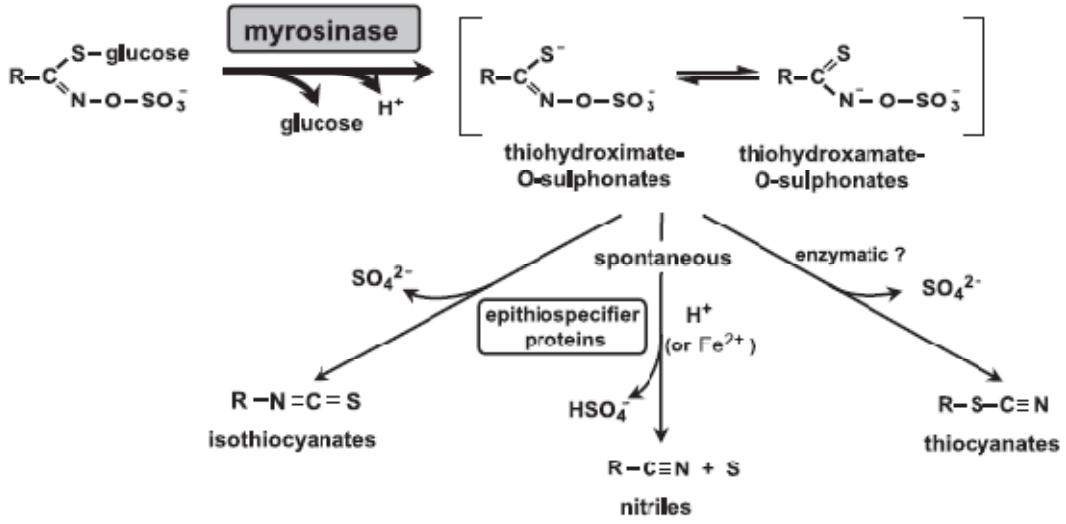


Figure 2.4 -Hydrolytic cleavage of glucosinolates by myrosinase, and formation of breakdown products (Kleinwächter & Selmar, 2004).

As referred before, the breakdown products formed depend on environment reaction conditions and glucosinolates nature. For example, if a formed isothiocyanate contains a double bond, and a epithiospecifier protein is present, it may be rearranged to produce an epithionitrile (Figure 2.5a). Aglycones from glucosinolates containing β-hydroxylated side-chains, such as progoitrin can spontaneously cyclise to form the corresponding oxazolidine-2-thiones (Figure 2.5b). Indolyl glucosinolates can also form unstable isothiocyanates, which are degraded to a corresponding alcohol, and by condensation can form diindolylmethane. Another environmental dependent reaction is the production of indolyl-3-acetonitrile and elemental sulphur, from indolyl glucosinolates, at lower pH values (Figure 2.5c), which has auxin activity, and can further be converted to indole-3-acetic acid (Mithen *et al.*, 2000).

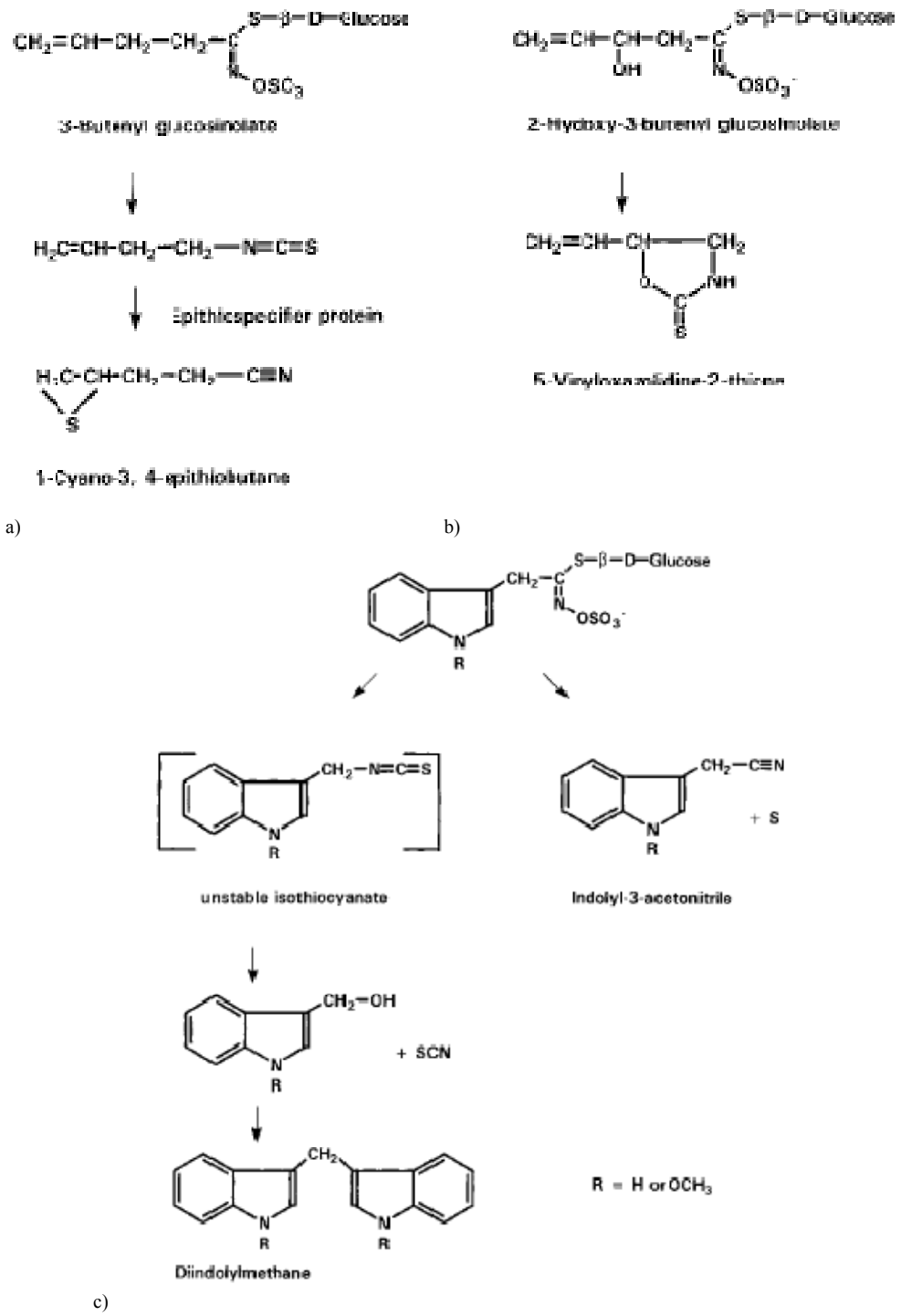


Figure 2. 5. (a) Formation of epithionitriles. (b) Isothiocyanates with a β-hydroxylated side-chain, for, spontaneously cyclise to form the corresponding oxazolidine-2-thione. (c) Indolyl glucosinolates also form unstable isothiocyanates, which degrade to the corresponding alcohol and may condense to form diindolylmethane (left). At more acidic pH, indolyl glucosinolates can form indolyl-3-acetonitrile and elemental sulphur (right).

2.5 Properties of Myrosinase

The myrosinase activity depends, in addition to the conformational structure, on intrinsic factors, like the presence of ascorbic acid, epithiospecifer protein (ESP), ions (Mg^{2+}), pH and water activity and extrinsic factors, as temperature, pressure or salts concentration.
In the case of ascorbic acid was shown that it induces a conformational change on the active centre of myrosinase, and increases the activity in low or moderated concentrations, decreasing it when present in high concentrations, acting as inhibition competitor. $MgCl_2$ itself does not act as an activator, but when combined with ascorbic acid enhances the enzyme activity (Ludikhuyze *et al.*, 2000).
The optimal pH of myrosinase depends on its origin. For broccoli, the optimal pH is the range of 6.5-7.0, which corresponds to fresh broccoli juice pH (Ludikhuyze *et al.*, 2000). Myrosinase from white and red cabbage was characterized by a pH optimum of 8, while white mustard and rapeseed myrosinase exerted maximal activity in the pH range 4.5 to 4.9. For myrosinase from Brussels sprouts on the other hand, two pH optima were found, 6.0 to 6.5 and 8.0 (Yen *et al.*, 1993; Ludikhuyze *et al.*, 2000).
Epithiospecifier is a small protein (30-40 KDa) that inhibits myrosinase non-competitively, which means that ESP acts at a site different from the substrate binding site, and it is inactivated in absence of ferrous ions (Bones *et al.*, 1996). ESP is more heat sensitive than myrosinase, and therefore a short time heating treatment (5 min, 60 °C) can inactivate it, but still leaves myrosinase activity. The combination of pH, presence of ESP and temperature will determine which breakdown products are formed (Bones *et al.*, 1996; Mathusheki *et al.*, 2006).

The activity of plant myrosinase and its isoenzymes can show significant differences between and within Brassica species and cultivars, in different parts of the plants and among seasons. 'Brigadier' broccoli and 'Emperor' broccoli showed almost 2-times higher activity in fall season than in spring season (Charron *et al.*, 2005). In the same study differences were also observed between the analyzed Brassica vegetables, specially for broccoli and Brussels sprouts, while for cabbage and cauliflower differences between seasons are not so significant. Activity-FW was highest in 'Emperor' broccoli and 'Dominant' cauliflower in fall 2000, 'Emperor' broccoli in Fall 2001, and 'Snow Crown' and 'Dominant' cauliflower and 'Emperor' and 'Brigadier' broccoli in both spring seasons. The highest activity-FW measured occurred in 'Emperor' broccoli grown in fall 2001 (151.7 U/g) and the lowest was in 'Winterbor' kale grown in spring 2000 (1.9 U/g). Activity-FW for each cultivar was highest in either or both of the fall seasons. Between cultivars inside the same group of vegetables significant differences were identified only for cauliflower.
Temperature, photosynthetic photon flux (PPF) and photoperiod can also influence myrosinase activity in different parts of the plants. Charron *et al.* (2005a) showed that temperature and PPF have a bigger influence on the enzymatic activity. From these data, climatic factors should also be taken into account when deciding planting time and location in order to optimize the cancer chemoprotective attributes of *Brassica* plants.

The heat stability and optimal temperature of myrosinase, like the optimal pH, is dependent on its source. Ludikhuyze *et al.*(2000) showed that myrosinase activity from broccoli increased with increasing temperature up to 30 °C and then decreased with further increase in temperature, becoming practically zero at 50 °C. At 50 °C, initial activity was reduced by 70% within 5 and 10 min, in crude extract and broccoli juice, respectively (Ludikhuyze *et al.*,

1999; Van Eylen *et al.*, 2007). In intact broccoli heads, the myrosinase stability is much higher than for broccoli juice and crude extract, where initial activity was reduced by 70% only after 50 minutes of heating treatment, at 50 °C (Van Eylen *et al.*, 2008). The optimal temperature found for activity of myrosinase from broccoli is rather low compared to myrosinase from other sources. For myrosinase from red/white cabbage, white mustard, and rapeseed the optimal temperature for enzyme activity was found to be 60 °C (Yen *et al.*, 1993), while 50 °C for myrosinase from brussels sprouts (Springett *et al.*, 1989). It is important to refer that Ludikhuyze *et al.*(2000) make reference to the fact that these differences might be attributed to the fact that different methods and sample preparations were used for the different studies.
Pressure has also influence on myrosinase activity and stability, as shown in Van Eylen *et al.* (2007). The activation energy of myrosinase inactivation was the highest at 200 MPa, thus the enzyme is more stable in this pressure conditions. The combined temperature/high pressure results showed that increasing both pressure and temperature the stability is decreased.

2.6 Health promoting effects

The lack of some essential nutrients and the presence of xenobiotics in diets can lead or enhance the risk to develop diseases, like some types of cancer or ulcers (Mithen *et al.*, 2000). For this reason, many components of human diets overlooked in the past are now considered to play an important role, mainly in prevention, but also in treatment of important diseases. Thus, due to a more efficient spread of scientific information, consumers are more aware of health-related effects of foods, and for that reason more research on the impact on human health of naturally occurring substances, is being conducted. This concern about healthy compounds led to the emergence of a fastest growing market – the nutraceutical market - in the last two decades. Nutraceutical is defined as a food or any part of food that gives health benefits beyond providing nutritients. Amongst the most important classes of nutraceuticals are the carotenoids, flavonols, saponins, phytosterols, glycoalkaloids and the glucosinolates, found in plant families of the order Capparales, including *Brassicas*, from which broccoli is part of.
Glucosinolates do not show biological activity by themselves, but the breakdown products formed, by myrosinase hydrolysis, may have important anticarcinogenic effects.
Epidemiological evidence suggests that consumption of brassica vegetables is associated with reduced risk of cancer at many sites, including the gut tract, which seems likely to have tissues which are greatly exposed of all to glucosinolate breakdown products
The World Cancer Research Fund (1997) concluded that diets rich in cruciferous vegetables probably protect human beings specifically against cancers of the colon, rectum and thyroid and, if consumed as part of diet high in other types of vegetable, generally against other types of cancers, in agreement with epidemiological evidences conducted since the 1960's.
It is known that cancer development is a multistage process, with several steps leading to the cancer formation. When the DNA of a susceptible cell is damaged reacting with electrophilic compounds and is not repaired, possible replication leads to permanent DNA injury, sometimes resulting in neoplastic transformation, in presence of tumor promotors. The transformed cells can gain growth advantage over the healthy surrounding cells and undergo expansion (Verhoeven *et al.*, 1997).
One of the principal sources of anti-carcinogenic activity from glucosinolates breakdown products, is thought to arise from isothiocyanates (Van Poppel *et al.*, 1999; Jones *et al.*, 2006).

Benzyl isothiocyanate, a breakdown product of the glucosinolate glucotropaeolin, is likely to inhibit the induction of mammary tumours by dimethylbenz(a)anthracene (DMBA) when administered orally to rats two hours before treatment with the carcinogen (Wattenberg, 1977). Seems that benzyl isothiocyanate can inhibite the carcinogen activation, so that the initial stage of the carcinogenic sequence associated with DNA damage is blocked and hence called as *blocking agent*. These group of substances protect against experimentally induced tumours when given before or in conjunction with a chemical carcinogen. On the other hand, substances which can inhibit or weaken the process of carcinogenesis, after administration and full metabolism of the carcinogen are termed *suppressing agents* (Mithen *et al.*, 2000).
Benzyl isothiocyanate exhibited suppressing activity against DMBA induced mammary tumours (Wattenberg, 1981) and was shown that whole plant tissue of broccoli and cabbage, fed to rats one week after treatment with the carcinogen, suppressed tumor development (Wattenberg, 1989).
The most important blocking mechanism induced by glucosinolate breakdown products is probably modulation of the activities of Phase I and Phase II biotransformation enzymes, inhibiting Phase I and inducing Phase II xenobiotic metabolizing enzyme activity.
Phase I enzymes catalyze hydrolytic, oxidation or reduction reactions. Then, the products formed are available for conjugation reactions, catalyzed by Phase II enzymes, followed by excretion. The most important Phase I enzymes are the hepatic microsomal cytochrome P450s which metabolize toxins or xenobiotic compounds, but can also activate some carcinogens, so that induction can lead to enhanced carcinogenic activity. Phase II enzymes such as glutathione-S-transferase and UDPglucuronyl transferase form conjugation products with the modified xenobiotics, more polar than the unconjugated species and hence more promptly excreted, so their induction is considered to be wholly protective (Mithen *et al.*, 2000)
Nowadays, there is ample evidence from animal models showing that certain isothiocyanates and their conjugates can inhibit the cytochrome p450 enzymes which activate nitrosamines to alkylating carcinogens responsible for induction of lung tumours (Mithen *et al.*, 2000).
An important example, specifically applied to broccoli, is sulphorophane, an isothiocyanate present at high levels in this vegetable, which seems to induce Phase II enzymes *in vitro* (Talalay *et al.*, 1995) but other isothiocyanates derived from common brassica vegetables may also exert comparable levels of biological activity.
Allyl isothiocyanate appears to be selectively cytotoxic to cancer cells *in vitro* (Musk *et al.*, 1993; Musk *et al.*, 1995) and it been established that phenethyl isothiocyanate induces cell death via an apoptotic pathway (Huang *et al.*, 1998).

Other health promoting effects of glucosinolate, than those described above, are reported, including inhibition of *Helycobacter pylori*, bacteria responsible for stomach ulcers or prevention of cardiovascular diseases (Jones *et al.*, 2006).

2.7 Food Production Chain – Influence on myrosinase-glucosinolate system

The food production chain consist of many steps, such as cultivation, storage, processing, packaging, consumer processing and consumption. All the steps have an influence on the quality, and therefore on the final levels of phytochemicals, more or less severe depending on how the food production chain is conducted, the types and quantity of processes along the way from cultivation to consumption.

In most cases, the consumption of raw and unprocessed fruits or vegetables is recommended in regard to the intake of bioactive compounds, although for some vegetables processing increases the bioavailability of some of these compounds.
The changes in the modern societies led to an increase of importance on the role of food industrial processing, for reasons of shelf life, costs, convenience, availability and consumer preferences. Included in the various industrial processes are washing, blanching, chopping, addition of preservatives and colorings, fermentation, freezing, canning or drying. Domestic treatments commonly include boiling, frying, steaming or microwaving. Figure 2.6 gives an overview of production steps that can affect the glucosinolate-myrosinase system.

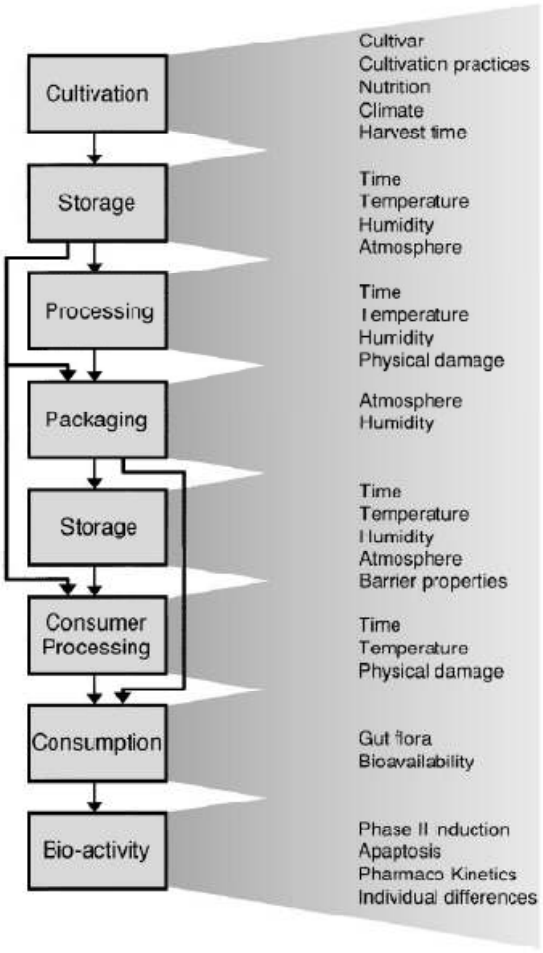


Figure 2.6 – Overview of the food production chain and possible causes for variation in levels of bioactive compounds (Dekker *et al.*, 2000).

Usually the myrosinase stability and activity decreases during most of theses processes, more specifically ones associated with heat and cell disruption. These effects influence the intake and bioavailability of glucosinolates and their breakdown products as demonstrated in various studies (Getahun & Chung, 1999; Conaway *et al.*, 2000).
Getahun & Chung (1999) have shown that the conversion of glucosinolates into isothiocyanates is more efficient in uncooked watercress (between 17 - 77 % of conversion) than in cooked (boiled) watercress, where only about 18 % of glucosinolates were converted. These findings suggest the existence of intestinal myrosinase likely for the hydrolysis of glucosinolates into isothiocyanates in humans, however is a lower extent than the plant myrosinase.

In a study conducted using fresh and steamed broccoli, results showed that the total of isothiocyanates released after ingestion of steamed broccoli was about one third of the amount excreted after ingestion of fresh broccoli (Conaway *et al.*, 2000), showing that steaming has a lower impact in the conversion of glucosinolates than cooking (boiling).
The effect of microwaving on red cabbage myrosinase activity seems to be dependent on exposure time and power combinations (Verkerk & Dekker, 2004). In this sudy, substantial myrosinase activity was retained after 24 minutes, at 180 W, while 4 minutes and 48 seconds, at 900 W resulted in complete loss of enzymatic activity.
Rungapamestry *et al.* (2006) compared the effects of microwaving and steaming on myrosinase activity concluding that microwaving has a higher negative impact on myrosinase activity than steaming. In Figure 2.7 are shown proposed processes occurring during cooking of cabbage. Although, generalizations should not be made, since the energy inputs of these two treatments are substantially different for this study.

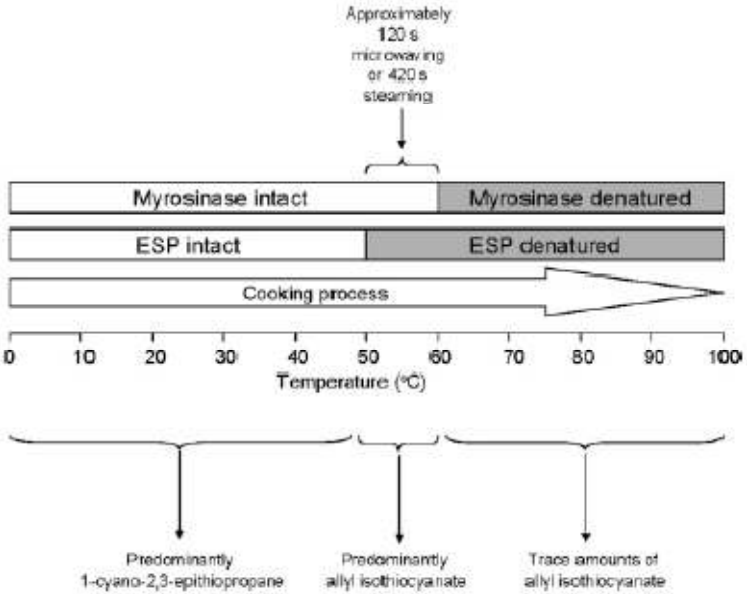


Figure 2.7. Scheme illustrating the proposed processes occurring during cooking of cabbage, leading to the production of different predominant breakdown products at different stages of cooking (Rungapamestry *et al.*, 2006).

The accumulation of processes in vegetables and other types of food can lead to positive or negative synergies. In blanch-frozen broccoli, the activity of myrosinase is about ten times lower than in fresh broccoli (Rungapamestry *et al.*, 2008), and after cooking both fresh and blanch-frozen broccoli, the decay is bigger for the fresh samples but still the activity still higher than in the blanch-frozen samples. Freezing damages plant cells and softens vegetables due to crystallisation of water in extra-cellular and intra-cellular spaces in the vegetable matrix. Posterior cooking or exposure to heat increases the disruption of cells and allows more heat to penetrate into the vegetable matrix which was cooked from frozen, rather than from fresh. Myrosinase activity remains stable after storage (-20 °C) of blanch-frozen broccoli over 90 days (Rungapamestry *et al.*, 2008).

In this way, it becomes evident that myrosinase plays an essential role in the conversion of glucosinolates in humans, and that inactivation of plant myrosinase sharply reduces the hydrolysis of glucosinolates, and consequently the dietary absorption of bioactive compounds. Summarizing, plant myrosinase should be kept intact, as much as possible to optimize the health advantages that come from the consumption of *Brassica* vegetables, including broccoli.

2.8 Enzyme Denaturation in Foods

Enzymes can have a large impact on food quality for the reason that they act as catalysts. The reactions catalysed by enzymes could be desirable or undesirable, depending on what is expected to obtain at the end of food processing, although, there are more cases where it is undesirable than desirable.
Enzymes activity and stability can be enhanced or worsen by heat treatments, application of high pressures, changes in chemical environment (pH, ionic strength, solvent quality) or macromolecular crowding.
Stability can be described as the resistance to denaturation, or unfolding. Denaturation does not change the primary structure, but it may change the secondary, terciary or quaternary structure. Denaturation result in loss or change of functional properties, at least partially, due to (partial) unfolding (Van Boekel, 2009). The unfolding can be reversible or irreversible, depending on the type and intensity of processing.

In food processing, heat treatments are commonly used to stop microorganisms growth, in a food safety context, and inactivate or inhibit enzymatic reactions, in the context of food quality. However, there are some enzymes that play important role on enhancing food quality, like myrosinase, and therefore should not be inactivated, which makes the food technologist job difficult and challenging. The food technologist should pursue the combination that better fits the safety and quality requirements, in order to get a safe final product, reducing as much as possible quality losses. In order to optimize the processes where enzymes play an important role, is essential to know how enzymes behave when submitted to the different treatments.
The effect of heating treatments in myrosinase stability was already discussed (see section 2.5), where various studies show that myrosinase stability is negatively affected by heat.
Since the main objective of this thesis is to check the effect of combination of heating treatments and different moisture contents on the stability of broccoli myrosinase, it seems important to describe possible effects of changing the moisture content of broccoli, and therefore the whole broccoli food matrix.
Heat stability of enzymes is known to be strongly influenced by the water content of the matrix in which enzymes are present. Usually a decrease of water content, means a strong increase on enzymes stability. The dependence of enzyme activity on water activity can be explained because pH, ionic strength and solute activity coefficients change with water activity, which can have large effects on protein conformation and thus on enzyme activity. At lower water content, also diffusion may be hindered, and thereby reaction rates slow down (Van Boekel, 2009). A possible explanation for this phenomenon is linked with the concept of volume exclusion or molecular “crowding”. Excluded volume theory predicts that at equilibrium, the presence of high concentrations of stable macromolecular cosolutes will stabilize a labile protein against denaturation by heat, cold, or denaturant (Minton, 2005) This means that due to the finite size of molecules, volume is excluded for other molecules. The consequence, at least theoretically, is that reactions that increase the available volume are favored. Applied to myrosinase case, less water available as solvent, becomes energetically unfavorable for proteins to unfold, increasing denaturation temperature, thus increasing the stability. The reason why there is a maximum of stability not corresponding to the lowest water content is unknown, as shown in Van Boekel (2009), for lipoxygenase. Should also be noted that at the same moisture content, water activity increases with temperature, a fact that could influence enzyme activity, although it is known that this effect is not very strong.

Generally, it is accepted that in dried foods, the heat stability of enzymes may be much higher than in aqueous systems, and this could have a large impact on the resulting quality.

3. Material and Methods

3.1 Sample preparation

A batch of 10 kilos of broccoli from the same lot, was bought in a fruit & vegetables shop in Wageningen. The original broccoli supplier was BrocoliCricket, Murcia, Spain, which gave the information about the variety and growing season The variety of broccoli used for the experiments was Parthenon, which is a cool season variety.

About 8 kg of the edible parts (stems and florets) of the broccoli were cut into small pieces, and immediately frozen with liquid nitrogen. Around 1000-1100 g were weighed into 7 different aluminum trays and freeze-dried (GRInstruments, Model GRI 20-85 MP 1996, The Netherlands), in order to achieve different moisture contents on each tray.
The trays were weighed every day, to check the evolution of moisture content of the samples.
Samples with different moisture content (a_w) were obtained (Table 1) and grounded into powder, using a Waring Blender (Waring Commercial, Torrington, Connecticut, USA) and kept at -22 °C, before further analysis. Samples with 82.5 %, 62.1 %, 26.1 % and 13.4 % of moisture, and water activities of 0.973, 0.947, 0.719, 0.115, respectively, were used for the myrosinase stability tests.

Table 3.1 – Relation between water activity and moisture content of fresh and freeze-dried samples

Water activity (a_w)	0.115	0.719	0.947	0.964	0.973	0.978	0.981	0.983
Moisture content (%)	13.4	26.1	62.1	70	82.5	86.1	88.6	90.1

3.2 Determination of D-glucose

For the reason that the glucose content in fresh broccoli could interfere with the myrosinase activity determination, and therefore the myrosinase activity could be overestimated, the glucose content was determined using a D-glucose determination kit Enzyplus® EZS 781+ (Biocontrol Europe, The Netherlands).
The principle of the kit is that D-glucose in presence of ATP (adenosine-5'-triphosphate is phosphorylated by HK (hexokinase) into (G-6-P), with the formation of ADP (adenosine-5'-diphosphate) (1).

$$\text{D-Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{G-6-P} + \text{ADP}$$

The enzyme G6PDH (glucose-6-phosphate dehydrogenase) in presence of NADP+ (nicotinamide-adenine dinucleotide phosphate) catalyses the G-6-P oxidation with formation of NADPH.

$$\text{G-6-P} + \text{NADP}^+ \xrightarrow{\text{G6PDH}} \text{gluconate-6-phosphate} + \text{NADPH} + \text{H}^+$$

The amount of NADPH formed in the reaction is stoichiometric with the amount of D-glucose formed. At the end is the NADPH which is measured by the increase in absorbance at 340 nm, in a UV Spectrophotometer.

To the determination 10 g of fresh broccoli were homogenized in a mixer Waring Blender (Waring Commercial, Torrington, Connecticut, USA). 0.5 g of the sample were weighed into a 100 ml volumetric flask, mixed with water using an Ultra Turrax (T25 Basic, IKA-Werke, Staufen, Germany). The mixture was filtrated, and the first 5 ml were discarded. The clear undiluted filtrate was directly used for the assay (100 µl).
Table 3.2 describes the procedure for glucose determination. A reaction mixture containing 3.000 ml of distilled water, 100 µl of buffer solution (R1 – Imidazole buffer, magnesium chloride and sodium azide as a preservative), 100 µl of ATP/$NADP^+$ (R2) and 100 µl of sample solution was prepared. The mixture was transferred to a glass cuvette of 1.00 cm light path and the absorbance (A1) was read after 3 minutes. After reading, 10 µl of hexokinase/glucose-6-P-dehydrogenase (R3) were added to the mixture and the absorbance (A2) read after 10 minutes.
A blank was also prepared, but instead of 100 µl of sample solution, 100 µl of distilled water were added.
To calculate the glucose content, the difference in absorbance for both blank and sample solution was determined. The absorbance difference of the blank was subtracted from the absorbance difference of the sample, thereby the $\Delta A_{D\text{-glucose}}$ was obtained.

Table 3.2 – Procedure of D-glucose Enzyplus® EZS 781+ kit

Pipette into cuvette	**BLANK**	**SAMPLE**
Distilled water	3.100 ml	3.000 ml
R1 (buffer solution)	0.100 ml	0.100 ml
R2 (ATP, NADP+)	0.100 ml	0.100 ml
Sample	-	0.100 ml
Mix, read A1 after 3 minutes and then add:		
R3 (HK/G6PDH)	0.010 ml	0.010 ml
Mix and read A2 after 10 minutes. If the reaction has not stopped after 10 min, continue to read the absorbance at 2 minutes intervals until the value remains stable.		

According to the method described by Biocontrol, the values of $\Delta A_{D\text{-glucose}}$ should, as rule, be at least 0.100 absorbance units to achieve sufficiently accurate results.
The concentration of D-glucose can be calculated as: $C = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A(g/L)$ where:

- V = final volume (ml);
- ν = Sample volume (ml);
- MW = Molecular weight of D-Glucose: 180.16 g/mol;
- ε = extinction coefficient of NADPH at 340 nm = 6.3 [l x $mmol^{-1}$ x cm^{-1}]
- d = light path (cm)

Therefore, it follows for D-glucose:

$$C = \frac{3.31 \times 180.16}{6.3 \times 1 \times 0.1 \times 1000} \times \Delta A(g/L) = 0.9466 \times \Delta A(g/L)$$

By the reason that broccoli is a semi-solid product, the D-glucose content (g/100g) was calculated from the weighed as follows:

$$C = \frac{cD-glu\cos e \ldots [g/L \ldots sample \ldots solution]}{weight \ldots sample \ldots [g/L \ldots sample \ldots sol.]} \times 100[g/100g]$$

3.3 Dry matter and Water Activity Determination

3.3.1 Dry matter content

Before freeze-drying the samples, dry matter content of fresh broccoli was measured in order to have a base for calculation of the dry matter of freeze-dried samples.
The dry matter content of a fresh broccoli juice was also measured, and therefore this dry matter content was used to calculate the weight of the samples and volume of water to be added for the myrosinase activity assays.
The dry matter content of fresh broccoli and freeze-dried broccoli samples were measured in triplicate. First, 3 aluminium boxes for both fresh and different freeze-dried samples were heated in a oven (100 °C), for 30 minutes, and then cooled to room temperature, using an excicator, for at least 20 minutes. The boxes were numbered and weighed, in an analytical balance (accuracy of 0,1 mg). Between 0.5 to 1 g of sample was weighed for each box. The samples were dried in an oven (100 °C), overnight, followed by 1 hour cooling in the excicator, and weighed using the analytical balance. The dry matter content was calculated using the following equation:

> % dm = weight after drying- weight box before drying)/ weight of the sample * 100 %.

The dry matter content of the fresh broccoli juice was measured using the same procedure as for the fresh and freeze-dried broccoli samples, except that 1 g of sea sand (Firma) was mixed with the juice using a glass spatula, to prevent the formation of a film. The glass spatula was also weighed together with the sea sand.
The sample were dried in an oven at 80 °C, overnight, and then at 100 °C, for 3 hours, followed by 1 hour cooling in the excicator.

3.3.2 Water Activity

The water activity was determined to build a relationship between the moisture content and the a_w of the tested samples.
This determination was conducted using a LabMaster-a_w (Novasina Lachen, Switzerland), at 25 °C. The powdered samples were placed in small plastic boxes suitable for this test, filling completely the bottom of the boxes, to ensure the correct measurement. The boxes were, then, placed one by one in the device and the water activity was measured until a stable value was reached. All the determinations were made in duplicate.

3.4 Heat Treatments

For the heating process, 3.38 g, 1.57 g , 0.8 g, 0.685 g from the 82.5, 62.1, 26.1, and 13.4 % of moisture samples, respectively, were weighed into metal tubes, and then firmly closed. The weights were all performed maintaining the samples frozen, using liquid nitrogen.

The heating treatments were preformed in duplicate, using a heating block (Labyrinth, Kerkdriel, Holland, Nr. 200406900), and the times used for each temperature/moisture content (a_w) are described as follow (Tables 3.3 to 3.6):

Table 3.3 – Experimental time (min) / temperature (°C) combinations for 82.5 % moisture samples

82.5 %moisture (aw = 0.973)				
30°C	33.5°C	37.5°C	40°C	45°C
0	0	0	0	0
5	5	5	5	5
20	20	15	15	9
45	45	35	30	27
70	60	55	60	45

Table 3.4 - Experimental time (min) / temperature (°C) combinations for 62.1% moisture samples

62.1 %moisture (aw = 0.947)				
30°C	33.5°C	37.5°C	40°C	45°C
0	0	0	0	0
5	5	5	5	5
20	20	15	15	9
45	45	35	30	27
70	60	55	60	45

Table 3.5 - Experimental time (min) / temperature (°C) combinations for 26.1 % moisture samples

26.1 %moisture (aw=0.719)			
30°C	33.5°C	37.5°C	40°C
0	0	0	0
5	5	5	5
20	20	15	15
45	45	35	30
70	60	55	60
26.1 %moisture			
45°C	52.5°C	60°C	70°C
0	0	0	0
3	3	10	5
9	8	30	15
27	18	45	25
45	35	55	35
90	60		
150	120		
225	--		

Table 3.6 - Experimental time (min) / temperature (°C) combinations for 13.4 % moisture samples

13.4 %moisture (aw = 0.115)				
30°C	33.5°C	37.5°C	40°C	45°C
0	0	0	0	0
5	5	5	5	5
20	20	15	15	9
45	45	35	30	27
70	60	55	60	45

13.4 %moisture			
52.5°C	60°C	70°C	80°C
0	0	0	0
3	15	10	10
9	35	30	30
27	60	50	60
45	120	80	75
90	180	110	
150	240	150	
225	300	200	
300			

The determination of the heating time for each experiment was based on the work developed by Ludikhuyze *et al* (1999) and Van Eylen *et al* (2007; 2008), and on the evolution of the results obtained.
The timer was started (2.5 - 3 minutes), when the samples achieved the desired temperature, for each heating treatment.

3.5 Determination of myrosinase activity

In this method, the myrosinase activity is determined by a coupled enzymatic procedure in which the glucose, released during the reaction between myrosinase and sinigrin is used as a substrate to transform $NADP^+$ to NADPH, which is followed spectrophotometrically at λ = 340 nm. Figure 3.1 shows a schematic overview of the glucose release method.
The enzyme activity is determined based on the slope of the linear part of the curve of absorbance vs. reaction time (Wilkinson *et al,* 1984).

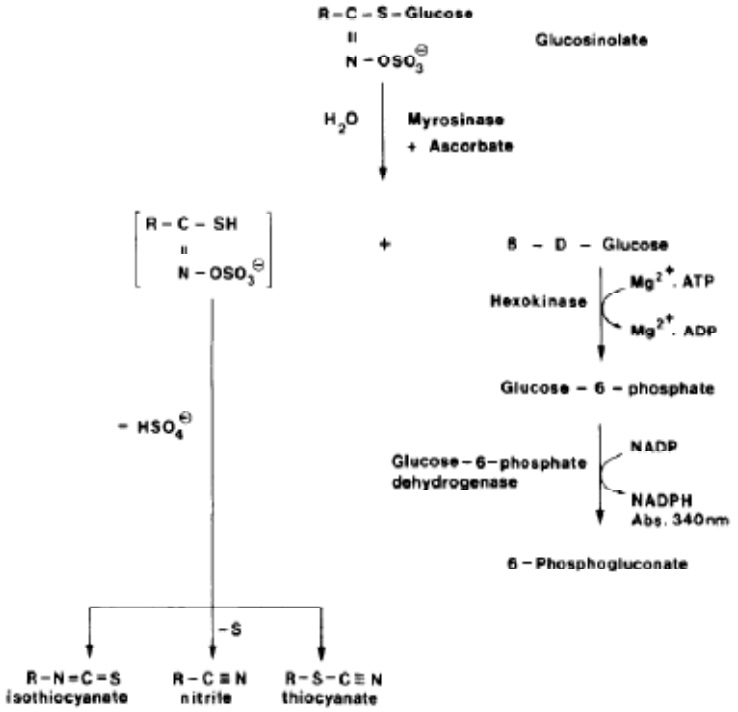


Figure 3.1 – Schematic overview of myrosinase activity measurement procedure (Wilkinson *et al.*, 1984)

A fresh broccoli juice was prepared with Oscar Juicer VitalMax 900 (Vitality 4 Life Pty. Ltd., Byron Bay, Australia) from 50.09 g of fresh broccoli. To raise the yield of juice the produced pellet was placed again on the juicer, obtaining 17.2 g of juice. The moisture content of the juice was calculated (92.605%) and used as a base to calculate the amount of each sample, for heating treatment, and the volume of Milli-Q water to be added after heating. The amount of sample for heat treatment was calculated relating the moisture content of each freeze dried sample with the moisture content of the broccoli juice, in order to have the same moisture content in all samples before enzyme extraction. An example of a calculation is shown in section 3.7 - Preliminary experiments.

After the heat treatments, the samples were mixed with Milli-Q water until 8 ml of volume were reached. All the samples were vortexed until homogeneous rehydration was visible (between 1-2 minutes) (Vortex VWR-VV3, VWR International B.V.) to rehydrate and achieve the same moisture content for all the samples. The myrosinase activity measurements were always performed in duplicate, for each (temperature vs. water activity) vs. time assay.
For each single determination, the tube containing the mixture of sample + Milli-Q water was centrifuged (Heraeus Multifuge X3R, DJB Labcare Ltd, England) at 4700 rpm, 4 °C, for 15 min to remove insoluble cell compounds, and minimize the loss of myrosinase. The centrifuge was pre-cooled before using. The supernatant was taken and centrifuged again in the same conditions (Hennig, 2008).

To avoid the detection of the original glucose contained in broccoli, which could interfere with activity measurement, the myrosinase was isolated by high speed centrifugation.
To precipitate the myrosinase, the supernatant of the last centrifugation was centrifuged in a pre-cooled centrifuge (Beckman High Speed Centrifuge Avanti J-26-XP), at 25000 rpm (rotor JA 25-15), 10 °C for 30 min (Henning, 2008). The resulting pellet was diluted in 4 ml of ice cold phosphate (mixture of potassium dihydrogen phosphate and di-potassium hydrogen

phosphate (Merck), 50 mM, pH 7.0), and the myrosinase was precipitated again under the same conditions. The obtained pellet was dissolved in 0.5 ml of ice cold phosphate buffer and the enzyme activity was measured. According to Rungapamestry *et al.* (2006), the whole procedure was performed as cool as possible to preserve the enzyme activity, keeping the samples on ice between the different steps.

3.5.1 UV Measurement

For the determination of the glucose released from the degradation of sinigrin by the myrosinase a D-glucose enzyme kit (Enzyplus® EZS 781+, Biocontrol Europe, The Netherlands) was used.
For the UV measurement, a reaction mixture (Milli-Q water, a water solution (every day freshly prepared) containing 1.63 g/l of ascorbic acid (Sigma-Aldrich) and 0.0825 g/l $MgCl_2$ (Merck), a buffer solution, a ATP/$NADP^+$ solution, hexokinase/glucose-6-P-dehydrogenase solution and 50 µl of sample solution containing the myrosinase) was prepared. The applied volumes from each different solution, during measurement with the enzyme kit are shown in Table 3.7.

Table 3.7 Procedure using the Enzyplus® EZS 781+, enzyme kit

Pipette into a tube:	**Volume (µl):**
$MgCl_2$ – ascorbic acid – solution:	986
Millipore water	464
Test kit solution R1: (imidazole buffer, magnesium chloride, sodium azide)	50
Test kit solution R2: *($NADP^+$, ATP)*	50
Sample solution:	50
Test kit solution R3: (hexokinase/ glucose-6-P-dehyrdogenase solution)	5

After mixing, 50 µl of sinigrin solution (30 mg/ml, (-)-sinigrin hydrate from horseradish, Sigma-Aldrich) were added, and the formation of NADPH was spectrophotometrically (Spectrophotometer Cary UV 50, Bergen op Zoom, The Netherlands) followed at 340 nm, at room temperature for 7 min, after a gap of 1 minute between the addition of sinigrin and the beginning of measurement, using an optical fibre immersion probe (661.302 UVS probe, Hellma, Müllheim, Germany).
The whole procedure had to be performed within one day, to keep the maximum of the enzyme activity as possible, since after storage, the enzyme activity can decrease.

3.5.2 Calibration curve

To establish a calibration curve the same measurement procedure which was used for the samples was applied, adding 50 µl myrosinase standard solution (thioglucosidase from sinapsis alba seed, Sigma-Aldrich) instead of samples solution.
Myrosinase standard solutions with concentrations in the range from 0.2 to 1.2 unit/ml were used.
A blank was measured by adding 50 µl water instead of sample solution.

Due to the fact that the sinigrin is an expensive compound, three calibration curve were built up by using three concentrations of sinigrin solution: 300 mg/ml, 30 mg/ml and 3 mg/ml, in order to reduce the amount used for the experiments .
After analyzing the obtained curves, the 30 mg/ml sinigrin solution was used for all further measurements, since good linearization and no cross between lines of different reference sinigrin concentrations was observed at that concentration. It was thus possible to reduce the cost of substrate by 90%.

3.5.3 Calculation

To calculate the enzyme activity (U/ml sample solution), the slope of the linear part of the curve absorbance vs. reaction time, for each sample, was calculated and using the equation of the calibration curve, the myrosinase activity was determined. The equation used to calculate the activity was the one obtained from the linear regression of the 30 mg/ml sinigrin calibration curve, as follow:

$$y = 0.101x + 0.0004$$

- y represents the slope of the line obtained from the released glucose, for each sample ;
- x represents the myrosinase activity of the samples.

To convert U/ml to U/g DM, the activity U/ml was transformed into mU/µl, obtaining an equivalent value which was converted to U/g DM using the following equation:

$$Activity(U/gDM) = (Activity(mU/\mu l) \times 50(\mu l) \times 10(-) \div 1000(mU)) \div 0.5916\ (gDM)$$

- 50 µl represents the amount of sample solution added to reaction mixture;
- 10 represents the dilution factor of the sample solution in the reaction mixture, or percentage of sample solution used for measurement. The isolated myrosinase of each sample was dissolved in 0.5ml, of which $1/10^{th}$ was used in the assay
- 0.5916 g represents the amount of dry matter in each and every sample for the heat treatment

3.6 Statistics and modeling the myrosinase stability of freeze-dried broccoli, during heating treatments

The effects of freeze-drying in myrosinase initial activity and the effects of interaction of moisture content and heating time for different temperatures were tested by single-factor ANOVA and two-factor with replication ANOVA, respectively. The probability was calculated for $\alpha = 0.05$.
Statistical analyses were performed using the Analysis ToolPak of Microsoft © Excel

The stability of myrosinase in freeze-dried broccoli could be affected by various factors. Thus, modeling the consequences of the heat treatment on myrosinase stability from freeze-dried broccoli becomes difficult to perform. Some mechanisms should be taken into account when modeling the behavior of myrosinase:

1) Heating up of broccoli samples;
2) Heat transfer from the heating tubes to the samples;
3) Thermal denaturation of myrosinase in the vegetable matrix;
4) Regeneration or refolding of myrosinase upon/ after the heat treatment.

These mechanisms could be described by mathematical equations, where parameters have to be estimated, as well the temperature dependence of the parameters. However, with the experimental data created in this study it is not possible to estimate accurately all the parameters to describe the mechanisms above. For that reason, simplification is necessary, neglecting the mechanisms that are expected to have little effect on the results.
Since the time for heating up the samples to the desired temperature, in each experiment, was almost the same (between 2.5 - 3 minutes) for all the different moisture content samples, and taking into account that different amount of sample were heated for each moisture content, mechanisms 1 and 2 were neglected. Only samples with heating times equal or longer that the heating up times were used for the modeling. Therefore the time zero sample for the modeling was in fact the sample that could have already somewhat lower activity due to denaturation during heating up.
Mechanism 4 was neglected based on the observation of results, where above denaturation temperature no myrosinase regeneration seems to occur.

In general, the decrease of enzyme activity (E) as a function of processing time (t) can be described by an n^{th} order kinetic model, as presented in equation (1):

$$\frac{dE}{dt} = -kE^n \tag{1}$$

where k is the inactivation rate constant and n represents the reaction order. Although enzyme inactivation is a complex chain of events, often a first order reaction kinetic model (n = 1) can be applied to describe inactivation kinetics.

Hence, two simplified models to describe inactivation of myrosinase were used to try to model the data available: first-order inactivation model and consecutive step inactivation model.
The irreversible first-order inactivation is the simplest mechanism to describe enzyme inactivation. In this model, it is assumed that the 3-D conformation of the active enzyme is changed irreversibly into an improper conformation, as shown schematically in scheme 3.6.1. In other words, the active enzyme, E is converted to an inactive form, E_i, in only one step.

$$E \xrightarrow{kd} Ei$$

The inactivation rate constant (r_{inact}) is given by equation (2), which was used directly for modeling purposes:

$$r_{inact} = \frac{dC_E}{dt} = -kd \cdot C_E \tag{2}$$

Separation of variables gives (3):

$$\frac{dC_E}{C_E} = -kd \cdot dt \tag{3}$$

Resulting in (4):

$$\ln \frac{C_E, t}{C_{E,0}} = -kd \cdot t \tag{4}$$

This results in the equation (5):

$$a_{rel} = \frac{C_{E,t}}{C_{E,0}} = e^{-kd \cdot t} \tag{5}$$

The consecutive step model has been used to describe thermal inactivation of broccoli myrosinase in a buffer system (Ludikhuyze *et al*., 1999) and in broccoli juice (Van Eylen *et al*., 2007) and can be applied to describe enzyme inactivation that occurs in two irreversible consecutive steps. In the first step, an intermediate (U_2) is formed that still has an active form (N_2) when returned to standard conditions. However, this activity can be different than the native form (N_1). In the second step, this intermediate (U_2) is turned over to the inactivated form (*I*).

$$N_1 \leftrightarrow U_1 \xrightarrow{k1} U_2 \xrightarrow{k2} I$$
$$\updownarrow$$
$$N_2$$

This model can also be applied when it is assumed that N_1 and N_2 are two enzyme forms, like free enzyme and enzyme interacting with impurities or substrate, both having an activity and where one form can be turned over in the other. In this case, the amount of N_2 can be dependent on temperature. The mathematical equation (7) describing the consecutive step model could be given by:

$$E = \left(E_1 - E_2\left(\frac{k_1}{k_1 + k_2}\right)\right)\exp(-k_1 t) + \left(E_2\left(\frac{k_1}{k_1 + k_2}\right)\right)\exp(-k_2 t) \qquad (7)$$

with E_1 the relative enzyme activity at t = 0, E_2 the relative enzyme activity of the second enzyme form (N_2) and k_1 and k_2 (min^{-1}) the inactivation rate constants respectively to obtain U_2 and *I* (min^{-1}). For modeling, the differential equations (8) and (9) based on the kinetics were used directly.

$$\frac{dE_1}{dt} = -k_1[E_1] \qquad (8)$$

$$\frac{dE_2}{dt} = k_1[E_1] - k_2[E_2] \qquad (9)$$

Temperature dependence of the time dependent models parameters (inactivation rate constant, k-values) was described using the Arrhenius equation (10):

$$\ln(k) = \ln(k_{ref}) + \frac{E_a}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right) \quad (R = 8.314 \text{ J mol}^{-1}\text{K}^{-1}) \qquad (10)$$

with k_{ref} the inactivation rate constant (min^{-1}) at the reference temperature T_{ref} (K) and E_a the activation energy (kJ/mol).

Integral fitting implies that the data sets from different incubation temperatures for each moisture content were fitted simultaneously (global fitting)and individually, to the degradation parameters. The software package Athena Visual Workbench (www.athenavisual.com) was used for numerical integration of differential equations as well as parameter estimation of the rate constants in the differential equations following minimization of the determinant criterion in order to obtain the reaction kinetic parameters (rate constants k_d, min^{-1}, and activation energies E_a, J/mol).

3.7 Preliminary Experiments on Myrosinase activity analysis

The aim of this thesis was to determine the stability of myrosinase in broccoli in samples with different moisture content, and consequently different water activity, when subjected to different heating treatments. To achieve this goal, preliminary experiments were conducted, in order to setup a method that could be reliable for myrosinase activity determination.
Different ratios sample/water, amount of sample to heat and preparation of samples were tested.
For the ratio sample/water, the moisture content of a broccoli juice was chosen as a base of calculation, for all the other samples. Due to limitations of available broccoli, related with the relative large amount of samples to test, a volume of 8 ml of mixture sample/water was established. Hence, the amount of sample for the heat treatment was calculated relating the dry weight of each sample with the dry weight of the juice, and the final volume for extraction. For example, for the driest samples (13.4 % moisture), the amount of sample was calculated, as follow:

$$\text{Amount of sample} = \frac{7.395\%DW}{86.6\%DW} \times 8$$

- 7.395 represents the amount of dry weight of broccoli juice;
- 86.6 represents the amount of dry weight of the driest (13.4% moisture) sample
- 8 represents the final volume of the mixture sample/water in the tubes before enzyme extraction.

For the extraction, a phosphate and a Tris-HCl buffer were tested for the two last centrifugations, as well as water and acetone as solvents, in the first two centrifugation steps. A phosphate buffer and water were selected for further analysis.
Minced and ground samples were also tested in order to define which one gives better and more stable results, and at the same time was more feasible. In this way, for all further experiments ground samples were used.
Also the time of measurement of the enzyme activity with the UV spectrophotometer was studied, to check if it was possible to reduce it. The measurement time for glucose release was reduced to 7 minutes, instead of 10 minutes, as no difference in the slopes of the curves from enzymatic activity, at 7 and 10 minutes was found.

4. Results and Discussion

4.1 Glucose content in fresh broccoli

The glucose background of broccoli can lead to a large background signal in the myrosinase activity measurement. The glucose content of fresh broccoli measured was 1.19×10^{-1} g/l sample solution ($1.082 \pm 6.085 \times 10^{-4}$ g/100g broccoli), as shown in Table 4.1, according to the equations described in section 3.2.
Hennig (2008) showed that a glucose background between 0-0.1 g/l sample solution does not disturb the enzyme activity measurement. According to Hennig (2008), this glucose background is slightly higher than the maximum glucose background concentration which does not seem to disturb myrosinase activity measurement by the glucose release method.

Table 4.1 - Glucose content of fresh broccoli samples

	[Glucose] (g/L sample solution)	[Glucose] (g/100g)
Fresh 1	0.1189	1.0817
Fresh 2	0.1190	1.0825

The minimum glucose background concentration tested by Hennig (2008) which disturbed the enzyme measurement was 0.3 g/l sample solution, however no experiments were made with glucose concentrations between 0.1-0.3 g/l sample solution. In this way, a possible disturb in enzyme measurements between 0.1-0.3 g/l is not described, leaving open the uncertainty if the measured glucose for the samples in the present study can disturb the method, *e.g.*, it is unknown what is the effect of a glucose background of 0.2 g/l sample solution in enzyme measurement.
The broccoli used for all the experiments was from Parthenon cultivar (F1 hybrid broccoli - *Brassica oleracea* L. convar *botrytis* (L.) Alef. Var. *cymosa* Duchesne), which is only grown in cool season. The glucose content of eleven cultivars of broccoli was always found to be substantially lower (2-4 fold) in plants grown in cool season than the same cultivars grown in warm season (Rosa *et al*., 2001). These findings show that to reduce the impact of glucose background on myrosinase activity determination, cool season grown plants should be used.
Another way to avoid or minimize the influence of glucose background is to separate the enzyme from the glucose using high-speed centrifugation, as was done in this project and described by van Eylen *et al.*, 2007.

4.2 Moisture Content and Water Activity

The freeze dried broccoli samples used to describe the myrosinase stability had 82.5, 62.1, 26.1 and 13.4% moisture content.
Since it is known that water activity is related to moisture content in a non-linear relationship and has influence on enzyme stability, water activity (25 ºC) was measured for the freeze dried samples. From Table 4.2 it is possible to concluded that the 82.5 and 62.1 moisture content (%) samples have a water activity value quite similar, and the driest ones, 13.4 and 26.1 moisture content (%), even with a 2- fold smaller difference between moisture contents than the higher moisture samples, show a large difference on water activity values obtained.

Table 4.2 - Water activity (25 ºC) *vs.* moisture content on freeze dried broccoli

a_w	0.115±0.004	0.719±0.008	0.947±0.001	0.973±0.005
Moisture content (%)	13.4±0.05	26.1±0.22	62.1±0.16	82.5±0.22

The non-linear relationship between moisture content and water activity is shown graphically in Figure 4.1.

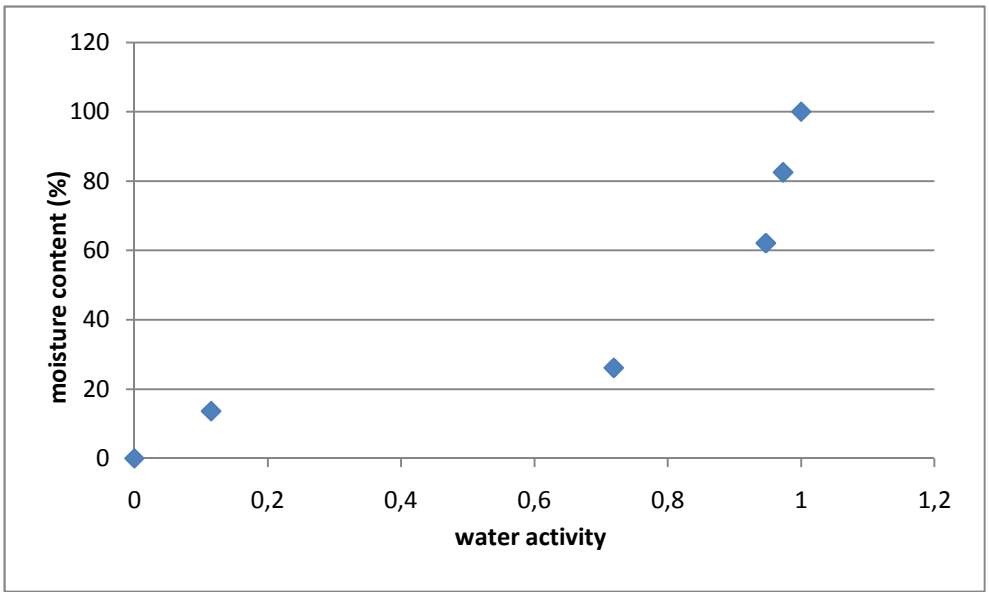


Figure 4.11 - Relation a_w *vs.* moisture content on freeze dried broccoli, at 25 ºC. Points (0,0) and (1,100) are represented as theoretical points.

This relationship is substance and temperature specific, meaning that these results should not be generalized for all broccoli cultivars, since variations in moisture content related to water activity are expected to exist between cultivars. However, it can give a good approach of this relationship.
Isotherms were not build for this project, but it could be of interest to have that information to help in prediction of product stability over time in different storage conditions, and specifically for myrosinase case, to find critical relationship points where enzyme stability increases or decreases.

4.3 Determination of myrosinase activity

4.3.1 Calibration Curves

In previous studies a 300 mg/ml sinigrin solution was used (Ludikhuyze *et al.*, 1999; van Eylen *et al.*, 2006; van Eylen *et al.*, 2007), a concentration of about 32 mg/ml sinigrin was used by Charron *et al.* (2005), and more recently van Eylen *et al.* (2008) used a 100 mg/ml sinigrin solution. Since around 300-350 myrosinase activity measurements were conducted, and due to the elevated price of sinigrin (220 € - 500 mg), used as substrate in this project, seems of extreme importance to use the less amount as possible. For that reason three calibration curves with sinigrin concentrations of 3, 30 and 300 mg/ml were build.
The obtained results show good linearization for all the three concentrations tested, and the R^2 obtained were 0.9724, 0.9938 and 0.9887 for the 3, 30 and 300 mg/ml of sinigrin calibration curves, respectively. However, in the calibration curve with the lowest concentration of sinigrin shown in Figure 4.2, even if good linearization was obtained, there are lines crossing, meaning that the initial absorbance was different for some of the myrosinase standard solutions. Thus, the lowest concentration was rejected for further experiments.

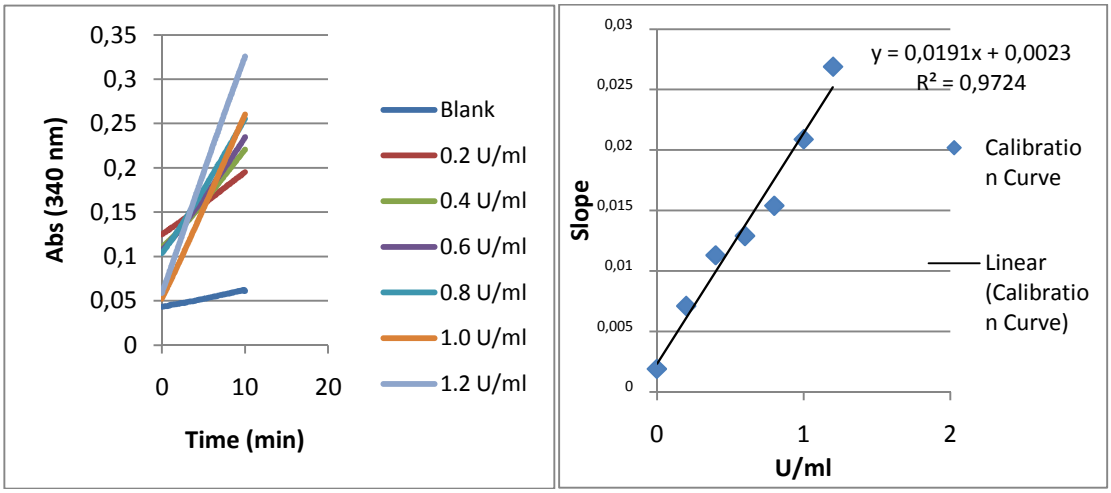


Figure 4.2 - Increase in UV absorbance shown on different myrosinase concentrations,and respective calibration curve [sinigrin] = 3 mg/ml

Comparing the calibration curves built with 30 (Figure 4.3) and 300 mg/ml of sinigrin (Figure 4.4), no significant differences in linearization are shown, as well as in myrosinase activity measurements performed with fresh broccoli. In Table I.1, from appendix I, myrosinase activity determined using the [sinigrin]=30 mg/ml calibration curve was 0.68 and 1.16 U/ml, respectively, for samples with a ratio broccoli/water 1:1 and 3:1, and for the higher sinigrin concentration curve, the values were 0.74 and 1.14 U/ml. These results show that no difference exists using the intermediate sinigrin concentration when compared to the highest one.
Charron *et al.* (2005)described 2.5 mM sinigrin as a saturation concentration for myrosinase activity determination. Hence, the final concentration of sinigrin used in the mixture for the myrosinase activity assay was calculated in 2.36 mM, when a 30 mg/ml sinigrin solution was used, which is only slightly lower than the concentration described by Charron *et al.* (2005). Consequently, a solution of 30 mg/ml sinigrin and the calibration curve built with a solution of same sinigrin amount were used to determine and calculate the myrosinase activity.

By conducting this experiment was possible to reduce in 90 % the economical and environmental costs of substrate used for the glucose release method, resulting in savings around 2000 €, and at least a lower release of CO_2 due to production and associated transportation.

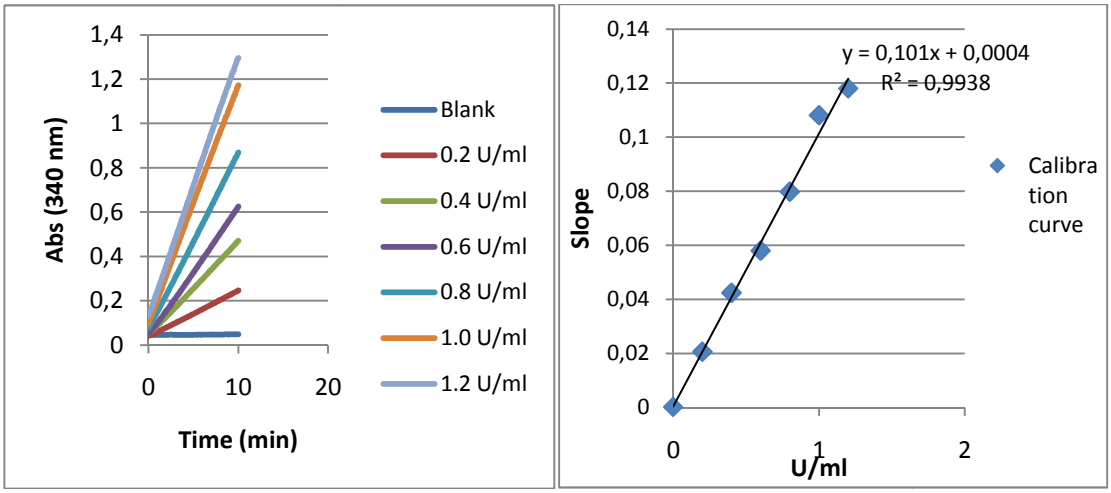


Figure 4.3 - Increase in UV absorbance shown on different myrosinase concentrations,and respective calibration curve [sinigrin] = 30 mg/ml

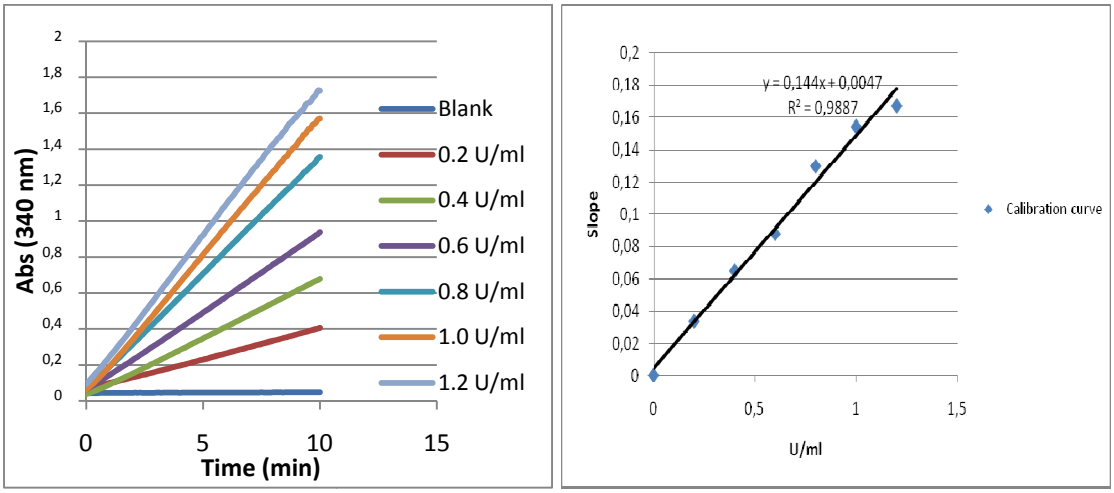


Figure 4.4 - Increase in UV absorbance shown on different myrosinase concentrations, and respective calibration curve [sinigrin] = 300 mg/ml

4.3.2 - Myrosinase initial activity

The myrosinase activity was determined using the glucose release method (chapter 3.4). Different amounts of samples, according to the different moisture contents were weighed into the metal tubes, in order to have the same amount of dry weight in all the samples, and therefore obtain activity results comparable between the different moisture content broccoli. In consequence, the amount of water added to the samples was different according to the moisture content of each group of samples.

The myrosinase initial activity (in this case, means that no heating treatments were performed) was determined to know if differences in myrosinase activity related to the moisture content of the samples were evident.
By the results shown in Figure 4.5 and ANOVA single factor analysis (Appendix IV, Table IV.1 and Table IV.2) is possible to conclude that no significant difference ($p < 0.05$) exists between the 62.1 and 70.1 % moisture content samples. Also for the 26.1, 82.5 and 90.1 % moisture content samples statistical differences were found ($p < 0.05$). The highest myrosinase activity was observed for the driest sample, and the lowest were observed for both 62.1 and 70.1 % moisture content samples.
The obtained results show that the initial myrosinase activity can be organized in three groups showing different enzymatic activity when compared to each other ($p > 0.05$). A group showing higher activity, where only the 13.4 % moisture content is included, an intermediate group, including the 26.1, 82.5 and 90.1 % moisture content samples showing slightly lower myrosinase activity than the first group, and a group showing lower activity, where the 62.1 and 70 % moisture content samples are included.

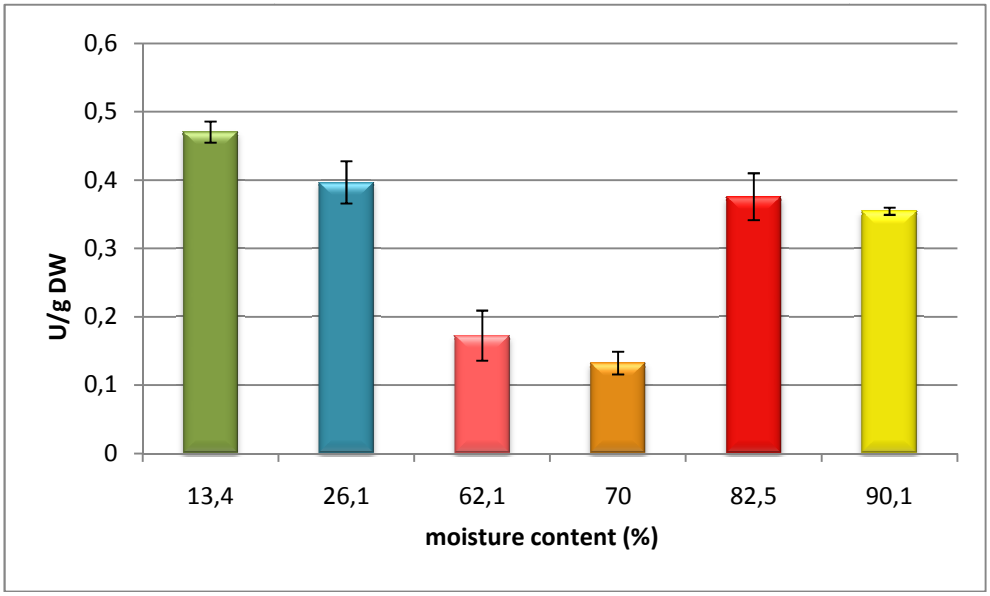


Figure 4.5 - Myrosinase initial activity(Units/g DW) (no heating) of freeze dried samples. Points are the average of two activity measurements.

From these results some speculation to help explaining these differences can be made.
The broccoli chopping step should not have an influence on these differences since before freeze-drying the whole broccoli batch was mixed, before division in the different trays. The

freeze-drying step is not likely to have influence, due to the fact that the 13.4 and 26.1 % moisture content samples showed higher myrosinase activity than the 62.1 and 70 % moisture samples, and to reach the final moisture content, the driest samples had to pass through the range between 60 - 70 % moisture. An inactivation - activation process dependent on moisture content could be proposed, although does not seem appropriated, since the water activities of the samples between 90.1 - 62.1 % moisture content are similar, between 0.983 - 0.947, moving in from the most humid to the 62.1 % moisture samples.

The grinding step seems to be the one that could have the major impact, for the reason that sensorial (visual) differences were noticed in the texture of the ground broccoli from the different moisture content samples. From the two driest samples a fine powder was obtained, while for the higher moisture content (82.5 and 90.1 %) a less fine powder was obtained. For the samples showing less enzymatic activity, a roughly powder, with "straw" aspect, was obtained. This factor may have an impact on the rehydration process of the samples, before the extraction and measurement steps. A different and less efficient rehydration process can be due to a lower superficial area available of the 62.1 and 70 % moisture content samples to react with water, which is essential for myrosinase to react, due to the fact that this enzyme is a hydrolase. Prolonged (5 min, instead of immediate start of extraction process) rehydration process for the 62.1 % moisture, however did not show differences in the measured enzyme activity.

The extraction and enzymatic activity measurements do not seem to influence the myrosinase enzymatic assay, once the exact same procedure was used for all the different samples tested.

4.3.3 - Degradation profile of myrosinase

The myrosinase activity was determined in freeze dried broccoli with four different moisture contents: 13.4, 26.1, 62.1 and 82.5 % moisture, after different heat treatments (ºC) for different times (minutes).
The driest samples were heated at nine different temperatures. Firstly, the 13.4 % samples were heated at temperatures from 30 - 45 ºC, for times between 0 and 70 minutes, depending on the heating treatment applied. The temperature/times applied are available in Table III.5, from Appendix III.
No myrosinase inactivation was shown for these temperatures/time treatments in the driest samples, shown in Figure VI.1 (appendix VI). For this reason, the heating temperatures and times were increased in order to determine the kinetical parameters of myrosinase inactivation for these samples. It is important to make reference for activation - inactivation and regeneration phenomenon, which are likely to occur when denaturation temperatures/time relation is not achieved (van Boekel, 2009). In other words no complete denaturation of myrosinase was reached for these treatments.
The lowest temperature used for heating treatment of the driest samples in which degradation was possible to be observed, though without much evidence was at 52.5 ºC, since after 240 minutes (4 hours) of heating, the myrosinase relative activity was 85 %.
Clear degradation of myrosinase was only possible to see when temperatures between 60-80 ºC were applied to the driest broccoli samples, as shown in figure 4.6. At 60 ºC, only after 240 minutes of treatment, relative activity decreased to 20 %. At 70 ºC, 200 minutes were needed to reduce the activity by 90%, while at 80 ºC, 60 minutes of heating were enough to reduce the relative activity to 10%.
Comparing these results with the studies conducted by van Eylen *et al.* (2007, 2008) and Ludikhuyze *et al*. (1999), is obvious the increase of myrosinase thermostability, in these conditions. In those studies, heating treatments between 5-7 minutes, at 60 ºC were enough to reduce the relative activity by 90 %.

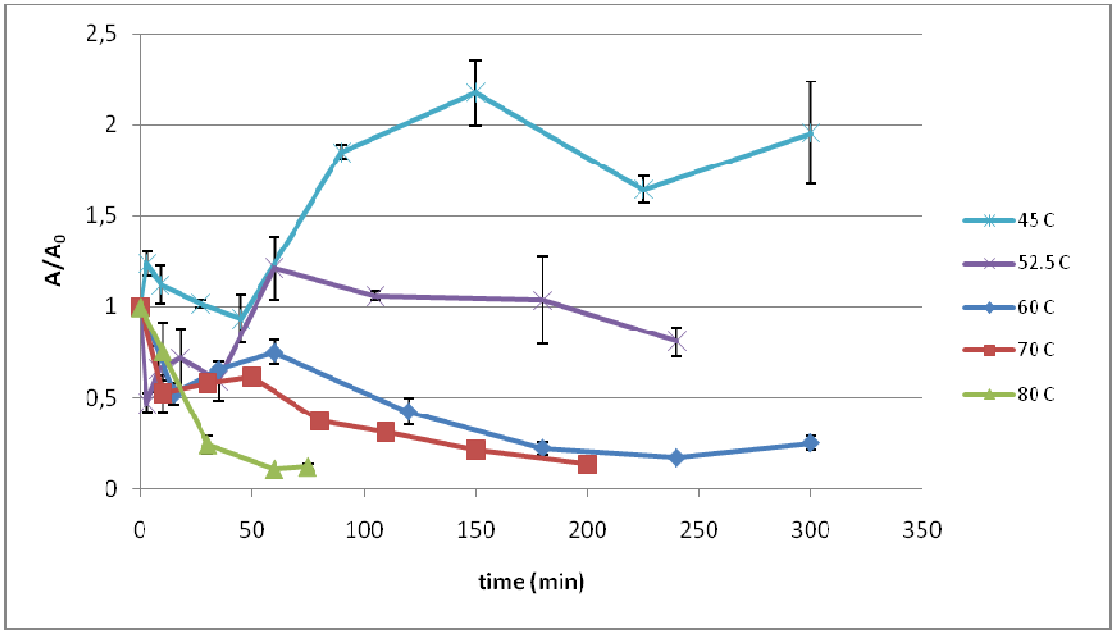


Figure 4.6 - Degradation profile of myrosinase on 13,4% moisture content freeze dried broccoli, at 60-80ºC. Points are the average of two activity measurements.

The 26.1 % moisture content samples, as the driest ones (13.4 %) were heated, firstly, at temperatures between 30 - 45 ºC, for different times (Table III.4, from appendix III). Only slight degradation was shown between 30 - 40 ºC, for the heating treatment applied times, as shown in Figure VI.2 (appendix VI).
Due to this fact, as for the 13.4 % moisture samples, experimental temperature/time relations were increased. The maximum temperature used for these samples was 70 ºC.
From Figure 4.7, it is possible to see a degradation profile in the range of temperatures between 45 and 70 ºC. At 45 ºC, a reduction of 70 % in relative activity was achieved after 150 minutes, while at 52.5 ºC for the same reduction in myrosinase activity only 45 minutes of heating were needed. At 60 and 70 ºC the myrosinase degradation occurred much faster, since heating times of 30 and 10 minutes, respectively, were sufficient to reduce the initial activity by 90 %.
These findings show that myrosinase is less thermostable in this condition, when compared to the driest samples (13.4% moisture). However, when compared with the stability of myrosinase in broccoli juice (van Eylen *et al.*, 2007) and in intact tissue (van Eylen *et al.*, 2008) is clear an increased thermostability, in the 26.1 % moisture freeze-dried broccoli powder. In those studies, heating treatments between 5-7 minutes, at 60 ºC were enough to reduce the relative activity by 90 %.

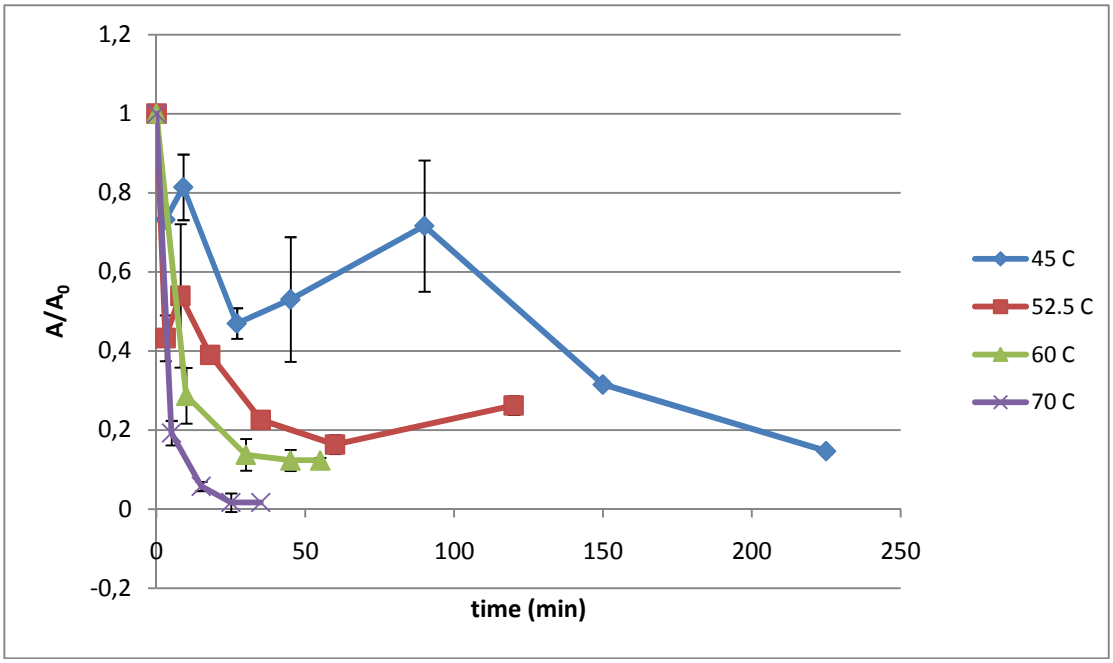


Figure 4.7 - Degradation profile of myrosinase on 26.1% moisture content freeze dried broccoli, at 45-70ºC. Points are the average of two activity measurements.

As it is visible in Figure 4.8, the myrosinase activity in 62.1 % moisture samples decreased sharply when subjected to heating treatments, especially in the range of temperatures between 37.5 - 45 ºC, where a reduction of enzymatic activity between 70 - 80 % is shown, after 5 minutes of heat treatment. Since the water activity of these samples is 0.947, a value close to the water activity of fresh broccoli (0.983), a comparison with previous studies should be possible. The degradation profile of the 62.1 % moisture samples has shown to be different from previous studies in broccoli in buffer solution (Ludikhuyze *et al.*, 1999), in broccoli juice (van Eylen *et al.*, 2007) and in intact tissues (van Eylen *et al.*, 2008). The degradation of myrosinase, in the present study, was faster than in those previous studies. At 40 ºC, the reduction in activity after 5 minutes, was about 80 %, while for broccoli in buffer solution,

only a decrease of 40% on myrosinase activity was observed. In broccoli juice (van Eylen *et al*., 2007) and in intact broccoli tissues (van Eylen *et al*., 2008) to reach the same degree of enzymatic degradation, 30 minutes were needed, at 50 ºC and 55 ºC, respectively for the juice and intact tissues.
In Figure VI.5 from appendix V, it is possible to observe that the initial activity for each temperature treatment of 62.1% moisture sample was always lower than for the other moisture content samples tested (Figures VI.3, VI.4, VI.6, in appendix VI), showing coherence with the results shown in 4.3.2, where the initial myrosinase activity of 62.1% moisture samples, with no heating treatment, has shown to be lower than for the other moisture content samples.

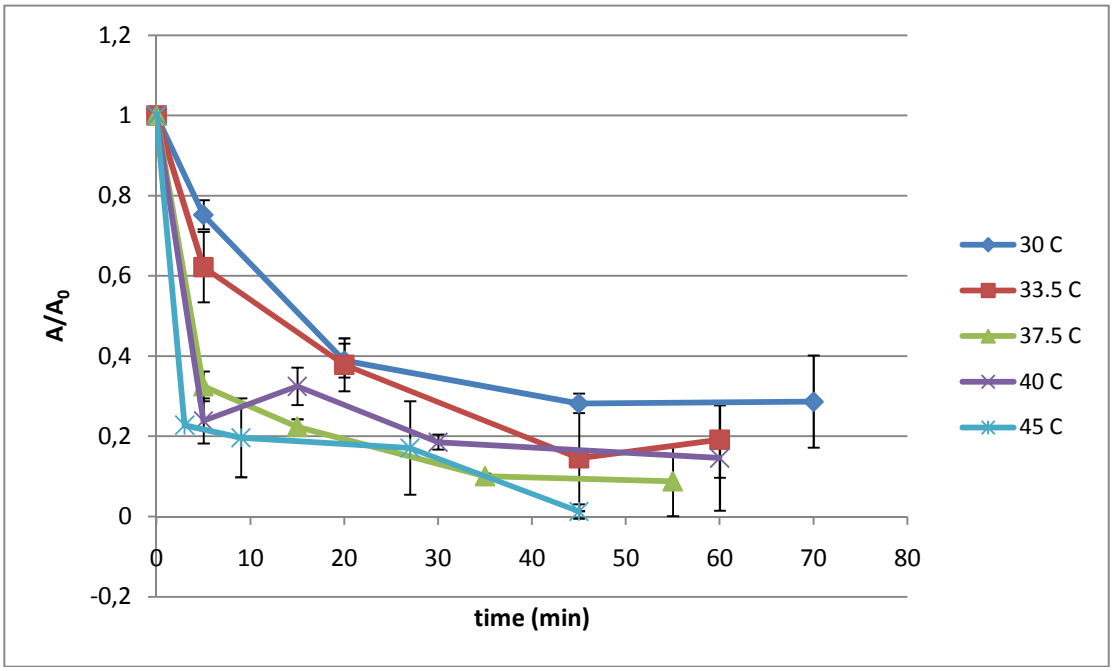


Figure 4.8 - Degradation profile of myrosinase on 62.1 % moisture content freeze dried broccoli, at 30-45ºC. Relative myrosinase activity (above) and myrosinase activity (Units/g DW). Points are the average of two activity measurements.

Figure 4.9 shows the degradation profile of myrosinase in 82.5 % moisture content freeze dried broccoli. The degradation profile of these samples is similar to one shown for 62.1 % moisture samples.
At 30 ºC, an activation-inactivation seems to occur, even if the variation between the two measurements, at 5 minutes, was relatively high.
As for the 62.1 % moisture samples, the myrosinase denaturation showed to be quicker than reported in previous studies in broccoli in buffer solution (Ludikhuyze *et al*., 1999), in broccoli juice (van Eylen *et al*., 2007) and in intact tissues (van Eylen *et al*., 2008). At 40 ºC, the reduction in activity after 5 minutes, was about 50 %, while for broccoli in buffer solution, only a decrease of 35% on myrosinase activity was observed. In broccoli juice (van Eylen *et al*., 2007) and in intact broccoli tissues (van Eylen *et al*., 2008) to reach the same enzymatic degradation degree, 10 minutes were needed, at 50 ºC and 55 ºC, respectively for the juice and intact tissues.
In this way, comparing figures 4.8 and 4.9, can be concluded that a slower myrosinase degradation for the 82.5 % moisture samples than for the 62.1 % moisture samples occured.

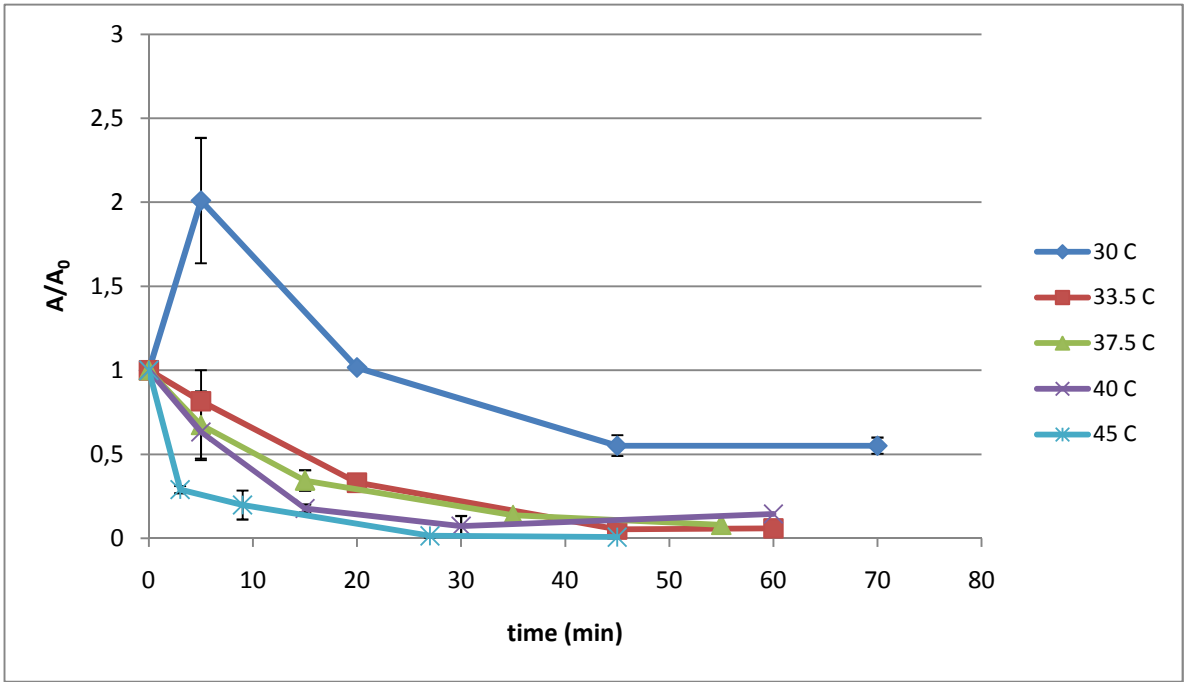


Figure 4.9 - Degradation profile of myrosinase on 82.5% moisture content freeze dried broccoli, at 30-45ºC. Points are the average of two activity measurements.

From Figure 4.8, is possible to speculate about the sensivity of the method when activity is measured in low enzyme activity samples. The stabilization of the relative activity at 20%, for the samples heated between 33.5 - 37.5 ºC, gives the impression that, caused by an already low initial myrosinase activity, even small increments in absorbance can affect significantly the myrosinase activity determination, reducing the measurement accuracy. The sensitivity seems to be lower for samples with less enzyme activity, also, by the fact that for 62.1 (Figure 4.8) and 82.5 % moisture content samples (Figure 4.9), some crosses between degradation lines at different temperatures were observed.

4.3.4 - Influence of moisture content / temperature related with time on the behavior of myrosinase.

The effect of broccoli moisture content on the behavior of myrosinase, at three different temperatures (30, 37.5 and 45 ºC), in relation with different heating times was analyzed by a two-factor with replication ANOVA test (Appendix V).
The activity of myrosinase in freeze dried broccoli was markedly influenced by these factors. Myrosinase activity was positively influenced ($p < 0.05$) by the decreasing of moisture content of samples, and negatively influenced ($p < 0.05$) by the increasing of cooking times, for all the temperatures. There is also a significant ($p < 0.05$) effect of the combination of moisture content and heating time, in myrosinase activity.

The statistical analysis was made for myrosinase relative activity (A/A_0). This analysis was made with relative activity and not with the absolute activities, since different results were expected to be obtained for the two different analysis, at each analyzed temperature. These difference results from the fact that measured initial myrosinase activities, for each moisture content samples at different temperatures were different, which makes comparison between moisture contents more difficult, and less accurate, when myrosinase absolute activity data is analysed.

Analysing the ANOVA values obtained, at 30 ºC, the effect of moisture content in myrosinase degradation was shown to have impact, as well as the influence of cooking times ($p<0.05$)
The same conclusion is possible for the combined effect of moisture content/cooking times, at 30 ºC ($p<0.05$). The results from the ANOVA analysis are shown in Appendix V.

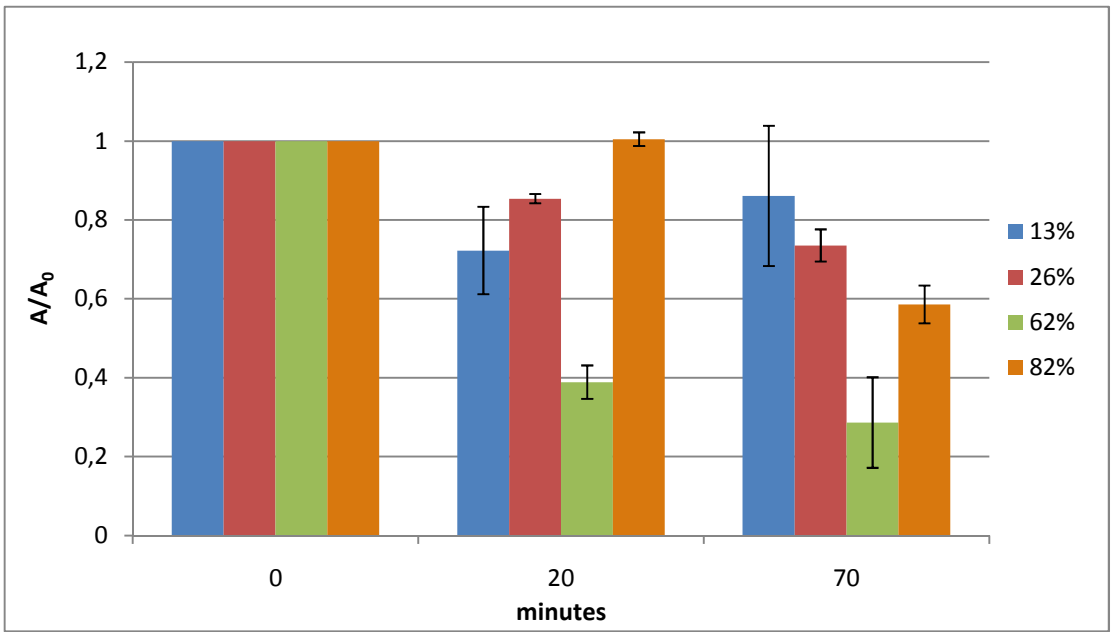


Figure 4.10 - Comparison of myrosinase behavior in freeze dried broccoli, with different moisture contents (13.4-82.5%) at 30ºC. Relative myrosinase activity (above) and myrosinase activity (Units/g DW). Points are the average of two activity measurements.

The effects of moisture content and time, and the combined effect of the two factors are even more evident with increasing temperature, as shown in figures 4.10, 4.11 and 4.12, and Appendix V. The p-value obtained for the effect of moisture content has increased 1000-fold for every temperature increase, when compared to the immediately preceding heat treatment temperature. The effect of cooking time has a much bigger influence on myrosinase activity, at 45 ºC, even if the influence of cooking time showed to be statistically significant, at 30 and 37.5 ºC ($p < 0.05$). The combined effect of moisture content/cooking time was 1000 and 100-fold bigger, at 45º C, in comparison to 30 and 37.5 ºC, respectively.

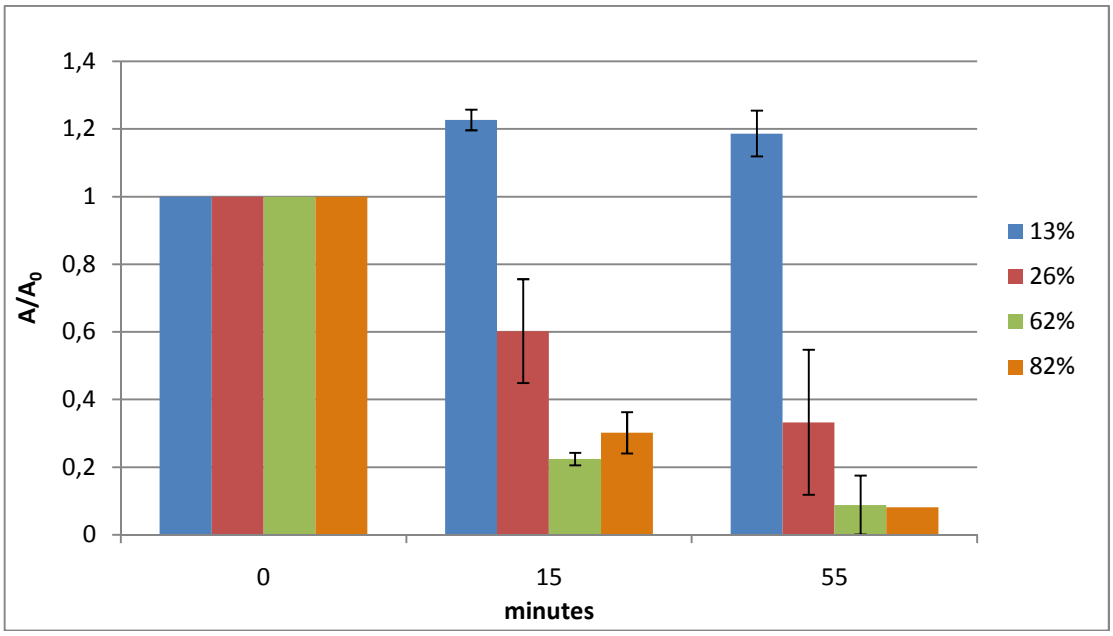


Figure 4.11 - Comparison of myrosinase behavior in freeze dried broccoli, with different moisture contents (13.4-82.5%) at 37.5ºC. Relative myrosinase activity (above) and myrosinase activity (Units/g DW). Points are the average of two activity measurements.

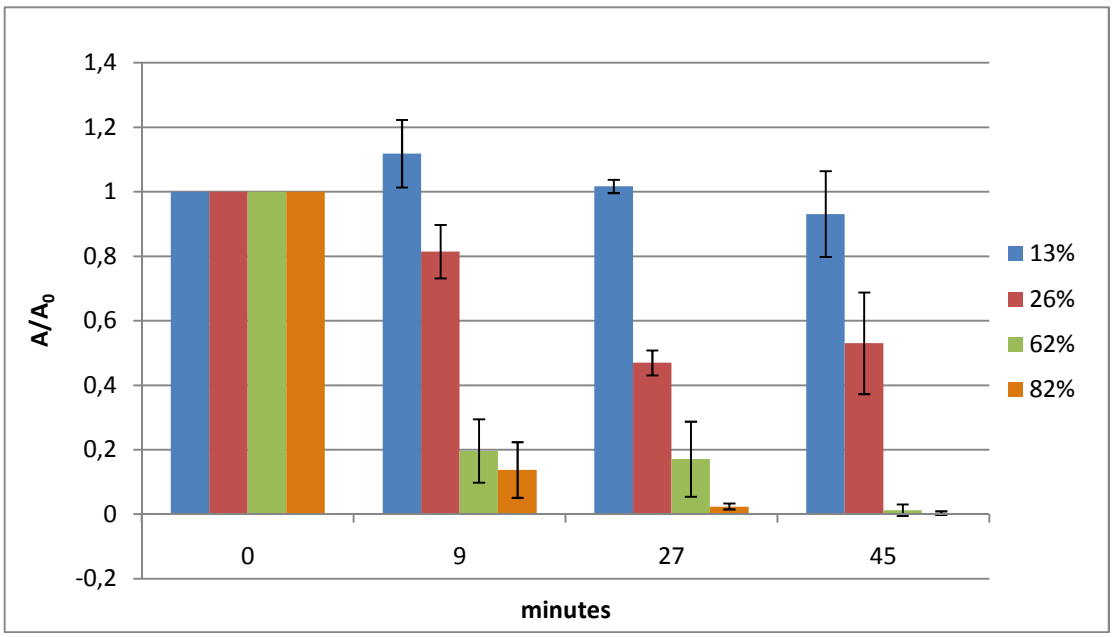


Figure 4.12 - Comparison of myrosinase behavior in freeze dried broccoli, with different moisture contents (13.4-82.5%) at 45ºC. Relative myrosinase activity (above) and myrosinase activity (Units/g DW). Points are the average of two activity measurements.

These findings are in accordance to the theoretical effects described of lowering water content in a system (Minton, 2005; van Boekel, 2009), and the discussed before in section 4.3.3, where an increase of myrosinase thermal stability was described for the driest samples.

4.4 Modeling the thermal stability of myrosinase in freeze dried samples.

Myrosinase activity was found for all the different moisture content of the heat treated freeze dried broccoli samples. The behavior of myrosinase in each different moisture content samples was modeled individually for each temperature where degradation was visible.
The modeling approach was based on the mathematical description of two possible models that can describe enzymatic inactivation. Both models included the thermal degradation of myrosinase in the vegetable matrix.
A first order kinetic model, which is frequently used to describe enzyme inactivation, and a consecutive step model were used to estimate the kinetic parameters (see chapter 3.5).
For the first order kinetic model it is assumed that the 3-D conformation of the active enzyme is changed irreversibly into an inactive conformation. In other words, the active enzyme is converted to an inactive form in only one step.
The consecutive step model can describe an inactivation process proceeding as a succession of two irreversible reaction steps. In the first step, the native enzyme converts to an intermediate form, with a reduced activity. On its turn, this intermediate form is converted to the inactive form.
With the mathematical equations describing the aforementioned models and the experimental data, the kinetic parameters were estimated. The results from the two models were compared to check which one fits better the experimental data.

Since no myrosinase degradation trends were visible for temperatures below 60 ºC, for the 13.4 % moisture content samples, only three temperatures were used to model the myrosinase stability of the driest samples.
At 80 ºC, the first order model seems to fit better the available data, once the response of the consecutive model for this temperature was the same as the first order model, confirmed by the same statistical parameters obtained for the two models. By the consecutive step model, no estimation of inactivation rate constant, k_{d2}, and A_2 (% initial activity) was possible.
At 70 and 60 ºC, neither the first order or the consecutive step model fit well the experimental data. This could be due to variation in the myrosinase activity measurements, possible enzyme regeneration or inactivation-activation processes occurred between 15 and 60 minutes of heat treatment for these two temperatures. However, the first order model fits better than the consecutive step model, as shown in Table 4.3 and 4.4, from the statistical parameters (R^2 and AIC) and due to the fact that some parameters were not estimated. The estimated inactivation rate constants, k_d, follow a coherent trend, increasing when temperature applied increases too.

Table 4.3 - Estimated kinetic parameters (± std. deviation) for thermal inactivation of broccoli myrosinase, in freeze dried broccoli (13.4% moisture) based on the 1st order model.

T (ºC)	k_d (min^{-1})	Initial Activity (U/g DW)	R^2	AIC
60	5.89 ±1.25 ($\times10^{-3}$)	5.74 ± 0.49 ($\times10^{-1}$)	0.724	-4.15
70	9.13 ± 2.22 ($\times10^{-3}$)	3.46 ± 0.36 ($\times10^{-1}$)	0.660	-4.87
80	3.55 ± 0.52 ($\times10^{-2}$)	4.50 ± 0.28 ($\times10^{-1}$)	0.936	-5.46

Table 4.4 - Estimated kinetic parameters (± std. deviation) for thermal inactivation of broccoli myrosinase, in freeze dried broccoli (13.4% moisture) based on the consecutive step model.

T (ºC)	k_{d1} (min^{-1})	Initial Activity (U/g DW)	k_{d2} (min^{-1})	A_2 [a](%)	R^2	AIC
60	8.89 ± 5.41($\times10^{-3}$)	5.92 ± 1.69 ($\times10^{-1}$)	NE	1.45 ± 0.60 ($\times10^{-1}$)	0.711	-3.95
70	9.15 ± 35.5 ($\times10^{-3}$)	3.46 ± 0.43 ($\times10^{-1}$)	NE	2.46 ± 4490 ($\times10^{-3}$)	0.634	-4.64
80	3.55 ± 0.52 ($\times10^{-2}$)	4.50 ± 0.28 ($\times10^{-1}$)	NE[b]	NE	0.936	-5.46

[a] given in percent of overall initial activity. [b] NE, non estimated.

For the 26.1 % moisture content samples, five from the eight temperatures used for the heating treatments were selected to be modeled by the two different models. As for the driest samples, the experimental data chosen to be modeled, was selected based on the temperatures where myrosinase degradation was observed. At 70 ºC, both first order and consecutive step model fit well the experimental data, as presented in Table 4.5 and 4.6. However, seems that the first order model fits better the data, due to a higher R^2 value and lower AIC. At 60 ºC, the consecutive step model seems to fit better, although, no estimation was possible for the activity of the intermediate form enzyme, A_2. The same can be concluded to the data available for heating treatments, at 52.5 ºC. In this case neither k_{d2} or A_2 were possible to estimate by the consecutive step model.
At 45 ºC, the first order model seems to fit better the available data, once the response of the consecutive model for this temperature was the same as the first order model, meaning that the consecutive step model was adjusted to a first order model, which is confirmed, also, by the same values obtained for the statistical parameters. From Table 4.5, it can be seen that, as demonstrated for the driest samples, the inactivation rate constants increased with increases of tested temperatures.

Table 4.5 - Estimated kinetic parameters (± std. deviation) for thermal inactivation of broccoli myrosinase, in freeze dried broccoli (26.1% moisture) based on the 1st order model.

T (ºC)	k_d (min^{-1})	Initial Activity (U/g DW)	R^2	AIC
40	7.57 ± 2.06 ($\times10^{-3}$)	3.06 ± 0.15 ($\times10^{-1}$)	0.612	-6.12
45	6.09 ± 1.59 ($\times10^{-3}$)	1.75 ± 0.14 ($\times10^{-1}$)	0.642	-6.31
52.5	3.52 ± 1.24 ($\times10^{-2}$)	2.25 ± 0.29 ($\times10^{-1}$)	0.542	-5.33
60	1.06 ± 0.22 ($\times10^{-1}$)	1.74 ± 0.13 ($\times10^{-1}$)	0.907	-7.14
70	3.23 ± 0.58 ($\times10^{-1}$)	1.52 ± 0.09 ($\times10^{-1}$)	0.960	-8.03

Table 4.6 - Estimated kinetic parameters (± std. deviation) for thermal inactivation of broccoli myrosinase, in freeze dried broccoli (26.1% moisture) based on the consecutive step model.

T (ºC)	k_{d1} (min^{-1})	Initial Activity (U/g DW)	k_{d2} (min^{-1})	A_2 [a](%)	R^2	AIC
40	4.92 ± 18.7 ($\times10^{-2}$)	3.20 ± 0.22 ($\times10^{-1}$)	2.71± 295 ($\times10^{-4}$)	6.37 ± 10.1 ($\times10^{-1}$)	0.583	-4.84
45	6.09 ± 1.59 ($\times10^{-3}$)	1.75 ± 0.14 ($\times10^{-1}$)	NE	NE	0.641	-6.31
52.5	1.11 ± 0.41 ($\times10^{-2}$)	2.97 ± 0.23 ($\times10^{-1}$)	NE[b]	NE	0.840	-6.18
60	1.77 ± 0.50 ($\times10^{-1}$)	1.77 ± 0.06 ($\times10^{-1}$)	3.31± 16.3 ($\times10^{-3}$)	NE	0.983	-7.63
70	5.40 ± 14 ($\times10^{-1}$)	1.52 ± 0.09 ($\times10^{-1}$)	9.13 ± 24.0 ($\times10^{-2}$)	1.84 ± 7.75 ($\times10^{-1}$)	0.953	-6.65

[a] given in percent of overall initial activity. [b] NE, non estimated.

The inactivation rate constants obtained by modeling individually the experimental data at each temperature, for the 62.1 % moisture content samples were the highest overall the different moisture content samples, in agreement with figures 4.8 and 4.11. An inactivation rate constant 100-fold higher than the inactivation rate constant for the 26.1 % moisture samples was obtained, at 45 ºC, which shows the significant difference in myrosinase thermal stability .
Analyzing the statistical parameters given in Table 4.7 and 4.8, seems that the consecutive step model fits better the data than the first order model, shown by high R^2 (> 90%, except at 40 ºC = 0.824), and lower AIC values obtained, at each temperature. However, some parameters were not estimated and high standard deviations, sometimes higher than the correspondent estimated parameter value, were obtained by the consecutive step model.
The estimated parameters using the first order model show a constant increase in inactivation rate constant as heat temperature increases.

The AIC values obtained, at 45 ºC by the first order model are lower than obtained for the 26.1% moisture content samples. For all the reasons aforesaid, the model that fits better the data available is the first order kinetic model.

Table 4.7 - Estimated kinetic parameters (± std. deviation) for thermal inactivation of broccoli myrosinase, in freeze dried broccoli (62.1% moisture) based on the 1st order model.

T (ºC)	k_d (min^{-1})	Initial Activity (U/g DW)	R^2	AIC
30	2.72 ± 0.59 ($\times10^{-2}$)	8.91 ± 0.75 ($\times10^{-2}$)	0.801	-7.82
33.5	4.19 ± 0.84 ($\times10^{-2}$)	7.35 ± 056 ($\times10^{-2}$)	0.880	-8.54
37.5	1.76 ± 0.43 ($\times10^{-1}$)	9.32 ± 0.85 ($\times10^{-2}$)	0.877	-8.06
40	1.88 ± 0.81 ($\times10^{-1}$)	6.08 ± 1.01 ($\times10^{-2}$)	0.587	-7.72
45	4.41 ± 1.33 ($\times10^{-1}$)	6.57± 0.68 ($\times10^{-2}$)	0.848	-8.52

Table 4.8 - Estimated kinetic parameters (± std. deviation) for thermal inactivation of broccoli myrosinase, in freeze dried broccoli (62.1% moisture) based on the consecutive step model.

T (ºC)	k_{d1} (min^{-1})	Initial Activity (U/g DW)	k_{d2} (min^{-1})	A_2 [a] (%)	R^2	AIC
30	8.94 ± 2.47 ($\times10^{-2}$)	9.84 ± 0.56 ($\times10^{-2}$)	NE	NE	0.925	-8.34
33.5	2.69 ± 2.44 ($\times10^{-1}$)	8.07 ± 0.52 ($\times10^{-2}$)	2.34 ± 1.08 ($\times10^{-2}$)	4.32 ± 2.16 ($\times10^{-1}$)	0.927	-7.82
37.5	5.73 ± 5.74 ($\times10^{-1}$)	9.54 ± 0.50 ($\times10^{-2}$)	2.87 ± 1.71 ($\times10^{-2}$)	2.42 ± 1.23 ($\times10^{-1}$)	0.957	-7.91
40	7.35 ± 10.6 ($\times10^{-1}$)	6.32 ± 0.66 ($\times10^{-2}$)	NE	2.18 ± 6.16 ($\times10^{-1}$)	0.824	-8.1
45	7.02 ± 2.75 ($\times10^{-1}$)	6.61 ± 0.51 ($\times10^{-2}$)	NE[b]	NE	0.915	-8.63

[a] given in percent of overall initial activity. [b] NE, non estimated.

The highest moisture content samples (82.5 %) show a slower, although similar, degradation of myrosinase, when compared to the 62.1 % moisture samples, at each one of the tested temperatures.
Similarly to what observed in the 62.1 % moisture samples, relatively good values for the statistical parameters were obtained by the consecutive step model (Table 4.10), however, the estimated parameters show high standard deviations, and some were not estimated, specifically the cases of k_{d2} (min^{-1}) and A_2 (% of overall initial activity).
The statistical parameters obtained from individual modeling by the first order model show relative high R^2 values and low AIC values (Table 4.9) comparing to those obtained by the consecutive step model (Table 4.10). An exception is made to the obtained parameters at 30 °C. Myrosinase degradation only occurred after an activation period (Figure 4.9) of 5 minutes, and neither the first order or the consecutive step model take into account mechanisms of enzyme activation – inactivation. The variation in myrosinase activity measured after 5 minutes, at 30 °C might be a reason to explain the poor relationship between experimental data and the values estimated by the model.
The first order model seems to fit better the experimental data obtained from the 82.5 % moisture content samples, than the consecutive step model comparing the AIC values obtained at each temperature, for the two models.

It could be hypothesized that the initial absolute enzyme activity has little or no influence on the degradation profile of myrosinase, since the two highest moisture content samples (82.5 and 62.1%) show different initial absolute myrosinase activity at each temperature (Appendix III, Table III.1 and III.2), but the estimated parameters are similar, for each tested temperature.

Table 4.9 - Estimated kinetic parameters (± std. deviation) for thermal inactivation of broccoli myrosinase, in freeze dried broccoli (82.5% moisture) based on the 1st order model.

T (ºC)	k_d (min^{-1})	Initial Activity (U/g DW)	R^2	AIC
30	1.37 ± 0.54 ($\times10^{-2}$)	2.36 ± 0.30 ($\times10^{-1}$)	0.471	-4.91
33.5	5.58 ± 0.81 ($\times10^{-2}$)	1.37 ± 0.07 ($\times10^{-1}$)	0.953	-8.11
37.5	8.20 ± 1.75 ($\times10^{-2}$)	1.66 ± 0.14 ($\times10^{-1}$)	0.891	-6.96
40	8.95 ± 2.38 ($\times10^{-2}$)	1.31 ± 0.14 ($\times10^{-1}$)	0.833	-6.94
45	3.57 ± 0.43 ($\times10^{-1}$)	1.09 ± 0.05 ($\times10^{-1}$)	0.974	-9.19

Table 4.10 - Estimated kinetic parameters (± std. deviation) for thermal inactivation of broccoli myrosinase, in freeze dried broccoli (82.5% moisture) based on the consecutive step model.

T (ºC)	k_{d1} (min^{-1})	Initial Activity (U/g DW)	k_{d2} (min^{-1})	A_2 [a](%)	R^2	AIC
30	9.36 ± 6.21($\times10^{-3}$)	2.16 ± 0.37 ($\times10^{-1}$)	NE	NE	0.337	-4.22
33.5	6.95 ± 1.63 ($\times10^{-2}$)	1.39 ± 0.07 ($\times10^{-1}$)	4.90 ± NE ($\times10^{-4}$)	6.85 ± 5.58 ($\times10^{-2}$)	0.953	-7.65
37.5	4.52 ± 1.35 ($\times10^{-2}$)	1.75 ± 0.12 ($\times10^{-1}$)	NE	NE	0.926	-6.89
40	1.04 ± 0.42 ($\times10^{-2}$)	1.32 ± 0.14 ($\times10^{-1}$)	NE[b]	NE	0.806	-6.33
45	8.21± 5.75 ($\times10^{-1}$)	1.10 ± 0.03 ($\times10^{-1}$)	9.94 ± 6.32 ($\times10^{-2}$)	2.95 ± 1.95 ($\times10^{-1}$)	0.990	-8.99

[a] given in percent of overall initial activity. [b] NE, non estimated.

A plot of ln k_d versus 1/T (K), using the estimated parameters from the individual data fitting with the first order model, was made in order check if the temperature dependence of the inactivation rate constant (k_d (min^{-1})) follows linearly the Arrhenius equation. A good linearization was obtained for all the different moisture content samples, confirmed by the obtained correlation coefficients (R^2) which varied between 0.910-0.957. From the obtained equations of linear regression activation energies (E_a) were calculated for the different moisture content samples, as shown in Table 4.11.

Table 4.11 - Activation energy of inactivation obtained from Arrhenius plot (ln k_d vs 1/T (K)), for the different moisture content freeze dried broccoli.

Moisture content (%)	82.5±0.22	62.1±0.16	26.1±0.22	13.4±0.05
E_a (kJ/mol)	155.2	155.9	124.3	87.3

Globally, the obtained results show a similar myrosinase thermo stability for the higher moisture content samples (82.5 and 62.1 %). Broccoli myrosinase in these samples is more thermolabile in comparison to the crude extract myrosinase from broccoli (Ludikhuyze *et al.*, 1999), to broccoli juice myrosinase (van Eylen *et al*., 2007) and to myrosinase from broccoli intact tissues (van Eylen *et al*., 2008). The results from literature should not be compared making generalizations, due to the fact that different food matrix were used for each test. However, the higher moisture content samples have water activities close to those obtained for fresh broccoli, and therefore an approximated comparison is possible for these two types of samples.

A substantial increase of thermal stability was noticed when the moisture content was reduced to 26.1 %, and more markedly to 13.4%. The inactivation rate constant at 80 °C, for the 13.4 % moisture samples (3.55 ± 0.52 ($\times10^{-2}$)min^{-1}) was approximately the same as the inactivation rate constant, at 30 °C, for the highest moisture content (82.5) samples (1.37 ± 0.54 ($\times10^{-2}$) min^{-1}), which shows the enormous increase of myrosinase thermal stability in the driest samples. These finding are graphically described in figure 4.13.

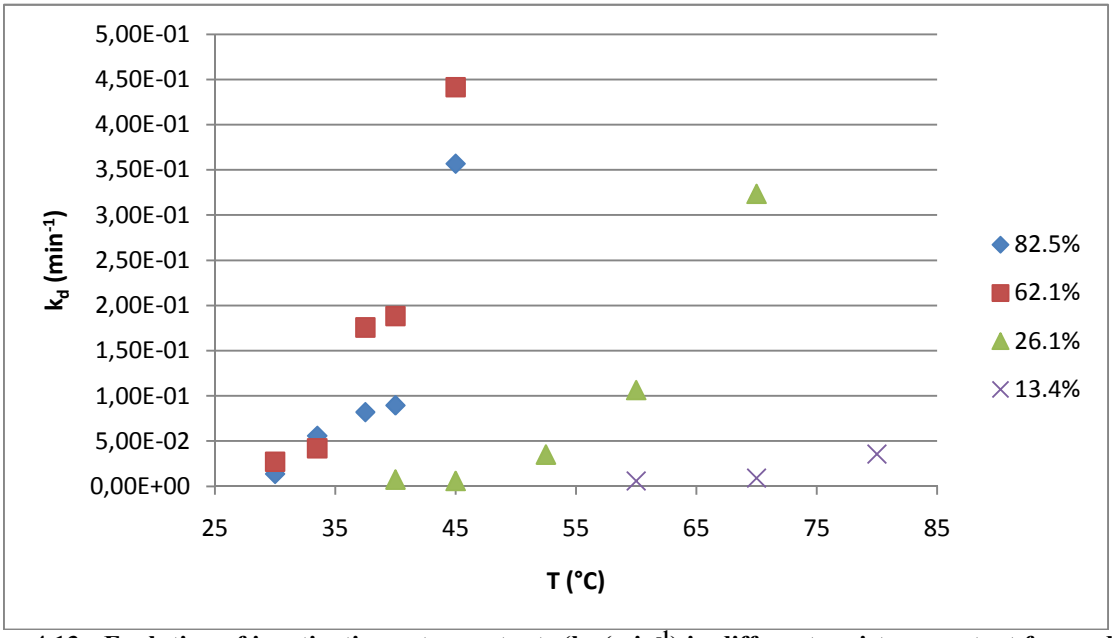


Figure 4.13 - Evolution of inactivation rate constants (k_d (min^{-1})) in different moisture content freeze dried broccoli in relation with temperature.

The temperature stability of myrosinase in the driest samples found in this study was slightly higher, but still similar to the stability of myrosinase from Brassica plants, like *S. alba* L. seeds (van Eylen *et al.*, 2006), red cabbage (Yen & Wei, 1993) and Brussels sprouts (Springett & Adams, 1989).

Despite the statistical and kinetic parameters obtained were not really accurate, modeling individually all temperatures for the different samples, a global fitting of the first order kinetic model was conducted, since a much lower accuracy was found using the consecutive step model, due to no estimation of some of the parameters and high standard deviations obtained.

4.5 Global fitting 1st order kinetic model

4.5.1 Fitting the model

An interesting approach, in the pursuit for suitable models is available in global fitting models to experimental data. This can be done when measurements are made at various temperatures, and it assumes that the temperature effect on enzyme inactivation can be described by Arrhenius' equation. Usually individual fitting gives better results than when overall data is fitted at once. However, global fitting is more correct to describe the overall behavior of an enzyme, in a range of temperatures, due to the higher number of existing interactions, and that is one of the reasons why individual modeling, generally, fits better the experimental data.

All the different moisture content samples were globally modeled using the first order kinetic model, and kinetic parameters were estimated
The global fitting of the model to the experimental data obtained from the 82.5 % moisture content samples has shown to be a reasonably good approach, when the global fitting was only made with four (Figure 4.14) of the five tested temperatures. The statistical parameters given in this case, R^2 = 0,913 and AIC = -7.89, in Table 4.12, explain in a bigger extent the variation of experimental data, than when 5 temperatures (Figure 4.15) were used in the global fitting, R^2 = 0.788 and AIC = -6.38. A worse fitting was due to variation in measurements at 30 ºC, after 5 minutes, probably due to an activation-inactivation process, wherein the myrosinase relative activity obtained was higher than 100%. In this way, and since the model do not describe these kind of processes, a good approach was not possible, confirmed by the low R^2, at 30 ºC (Tables 4.9 and 4.10).

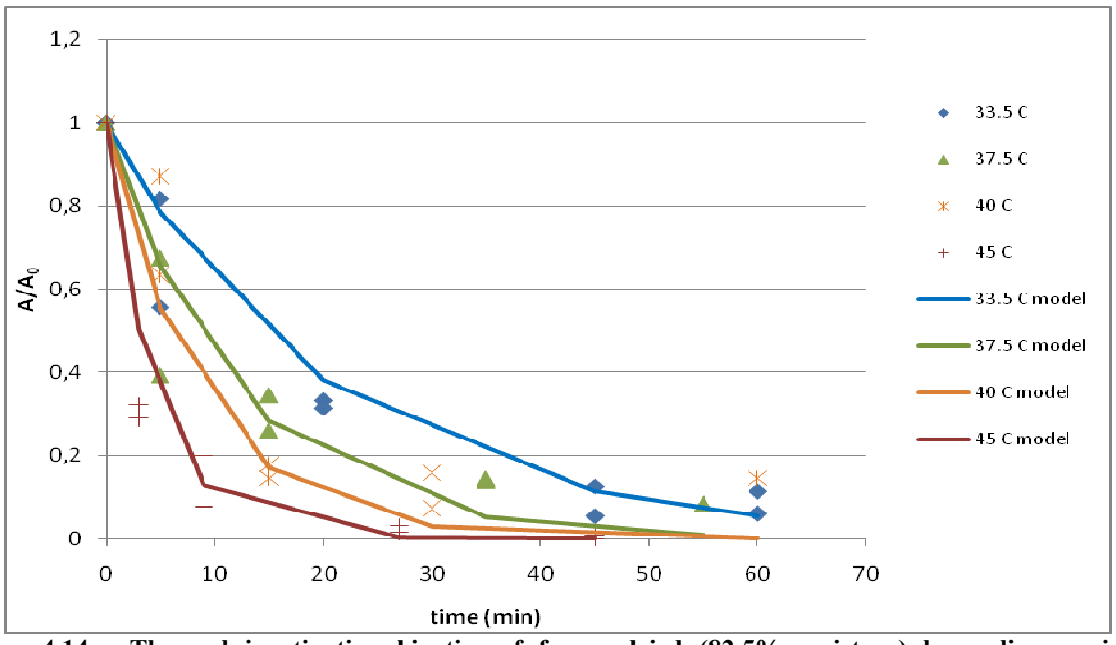


Figure 4.14 - Thermal inactivation kinetics of freeze dried (82.5% moisture) broccoli myrosinase described by a first order model, using 4 temperatures

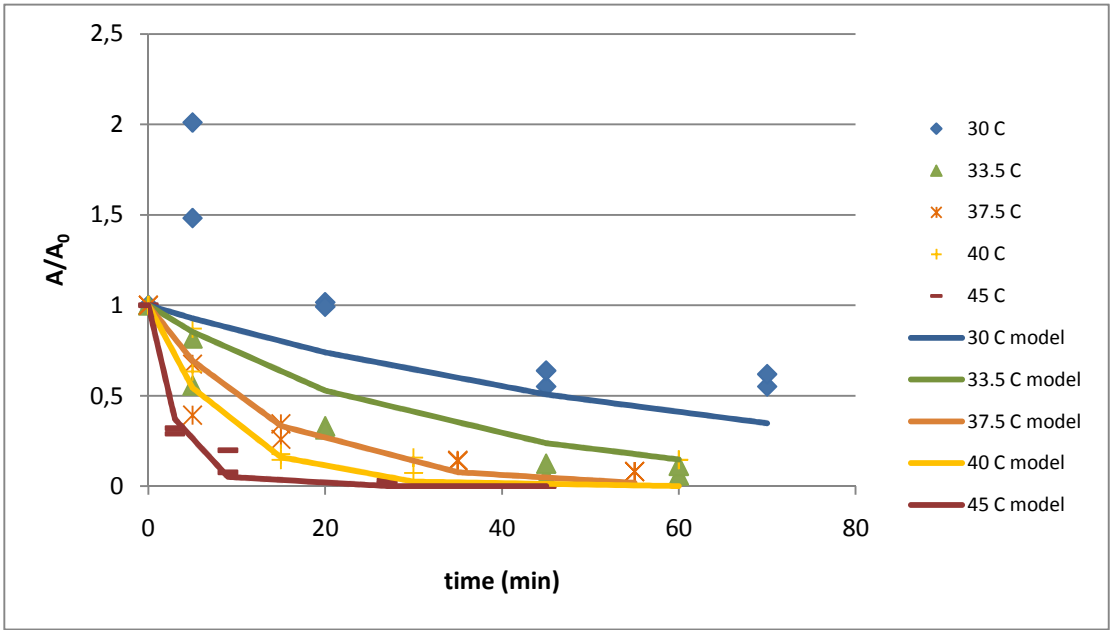


Figure 4.15 - Thermal inactivation kinetics of freeze dried (82.5% moisture) broccoli myrosinase described by a first order model, using 5 temperatures.

From Figure 4.16, is possible to observe that a good, although not perfect fitting was achievable applying the first order kinetic model to the experimental data available from the 62.1 % moisture content samples. The bigger maladjustments of the model to the data, seem to be caused by variation of measured enzymatic activity, at 30 and 40 ºC. In general, the first order model showed to overestimate the myrosinase activity in the early minutes of heating, and underestimate, at longer heating time. However, the R^2 (0.856) value obtained is relatively high.

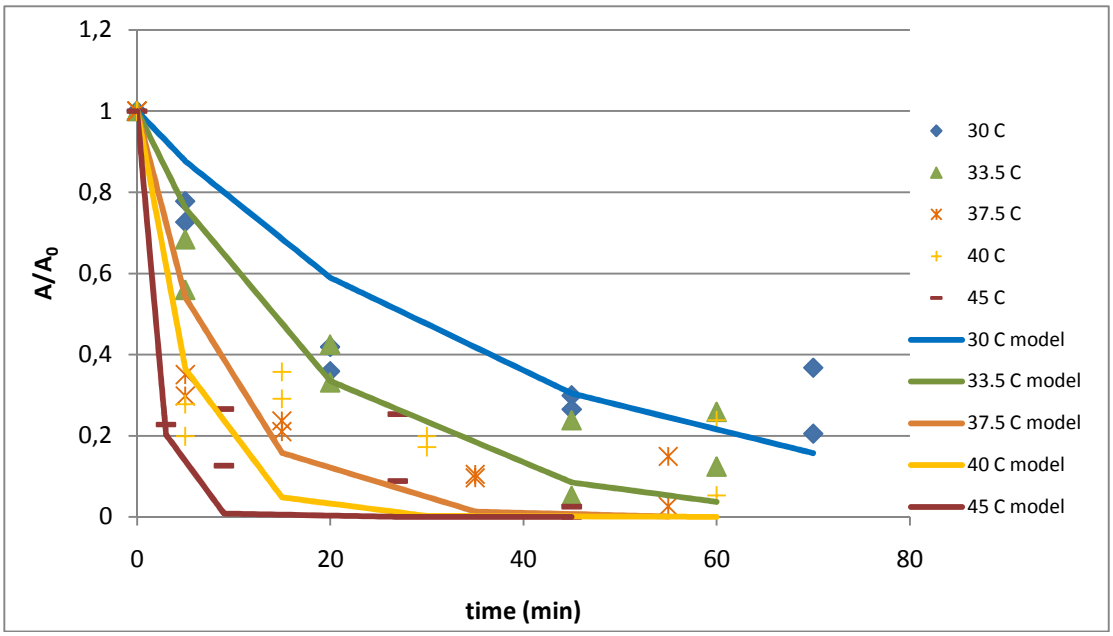


Figure 4.16 - Thermal inactivation kinetics of freeze dried (62.1% moisture) broccoli myrosinase described by a first order model.

The global fitting of measured myrosinase activities for the 26.1% mositure content samples has shown to fairly well fit the experimental data. The plot of experimental data and model estimation is shown in Figure 4.17. Some variations in measurements were obtained specially at 40, 45 and 52.5 ºC. At 52.5 ºC, the measured myrosinase activity after 120 minutes was the focal cause of variation in the model, for the reason that a higher myrosinase activity was obtained in comparison to previous point of measurement (60 minutes). Enzymatic regeneration seems to be the main cause for the increase of enzymatic activity, as little variation between the two measured enzyme activities was obtained. Additionally, it appears that an activation-inactivation of the enzyme occurred during the experiments, at 45 ºC, between 30 and 90 minutes of heating.

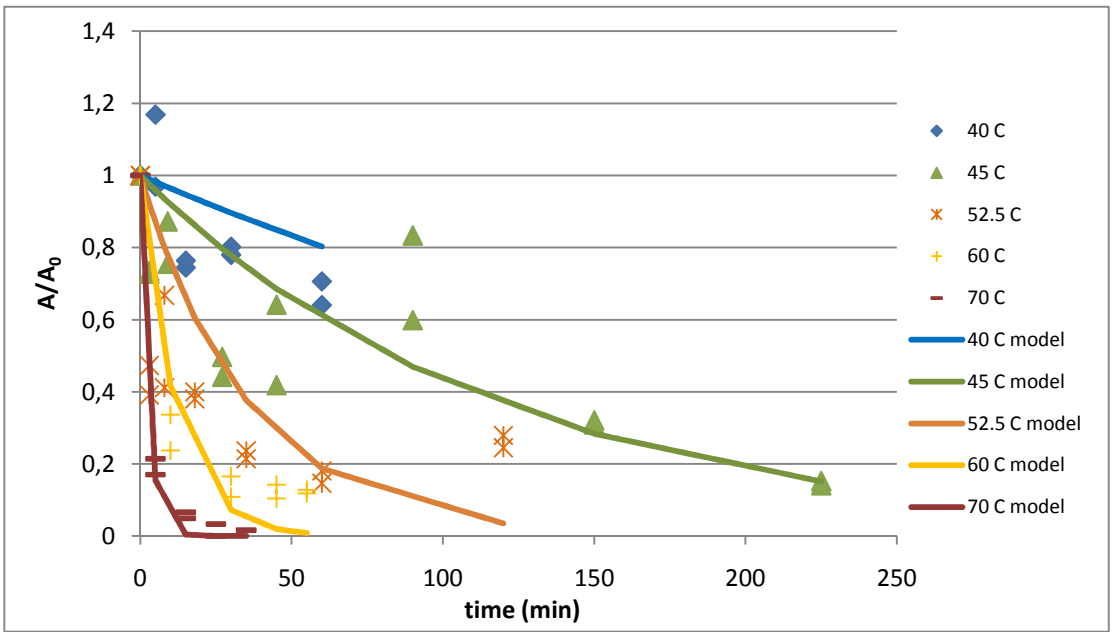


Figure 4.17 - Thermal inactivation kinetics of freeze dried (26.1% moisture) broccoli myrosinase described by a first order model.

The driest moisture content samples data (13.4%) amongst all the modeled data were the ones that more poorly were fitted by the first order global model. Only data from three temperatures was possible to use for modeling purposes, as myrosinase remained stable at temperatures lower than 60 ºC, for the heating times tested. The visual fit of the model is shown in Figure 4.18.

The experimental data, at 80 ºC was well fitted by the model (R^2 = 0.936), unlike to what occured at 70 and 60 ºC, as confirmed from the outcome statistical parameters from table 4.3, R^2 = 0.660 and 0.724, respectively for 70 and 60 ºC.

A sharp decline in myrosinase activity followed by a quick increase on enzymatic activity, between 0 and 30 minutes, for both temperatures (60 and 70 ºC) was responsible for the poor individual fitting of first order and consecutive step models. Due to this, the global fitting using the first order model was negatively affected, resulting in a penalty in estimated statistical parameters values. A global fitting R^2 = 0.799 was obtained, as shown in Table 4.12 (section 4.5.2).

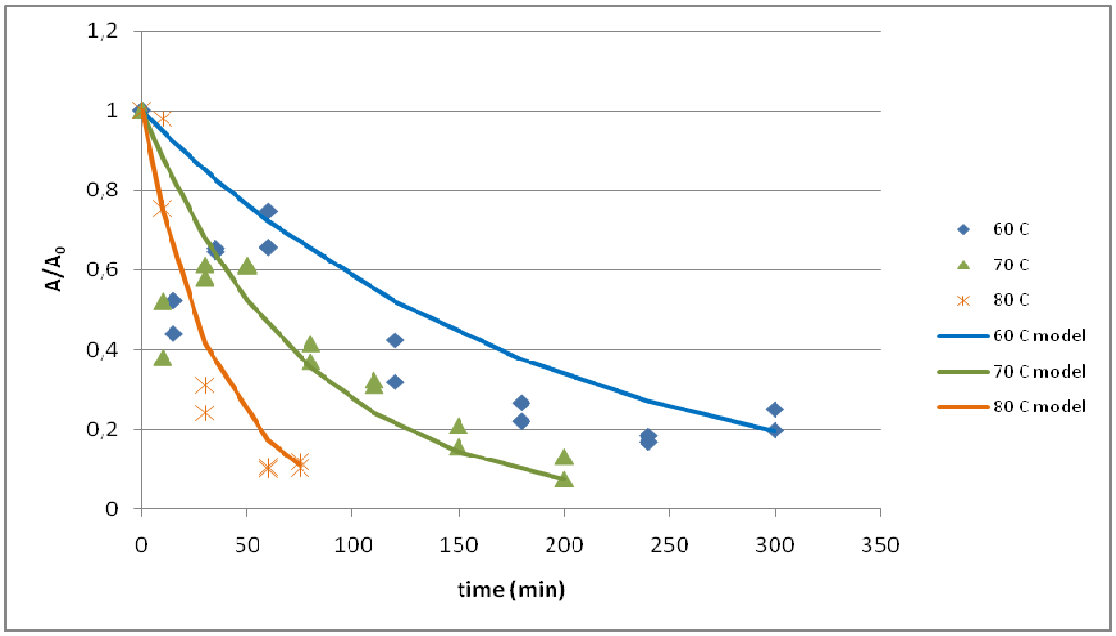


Figure 4.18 - Thermal inactivation kinetics of freeze dried (13.4% moisture) broccoli myrosinase described by a first order model.

Further work at longer heating times or higher temperatures at which visible degradation is shown are needed to the quest for a more suitable model. Two possible types of mechanism could have led to the imperfect fit of first order model, a parallel-type which involves the concurrent inactivation of isoenzymes with different heat stabilities and a series-type involving the conversion of the native enzyme to intermediate forms with lower specific activities and enhanced heat stabilities. With the data available from this study, the choice of heating times and temperatures should take into account the degradation profiles obtained for the different moisture content/water activity samples in this project.

4.5.2 Estimated kinetic parameters

Although, the model does not fit perfectly the experimental data, the kinetic parameters, inactivation rate constant - k_d (min^{-1}) and activation energy of inactivation - E_a (kJ/mol) were estimated, setting 50 ºC as the reference temperature.
The estimated parameters were calculated to give an approximation of the real behavior of myrosinase in freeze dried broccoli with different moisture contents. Fitting separately all data of each different moisture content samples to the model resulted in inactivation rate constant - k_d (min^{-1}) and activation energy - E_a (kJ/mol) shown in Table 4.12:

Table 4.12 - Estimated kinetic parameters for thermal inactivation of different moisture content/ water activity freeze dried broccoli myrosinase based on the global first order model, and respective statistical data (T_{ref} = 50ºC).

% moisture	82.5 % - 5 T (ºC)	82.5 % - 4 T (ºC)	62.1 - 5 T (ºC)	26.1 - 5 T (ºC)	13.4 % - 3 T (ºC)
a_w	0.973	-	0.947	0.719	0.115
k_d (min^{-1})	8.67±3.92 ($\times10^{-1}$)	4.34±1.57 ($\times10^{-1}$)	1.36± 0.51($\times10^{0}$)	1,9±0.29 ($\times10^{-2}$)	2.17±0.67 ($\times10^{-3}$)
E_a (kJ/mol)	165±22	110±24	160±20	138±18	82±16
R^2	0.788	0.913	0.856	0.863	0.799
AIC	-6.38	-7.89	-8.63	-6.39	-4.74

The inactivation rate constants (k_{d50}) for the highest moisture content samples were the highest amongst the different types of samples. The inactivation rate constant for the 62.1% moisture samples was slightly higher than for 82.5 % moisture samples, however due to the standard deviations of the inactivation rate, this difference is not sufficient to conclude that the enzyme inactivation profile is different between these two types of samples (Figure 4.19).
In previous studies about thermal stability of myrosinase in broccoli using the consecutive step model, inactivation rate constants (50 ºC), $k_1 = (9.3 \pm 0.1)\times10^{-1}$ and $k_2 = (1.5 \pm 0.2)\times10^{-2}$ min^{-1}, for crude extract enzyme (Ludikhuyze *et al.*, 1999) and $k_1 = (6.42 \pm 4.90)\times10^{-2}$ and $k_2 = (1.10 \pm 0.81)\times10^{-2}$ min^{-1} in broccoli intact tissue (van Eylen *et al.*, 2008) were obtained. In broccoli juice, using 52.5 ºC as reference temperature, the inactivation rate constants were $k_1 = (4.35 \pm 0.77)\times10^{-1}$ and $k_2 = (5.58 \pm 0.91)\times10^{-2}$ min^{-1} (van Eylen *et al.*, 2007).
The myrosinase thermal stability of the higher moisture content samples (82.5%) seems to be similar to the thermo stability in crude extract (Ludikhuyze *et al.*, 1999), and lower than the verified in broccoli juice and in intact tissues (van Eylen *et al.*, 2007, 2008).
The myrosinase thermal stability in the 26.1 % moisture samples is likely to be higher than the showed in the aforementioned studies, resulting from lower moisture content and water activity.
A remarkable decrease in inactivation rate constant in 13.4 % moisture samples was observed. The inactivation rate constant in these samples was around 1000 and 10 times lower than for the two highest moisture content samples (82.5 and 62.1%) and 26.1%, respectively.
The increase of myrosinase thermal stability might be related to the macromolecular crowding or volume exclusion theory. Volume exclusion theory predicts that at equilibrium, the presence of high concentrations of stable macromolecular cosolutes will stabilize a labile protein against denaturation by heat, cold, or denaturant (Minton, 2005) This means that due to the finite size of molecules, les volume is available for other molecules. Applied to myrosinase case, with much less water available as solvent, becomes energetically unfavorable for the enzyme to unfold, increasing denaturation temperature, thus increasing the stability.

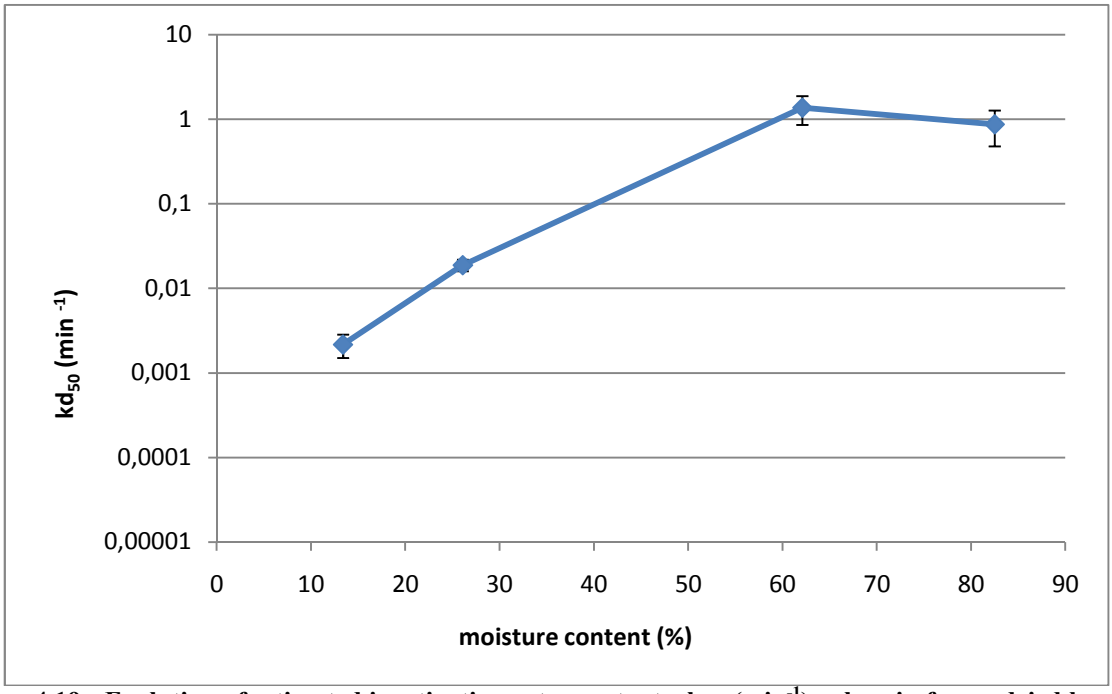


Figure 4.19 - Evolution of estimated inactivation rate constants k_{d50} (min^{-1}) values in freeze dried broccoli in relation to moisture content (logarithmic scale).

Activation energy gives a measure of how an inactivation rate constant increases when temperature also increases. The activation energies of inactivation for the different moisture content samples, obtained from global fitting are shown in Table 4.12, and the evolution visually described in Figure 4.20.

Comparing the values obtained from global fiiting to those obtained by the Arrhenius equation, shown in Table 4.11 (section 4.4) no significant difference was found between the values obtained for each different moisture content. From individual modeled data, activation energies of 155.2, 155.9, 124.3 and 87.3 kJ/mol were obtained, respectively for 82.5, 62.1, 26.1 and 13.4% moisture content samples, as shown in Table 4.11. Since the obtained values are quite similar to those obatined with the global fitting (Table 4.12), the trend of the evolution of the activation energies in relation to the moisture content of the different samples is the same for both situation. These findings confirm that the temperature dependence of the inactivation rate constant (k_d) follows the Arrhenius equation.

Higher activation energy were obtained for the 82.5 and 62.1% moisture content samples, 165±22 and 160±20 kJ/mol, showing that the myrosinase stability to heat is similar for these two types of samples. The 26.1% moisture samples showed an activation energy of 138± 18 kJ/mol, while the lowest activation energy was obtained for the driest sample, 82±16 kJ/mol.
The results from the two higher moisture content samples are similar to the activation energies reported for broccoli juice (van Eylen *et al.*, 2007), although a consecutive step model was used in that study, thus two different activation energies were obtained, E_{a1} = 185.4 kJ/mol and E_{a2} = 161.8 kJ/mol. The inactivation kinetics of myrosinase in red cabbage juice, was reported by Dekker *et al.*, (2004). The red cabbage myrosinase inactivation studied at temperatures between 25 and 70 °C is described by a first-order model, and the estimated *E*a value was 155 kJ/mol (comparable to the highest moisture content samples in this study). In intact broccoli tissue, higher E_a were estimated, around 200 kJ/mol (van Eylen *et al.*, 2008). The lower E_a values from the driest samples mean that besides showing higher myrosinase thermal stability, driest samples show a lower increase in degradation rate when temperature

increases, thus the difference in inactivation rate constants between dry and high moisture content samples is enlarged by thermal increase.

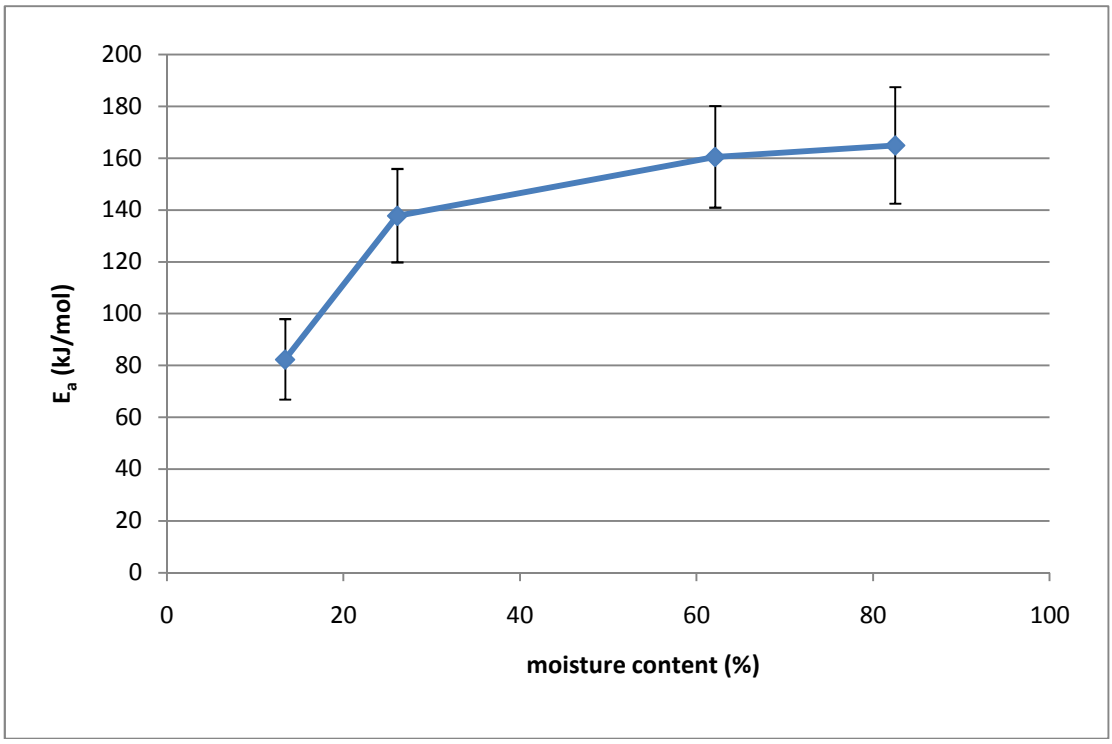


Figure 4.20 - Evolution of estimated activation energy of inactivation (E_a (kJ/mol)) in freeze dried broccoli in relation to moisture content.

5.Conclusions

Myrosinase activity was found in all the different moisture content (water activity) freeze dried powder broccoli samples, obtained from the edible parts of broccoli (stems and florets).

The glucose background of the batch of broccoli seems to have little or no influence in the myrosinase activity measurement.
A 90% concentration reduction of sinigrin solution used for myrosinase activity measurement was possible, since no difference on measured myrosinase concentrations was found for a 30 mg/ml and 300 mg/ml sinigrin solution, which represented a saving in economical costs.

There are substantial differences in initial myrosinase activity (no heating treatment), from different moisture content samples. The driest ones (13.4 and 26.1 % moisture) and the most moisted (82.5%) showed higher enzymatic activity than the samples in the range of 60 – 70% moisture content. Along the heating experiments, myrosinase activity values behaved in accordance with the trend described before for initial activity. The experimental data showed that myrosinase degradation starts at 30 ºC, for the 82.5 and 62.1 % moisture samples, at 45 ºC for the 26.1 % moisture samples, and at 60 ºC for the driest samples (13.4%), for heating times tested.

The influence of the differences in moisture content/water activity in myrosinase degradation increases with temperature increase, as well as the influence of heating times ($\alpha = 0.05$). Consequently, the combined influence of moisture content in relation to cooking times increases with the escalation of temperatures ($\alpha = 0.05$).
Globally, individual modeling of the experimental data obtained at different temperatures, for the different moisture content samples shows that the first order kinetic model fits better the data, than the consecutive step model. Some of the samples show to be better explained by the consecutive step model, however no estimation of parameters or high standard deviation was obtained for the majority of the samples using this model. Thus, a global fitting of the first order kinetic model to the data was conducted. In general, the global first order model fits reasonably well to the experimental data, nevertheless possible enzymatic regeneration and activation-inactivation processes should be taken into account for the modeling approach, according to the results obtained, at 45 and 52.5 ºC, for the 13.4 and 26.1 % moisture samples. Also, in the 82.5 % moisture samples, at 30 ºC, an activation-inactivation process seems to occur. The inactivation rate constants, at 50 ºC (k_d) and activation energy (E_a) for all the different moisture content samples have been determined to:

$k_{d,\,50\,°C,\,82.5\%} = 8{,}67 \pm 3{,}92\ (\times 10^{-1})\ min^{-1}$; $E_{a,\,82.5\%} = 165 \pm 22$ kJ/mol

$k_{d,\,50\,°C,\,62.1\%} = 1.36 \pm 0.51\ (\times 10^{0})\ min^{-1}$; $E_{a,\,62.1\%} = 160 \pm 20$ kJ/mol

$k_{d,\,50\,°C,\,26.1\%} = 1{,}90 \pm 0.29\ (\times 10^{-2})\ min^{-1}$; $E_{a,\,26.1\%} = 138 \pm 18$ kJ/mol

$k_{d,\,50\,°C,\,13.4\%} = 2.17 \pm 0.67\ (\times 10^{-3})\ min^{-1}$; $E_{a,\,13.4\%} = 82 \pm 16$ kJ/mol

The inactivation rate constant of the driest samples is around 1000 and 10 times lower than for the two higher moisture content samples (82.5 and 62.1%) and 26.1%, respectively.
The lower activation energy values from the driest samples, specially 13.4% moisture samples, means that besides showing higher myrosinase thermal stability, driest samples have a lower increase of rate degradation when temperature increases, and therefore the difference

of myrosinase stability at higher temperatures should will be even bigger, between lower and higher moisture content samples.
The imperfect fitting of the models can be due to variation in measurements, enzymatic regeneration, and possible inaccurate measurements of samples with low activity. The statistical parameters (R^2 and AIC) obtained and the existence of non-randomly distributed residuals for some samples, by global fitting, show that a first order model is not the most accurate model, and therefore the quest for a better model should proceed.

6. Recommendations and Furthermore Investigation

Further research on the influence of moisture content/water activity and temperature on the thermal stability of myrosinase should be conducted.
Since the temperature of the freeze-drying process was gradually increased from -15 ºC until 5 ºC, at the end of the process, the different samples were subjected to different temperatures, however, low ones during the process. This means that the higher moisture content samples were subjected to lower temperatures than the samples with lowest moisture content. For this reason the temperature of the freeze dryer could be maintained the same for the whole step, even no or little impact in myrosinase activity is expected, in order to eliminate a possible source of variation, even if is just hypothetical.
The sample grinding step should be improved, as well as the sample rehydration after heating. It seems that these two steps could have a significant influence on myrosinase extraction and variation in measurements, respectively. The broccoli powder should be more texturally homogeneous, as the difference of superficial area could affect the extractability, and thus the measured enzyme activity. The vortexing of samples after water addition is made manually, hence it might be a source of variation. Eventually, a standardised procedure with set times could be tested.
More research could be conducted about the time gap between heating experiments and enzymatic activity measurements. Conducting the heating procedure in a reverse order could be a solution to avoid enzymatic regeneration or at least give the same repose time to all the samples. In other words, all samples are taken at the same time from the heating block, instead of being placed at the same time. This might have an impact on myrosinase activity measurement variation. Additional experiments could be executed on high speed centrifugation, in an effort to reduce the procedure time.
The resuspension of the pellet in buffer may be reviewed, as it is done manually, and thus it can be a source of variation. Possibly, resuspend the pellet with an Ultra Turrax at very low speed might be a solution.

The UV measurements should be conducted in a stabilized temperature room, even if all the solutions are kept on ice until mixture solution preparation. During the measurement, bigger or lower temperature gradient can induce variation. Might be interesting to create the possibility of measuring more than one sample at the same time. Additionally, trials on the enhancement of the sensitivity of the method should be performed, especially in the case of low myrosinase concentration samples. Improvement could be done by using an higher amount of sample for the experiments.

Furthermore research has to be conducted for the heating temperatures and times to describe to better describe the behavior and thermal stability of myrosinase in freeze dried broccoli. Some of the heating times should be extended, while others shortened, depending on the moisture content and water activity of the samples, as well as some of the temperatures. Samples with lower water activity than the lowest one used in this study could be obtained and the myrosinase activity tested, as well as samples with intermediate water activities, between those used in the present project. The experimental data available from this study might be useful for that purpose. Doing so, might provide an extended comprehension of the mechanism of myrosinase denaturation, and a better modeling approach could be set, using the same or different models than those used in this project. A fractional conversion model might be an option for modeling, or models that can predict possible occurence of regeneration and activation-inactivation phenomenon.

Afterwards of an excellent understanding about the influence of moisture content/ water activity and temperature on broccoli myrosinase stability, further research could be conducted in previously high-pressure treated or blanched broccoli and other *Brassica* vegetables, towards a more realistic understanding about the whole impact of the food processing steps in myrosinase stability and degradation.

7. Literature (References)

Andreasson, E.; Jørgensen, L.B.; Höglund, A.S. (2001). Different myrosinase and idioblast distribution in *Arabidopsis* and *Brassica napus*. *Plant Physiol*ogy 127: 1750–1763.

Bellostas, N.; Petersen, I.L.; Sorensen, J.C. (2008). A fast and gentle method for the isolation of myrosinase complexes from Brassicaceous seeds. *Journal of Biochemical and Biophysical Methods* 70: 918–925.

Björkman, R. & Janson, J. C. 1972. Studies on myrosinases. I. Purification and characterization of a myrosinase from white mustard seed (Sinapis alba L.). *Biochemistry and Biophysics Acta* 276: 508–518.

Bones, A. M. & Slupphaug, G. (1989). Purification, characterization and partial amino acid sequencing of β-thioglucosidase from Brassica napus L. *Journal of Plant Physiol*ogy 134: 722–729.

Bones, A.M.,; Thangstad, O.P.; Haugen, O.A. (1991). Fate of myrosin cells: characterization of monoclonal antibodies against myrosinase. Journal of Experimental Botany 42: 1541–1550.

Bones, A. M.; Rossiter, J. T. (1996). The Myrosinase-glucosinolate System, its Organization and Biochemistry. *Physiologia Plantarum* 97: 194-208.

Bridges, M.; Jones, A.M.E.; Bones, A.M. (2002). Spatial organization of the glucosinolate-myrosinase system in brassica specialist aphids is similar to that of the host plant. *Proceedings of the Royal Society: Biological Sciences* 269:187–191.

Charron, C. S.; Saxton, A. M.; Sams, C. E. (2005) Relationship of climate and genotype to seasonal variation in the glucosinolate-myrosinase system. II. Myrosinase activity in ten cultivars of Brassica oleracea grown in fall and spring seasons. *Journal of the Science of Food and Agriculture* 85: 682-690.

Conaway, C. C.; Getahun, M. S.; Liebes, L. L.; Pusateri, D. J.; Topham, D. K. W.; Botero-Omary, M.; Chung, F-L. (2000). Disposition of Glucosinolates and Sulforphane in Humans After Ingestion of Steamed and Fresh Broccoli. *Nutrition and Cancer* 38: 168-178.

Dekker, M.; Verkerk, R.; Jongen, W. M. F. (2000). *Predictive modelling of health aspects in the food production chain: a case study on glucosinolates in cabbage, Trends in Food Science & Technology* 11: 174-181

Dekker, M.; Verkerk, R. (2004). Glucosinolates and Myrosinase Activity in red Cabbage (Brassica oleracea L. var. capitata f. rubra DC.) after Various Microwave Treatments. *Journal of Food and Agricultural Chemistry* 52: 7318-7323.

Fahey, J. W.; Zalcmann, A. T.; Talalay, P. (2001) The chemical diversity and distribution of glucosinolates and isothiocyanates among plants, *Phytochemistry* 56: 5-51.

Fenwick, R.; Griffiths, N. M.; Heaney, R. K. (1983). Bitterness in brussels sprouts (Brassica oleracea L. var. gemmifera): The role of glucosinolates and their breakdown products. *Journal of the Science of Food and Agriculture* 34: 73–80.

Getahun, S.M. Chung, F-L.; (1999). Conversion of Glucosinolates to Isothiocyanates in Humans After Ingestion of Cooked Watercress. *Cancer Epidemiology, Biomarkers & Prevention* 8: 447-45.

Hamauzu, Y.; Zhang, D. (2004). Phenolics, ascorbic acid, carotenoids and antioxidant activity of broccoli and their changes during conventional and microwave cooking. *Food Chemistry* 88: 503-509

Hasapis, X. & MaCleod, A. J. (1982). Benzylglucosinolate degradation in heat-treated *Lepidium .lativum* seeds and detection of a thiocyanate-forming factor. *Phytochemistry* 21: 1009- 1013.

Helmlinger, J.; Rausch, T.; Hilgenberg, W. (1983). Localization of newly synthesized indole-3-methylglucosinolate (=glucobrassicin) in vacuoles from horseradish (*Armoracia rusticana*). *Physiology Plantarum* 58: 302–310.

Hennig, K. (2008). Fate of glucosinolates during mastication – study on broccoli. Agrotechnology and Food Sciences – Product Design and Quality Management Group, WUR.

Höglund, A-S.; Lenman, M.; Falk, A. (1991). Distribution of myrosinase in rapeseed tissues. *Plant Physiology* 95: 213–221.

Huang, C,.; Ma, W.Y.; Li, J.; Hecht, S.S.; Dong, Z. (1998). Essential role of p53 in phenethyl isothiocyanate-induced apoptosis. *Cancer Research* 58: 4102-4106.

Husebye, H.: Chadchawan, S.; Winge, P. (2002). Guard cell and phloem idioblast-specific expression of thioglucoside glucohydrolase 1 (myrosinase) in *Arabidopsis*. *Plant Physiology* 128: 1180–1188.

James, D. & Rossiter, J. T. 1991. Development and characteristics of myrosinase in Brassica napus during early seedling growth. *Physiology Plant*arum 82: 163–170.

Jones, R. B.; Faragher, J. D.; Winkler, S. (2006). A review of the influence of postharvest treatments on quality and glucosinolate content in broccoli (Brassica oleracea var. Italica) heads. *Postharvest Biology and Technology* 41: 1–8.

Kelly, P.J.; Bones, A.; Rossiter, J.T. (1998). Sub-cellular immunolocalization of the glucosinolate sinigrin in seedlings of Brassica juncea. *Plantarum* 206: 370–377.

Kissen, R.; Rossiter, J.; Bones, A.(2009). The 'mustard oil bomb': not so easy to assemble?! Localization, expression and distribution of the components of the myrosinase enzyme system. *Phytochemistry Reviews* 8: 69-86.

Kleinwächter, M.; Selmar, D. (2004). A novel approach for reliable activity determination of ascorbic acid depending myrosinases. *Journal of Biochemical and Biophysical Methods* 59: 253-265.

Koroleva, O.A.; Davies, A.; Deeken, R.; Thorpe, M.R.; Tomos, A.D.; Hedrich, R. (2000). Identification of a new glucosinolate-rich cell type in Arabidopsis flower stalk. *Plant Physiology* 124:599–608.

Ludikhuyze, L.; Oooms, V.; Weemaes, C.; Hendrickx, M. (1999). Kinetic study of the Irreversible Thermal and Pressure Inactivation of Myrosinase from Broccoli (Brassica oleracea L. var. italica). *Journal of Agriculture and Food Chemistry* 47: 1794-1800.

Ludikhuyze, L.; Rodrigo, L.; Hendrickx, M. (2000). The Activity of Myrosinase from Broccoli (Brassica oleracea L. var. italica): Influence of Intrinsic and Extrinsic Factors. *Journal of Food Protection* 63: 400-403.

Lüthy, B. & Matile, Ph. 1984. The mustard oil bomb: Rectified analysis of the subcellular organisation of the myrosinase system. *Biochemistry Physiology Pflanz*. 179: 5–12.

Maheshwari, P.N.; Stanley, D.W.; Beveridge, T.J.; van de Vort, F.R. (1981). Localization of myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) in cotelydon cells of rapeseed. *Journal of Food Biochemistry* 5: 39-61

Matile. Ph. 1980. "Die Senfölbombe": Zur Kompartimentierungdes Myrosinase systems. *Biochemistry Physiology Pflanz*. 175: 722–731

Matusheski, N.V.; Swarup, R.; Juvik, J.A. (2006). Epithiospecifier protein from broccoli (*Brassica oleracea* L. ssp italica) inhibits formation of the anticancer agent sulforaphane. *Journal of Agriculture and Food Chemistry* 54: 2069–2076

Milner, J. A.; (1999). Functional Foods and Health Promotion. *Journal of Nutrition* 129: 1395-1397

Minton, A. P. (2005). Influence of macromolecular crowding upon the stability and state of association of proteins: Predictions and observations. *Journal of Pharmaceutical Sciences* 94: 1668–1675

Mithen, R. F.; Dekker, M.; Verkerk, R.; Rabot, S.; Johnson, I. T (2000). The nutritional significance, biosynthesis and bioavailability of glucosinolates in human foods. *Journal of the Science of Food and Agriculture* 80: 1097-0010

Musk, S.R.; Johnson, I.T. (1993). Allyl isothiocyanate is selectively toxic to transformed cells of the human colorectal tumour line HT29. *Carcinogenesis* 14: 2079-2083.

Musk, S.R.; Stephenson, P.; Smith, T.K.; Stening, P.; Fyfe, D.; Johnson, I.T. (1995). Selective toxicity of compounds naturally present in food toward the transformed phenotype of human colorectal cell line HT29. *Nutrition and Cancer* 24: 289-298.

Oerlemans, K.; Barrett, D. M.; Suades, C. B.; Verkerk, R.; Dekker, M. (2006). Thermal degradation of glucosinolates in red cabbage. *Food Chemistry* 95:19-29

Podsedek, A. (2007). Natural antioxidants and antioxidant capacity of Brassica vegetables: A review. *Food Science and Technology* 40: 1-11

Rask, L.; Andreasson, E.; Ekbom, B. (2000). Myrosinase: gene family evolution and herbivore defense in Brassicaceae. *Plant Molecular Biology* 42: 93–113.

Rest, J. A. & Vaughan, J. G. (1972). The development of protein and oil bodies in the seed *of Sinapis alba* L,. *Plantarum* 105: 245-262.

Rosa, E. A. S.; Heaney, R. K.; Fenwick, G. R.; Portas, C. A. M. (1997). Glucosinolates in crop plants, *Horticultural Reviews* 19: 99– 125.

Rosa, E.; David, M.; Gomes, M. H. (2001). Glucose, fructose and sucrose content in broccoli, white cabbage and Portuguese cabbage grown in early and late seasons. *Journal of the Science of food and Agriculture* 81: 1145-1149.

Rungapamestry, V.; Duncan, A. J.; Fuller, Z.; Ratcliffe, B. (2006). Changes in Glucosinolates concentrations, Myrosinase Activity, and Production of Glucosinolates in Cabbage (Brassica oleracea var. capitata) Cooked for Different Durations. *Journal of Food and Agricultural Chemis*try 54: 7628-7634.

Rungapamestry, V.; Duncan, A.; Fuller, Z.; Ratcliffe, B. (2008).Influence of blanching and freezing broccoli prior to storage and cooking on glucosinolate concentrations and myrosinase activity *European Food Research and Technology* 227: 37-44.

Springett, M.B.; Adams, J.B. (1989). Properties of Brussels sprouts thioglucosidase. *Food Chemistry* 33: 173-186.

Talalay, P.; Fahey, J.W.; Holtzclaw, W.D.; Prestera, T.; Zhang. Y. (1995). Chemoprotection against cancer by phase 2 enzyme induction. *Toxicology Letters* 82: 173-179.

Thangstad, O.P.; Evjen, K.; Bones, A. (1991). Immunogold-EM localization of myrosinase in Brassicaceae. *Protoplasma* 161:85–93.

Tookey, H, L. (1973). *Crambe* thioglucoside glucohydrolase (EC 3,2.3.1): Separation of a protein required for epithiobutane formation. Canadian Journal of Biochemistry, 51: 1654-1660.

van Boekel, M. A. J. S. (2009). Kinetic Modeling Of Reactions In Foods. *CRC Press, Taylor & Francis Group, LLC*; United States of America.

van Poppel, G.A.; Verhoeven, D.T.; Verhagen, H.; Goldbohm, R.A. (1999). Brassica vegetables and cancer prevention – Epidemiology and mechanisms. *Advances in Nutrition and Cancer* 2: 159-167.

van Eylen, D.; Indrawati; Hendrickx, M.; Van Loey, A. (2006). Temperature and pressure stability of mustard seed (*Sinapsis alba* L.) myrosinase. *Food Chemistry* 97; 263-271.

van Eylen, D.; Oey, I.; Hendrickx, M.; Van Loey, A. (2007). Kinetics of the stability of broccoli (Brassica oleracea cv Italica) myrosinase and isothiocyanates in broccoli juice during pressure/temperature treatments. *Journal of Agricultural and Food Chemistry* 55: 2163–2170.

van Eylen, D.; Oey, I.; Hendrickx, M.; van Loey, A. (2008). Effects of pressure/temperature treatments on stability and activity of endogenous broccoli (*Brassica oleracea* L. cv. Italica) myrosinase and on cell permeability. *Journal of Food Engineering* 89: 178-186.

Verhoeven, D.T.; Verhagen, H.; Goldbohm, R.A.; van den Brandt, P.A.; van Poppel, G.A. (1997). Review of mechanisms underlying anticarcinogenicity by brassica vegetables. Chemico-Biological Interactions 103:79-129.

Wattenberg, L.W. (1977). Inhibition of carcinogenic effects of polycyclic hydrocarbons by benzyl isothiocyanate and related compounds. *Journal of the National Cancer Institute 58*: 395-398.

Wattenberg, L.W. (1981). Inhibition of carcinogen-induced neoplasia by sodium cyanate, tert-butylisocyanate and benzyl isothiocyanate administered subsequent to carcinogen exposure. *Cancer Research* 41: 2991-2994.

Wattenberg, L.W. (1989). Inhibition of mammary tumor formation by broccoli and cabbage. *Proceedings of the American Association for Cancer Research* 30: 181-189.

Werker, E.; Vaughan, J.G. (1974). Anatomical and ultrastructural changes in aleurone and myrosin cells of *Sinapis alba* during germination. *Plantarum* 116: 243-255

Werker, E.; Vaughan, J.G. (1976). Ontogeny and distribution of myrosin cells in the shoot of *Sinapis alba* L. A light- and electron microscope study. *Israel Journal of Botany* 25: 140-151

Wilkinson, A. P.; Rhodes, M. J. C; Fenwick, R. G. (1984). Myrosinase Activity of Cruciferous Vegetables. *Journal of the Food Science and Agriculture*, 35: 543-552.

World Cancer Research Fund, Food, Nutrition and the Prevention of Cancer: a Global Perspective, American Institute of Cancer Research, Washington (1997).

Appendices

Appendix I - Preliminary Experiments: Results

The preliminary experiments were conducted in fresh broccoli samples. Fresh broccoli was ground using a Waring Blender (Waring Commercial, Torrington, Connecticut, USA) into powder. These experiments were conducted to check which sample/water ratio was more suitable for myrosinase extraction and posterior measurement. It was concluded that a bigger amount of sample should be added for extraction, in order to increase the amount of myrosinase extracted, as the data provided in Table I.1 shows. A bigger amount of myrosinase extracted is important to avoid a possible lack of method sensitivity when broccoli samples with lower myrosinase concentration are measured.

The extraction buffer used for high speed centrifugation was tested. The results from the use of a phosphate buffer (50 mM, pH 7.0) and a Tris-HCl (25 mM, pH 7.0) were compared and are available in Table I.2. *F samples* myrosinase activity values were obtained using a phosphate buffer, while *Tris samples* used the Tris-HCl buffer. From this table it is easy to conclude that the phosphate buffer was tremendously more efficient than the Tris-HCl bufeer. Therefore, all the myrosinase extraction was conducted with a phosphate buffer, for the high speed centrifugation steps, as described in section 3.4.

Lastly, the myrosinase activity of sample solutions subjected to a short centrifugation (Spectrafuge 16 M, National Labnet Co., USA) at 14000 rpm for 1 minute was measured. In Table I.2, *mix* samples means that those samples were subjected to this treatment. From the results, it was concluded that resuspension of the pellet provided better results than when the short centrifugation was applied. Could be hypothesized that myrosinase was precipitated again in this conditions.

Table I.1 - Myrosinase activity on fresh broccoli related to sinigrin concentration and different water/broccoli ratio.

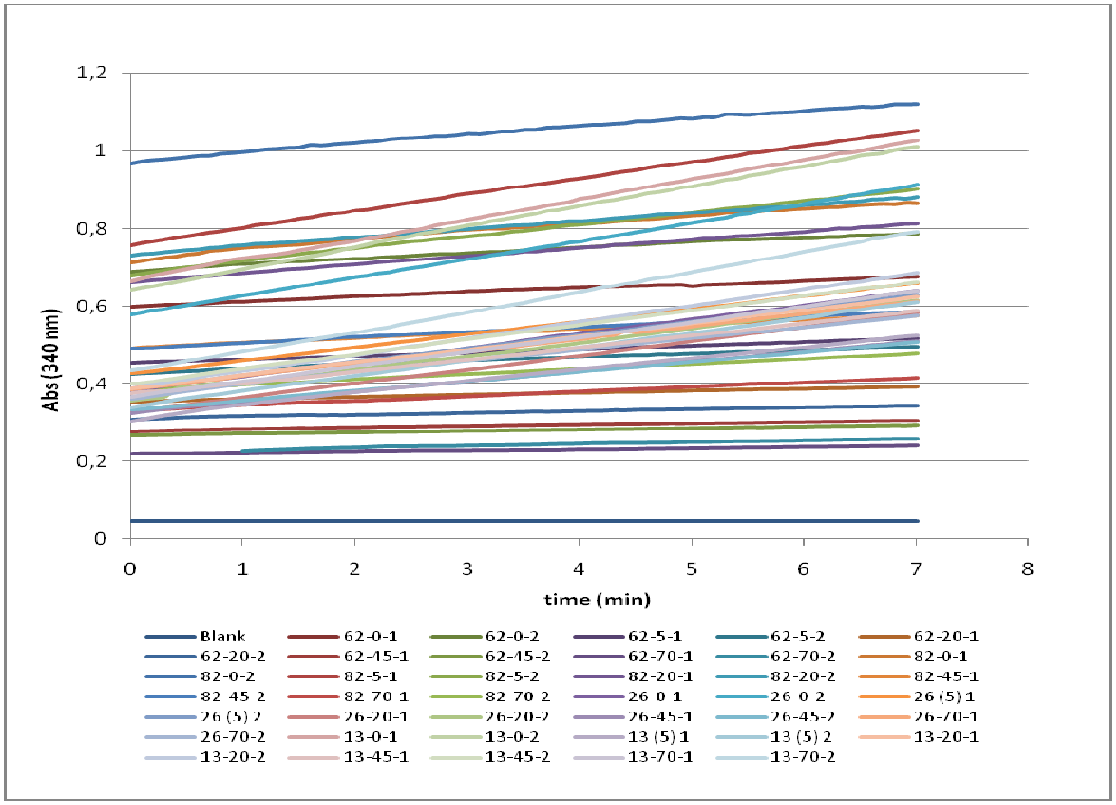
[Sinigrin] mg/ml	300			30			3	
Myrosinase Activity	Blank	F 1:1	F 3:1	Blank	F 1:1	F 3:1	Blank	F 3:1
Slope	0,0001	0,1124	0,1696	0,0004	0,07	0,1176	0,0004	0,0222
U/ml	0	0,7479	1,1451	0	0,6891	1,1603	0	1,0418

Table I.2 - Myrosinase activity ([sinigrin]=30 mg/ml) in relation to the extraction buffer used.

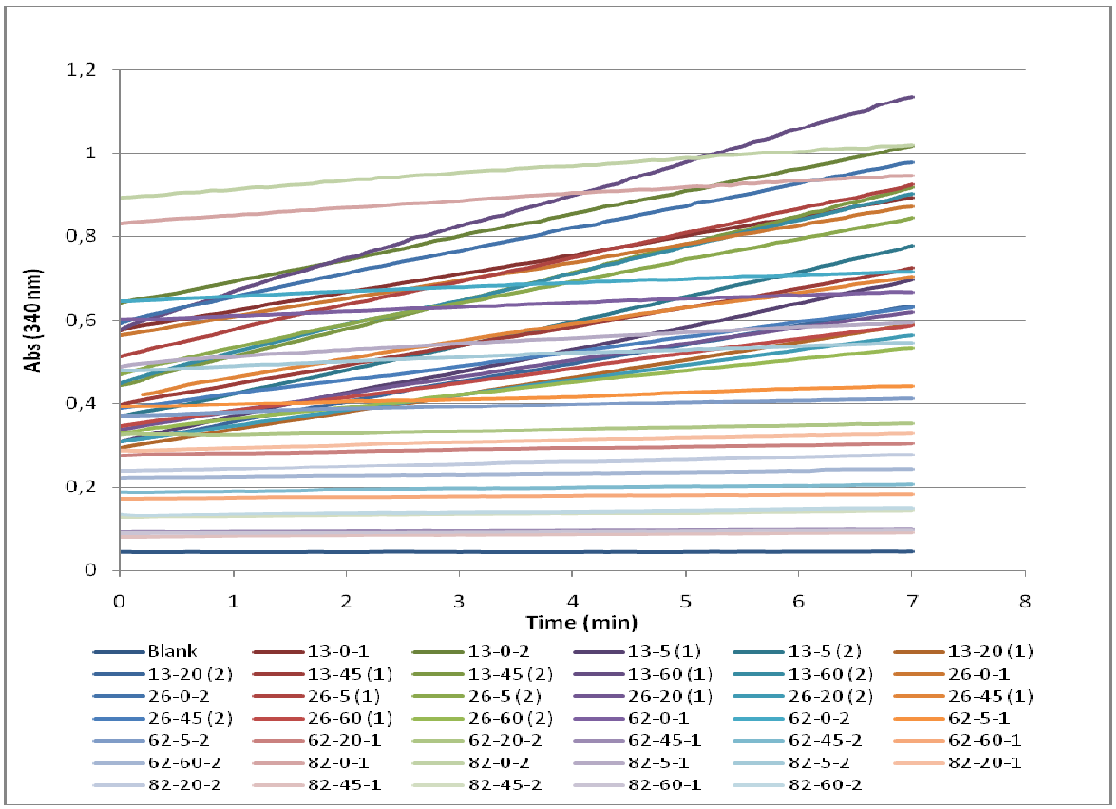
Myrosinase Activity	Blank	F 1:1	F 3:1	F 3:1 mix	Tris 1	Tris 2	Tris 1 mix	Tris 2 mix
Slope	0,0004	0,07	0,1176	0,0152	0,0096	0,0103	0,0119	0,0104
U/ml	0	0,6891	1,1603	0,1465	0,0910	0,0980	0,1138	0,0990

Appendix II - Charts of UV measurements of myrosinase activity for the different moisture content freeze dried broccoli.

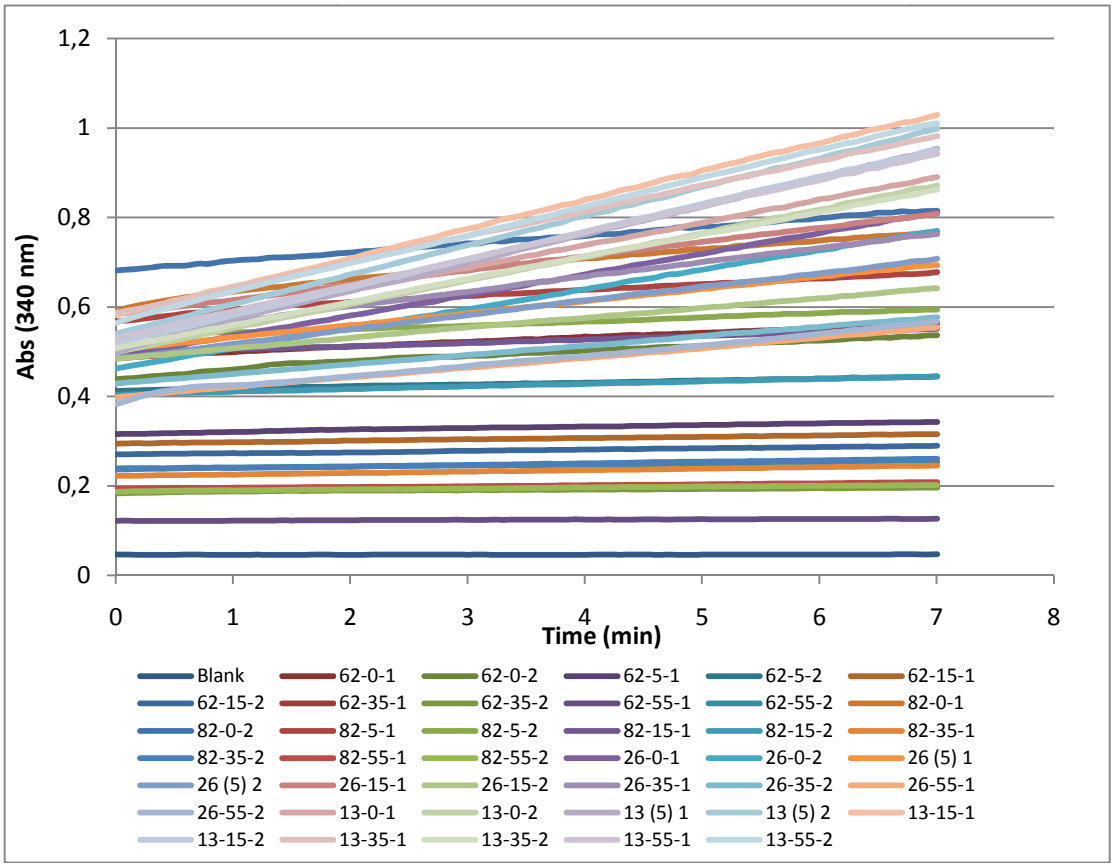
30 ºC



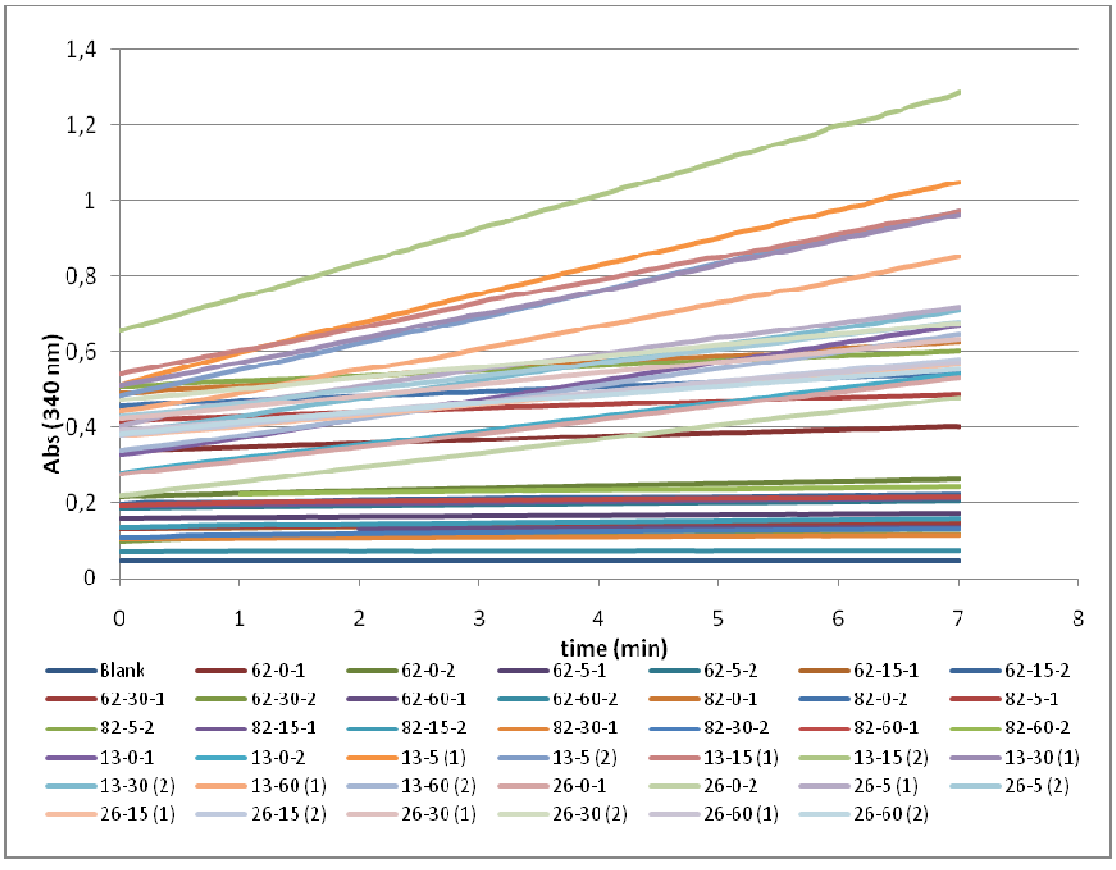
33.5 ºC



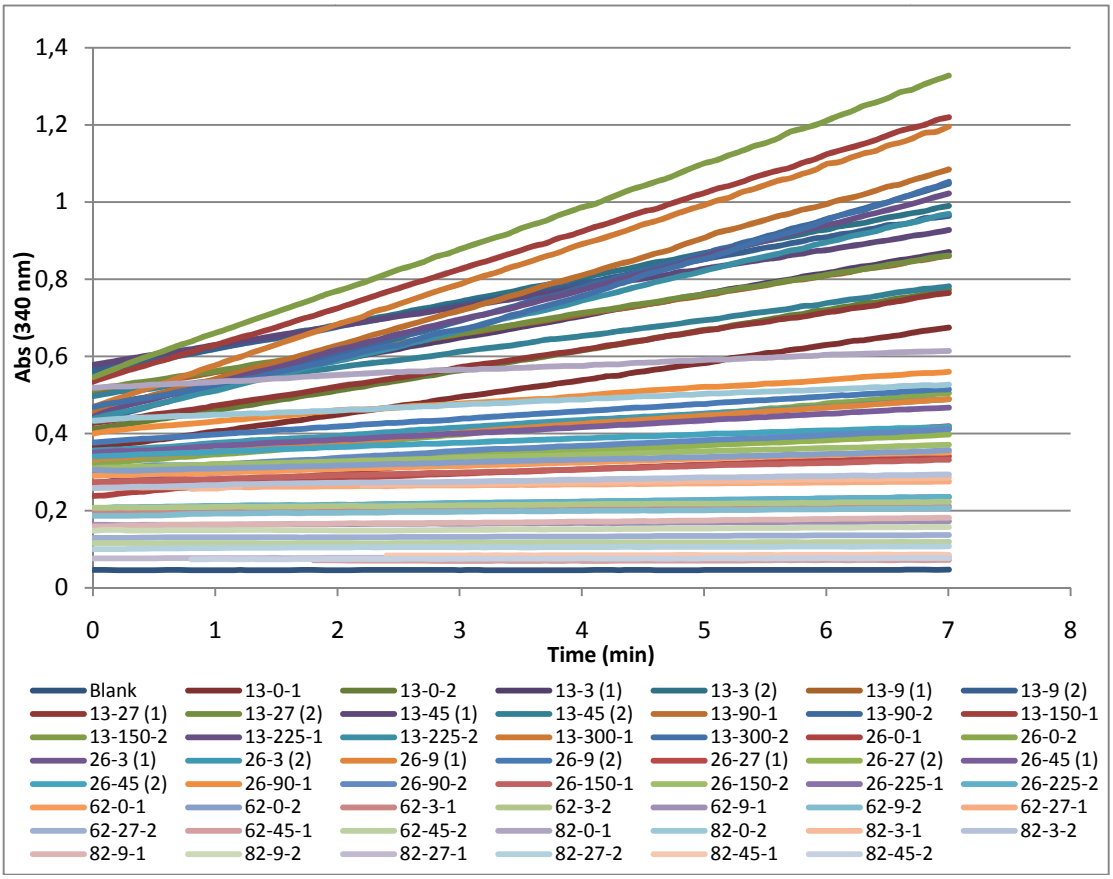
37.5 ºC


1,2
1
0,8
0,6
0,4
0,2
0
Abs (340 nm)
0
1
2
3
4
5
6
7
8
Time (min)
Blank
62-0-1
62-0-2
62-5-1
62-5-2
62-15-1
62-15-2
62-35-1
62-35-2
62-55-1
62-55-2
82-0-1
82-0-2
82-5-1
82-5-2
82-15-1
82-15-2
82-35-1
82-35-2
82-55-1
82-55-2
26-0-1
26-0-2
26 (5) 1
26 (5) 2
26-15-1
26-15-2
26-35-1
26-35-2
26-55-1
26-55-2
13-0-1
13-0-2
13 (5) 1
13 (5) 2
13-15-1
13-15-2
13-35-1
13-35-2
13-55-1
13-55-2

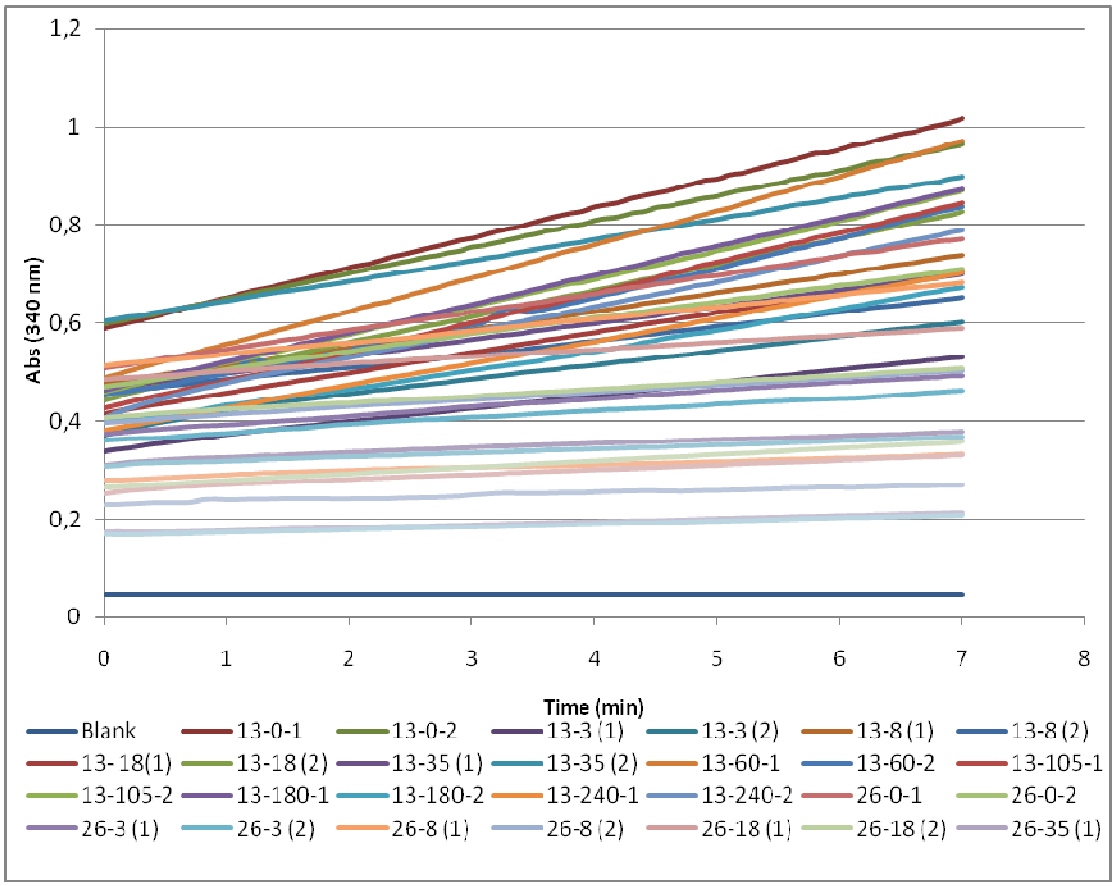
40 ºC


1,4
1,2
1
0,8
0,6
0,4
0,2
0
Abs (340 nm)
0
1
2
3
4
5
6
7
8
time (min)
Blank
62-0-1
62-0-2
62-5-1
62-5-2
62-15-1
62-15-2
62-30-1
62-30-2
62-60-1
62-60-2
82-0-1
82-0-2
82-5-1
82-5-2
82-15-1
82-15-2
82-30-1
82-30-2
82-60-1
82-60-2
13-0-1
13-0-2
13-5 (1)
13-5 (2)
13-15 (1)
13-15 (2)
13-30 (1)
13-30 (2)
13-60 (1)
13-60 (2)
26-0-1
26-0-2
26-5 (1)
26-5 (2)
26-15 (1)
26-15 (2)
26-30 (1)
26-30 (2)
26-60 (1)
26-60 (2)

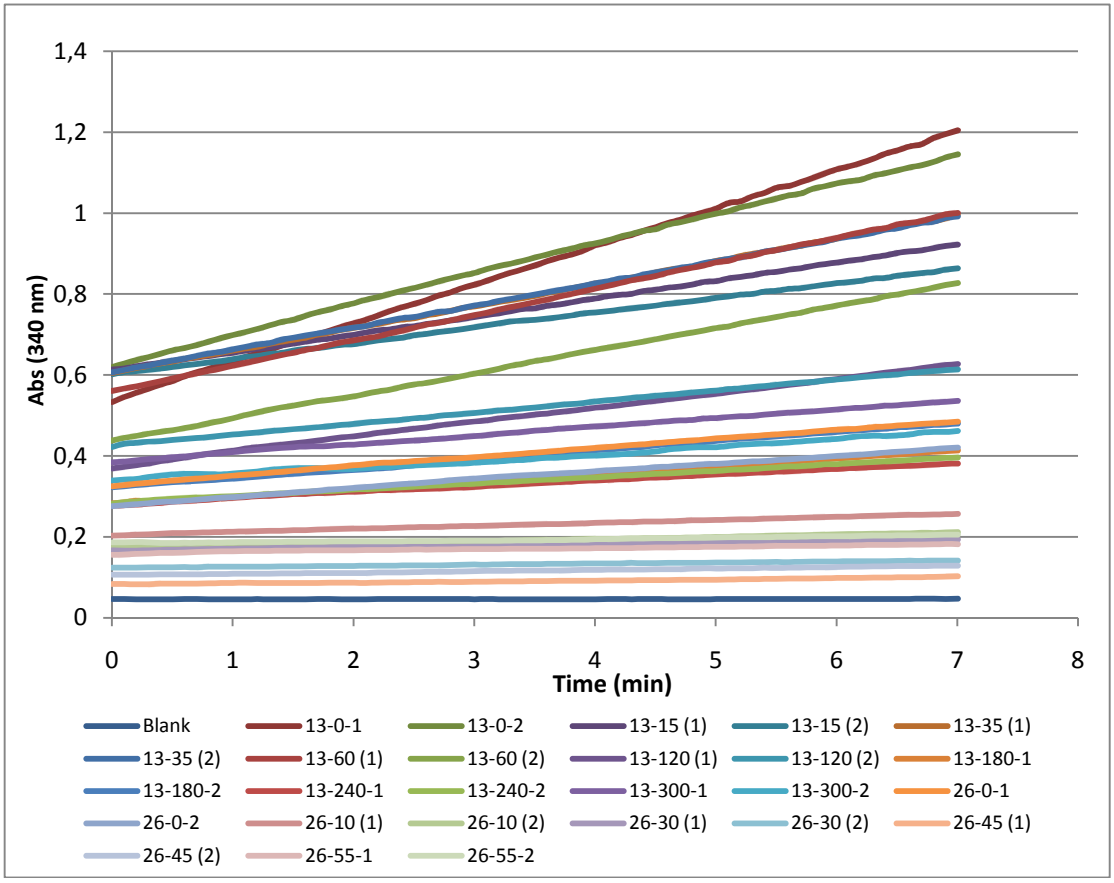
45 ºC


1,4
1,2
1
0,8
0,6
0,4
0,2
0
Abs (340 nm)
0
1
2
3
4
5
6
7
8
Time (min)
Blank
13-0-1
13-0-2
13-3 (1)
13-3 (2)
13-9 (1)
13-9 (2)
13-27 (1)
13-27 (2)
13-45 (1)
13-45 (2)
13-90-1
13-90-2
13-150-1
13-150-2
13-225-1
13-225-2
13-300-1
13-300-2
26-0-1
26-0-2
26-3 (1)
26-3 (2)
26-9 (1)
26-9 (2)
26-27 (1)
26-27 (2)
26-45 (1)
26-45 (2)
26-90-1
26-90-2
26-150-1
26-150-2
26-225-1
26-225-2
62-0-1
62-0-2
62-3-1
62-3-2
62-9-1
62-9-2
62-27-1
62-27-2
62-45-1
62-45-2
82-0-1
82-0-2
82-3-1
82-3-2
82-9-1
82-9-2
82-27-1
82-27-2
82-45-1
82-45-2

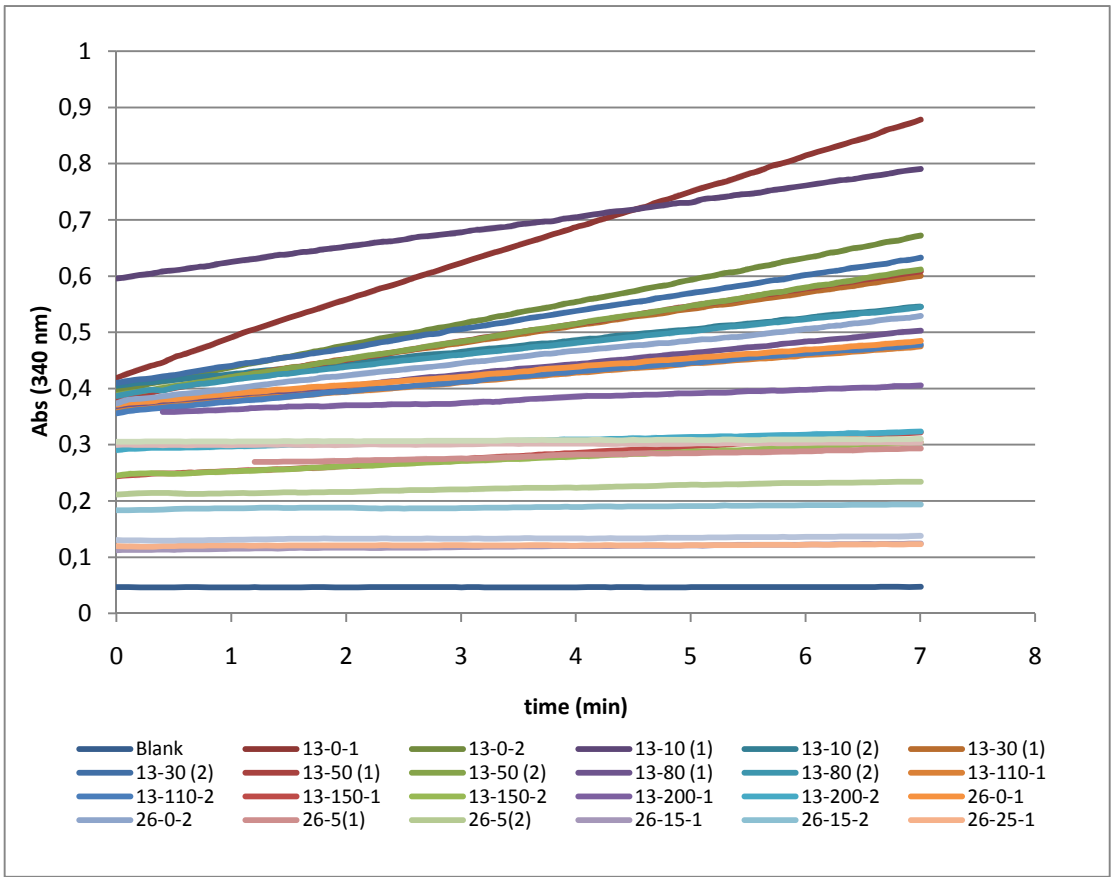
52.5 ºC


1,2
1
0,8
0,6
0,4
0,2
0
Abs (340 nm)
0
1
2
3
4
5
6
7
8
Time (min)
Blank
13-0-1
13-0-2
13-3 (1)
13-3 (2)
13-8 (1)
13-8 (2)
13-18(1)
13-18 (2)
13-35 (1)
13-35 (2)
13-60-1
13-60-2
13-105-1
13-105-2
13-180-1
13-180-2
13-240-1
13-240-2
26-0-1
26-0-2
26-3 (1)
26-3 (2)
26-8 (1)
26-8 (2)
26-18 (1)
26-18 (2)
26-35 (1)

60 ºC


1,4
1,2
1
0,8
0,6
0,4
0,2
0
Abs (340 nm)
0
1
2
3
4
5
6
7
8
Time (min)
Blank
13-0-1
13-0-2
13-15 (1)
13-15 (2)
13-35 (1)
13-35 (2)
13-60 (1)
13-60 (2)
13-120 (1)
13-120 (2)
13-180-1
13-180-2
13-240-1
13-240-2
13-300-1
13-300-2
26-0-1
26-0-2
26-10 (1)
26-10 (2)
26-30 (1)
26-30 (2)
26-45 (1)
26-45 (2)
26-55-1
26-55-2

70 ºC


1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
Abs (340 nm)
0
1
2
3
4
5
6
7
8
time (min)
Blank
13-0-1
13-0-2
13-10 (1)
13-10 (2)
13-30 (1)
13-30 (2)
13-50 (1)
13-50 (2)
13-80 (1)
13-80 (2)
13-110-1
13-110-2
13-150-1
13-150-2
13-200-1
13-200-2
26-0-1
26-0-2
26-5(1)
26-5(2)
26-15-1
26-15-2
26-25-1

80 ºC

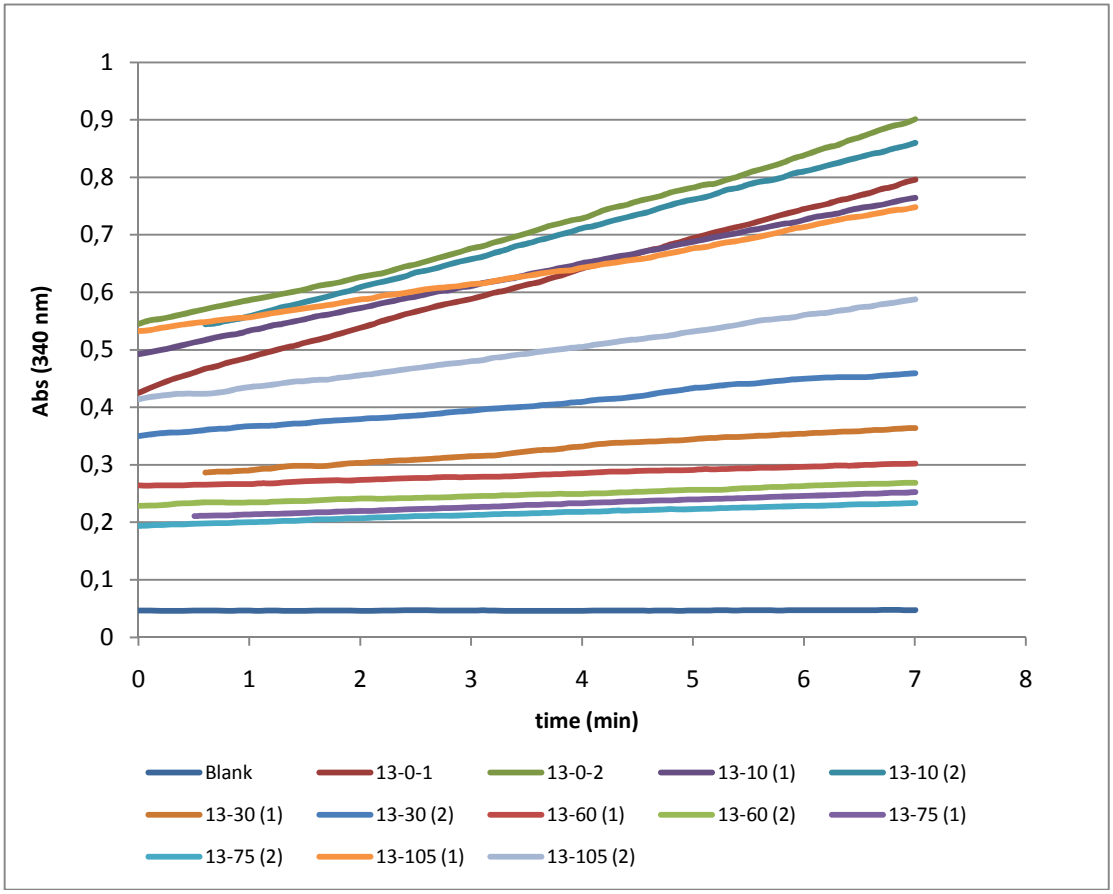


Figure II.1 - Released glucose measured by UV absorbance (340 nm), at various temperatures (30-80 ºC) in different moisture content freeze dried broccoli.

Appendix III - Experimental data of myrosinase degradation in freeze dried broccoli (U/ml; U/g DW; Relative activity)

Table III.1 - Initial (no heating) myrosinase activity in different moisture content freeze dried and fresh broccoli.

Moisture Content (%)	13.4	26.1	62.1	70	82.5	90.1
Sample 1 (U/g DW)	0.481159	0.418399	0.198321	0.120499	0.351455	0.358149
Sample 2 (U/g DW)	0.459402	0.374885	0.14644	0.143929	0.399989	0.350618
Average	0.47028	0.396642	0.17238	0.132214	0.375722	0.354384
Std. Dev	0.015384	0.030769	0.036686	0.016568	0.034319	0.005325

Table III.2 - Experimental data of 82.5% moisture content freeze dried broccoli myrosinase at 30 ºC (green), 33.5 ºC (blue), 37.5 ºC (red), 40 ºC (yellow), 45 ºC (grey).

time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,204455	0,20297	0,0021	0,171544	0,172799	0,001775	1	1	0
0		0,205941		0,174054			1		
5	0,356931	0,410891	0,076312	0,347271	0,301666	0,064496	2,009685	1,745763	0,373243
5		0,30297		0,25606			1,48184		
20	0,205446	0,207921	0,003501	0,175728	0,173636	0,002959	1,016949	1,004843	0,017121
20		0,20297		0,171544			0,992736		
45	0,121782	0,112871	0,012602	0,095395	0,102926	0,010651	0,552058	0,595642	0,061636
45		0,130693		0,110457			0,639225		
70	0,119802	0,112871	0,009801	0,095395	0,101253	0,008284	0,552058	0,585956	0,047939
70		0,126733		0,10711			0,619855		
time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,167327	0,158416	0,012602	0,133888	0,141419	0,010651	1	1	0
0		0,176238		0,14895			1		
5	0,114851	0,136634	0,030805	0,115478	0,097069	0,026035	0,816568	0,686391	0,184099
5		0,093069		0,078659			0,556213		
20	0,05396	0,055446	0,0021	0,046861	0,045605	0,001775	0,331361	0,322485	0,012552
20		0,052475		0,04435			0,313609		
45	0,014851	0,008911	0,008401	0,007531	0,012552	0,0071	0,053254	0,088757	0,050209
45		0,020792		0,017573			0,12426		
60	0,014356	0,009901	0,006301	0,008368	0,012134	0,005325	0,059172	0,085799	0,037657
60		0,018812		0,015899			0,112426		
time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,206931	0,227723	0,029404	0,192463	0,174891	0,024852	1	1	0
0		0,186139		0,157318			1		
5	0,110396	0,139604	0,041306	0,117988	0,093303	0,034911	0,674641	0,533493	0,199614
5		0,081188		0,068617			0,392344		
15	0,062376	0,071287	0,012602	0,060249	0,052718	0,010651	0,344498	0,301435	0,060899
15		0,053465		0,045187			0,258373		
35	0,029208	0,028713	0,0007	0,024267	0,024686	0,000592	0,138756	0,141148	0,003383
35		0,029703		0,025104			0,143541		
55	0,016832	0,016832	0	0,014226	0,014226	0	0,08134	0,08134	0
55		0,016832		0,014226			0,08134		

time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,15	0,184158	0,048307	0,155644	0,126775	0,040828	1	1	0
0		0,115842		0,097905			1		
5	0,112871	0,09505	0,025204	0,080333	0,095395	0,021301	0,633663	0,752475	0,168025
5		0,130693		0,110457			0,871287		
15	0,024257	0,026733	0,003501	0,022594	0,020502	0,002959	0,178218	0,161716	0,023337
15		0,021782		0,01841			0,145215		
30	0,017327	0,010891	0,009101	0,009205	0,014644	0,007692	0,072607	0,115512	0,060676
30		0,023762		0,020083			0,158416		
60	0,021782	0,021782	0	0,01841	0,01841	0	0,145215	0,145215	0
60		0,021782		0,01841			0,145215		
time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,129703	0,131683	0,0028	0,111294	0,10962	0,002367	1	1	0
0		0,127723		0,107947			1		
3	0,039604	0,037624	0,0028	0,031798	0,033472	0,002367	0,290076	0,305344	0,021591
3		0,041584		0,035146			0,320611		
9	0,017822	0,025743	0,011202	0,021757	0,015062	0,009467	0,198473	0,137405	0,086364
9		0,009901		0,008368			0,076336		
27	0,00297	0,00198	0,0014	0,001674	0,00251	0,001183	0,015267	0,022901	0,010796
27		0,00396		0,003347			0,030534		
45	0,000495	0,00099	0,0007	0,000837	0,000418	0,000592	0,007634	0,003817	0,005398
45		0		0			0		

Table III.3 - Experimental data of 62.1% moisture content freeze dried broccoli myrosinase at 30 ºC (green), 33.5 ºC (blue), 37.5 ºC (red), 40 ºC (yellow), 45 ºC (grey).

time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,115842	0,10099	0,021003	0,085353	0,097905	0,017751	1	1	0
0		0,130693		0,110457				1	
5	0,087129	0,084158	0,004201	0,071128	0,073638	0,00355	0,752137	0,726496	0,036262
5		0,090099		0,076149				0,777778	
20	0,04505	0,048515	0,004901	0,041003	0,038074	0,004142	0,388889	0,418803	0,042306
20		0,041584		0,035146				0,358974	
45	0,032673	0,034653	0,0028	0,029288	0,027614	0,002367	0,282051	0,299145	0,024175
45		0,030693		0,025941				0,264957	
70	0,033168	0,023762	0,013302	0,020083	0,028033	0,011242	0,286325	0,205128	0,114829
70		0,042574		0,035982				0,367521	

time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,095545	0,09604	0,0007	0,081169	0,080751	0,000592	1	1	0
0		0,09505		0,080333				1	
5	0,059406	0,065347	0,008401	0,055229	0,050208	0,0071	0,621762	0,683938	0,08793
5		0,053465		0,045187				0,559585	
20	0,036139	0,031683	0,006301	0,026778	0,030543	0,005325	0,378238	0,331606	0,065948
20		0,040594		0,034309				0,42487	
45	0,013861	0,00495	0,012602	0,004184	0,011715	0,010651	0,145078	0,051813	0,131896
45		0,022772		0,019246				0,238342	
60	0,018317	0,011881	0,009101	0,010042	0,015481	0,007692	0,19171	0,124352	0,095258
60		0,024752		0,02092				0,259067	
time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,112871	0,10099	0,016803	0,085353	0,095395	0,014201	1	1	0
0		0,124752		0,105437				1	
5	0,036634	0,033663	0,004201	0,028451	0,030962	0,00355	0,324561	0,298246	0,037216
5		0,039604		0,033472				0,350877	
15	0,025248	0,026733	0,0021	0,022594	0,021338	0,001775	0,223684	0,236842	0,018608
15		0,023762		0,020083				0,210526	
35	0,011386	0,011881	0,0007	0,010042	0,009623	0,000592	0,100877	0,105263	0,006203
35		0,010891		0,009205				0,096491	
55	0,009901	0,00297	0,009801	0,00251	0,008368	0,008284	0,087719	0,026316	0,086838
55		0,016832		0,014226				0,149123	
time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,074752	0,091089	0,023103	0,076985	0,063178	0,019526	1	1	0
0		0,058416		0,049371				1	
5	0,017822	0,014851	0,004201	0,012552	0,015062	0,00355	0,238411	0,198675	0,056194
5		0,020792		0,017573				0,278146	
15	0,024257	0,021782	0,003501	0,01841	0,020502	0,002959	0,324503	0,291391	0,046828
15		0,026733		0,022594				0,357616	
30	0,013861	0,012871	0,0014	0,010878	0,011715	0,001183	0,18543	0,172185	0,018731
30		0,014851		0,012552				0,198675	
60	0,010891	0,017822	0,009801	0,015062	0,009205	0,008284	0,145695	0,238411	0,131119
60		0,00396		0,003347				0,05298	
time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,078218	0,086139	0,011202	0,072801	0,066107	0,009467	1	1	0
0		0,070297		0,059413				1	
3	0,017822	0,017822	0	0,015062	0,015062	0	0,227848	0,227848	0
3		0,017822		0,015062				0,227848	
9	0,015347	0,009901	0,007701	0,008368	0,01297	0,006509	0,196203	0,126582	0,098458
9		0,020792		0,017573				0,265823	
27	0,013366	0,019802	0,009101	0,016736	0,011297	0,007692	0,170886	0,253165	0,116359
27		0,006931		0,005858				0,088608	
45	0,00099	0	0,0014	0	0,000837	0,001183	0,012658	0	0,017901
45		0,00198		0,001674				0,025316	

Table III.4 - Experimental data of 26.1% moisture content freeze dried broccoli myrosinase at 30 ºC (green), 33.5 ºC (blue), 37.5 ºC (red), 40 ºC (yellow), 45 ºC (grey), 52.5 ºC (light-green), 60º C (orange), 70 ºC (brown).

time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,413366	0,361386	0,073511	0,305431	0,349363	0,062129	1	1	0
0		0,465347		0,393295				1	
5	0,332178	0,327723	0,006301	0,27698	0,280746	0,005325	0,803593	0,792814	0,015243
5		0,336634		0,284511				0,814371	
20	0,35297	0,356436	0,004901	0,301247	0,298318	0,004142	0,853892	0,862275	0,011856
20		0,349505		0,29539				0,845509	
45	0,247525	0,254455	0,009801	0,215057	0,209199	0,008284	0,598802	0,615569	0,023711
45		0,240594		0,203342				0,582036	
70	0,30396	0,315842	0,016803	0,266938	0,256897	0,014201	0,735329	0,764072	0,040648
70		0,292079		0,246855				0,706587	
time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,481188	0,427723	0,075611	0,361497	0,406684	0,063904	1	1	0
0		0,534653		0,451871				1	
5	0,542574	0,571287	0,040606	0,482832	0,458565	0,034319	1,127572	1,187243	0,084387
5		0,513861		0,434298				1,067901	
20	0,374257	0,39505	0,029404	0,333882	0,31631	0,024852	0,777778	0,820988	0,061108
20		0,353465		0,298737				0,734568	
45	0,367327	0,39802	0,043407	0,336393	0,310452	0,036686	0,763374	0,82716	0,090207
45		0,336634		0,284511				0,699588	
60	0,311386	0,339604	0,039906	0,287022	0,263173	0,033727	0,647119	0,705761	0,082932
60		0,283168		0,239324				0,588477	
time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,441584	0,452475	0,015402	0,382417	0,373212	0,013018	1	1	0
0		0,430693		0,364007				1	
5	0,287129	0,264356	0,032205	0,223425	0,242671	0,027218	0,650224	0,598655	0,07293
5		0,309901		0,261918				0,701794	
15	0,265842	0,313861	0,06791	0,265265	0,22468	0,057395	0,602018	0,710762	0,153788
15		0,217822		0,184095				0,493274	
35	0,265842	0,326733	0,086113	0,276143	0,22468	0,07278	0,602018	0,73991	0,195009
35		0,20495		0,173217				0,464126	
55	0,222277	0,213861	0,011902	0,180748	0,187861	0,010059	0,503363	0,484305	0,026953
55		0,230693		0,194974				0,522422	

time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,361881	0,360396	0,0021	0,304594	0,30585	0,001775	1	1	0
0		0,363366		0,307105				1	
5	0,386634	0,422772	0,051108	0,357313	0,326769	0,043194	1,068399	1,168263	0,141228
5		0,350495		0,296226				0,968536	
15	0,272772	0,269307	0,004901	0,227609	0,230538	0,004142	0,753762	0,744186	0,013542
15		0,276238		0,233467				0,763338	
30	0,286139	0,290099	0,005601	0,245182	0,241835	0,004734	0,790698	0,801642	0,015477
30		0,282178		0,238487				0,779754	
60	0,243564	0,255446	0,016803	0,215894	0,205852	0,014201	0,673051	0,705882	0,046431
60		0,231683		0,195811				0,640219	
time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,25297	0,249505	0,004901	0,210873	0,213802	0,004142	1	1	0
0		0,256436		0,216731				1	
3	0,185149	0,186139	0,0014	0,157318	0,156481	0,001183	0,731898	0,735812	0,005535
3		0,184158		0,155644				0,727984	
9	0,205941	0,220792	0,021003	0,186606	0,174054	0,017751	0,81409	0,872798	0,083026
9		0,191089		0,161502				0,755382	
27	0,118812	0,111881	0,009801	0,094558	0,100416	0,008284	0,469667	0,44227	0,038746
27		0,125743		0,106273				0,497065	
45	0,134158	0,162376	0,039906	0,137235	0,113386	0,033727	0,530333	0,641879	0,15775
45		0,105941		0,089537				0,418787	
90	0,181188	0,210891	0,042006	0,178238	0,153134	0,035502	0,716243	0,833659	0,166052
90		0,151485		0,12803				0,598826	
150	0,079703	0,078218	0,0021	0,066107	0,067362	0,001775	0,315068	0,309198	0,008303
150		0,081188		0,068617				0,320939	
225	0,037129	0,038614	0,0021	0,032635	0,03138	0,001775	0,146771	0,152642	0,008303
225		0,035644		0,030125				0,1409	
time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,351485	0,369307	0,025204	0,312126	0,297063	0,021301	1	1	0
0		0,333663		0,282001				1	
3	0,15198	0,166337	0,020303	0,140582	0,128448	0,017159	0,432394	0,473239	0,057764
3		0,137624		0,116315				0,391549	
8	0,189604	0,234653	0,06371	0,198321	0,160247	0,053845	0,539437	0,667606	0,181258
8		0,144554		0,122172				0,411268	
18	0,137129	0,140594	0,004901	0,118825	0,115896	0,004142	0,390141	0,4	0,013943
18		0,133663		0,112968				0,380282	
35	0,079208	0,083168	0,005601	0,070291	0,066944	0,004734	0,225352	0,23662	0,015935
35		0,075248		0,063597				0,214085	
60	0,057426	0,063366	0,008401	0,053555	0,048534	0,0071	0,16338	0,180282	0,023902
60		0,051485		0,043513				0,146479	
120	0,092079	0,09802	0,008401	0,082843	0,077822	0,0071	0,261972	0,278873	0,023902
120		0,086139		0,072801				0,24507	

time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,208911	0,219802	0,015402	0,185769	0,176564	0,013018	1	1	0
0		0,19802		0,16736				1	
10	0,059901	0,070297	0,014702	0,059413	0,050626	0,012426	0,28673	0,336493	0,070376
10		0,049505		0,04184				0,236967	
30	0,028713	0,034653	0,008401	0,029288	0,024267	0,0071	0,137441	0,165877	0,040215
30		0,022772		0,019246				0,109005	
45	0,025743	0,021782	0,005601	0,01841	0,021757	0,004734	0,123223	0,104265	0,02681
45		0,029703		0,025104				0,14218	
55	0,025743	0,026733	0,0014	0,022594	0,021757	0,001183	0,123223	0,127962	0,006702
55		0,024752		0,02092				0,118483	
time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,180198	0,153465	0,037806	0,129704	0,152297	0,031952	1	1	0
0		0,206931		0,174891				1	
5	0,034653	0,038614	0,005601	0,032635	0,029288	0,004734	0,192308	0,214286	0,031082
5		0,030693		0,025941				0,17033	
15	0,010396	0,011881	0,0021	0,010042	0,008786	0,001775	0,057692	0,065934	0,011656
15		0,008911		0,007531				0,049451	
25	0,00297	0	0,004201	0	0,00251	0,00355	0,016484	0	0,023311
25		0,005941		0,005021				0,032967	
35	0,00297	0,00297	0	0,00251	0,00251	0	0,016484	0,016484	0
35		0,00297		0,00251				0,016484	

Table III.5 - Experimental data of 26.1% moisture content freeze dried broccoli myrosinase at 30 ºC (green), 33.5 ºC (blue), 37.5 ºC (red), 40 ºC (yellow), 45 ºC (grey), 52.5 ºC (light-green), 60º C (orange), 70 ºC (brown), 80 ºC (light-blue).

time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,511881	0,50396	0,011202	0,42593	0,432624	0,009467	1	1	0
0		0,519802		0,439319				1	
5	0,332178	0,294059	0,053908	0,248529	0,280746	0,045561	0,648936	0,574468	0,105314
5		0,370297		0,312962				0,723404	
20	0,369802	0,329703	0,056709	0,278654	0,312544	0,047928	0,722437	0,644101	0,110785
20		0,409901		0,346434				0,800774	
45	0,335149	0,30099	0,048307	0,254386	0,283256	0,040828	0,654739	0,588008	0,094372
45		0,369307		0,312126				0,72147	
70	0,440594	0,376238	0,091014	0,317983	0,372375	0,076922	0,860735	0,73501	0,177802
70		0,50495		0,426767				0,98646	

time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,486634	0,440594	0,06511	0,372375	0,411286	0,055029	1	1	0
0		0,532673		0,450197				1	
5	0,554455	0,532673	0,030805	0,450197	0,468607	0,026035	1,139369	1,094608	0,063302
5		0,576238		0,487016				1,18413	
20	0,432673	0,409901	0,032205	0,346434	0,365681	0,027218	0,889115	0,842319	0,066179
20		0,455446		0,384927				0,93591	
45	0,558416	0,454455	0,147022	0,38409	0,471954	0,124258	1,147508	0,933876	0,302121
45		0,662376		0,559818				1,361139	
60	0,69802	0,769307	0,100815	0,650192	0,589942	0,085206	1,434385	1,580875	0,207169
60		0,626733		0,529693				1,287894	
time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,506931	0,490099	0,023804	0,414215	0,42844	0,020118	1	1	0
0		0,523762		0,442666				1	
5	0,635149	0,627723	0,010502	0,53053	0,536806	0,008876	1,25293	1,238281	0,020716
5		0,642574		0,543082				1,267578	
15	0,621782	0,632673	0,015402	0,534714	0,525509	0,013018	1,226563	1,248047	0,030383
15		0,610891		0,516304				1,205078	
35	0,526733	0,557426	0,043407	0,471117	0,445176	0,036686	1,039063	1,099609	0,085626
35		0,49604		0,419236				0,978516	
55	0,601485	0,577228	0,034305	0,487853	0,508355	0,028994	1,186523	1,138672	0,067672
55		0,625743		0,528856				1,234375	
time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,425743	0,485149	0,084013	0,410031	0,359823	0,071005	1	1	0
0		0,366337		0,309615				1	
5	0,720297	0,747525	0,038506	0,631782	0,60877	0,032544	1,69186	1,755814	0,090444
5		0,693069		0,585758				1,627907	
15	0,746535	0,606931	0,19743	0,512957	0,630945	0,166861	1,753488	1,425581	0,46373
15		0,886139		0,748934				2,081395	
30	0,54703	0,635644	0,125319	0,537224	0,462331	0,105915	1,284884	1,493023	0,294354
30		0,458416		0,387437				1,076744	
60	0,507921	0,582178	0,105016	0,492037	0,429277	0,088756	1,193023	1,367442	0,246665
60		0,433663		0,366517				1,018605	
time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,474257	0,439604	0,049007	0,371538	0,400826	0,041419	1	1	0
0		0,508911		0,430114				1	
3	0,586139	0,564356	0,030805	0,476975	0,495384	0,026035	1,235908	1,189979	0,064953
3		0,607921		0,513794				1,281837	
9	0,530198	0,49505	0,049708	0,418399	0,448105	0,042011	1,117954	1,043841	0,104811
9		0,565347		0,477811				1,192067	
27	0,482178	0,475248	0,009801	0,401663	0,40752	0,008284	1,016701	1,002088	0,020667
27		0,489109		0,413378				1,031315	
45	0,441584	0,486139	0,06301	0,410868	0,373212	0,053253	0,931106	1,025052	0,132859
45		0,39703		0,335556				0,837161	
90	0,877723	0,891089	0,018903	0,753118	0,741821	0,015976	1,850731	1,878914	0,039858

90		0,864356		0,730524				1,822547	
150	1,030693	0,970297	0,085413	0,820062	0,871106	0,072188	2,173278	2,045929	0,180098
150		1,091089		0,922151				2,300626	
225	0,779208	0,80396	0,035005	0,67948	0,65856	0,029585	1,643006	1,695198	0,073811
225		0,754455		0,63764				1,590814	
300	0,927228	1,020792	0,13232	0,862738	0,783661	0,111832	1,955115	2,152401	0,279005
300		0,833663		0,704584				1,757829	
time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,558911	0,59901	0,056709	0,506263	0,472372	0,047928	1	1	0
0		0,518812		0,438482				1	
3	0,282673	0,262376	0,028704	0,221751	0,238906	0,02426	0,505757	0,469442	0,051358
3		0,30297		0,25606				0,542073	
8	0,316337	0,366337	0,070711	0,309615	0,267357	0,059762	0,565988	0,655447	0,126515
8		0,266337		0,225099				0,476528	
18	0,464851	0,4	0,091714	0,338066	0,392877	0,077513	0,831709	0,715678	0,164094
18		0,529703		0,447687				0,947741	
35	0,372772	0,330693	0,059509	0,27949	0,315054	0,050295	0,666962	0,591674	0,106473
35		0,414851		0,350618				0,74225	
60	0,607426	0,676238	0,097315	0,571533	0,513375	0,082247	1,086802	1,20992	0,174115
60		0,538614		0,455218				0,963685	
105	0,582178	0,592079	0,014002	0,500405	0,492037	0,011834	1,04163	1,059345	0,025052
105		0,572277		0,483669				1,023915	
180	0,482673	0,578218	0,13512	0,48869	0,407939	0,114199	0,863596	1,034544	0,241757
180		0,387129		0,327188				0,692648	
240	0,482178	0,450495	0,044807	0,380743	0,40752	0,037869	0,86271	0,806023	0,080168
240		0,513861		0,434298				0,919398	
time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,833663	0,934653	0,142822	0,789937	0,704584	0,120708	1	1	0
0		0,732673		0,61923				1	
15	0,40099	0,435644	0,049007	0,368191	0,338903	0,041419	0,480998	0,522565	0,058786
15		0,366337		0,309615				0,43943	
35	0,541089	0,544554	0,004901	0,460239	0,45731	0,004142	0,64905	0,653207	0,005879
35		0,537624		0,454381				0,644893	
60	0,584653	0,622772	0,053908	0,526346	0,494129	0,045561	0,701306	0,747031	0,064664
60		0,546535		0,461912				0,655582	
120	0,308911	0,352475	0,061609	0,2979	0,261081	0,05207	0,370546	0,422803	0,073902
120		0,265347		0,224262				0,31829	
180	0,202475	0,183168	0,027304	0,154808	0,171125	0,023077	0,242874	0,219715	0,032752
180		0,221782		0,187443				0,266033	
240	0,14604	0,139604	0,009101	0,117988	0,123428	0,007692	0,175178	0,167458	0,010917
240		0,152475		0,128867				0,182898	
300	0,186139	0,207921	0,030805	0,175728	0,157318	0,026035	0,223278	0,249406	0,036951
300		0,164356		0,138908				0,19715	

time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,510396	0,637624	0,179927	0,538898	0,431369	0,152068	1	1	0
0		0,383168		0,323841				1	
10	0,230198	0,266337	0,051108	0,225099	0,194555	0,043194	0,451018	0,521823	0,100133
10		0,194059		0,164012				0,380213	
30	0,304455	0,29604	0,011902	0,250203	0,257315	0,010059	0,596508	0,580019	0,023319
30		0,312871		0,264428				0,612997	
50	0,311881	0,312871	0,0014	0,264428	0,263591	0,001183	0,611057	0,612997	0,002743
50		0,310891		0,262754				0,609117	
80	0,200495	0,189109	0,016102	0,159828	0,169452	0,013609	0,392823	0,370514	0,031549
80		0,211881		0,179075				0,415131	
110	0,161881	0,158416	0,004901	0,133888	0,136816	0,004142	0,317168	0,310378	0,009602
110		0,165347		0,139745				0,323957	
150	0,093564	0,106931	0,018903	0,090374	0,079077	0,015976	0,183317	0,209505	0,037036
150		0,080198		0,067781				0,157129	
200	0,05297	0,067327	0,020303	0,056902	0,044769	0,017159	0,103783	0,131911	0,039779
200		0,038614		0,032635				0,075655	
time (min)	U/ml (avg)	U/ml	Std. Dev.	U/g DW	U/g DW (avg)	Std. Dev	A/A_0 (avg)	A/A_0	Std. Dev
0	0,50396	0,507921	0,005601	0,429277	0,42593	0,004734	1	1	0
0		0,5		0,422583				1	
10	0,437129	0,380198	0,080512	0,32133	0,369446	0,068046	0,867387	0,75442	0,159759
10		0,494059		0,417562				0,980354	
30	0,139109	0,121782	0,024504	0,102926	0,11757	0,02071	0,276031	0,24165	0,048622
30		0,156436		0,132214				0,310413	
60	0,05198	0,053465	0,0021	0,045187	0,043932	0,001775	0,103143	0,10609	0,004168
60		0,050495		0,042677				0,100196	
75	0,055941	0,060396	0,006301	0,051045	0,047279	0,005325	0,111002	0,119843	0,012503
75		0,051485		0,043513				0,102161	

Appendix IV - ANOVA analysis (single-factor) of initial myrosinase activity on different moisture content freeze dried broccoli.

Table IV.1 - Statistical analysis of initial myrosinase activity of 26, 82 and 90% moisture content freeze dried broccoli

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.001786	2	0.000893	1.244267	0.404108	9.552094
Within Groups	0.002153	3	0.000718			
Total	0.003939	5				

Table IV.2 - Statistical analysis of initial myrosinase activity of 62 and 70% moisture content freeze dried broccoli

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.001613	1	0.001613	1.991357	0.293659	18.51282
Within Groups	0.00162	2	0.00081			
Total	0.003234	3				

Appendix V - ANOVA analysis (two-factor with replication) of the influence of moisture content and time, related to temperature, on myrosinase stability of different moisture content freeze dried broccoli.

30 ºC - Relative Activity

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,416365	3	0,138788	26,33282	1,45E-05	3,490295
Columns	0,609743	2	0,304872	57,84432	6,89E-07	3,885294
Interaction	0,364422	6	0,060737	11,52383	0,000223	2,99612
Within	0,063247	12	0,005271			
Total	1,453777	23				

37.5 ºC - Relative Activity

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	1,903861	3	0,63462	87,71283	1,98E-08	3,490295
Columns	1,416359	2	0,70818	97,87969	3,71E-08	3,885294
Interaction	0,982335	6	0,163722	22,62859	6,95E-06	2,99612
Within	0,086822	12	0,007235			
Total	4,389377	23				

45 ºC - Relative Activity

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	2,753388	3	0,917796	157,0525	4,45E-12	3,238872
Columns	1,96935	3	0,65645	112,3312	5,83E-11	3,238872
Interaction	0,981092	9	0,10901	18,65373	7,24E-07	2,537667
Within	0,093502	16	0,005844			
Total	5,797331	31				

Appendix VI - Degradation profile of myrosinase

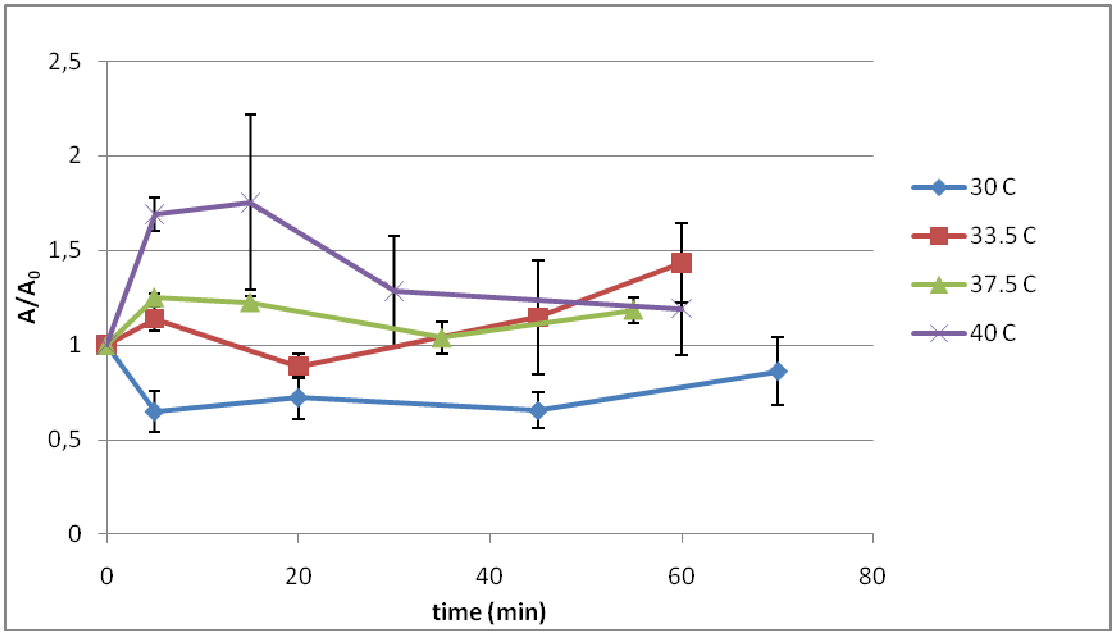


Figure VI.1 - Degradation profile of myrosinase (relative activity) on 13,4% moisture content freeze dried broccoli, at 30-40ºC. Points are the average of two activity measurements.

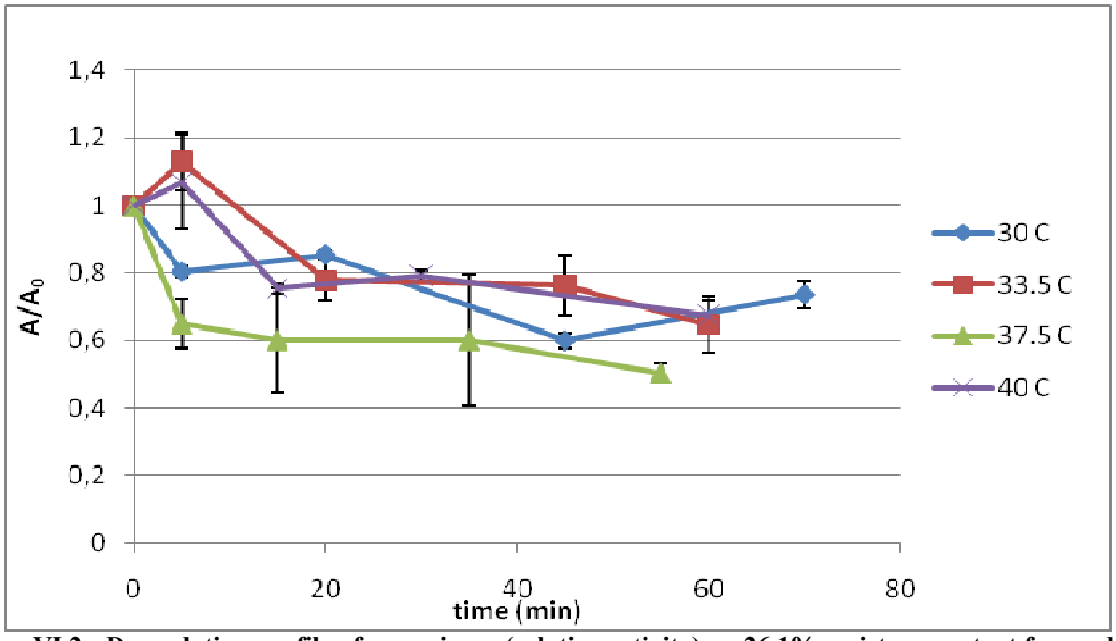


Figure VI.2 - Degradation profile of myrosinase (relative activity) on 26.1% moisture content freeze dried broccoli, at 30-40ºC. Points are the average of two activity measurements.

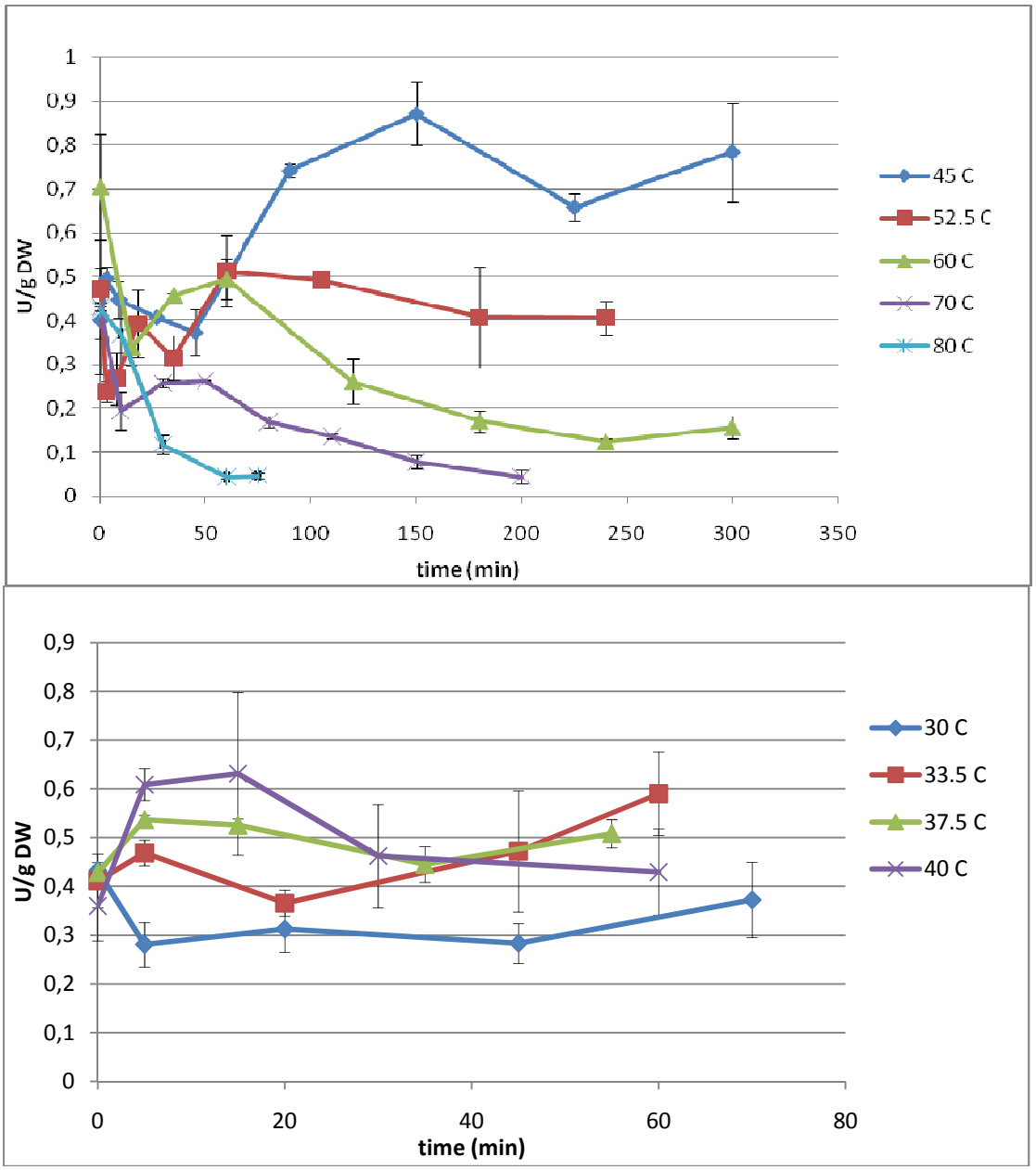


Figure VI.3 - Degradation profile of myrosinase (absolute activity) on 13.4% moisture content freeze dried broccoli, at 30-80ºC. Points are the average of two activity measurements.

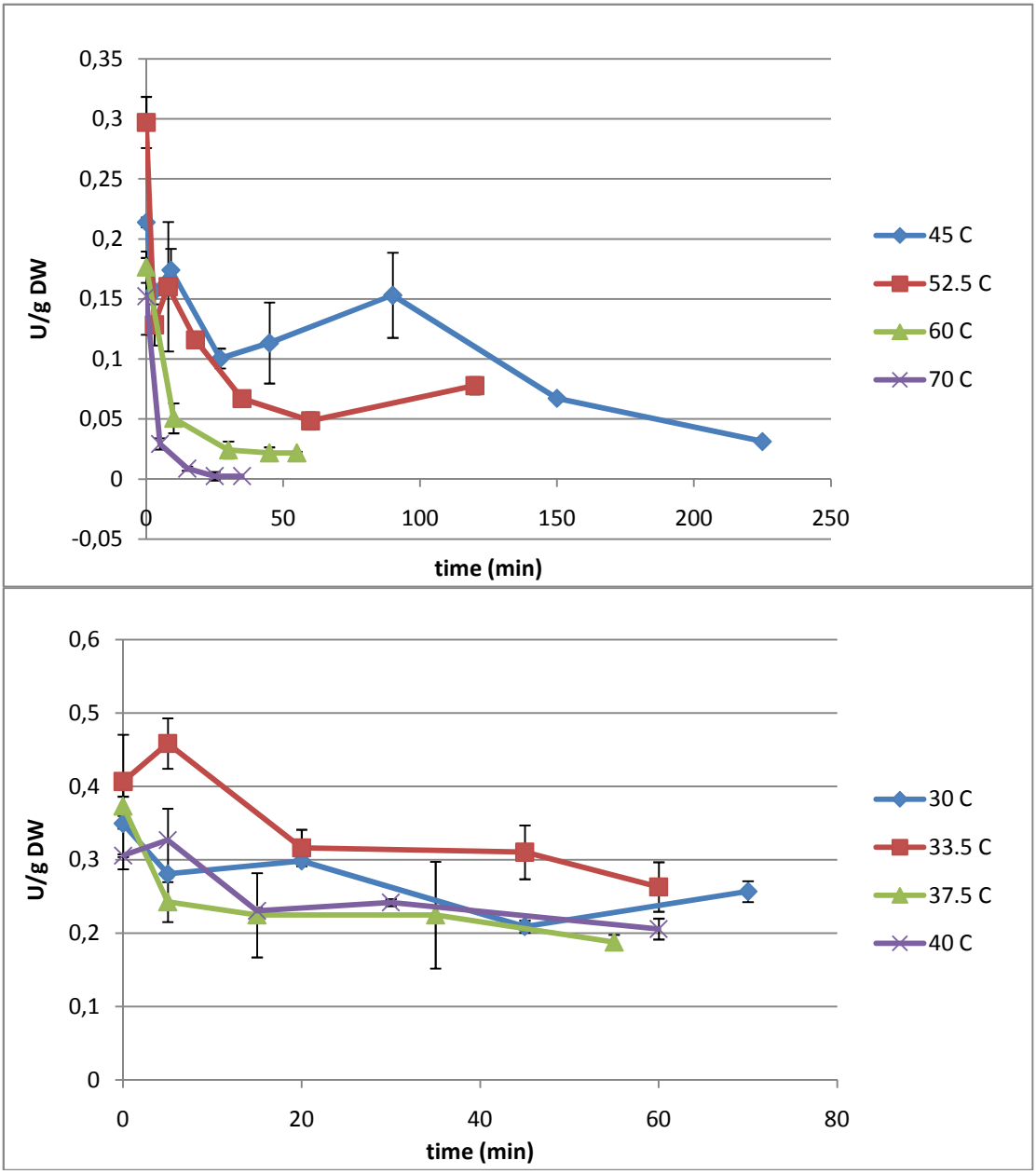


Figure VI.4 - Degradation profile of myrosinase (absolute activity) on 26.1 % moisture content freeze dried broccoli, at 30-70ºC. Points are the average of two activity measurements.

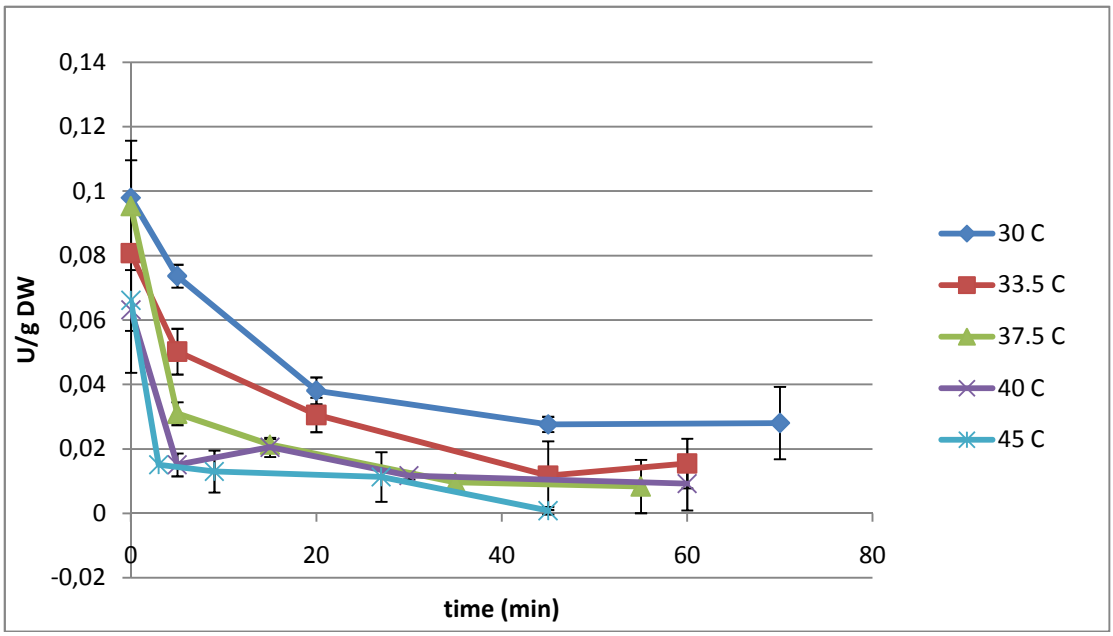


Figure VI.5 - Degradation profile of myrosinase (absolute activity) on 62.1% moisture content freeze dried broccoli, at 30-45°C. Points are the average of two activity measurements.

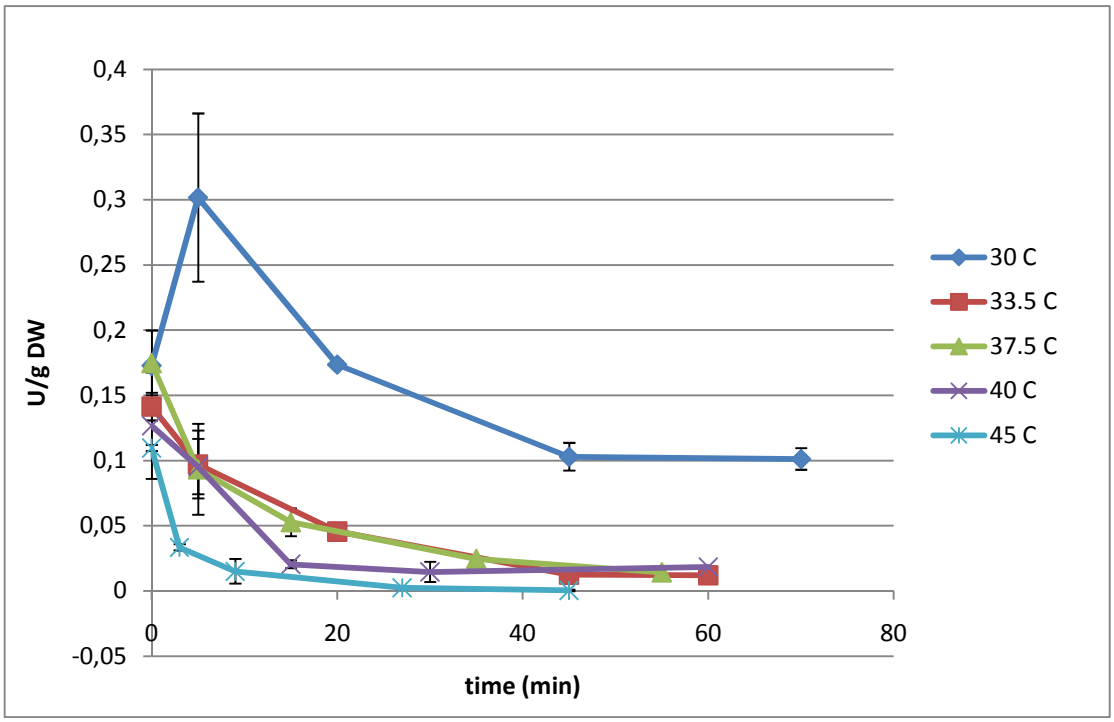


Figure VI.6 - Degradation profile of myrosinase (absolute activity) on 82.5% moisture content freeze dried broccoli, at 30-45°C. Points are the average of two activity measurements.