Physico-chemical properties and thermal aggregation of patatin, the major potato tuber protein

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Stellingen

1. Patatine is een eiwitfamilie die kan worden onderverdeeld in twee massa-isomeren en meerdere ladings-isomeren
   Dit proefschrift, hoofdstukken 2 en 3

2. Ondanks ladings- en massaverschillen hebben patatine-isovormen een sterk gelijkende structuur en conformationele stabiliteit
   Dit proefschrift, hoofdstuk 3

3. Patatine is bij kamertemperatuur en neutrale pH een hooggestructureerd globulair eiwit, dat gedeeltelijk ontvouwt door verhitten of door het verlagen van de pH
   Dit proefschrift, hoofdstukken 4 en 5

4. Visualisatie van reactieprocessen middels wiskundige of mechanistische modellen leidt tot betere en beter toetsbare modellen
   Dit proefschrift, hoofdstukken 6 en 7

5. Fouten in analytische oplossingen van differentiaalvergelijkingen van reactiemechanismen lijken soms meer regel dan uitzondering

6. De Franse keuken doet de aardappel recht door hem als groente te beschouwen, de Nederlandse door dat juist niet te doen

7. Allerlei reclames voor honden- of kattenvoer zijn meer gericht op het welbevinden van het baasje dan op dat van het dier zelf

8. Gezien haar chromosomale samenstelling is het logischer een eicel x-cel te noemen

Stellingen behorende bij het proefschrift

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Abstract

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Key words: *Solanum tuberosum*, potato tuber proteins, patatin, thermal structural stability, aggregation

In potato tubers patatin is the most abundant protein, it is a 43 kDa glycoprotein with a lipid acyl hydrolase activity. Next, different classes of potato protease inhibitors are present. The content and biological activity of patatin and a fraction of potato protease inhibitors of molecular size 20-22 kDa were monitored as a function of storage time of whole potato tubers. It was observed that the amount of buffer-extractable protein decreased gradually during storage of whole potatoes of the cultivars Bintje and Desiree whereas, for Elkana, after an initial decrease it increased after approximately 25 weeks.

Patatin can be divided into two mass isomers, that each can be divided into various isoforms with a slightly differing primary sequence (genetic variants). The isoforms appeared to be of highly homogenous character, therefore, patatin can be studied as a single protein species.

Isolated patatin at room temperature is a highly structured molecule at both secondary and tertiary level as indicated by fluorescence, circular dichroism and Fourier transform infrared spectroscopy. Patatin unfolds partly upon heating or lowering the pH. At low pH, when the starting conformation is already irreversibly unfolded to a certain extent, only minor changes occur upon heating. The unfolding of patatin coincides with its precipitation in the potato fruit juice. The acid or heat precipitation of patatin when present in this juice may be enhanced by so far unknown components.

The thermal aggregation of patatin was studied by dynamic lightscattering and chromatographic analysis of the proportions of non-aggregated and aggregated patatin as a function of incubation time and temperature. The aggregation of patatin requires the unfolding of the protein and can accurately be described quantitatively with a two-step model. The course of aggregation suggested a mechanism of slow coagulation, limited by both reaction and diffusion.

Abbreviations

ANS:	8-anilino-1-naphtalene-sulfonic acid
CE:	Capillary electrophoresis
CD:	Circular dichroism
c.i.	Confidence interval
CLA:	Chemically limited aggregation
CMC:	Carboxymethylcellulose
DLA:	Diffusion limited aggregation
DLS:	Dynamic light scattering
DSC:	Differential scanning calorimetry
FTIR:	Fourier transform infrared
K:	Equillibrium constant
LAH:	Lipid acyl hydrolase
MALDI-TOF MS:	Matrix-assisted laser desorption/ionisation time of flight mass spectrometry
MW:	Molar weight
NEM:	N-ethyl maleimide
NMR:	Nuclear magnetic resonance
RMS:	Root mean square
SANS:	Small-angle neutron scattering
SDS-PAGE:	Dodium dodecyl sulfate poly acryl amide electrophoresis
UV:	Ultra violet
W:	Retardation factor

Contents

Chapter 1

General introduction

Potato (*Solanum tuberosum* L.)

The potato plant is a species of the family Solanaceae. It has been cultivated for thousands of years in South America, mainly in the Andean mountain range before it was introduced in Western Europe in the second half of the 16th century. It lasted about a century before potato tubers (potatoes) were first used as a foodstuff, mainly in Ireland. In the 18th century potatoes became popular in the rest of Western Europe (Zwartz, 1967). Nowadays, about 290 million tons of potatoes are produced world wide annually (FAO, 1998). Potato ranks second to soy bean in the amount of protein produced per hectare and second to sugarcane in carbohydrate production (Johnson and Lay, 1974).

Potato is a major crop in the Netherlands also. It contributes about 5% to the total value of the Dutch agricultural production. In 1997 about 8 million tons of potatoes were traded, having a value of approximately 900 million dollar ($). Some 2.8 million tons (35%) were used for the industrial starch production (CBS, 1998). Potatoes contain about 1.5% of protein on a fresh weight basis (Lisinska and Leszcynski, 1989), therefore, there is an annual production of 40 to 50 thousand tons of potato proteins. However, it can only be used in animal feed, due to its poor solubility and functionality after the currently applied industrial process (Knorr *et al.*, 1977). For this reason the contribution of the proteins to the total value of the industrially used potato is limited. From 100 kg potatoes some 16-18 kg of starch is obtained, having a value of about 7-8$. Currently, the value of protein derived from 100 kg of potatoes is about 0.5$, which results in a contribution of 8% to the value of the potato constituents. Typical prizes of food proteins are 1-2$ for soy isolates and 2.3-5.5$ for whey powder (Van der Meer, 1996). If potato proteins could be applied in human food, similar to for example soy isolates, the contribution of the proteins to the value of the tuber constituents may increase from 8% to 14-28% of the value of the starch. This obviously would increase the economical importance of potato proteins as compared to potato starch and may improve the economic viability of potato as an agricultural commodity crop.

Potato tuber proteins

Soluble potato tuber proteins can be classified into three groups: First, the major tuber protein patatin; second, a group constituted of protease inhibitors; and a third group containing all other proteins. The proportions of proteins present in each class are uncertain, since by far not all of the potato proteins have been quantified yet.

Patatin

Patatin was given its trivial name by Racusen and Foote (1980), but the isolation and partial characterization of the enzyme activity of the 45 kDa potato glycoprotein, as it was denoted, had been described in the early 70's already (Galliard, 1971; Dennis and Galliard, 1974; Galliard and Dennis, 1974). Patatin represents 40-60% of all buffer-extractable tuber proteins and consists of a family of 43 kDa glycoproteins with their isoelectric point between 4.8 and 5.2 (Racusen and Foote, 1980; Park *et al.*, 1983; Racusen 1983A). It is considered to be a storage protein because of its high accumulation in the tuber (Racusen and Foote, 1980; Rosahl *et al.*, 1986), where it is localized mainly in the central vacuoles of the parenchyma cells (Sonnewald *et al.*, 1989).

Patatin has a lipid acyl hydrolase (LAH) activity for both lipid deacylation and wax ester formation (Galliard, 1971; Dennis and Galliard, 1974; Galliard and Dennis, 1974). It is active with phospholipids, monoacylglycerols and *p*-nitrophenyl esters, moderately active with galactolipids but is apparently inactive with di-acyl and tri-acyl glycerols (Andrews *et al.*, 1988). Considerable variation in LAH-activity is observed among cultivars (Racusen, 1985). The LAH-activity of the cultivar Desiree for example, is approximately by a factor 100 lower than that of the cultivar Kennebec (Racusen, 1985). It has been suggested that this LAH-activity may play a role in the plant defense mechanism. The LAH-activity releases fatty acids upon damaging of the vacuole. These fatty acids are oxidized by lipoxygenases that are also present in the tuber (Racusen, 1983B). Cell injury then might lead to the production of cytotoxic, oxidized fatty acid reaction products and water-insoluble waxes that inhibit microbial invasion (Racusen, 1983B). Considering that cultivars can show considerable variation in LAH-activity (Racusen, 1985), those containing patatin with a high LAH-activity may be more resistant towards insect or pathogen attack than cultivars with a low LAH-activity. Despite the relevance of this hypothesis, that could lead to use of cultivars allowing a lower level of pesticides during production, in the literature no information was found on this subject. Another possible defense mode could be the direct inhibition of growth of invasive organisms by the LAH-activity of patatin, as claimed by Strickland *et al.* (1995).

Patatin consists of proteins encoded by two multigene classes of 50-70 genes that are 98% homologous (Mignery *et al.*, 1984; Pikaard *et al.*, 1987; Twell and Ooms, 1988). The Class I genes are expressed exclusively in the tuber, in relatively high amounts; whereas Class II genes are expressed at a low level throughout the whole plant (Pikaard *et al.*, 1987). In the tuber the mRNA's for patatin are by a factor of 100 more abundant than in other parts of the plant (Pikaard *et al.*, 1987). The major source for the generation of diversity among the members of the classes would originate from rearrangements of the genes (Liu *et al.*, 1991).

The primary sequence of patatin (362 amino acids) shows neither extended hydrophilic nor hydrophobic amino acid sequences (Figure 1; Stiekema *et al.*, 1988). The positive and negative charges of the side-chains are randomly distributed over the sequence. The protein contains 17 tyrosines and 2 tryptophans, the latter being positioned closely together in the primary sequence (residues 254 and 259).

Based on the high degree of homology between the gene families and the identical immunological responses (Park *et al.*, 1983; Mignery *et al.*, 1984), patatin was considered a single protein species. In the literature no physico-chemical description of isolated isoforms can be found.

1
Lys-Leu-Glu-Glu-Met-Val-Thr-Val-Leu-Ser-Ile-Asp-Gly-Gly-Gly-Ile-Lys-Gly-Ile-Ile-Pro-Ala-Ile-Ile- Leu
26
Glu-Phe-Leu-Glu-Gly-Gln-Leu-Gln-Glu-Val-Asp-Asn-Asn-Lys-Asp-Ala-Arg-Leu-Ala-Asp-Tyr-Phe-Asp-Val- Ile
51
Gly-Gly-Thr-Ser-Thr-Gly-Gly-Leu-Leu-Thr-Ala-Met-Ile-Thr-Thr-Pro-Asn-Glu-Asn-Asn-Arg-Pro-Phe-Ala- Ala-
76
Ala-Lys-Asp-Ile-Val-Pro-Phe-Tyr-Phe-Glu-His-Gly-Pro-His-Ile-Phe-Asn-Tyr-Ser-Gly-Ser-Ile-Leu-Gly- Pro
101
Met-Tyr-Asp-Gly-Lys-Tyr-Leu-Leu-Gln-Val-Leu-Gln-Glu-Lys-Leu-Gly-Glu-Thr-Arg-Val-His-Gln-Ala-Leu- Thr
126
Glu-Val-Ala-Ile-Ser-Ser-Phe-Asp-Ile-Lys-Thr-Asn-Lys-Pro-Val-Ile-Phe-Thr-Lys-Ser-Asn-Leu-Ala-Lys- Ser
151
Pro-Glu-Leu-Asp-Ala-Lys-Met-Tyr-Asp-Ile-Cys-Tyr-Ser-Thr-Ala-Ala-Ala-Pro-Ile-Tyr-Phe-Pro-Pro- His- His Phe-
176
Val-Thr-His-Thr-Ser-Asn-Gly-Ala-Arg-Tyr-Glu-Phe-Asn-Leu-Val-Asp-Gly-Ala-Val-Ala-Thr-Val-Gly- Asp- Pro
201
Ala-Leu-Leu-Ser-Leu-Ser-Val-Ala-Thr-Arg-Leu-Ala-Gln-Glu-Asp-Pro-Ala-Phe-Ser-Ser-Ile-Lys-Ser- Leu- Asp
226
Tyr-Lys-Gln-Met-Leu-Leu-Leu-Ser-Leu-Gly-Thr-Gly-Thr-Asn-Ser-Glu-Phe-Asp-Lys-Thr-Tyr-Thr-Ala- Glu- Glu
251
Ala-Ala-Lys-Trp-Gly-Pro-Leu-Arg-Trp-Met-Leu-Ala-Ile-Gln-Gln-Met-Thr-Asn-Ala-Ala-Ser-Ser-Tyr- Met- Thr
276
Asp-Tyr-Tyr-Ile-Ser-Thr-Val-Phe-Gln-Ala-Arg-His-Ser-Gln-Asn-Asn-Tyr-Leu-Arg-Val-Gln-Glu-Asn- Ala- Leu
301
Asn-Gly-Thr-Thr-Thr-Glu-Met-Asp-Asp-Ala-Ser-Glu-Ala-Asn-Met-Glu-Leu-Leu-Val-Gln-Val-Gly-Glu- Thr- Leu
326
Leu-Lys-Lys-Pro-Val-Ser-Lys-Asp-Ser-Pro-Glu-Thr-Tyr-Glu-Glu-Ala-Leu-Lys-Arg-Phe-Ala-Lys-Leu- Leu- Ser
351 362
Asp-Arg-Lys-Lys-Leu-Arg-Ala-Asn-Lys-Ala-Ser-His

Figure 1 Primary structure of a patatin isoform expressed in tobacco (Stiekema *et al.*, 1988).

Patatin isolated from transgenic tobacco plants in which a single patatin gene of the cultivar Berolina was expressed, has three glycosylation sites at 37, 67 and 181 asparagine residues, two of which where found to be actually glycosylated (Sonnewald *et al.*, 1989). The consensus sequence for N-linked glycosylation is Asn-X-Ser/Thr, where X may be any amino acid other then proline or asparagine (Katsube *et al.*, 1998). At the sequence as presented by Stiekema *et al.* (1988) patatin has only two glycosylation sites, at the 92 and 301 asparagine residues, respectively.

A carbohydrate structure was suggested based on the structure generally observed in plants, as: Manα3[Manα6][Xylβ2]Manβ4GlcNacβ4[Fucα3] GlcNAc (MW 1169 Da), assuming that the glycosylation in the tobacco is identical to that in potato (Sonnewald *et al.*, 1989). The molar mass of a patatin isoform from the cultivar Bintje, without the carbohydrate contributions, is 39745 Da, as calculated from its primary sequence (Stiekema *et al.*, 1988).

Adding the mass of one, two or three carbohydrate chains would increase the total mass to 40 914, 42 083 and 43 252 Da. The apparent molar mass of patatin is 43 kDa determined using SDS-PAGE, whereas in media without SDS or urea it appears as a dimer with an apparent molar mass of about 80 000 Da (Racusen and Weller, 1984), a value which was also found with analytical ultracentrifugation under the same conditions (Racusen and Weller, 1984). These techniques are, however, not sufficiently accurate to determine the degree of glycosylation.

Protease inhibitors

Numerous papers describe the presence and importance of protease inhibitors (e.g: Melville and Ryan, 1972; Bryant *et al.*, 1976; Suh *et al.*, 1990, 1991; Dao and Friedman, 1994; Jongsma *et al.*, 1994). They inhibit proteolytic enzymes from a wide variety of micro-organisms and insects, as a part of the plants defense mechanism against environmental stress, pathogen or insect attack (Jongsma, 1995). Plant proteases are only rarely inhibited (Kaiser *et al.*, 1974; Sanchez-Serrano *et al.*, 1986). Potato protease inhibitors represent 20 to 30% of all extractable tuber proteins. Generally, plant protease inhibitors are classified based on the composition of the active site of the protease they inhibit; for example, serine, cysteine, aspartic or metallo protease inhibitors are distinguished (Jongsma, 1995). Potato protease inhibitors, however, are not categorized this way, and they are currently divided into three subclasses:

Subclass I: The Potato Protease Inhibitor I, a serine protease inhibitor that is constituted of an iso-inhibitor family. It represents 2% of the soluble proteins of the tubers and it accumulates in the central vacuoles (Graham *et al.*, 1985; Cleveland *et al.*, 1987). It consists of four subunits of 9-10 kDa each, which can agglomerate to a complex of 39000 +/- 2000 Da (Melville and Ryan, 1972; Richardson and Cossins, 1974). Based on their proportional presence, the subunits are divided into two major and two minor variants. The N and C-termini of the proteins are highly homologous (>95%; Richardson and Cossins, 1974; Cleveland *et al.*, 1987), although it should be noted that the sequence as presented by Richardson and Cossins (1974, from cultivar Ulster Prince) has an insert of 14 amino acids, starting at residue 60 of the sequence as observed by Cleveland and co-workers (1987, from cultivar Russet Burbank).

Based on a residual solubility of over 90% after a heat-treatment of 10 min at 80°C at pH values of 3-9, the Protease Inhibitor I is denoted as heat stable (Melville and Ryan, 1972).

Subclass II: The Potato Protease Inhibitor II represents 5% of the soluble proteins of the tubers (Cleveland *et al.*, 1987). It is a serine protease inhibitor consisting of 154 amino acids with a molar mass of 12.3 kDa and it accumulates in the central vacuole (Graham *et al.*, 1985; Sanchez-Serrano *et al.*, 1986; Cleveland *et al.*, 1987).

The genes encoding for the Potato Protease Inhibitor II can be subdivided in two groups. One gene codes for a developmentally controlled (not wound-inducible) tuber specific protein. Upon wounding of the tuber no extra mRNA for Inhibitor II was observed in the tuber. This

in contrast to the environmentally inducible (wound-inducible) second gene, that is found in the leaves (Sanchez-Serrano *et al.*, 1986).

Subclass III: In Subclass III are categorized all identified protease inhibitors that do not belong to the Subclasses I or II. It consists of numerous protease inhibitors present in the potato, such as a 22 kDa wound inducible protease inhibitor (Suh *et al.*, 1991), a cathepsin D inhibitor of 20.4 kDa (Ritonja *et al.*, 1990), and cysteine proteinase inhibitors of 25 000 and 22 000 kDa (Brzin *et al.*, 1988).

Other proteins

All identified potato proteins other than patatin and protease inhibitors belong to this class, each representing a minor fraction, but in total accounting for 20-30% of all protein. Examples are protein kinases, e.g. those that are part of the signaling cascades regulating responses to external stimuli (Man *et al.*, 1997; Subramaniam *et al.*, 1997). Another group in this class are the enzymes involved in starch synthesis; such as the major starch synthase (140 kDa) or high-molar mass (ranging from 180 to 600 kDa) potato phosphorylase isoenzymes (Gerbrandy and Doorgeest, 1972; Shivaram, 1976; Marshall *et al.*, 1996). Further examples of a subgroups are polyphenol oxidases, consisting of 60 and 69 kDa proteins (Partington and Bolwell, 1995) and a potato lectin of 65.5 kDa that is for 50 % (w/w) glycosylated (Allen *et al.*, 1996).

Potato proteins from the starch industry

After industrial processing of potatoes to potato starch the potato proteins are present in the so called potato fruit juice. In Figure 2 the industrial production of potato starch and fruit juice is outlined. The potatoes are ground and the insoluble material, mainly starch and cell wall material, is removed by hydrocyclones. The remaining fluid is the potato fruit juice. This has a pH of approximately 5.6 and contains 2-5 % (w/w) dry matter, which constitute of about 35% protein and amino acids, 35% sugars, 20% minerals, 4% organic acids and 6% other compounds, such as phenolic compounds (Knorr *et al.*, 1977; Knorr, 1980). The potato proteins are recovered by an acidic heat-treatment of the potato juice. This treatment results in irreversibly precipitated proteins, which have lost all functionality in terms of water holding or sensory properties (Knorr *et al.*, 1977; Boruch *et al.*, 1989). As a consequence, the proteins can only be used in low-value applications such as feed or fertilizer (De Noord, 1975; Ahldén and Trägård, 1992).

Undenatured potato proteins are claimed to exhibit promising functional properties, such as the capacity to form and stabilize emulsions and foams (Holm and Eriksen, 1980; Jackman and Yada, 1988). Furthermore, they have a high nutritional value, derived from amino acid analysis and bio-assays (Kapoor *et al.*, 1975; Liedl *et al.*, 1987). The nutritional value of potato protein in humans was shown to be comparable to that of whole egg. The lysine content of potato protein (7.5 %) is higher than that of most plant proteins, e.g those of wheat

or beans. Therefore, it would be an excellent supplement for lysine-poor proteins in the human diet, such as cereal proteins in bread (Knorr, 1978). In addition, allergic reactions to potato proteins are uncommon (Castells *et al.*, 1986; Quirce *et al.*, 1989). This was confirmed by recent research (Zock *et al.*, 1996, 1998), which has shown that the alleged allergic reactions of people working in the potato processing industry (Hollander *et al.*, 1994; Dutkiewicz, 1994) were in fact due to exposure to microbial endotoxins.

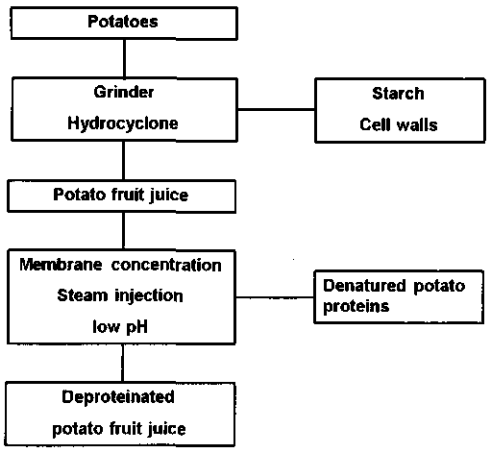

Figure 2 Simplified flow diagram for the production of potato starch and protein.

For these reasons, potato proteins are a promising source of plant proteins for human consumption. Therefore, a considerable amount of work has been performed to obtain undenatured potato proteins from the potato fruit juice in industrial processes. This was tried with combinations of various precipitation conditions and temperature regimes. The influence of weak and strong acids, such as acetic, citric, hydrochloric or sulfuric acid was examined. Furthermore, precipitation was induced with specific coagulants such as carboxymethylcellulose (CMC) or bentonite as well as salts as $FeCl_3$ or $Al_2(SO_4)_3$ (e.g: Knorr *et al.*, 1977; Knorr, 1977, 1980; Lindner *et al.*, 1980, 1981; Shomer *et al.*, 1982; Boruch *et al.*, 1989; Gonzalez *et al.*, 1991). With these methods a high efficiency of protein precipitation was obtained. Nevertheless, in all cases the precipitation was highly irreversible. Consequently, no industrial method for the production of a resoluble potato protein preparation has been developed and no potato protein product is available that can be applied in food products so far.

The mechanism of the irreversible heat-precipitation in the potato juice is unknown. A reason for this could be the fact that this work generally not was performed on a well defined system of isolated proteins, but on protein mixtures. Moreover, it was done in a matrix containing unknown potato fruit juice components of non-protein origin. An indication for the relevance of these components for the behavior of the proteins is that a minimal protein solubility was

observed below pH 3 (Lindner *et al.*, 1980; Ahldén and Trägårdh, 1992), whereas the isoelectric pH of the potato proteins was observed to be between approximately 5 and 8 (Ahldén and Trägårdh, 1992). In the literature this discrepancy is not noted, therefore, no explanation is available.

In this perspective, information on the structural stability of isolated potato protein may be of help in establishing a link between the observed irreversible precipitation in the potato fruit juice and possible heat-induced structural properties of the proteins. To that end, the conformational stability of the proteins should be established as a function of temperature and pH, in a well defined system with isolated protein. The literature contains only limited information on the structural properties of potato proteins on a molecular level. Lindner and co-workers (1980) recorded a far-ultra violet CD spectrum at ambient temperatures of a patatin-rich fraction and concluded qualitatively that the proteins present in this fraction have a significant amount of α-helical and β-stranded contributions.

Structural properties and stability of proteins

Protein structure

The amino acid sequence of a protein (the primary structure) determines its secondary, tertiary and quaternary structure, i.e. the molecular conformation (Creighton, 1996). The secondary structure is the local conformation of the polypeptide backbone, and can generally been divided into 3 main structure types. The random coiled conformation may be considered as the natural unordered state of a polymer, whereas the α-helix and the β-strand present structured conformations. The α-helix has a helical shape where the stairway-like β-strand usually is incorporated into β-sheets, consisting of closely interacting β-strands (Creighton, 1996). The tertiary structure is the overall topology of the folded polypeptide chain and the quaternary structure is the association of the separate polypeptide chains (Creighton, 1996).

The conformation of a protein can only be obtained exactly by nuclear magnetic resonance (NMR) or X-ray scattering. These techniques, however, are both highly time consuming. Various alternative techniques, such as circular dichroism (CD), fluorescence and Fourier transform infrared spectroscopy (FT-IR), are applied widely to study structural properties and stability of proteins (e.g: Brouilette *et al.*, 1987; Yang *et al.*, 1987; Cladera *et al.*, 1992; De Wolf and Keller, 1996). Though less detailed information is presented as compared to NMR or X-ray analysis, spectral methods provide useful information on the secondary and tertiary structure of a protein, and the changes therein due to for example heat-treatment. They are the methods of choice to investigate changes in behavior of a protein under various solvent conditions, and to monitor structural transitions such as unfolding under a variety of conditions (Johnson, 1988; Pace *et al.*, 1989; Schmid, 1989; Goormaghtigh *et al.*, 1994; Greenfield, 1996; Vuilleumier *et al.*, 1993; Woody and Dunker, 1996).

Heat-induced changes of proteins

Structural stability and unfolding

The stability of the protein structure is related to the small difference in free energy between the relatively small ensemble of folded conformations and the immense ensemble of unfolded alternatives. Physical forces that underlie this balance include the hydrophobic interactions, van der Waals forces, electrostatic interactions, hydrogen bonds, hydration of polar groups and covalent cross links. (Alber, 1990). Generally, the hydrophobic interaction is considered a major contributor, though the phenomenon is complex and there is some controversy (Creighton, 1990; Ben-Naim, 1995).

Generally, food products or its components are heat-treated in the industry, primarily for safety reasons and to achieve desired product properties, such as digestibility or specific sensory characteristics. Heating of proteins in an aqueous environment above their denaturation temperature results in unfolding, which can furthermore be induced by changing the solvent quality or by bringing the pH away from the isoelectric pH of the protein (Creighton, 1990). Unfolding of a protein, or denaturation, is characterized by a rearrangement of the polypeptide chain, resulting in a loss of the native conformation without the disruption of the peptide bond. This can result in the exposure of hydrophobic groups to the solvent (Tanford, 1961). Generally, unfolding is a reversible process (Creighton, 1990). It can become irreversible, however, when reactions occurring in the unfolded state obstruct refolding. This irreversible unfolding can for example be caused by association of the protein molecules (Boye *et al.*, 1997).

Aggregation

Clusters of molecules, also denoted aggregates or coagulates, associate through intermolecular interactions. The forces underlying these intermolecular interactions are basically the same as those involved in the intramolecular structure. By associating, the proteins can, for example, minimize the hydration of hydrophobic areas, or give rise to electrostatic atttractions. Generally, two main mechanisms of aggregation are distinguished (Lin *et al.*, 1989): Diffusion limited aggregation (DLA), with a relatively high proportion of permanent contacts upon collisions (Meakin, 1989) and chemically limited aggregation (CLA), which is characterized by a relatively low sticking probability (Rouw and de Kruif, 1989). Computer models simulating these mechanisms have been developed and applied to validate the assumption concerning a specific type of coagulation (Meakin, 1989; Einarson and Berg, 1993; Elofsson *et al.*, 1996; Kyriakidis *et al.*, 1997).

Aggregation of proteins can considerably affect the rheological properties of a food system (Boye *et al.*, 1997; Oakenfull *et al.*, 1997). To be able to distinguish general pathways of aggregation mechanisms of (food)proteins fundamental knowledge on the physico-chemical properties of these proteins must be combined with a description of the aggregation mechanism. By this means the understanding of the structure-function relationships may be

enhanced. This provides a knowledge base for the control of aggregation, possibly leading to protein products that better meet the properties, required for application in a food system.

Aim

The goal of this work was, first, to extend the knowledge on the presence and characteristics of soluble potato tuber proteins. Next, the objective was to examine the thermal structural stability of isolated patatin and its thermal aggregation mechanism in a well defined system. The information on the structural stability may be of help to elucidate the mechanism of the irreversible precipitation as occurring in the industrial process. Furthermore, when combined with the mechanism of aggregation, it would allow us to relate its functional behavior to the structural properties of the protein.

Outline

Chapter 2 describes the effects of storage of whole potatoes of the cultivars Bintje, Desiree and Elkana on the proportion, molar masses and bio-activity of patatin and a protease inhibitor fraction using matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI TOF MS), capillary electrophoresis (CE) and enzyme (inhibition) assays. Bintje and Desiree are commonly used for human consumption in Europe while Elkana is the most frequently used cultivar for the industrial production of potato starch, and hence, the most important source of potato protein in the Netherlands. In Chapter 3 the isolation and physico-chemical characterization of three isoform pools of patatin isolated from Bintje is reported, to allow comparing of their properties with those of unfractionated patatin. The thermal structural stability of unfractionated patatin as a function of pH and ionic strength is presented in chapters 4 and 5, making use of various spectroscopic techniques. In chapter 6 a kinetic model is proposed that is based on quantitative chromatographic analysis of the amounts of aggregated and non-aggregated protein after heat-treatments. Chapter 7 presents a physical mechanism for the thermal aggregation of patatin *in situ*, based on results as obtained using dynamic light scattering. The structural properties of the protein as obtained in chapters 4 and 5 are linked to the aggregation data from the chapters 6 and 7. In chapter 8 a general discussion of the work is presented.

Chapter 2

The effect of storage of whole potatoes of three cultivars on the patatin and protease inhibitor content; a study using capillary electrophoresis and MALDI-TOF mass spectrometry*

Abstract

The content and biological activity of patatin and the protease inhibitors of molecular size 20-22 kDa present in whole potato tubers were investigated as a function of storage time. The amount of buffer-extractable protein decreased gradually during storage of whole potatoes of the cultivars Bintje and Desiree for 47 weeks whereas, for Elkana, it increased after approximately 25 weeks.

The patatin proportion of the extractable protein did not decrease significantly during storage, whereas the proportion of PP_{20-22} protease inhibitors decreased. All cultivars contained several different patatin isoforms. Bintje and Desiree showed patatin populations with two masses, whereas for Elkana only one molar mass was found. Patatin isoforms of the three examined cultivars showed no significant differences in stability towards degradation as was concluded from capillary electrophoresis analysis.

No inactivation of patatin or protease inhibitors by partial degradation of these proteins was observed using matrix assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS), whereas enzyme-activity assays suggested that the biological activity, especially the cultivar Bintje, decreased markedly at the break of dormancy.

* This chapter has been submitted as:
The effect of storage of whole potatoes of three cultivars on the patatin and protease inhibitor content; a study using capillary electrophoresis and MALDI-TOF mass spectrometry
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Introduction

Potato (*Solanum tuberosum*) tubers contain about 15 g protein·Kg^{-1} on a fresh weight basis (Lisinska and Leszcynski, 1989). A tentative classification of potato proteins is as follows:
I) Patatin, a highly homologous group of isoforms consisting of 43 kDa glycoproteins with a storage function and lipid acyl hydrolase (LAH) activity (Racusen and Foote, 1980; Racusen, 1983A). It represents 40-60% of all buffer-extractable potato proteins.
II) Protease inhibitors (20-30%), divided into subclass I, containing an 8.1 kDa protein; subclass II with e.g. a 12.3 kDa protein (Cleveland *et al.*, 1987; Sanchez-Serrano *et al.*, 1986) and a third subclass containing protease inhibitors of various molecular weights (22-25 kDa) (Ritonja *et al.*, 1990; Suh *et al.*, 1990; Brzin *et al.*, 1988).
III) Other proteins (20-30%), like high-molecular weight proteins involved in starch synthesis such as an 80 kDa phosphorylase (Gerbrandy and Doorgeest, 1972).
These proteins are a by-product of the potato starch industry, recovered by a combined acidic heat-treatment of the so-called potato fruit juice (Knorr *et al.*, 1977; Boruch *et al.*, 1989) and can only be used as low-value cattle-fodder. Potato protein has a high nutritional value (Kapoor *et al.*, 1975; Knorr, 1978). Therefore, it is a promising source of plant proteins for human consumption. Investigations on the physico-chemical behaviour of patatin (Chapter 4) showed that the major drawback for food use, namely insolubility after the acidic heat-treatment, was not necessarily related to the properties of the protein but to the isolation process.
When potato protein isolates are to be used for food purposes, knowledge on the quantities, relative proportions and biochemical characteristics of their individual components becomes important. Another research objective is to know for how long potatoes can be stored with respect to the biochemical characteristics of the proteins.
The amount of patatin present during growth and storage of whole potato tubers has been described (Racusen, 1983b), though biochemical characterisation of the protein is lacking. So far, no attention has been paid to the effect of prolonged storage of whole potatoes on their protease inhibitor content and specific activity, although numerous papers describe the presence and importance of these proteins (Melville and Ryan, 1971; Bryant *et al.*, 1976; Suh *et al.*, 1991; Dao and Friedman, 1994). They inhibit proteolytic enzymes from a wide variety of micro-organisms and insects whereas proteases from plants are only rarely inhibited. Therefore, they are not playing a role in the nitrogen balance of the plant during storage and sprouting.
With regards to biochemical characterisation, (free zone) capillary electrophoresis (CE) and matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS) have become available as tools to analyse biopolymers (Bahr *et al.*, 1994; Kaufmann, 1995; Wehr *et al.*, 1997). Therefore, CE can be of use for the analysis of potato protein, especially to study the patatin isoforms. MALDI-TOF MS can be used as an additional tool

to identify proteins. Furthermore, it allows the breakdown of proteins to be monitored as could occur during storage or sprouting of potatoes.

In this study CE and MALDI-TOF MS were used to analyse the effect of prolonged storage on the amount and biological activity of patatin as well as of specific protease inhibitors in three potato cultivars. These were the cultivar Elkana, the most frequently used cultivar for the industrial production of potato starch and hence the most important source of potato protein in the Netherlands, and cultivars Bintje and Desiree that are commonly used for human consumption in Europe.

Experimental procedures

Extraction and fractionation

Storage Mature potatoes (*Solanum tuberosum)* of the cultivars Bintje, Desiree and Elkana (1995 harvest) were stored without anti-sprouting treatments at 4°C in the dark at a relative humidity between 95 and 100%.

Extraction and isolation At various time-intervals, spanning 47 weeks of storage, protein was extracted from 50 g of potatoes using a Tris/HCl buffer pH 8 as described in Chapter 4. The extract was applied to a DEAE sepharose anion exchange column and fractionated as described in Chapter 4. This yielded a non-bound and a NaCl-eluted fraction; the former was not studied further. Volumes of 5-10 ml (3-4 mg protein·ml^{-1}) of NaCl-eluted protein were applied to a Sephacryl S-100 HR gel filtration column as described previously (Chapter 4). Proteins were detected by absorbance at 280 nm and fractions (10 ml) were collected and pooled as appropriate. This resulted in 2 pools; one contained mainly patatin and the other contained proteins expressing protease inhibiting activity (Mw 20-22 kDa, denoted as PP_{20-22}). The PP_{20-22} protein pool was studied to monitor the protease inhibitor content in potatoes as a function of storage time. Samples and extracts were stored at -20°C prior to further analysis.

The patatin and PP_{20-22} content during the storage of whole potatoes was related to the amount of buffer-extractable protein.

Analysis

Dry matter and protein content The dry matter content of potatoes was determined using the weight difference between fresh and lyophilised potatoes which were subsequently dried for 24 h at 105°C. The potato extracts were extensively dialysed at 4°C (Visking V20, Carl Roth GmbH + Co, Karlsruhe, Germany) against distilled water prior to the determination of the nitrogen content (N) using a Micro Kjeldahl assay (AOAC, 1980). All N in the extracts after dialysis was assumed to be of protein origin and the protein content was calculated as 6.25*N. The protein contents of chromatographic fractions was determined using the Bradford assay (Bradford, 1976) taking BSA (Sigma A-7511) as reference.

Polyacrylamide gel electrophoresis (PAGE) Sodium dodecyl sulphate (SDS) PAGE was performed using Gradient 8-25 Phastgels with a Pharmacia PhastSystem according to the description of the manufacturer (Pharmacia Biotech, Uppsala, Sweden).

Capillary zone electrophoresis (CE) was performed with a Beckman P/Ace System 5500 equipped with a Diode Array Detector controlled by Cesight for Windows (Beckman Instruments, Fullerton, USA). Separation was obtained using a 50 µm hydrophilic coated capillary of 57 cm (Celect p150, Supelco, Bellefonte, USA). Pooled fractions after gel filtration were analysed without further sample treatment in a 150 mM sodium citrate buffer pH 3 containing 6 M urea and 0.05% (w/w) methylhydroxyethyl cellulose (MHEC E111-10248; Hoechst, Frankfurt am Main, Germany). The separating voltage was 20 kV, the temperature 30°C, detection was at 214 nm (data collection rate 1 Hz) and injections were carried out by pressure (injection time 5 s). Electropherograms were corrected for background by means of an electropherogram of a protein-free sample obtained under identical conditions.

Optimisation experiments using other eluents such as sodium acetate or sodium phosphate buffers of several strengths under acidic conditions, with various concentrations of urea or MHEC and several temperatures, voltages and injection times as well as basic conditions with reversed polarity of the voltage did not improve the quality of the separation.

Mass spectrometry MALDI-TOF MS analysis in the linear mode was performed using a Voyager DE RP instrument (Perseptive Biosystems, Framingham, USA). Pooled fractions obtained after gel filtration and dialysed (Visking V20, see above) against distilled water were diluted to 10, 1 and 0.1 µmol protein·ml^{-1} and analysed. To this end a mixture of 1 µl sample and 9 µl matrix solution was set to crystallise for 30 min at ambient temperature on gold plated welled plates. The matrix contained 10 mg·ml^{-1} 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, Sigma D-7927, St Louis, USA) in 0.6% (v/v) aqueous trifluoroacetic acid and acetonitrile in a 7:3 (by volume) mixture. External calibration under identical conditions on the same plate as that of the samples was performed according to the description of the manufacturer using insulin, thioredoxin and myoglobulin (Mw 5733, 11673 and 16951 Da, respectively).

Trypsin inhibitor activity Trypsin inhibitor activity of $PP_{20\text{-}22}$ protein was measured in four-fold using the modified Kakade method (Smith *et al.*, 1980).

Lipid acyl hydrolase (LAH) measurements Patatin solutions were diluted to a final concentration of 0.65 µM (0.28 mg·ml^{-1}) in a 30 mM Tris/HCl buffer pH 8.2. In a microtiter plate well 200 µl of a dilution was equilibrated at 30°C for 5 min prior to the addition of 50 µl of a 5.26 µM *p*-nitrophenyl laurate solution in the same buffer (Racusen, 1985). After incubation at 30°C for 5 min the absorbance at 410 nm was measured. The measurement was performed four-fold and the LAH-activity was expressed as specific activity.

Results and discussion

The aim of this study was to extend the knowledge on the presence and biochemical characteristics of individual classes of potato proteins during storage of whole potatoes. This is important for future food applications of potato proteins since it can facilitate processing required to inactivate anti-nutritional factors.
It was suggested (Brierley *et al.*, 1996; Racusen, 1983A) that sprouting is correlated with the protein content of the tubers during storage. Protease activity during storage (Racusen, 1983A) provides the plant with a pool of free amino acids as nitrogen source for sprout formation at the end of dormancy.
Visual observation revealed that the potatoes had not sprouted up to 30 weeks of storage though they had lost firmness. From about 40 weeks of storage, the tubers of all cultivars started sprouting and they were heavily sprouted with a spongy texture at week 47. Thereafter rotting of the tubers occurred.

Extractable protein

The amount of extractable protein as a function of storage time related to the dry matter content for whole potatoes of Bintje, Desiree and Elkana is presented in Fig. 1. Routinely, the extraction procedure resulted in extraction of about 60% of all tuber nitrogen from Bintje and Desiree, whereas from Elkana approximately 55% could be extracted. Both Bintje and Desiree exhibited a gradual decrease of the amount of extractable protein from 0.03-0.035 g per g of dry weight of whole potato to 0.01-0.018 $g \cdot g^{-1}$ over a period of 1 to 47 weeks. Up to about 20 weeks of storage, a similar behaviour was observed for the extractable protein content of Elkana. However, after 20 weeks the amount of extractable protein increased linearly to 0.04 $g \cdot g^{-1}$. About 75% of the extracted nitrogen from Bintje and Desiree was nitrogen of protein origin, whereas for Elkana that value was about 60%. Extraction of 0.03-0.035 g protein$\cdot g^{-1}$ dry weight, from each cultivar, implied an extraction efficiency of approximately 50-60% of the proteins present in the tuber. This may suggest that the extraction efficiency is limited by the disruption of the tuber cells, since the protein-nitrogen is extracted approximately as efficient as the total of protein and non-protein nitrogen. The decrease of extractable protein content of the potatoes was in accordance with literature (Racusen, 1983A), and is reportedly due to metabolic activity. Results of other storage experiments with whole potatoes, not focused on protein components, confirm that potatoes exhibit metabolic activity during dormancy (Kolbe *et al.*, 1994; Gichohi and Pritchard, 1995). Sprouting, occurring from week 40 onward, did not result in an additional decrease of the amount of extractable protein of Bintje and Desiree.
In contrast to the expected decrease, the amount of extractable protein from Elkana increased after week 25. The protein content of Elkana directly after harvest was higher than that of

Bintje and Desiree (results not shown), whereas at short storage times no higher protein extractability was observed (Fig 1).

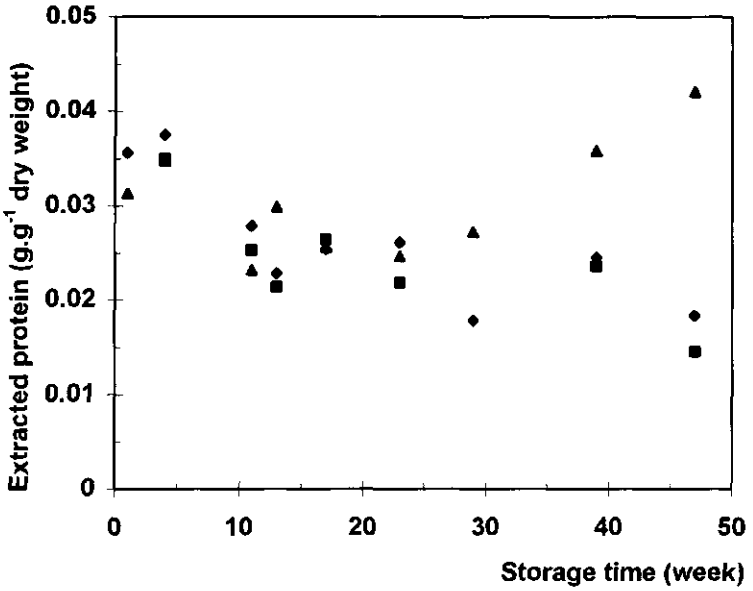

Figure 1 Extractable protein, expressed as non-dialysable N*6.25 per g of dry weight of potato, as a function of storage time of whole potatoes of Bintje (◆), Desiree (■) and Elkana (▲). The error in the measurements is about 10%.

Patatin and protease inhibitor containing fractions

SDS-PAGE analysis revealed that the patatin fractions of each cultivar contained over 95% of a 43 kDa protein (patatin; Racusen and Foote, 1980). The PP_{20-22} protein pools consisted, depending on the cultivar, of one or two proteins in the 18-22 kDa region. Bintje showed a strong 20 kDa band and a weak 23 kDa band, while Desiree exhibited only a 23 kDa band and Elkana showed weak bands at 18 and 22 kDa.

The proportion of patatin and PP_{20-22} in the extracted protein during storage of whole potatoes is presented in Fig. 2. The combined patatin and PP_{20-22} content made up 70 to 80% of all extracted protein maximally. The residual 20-30% of the proteins are removed in the DEAE-anion exchange chromatography step and were not studied further. The proportion of patatin and PP_{20-22} varied between 50-70% and 0-15%, respectively. No significant decrease in the proportion of patatin was observed as a function of storage time. The unexpectedly low patatin proportion of about 40% as observed for Desiree after 47 weeks (Fig. 2) was not interpreted as significant, since it was also seen that the proportion of patatin in Bintje at the same time had increased strangely. At these long storage times the proportions of patatin present in the tuber showed a strange, behaviour which could possibly be due to the sprouting of the potatoes. These results indicate that for the cultivars Bintje and Desiree the patatin

content decreased in time proportionally with all proteins, since the total amount of extractable protein decreased for these cultivars (Fig. 1). This is comparable to the behaviour observed for the cultivar Kennebec (Racusen, 1983A).

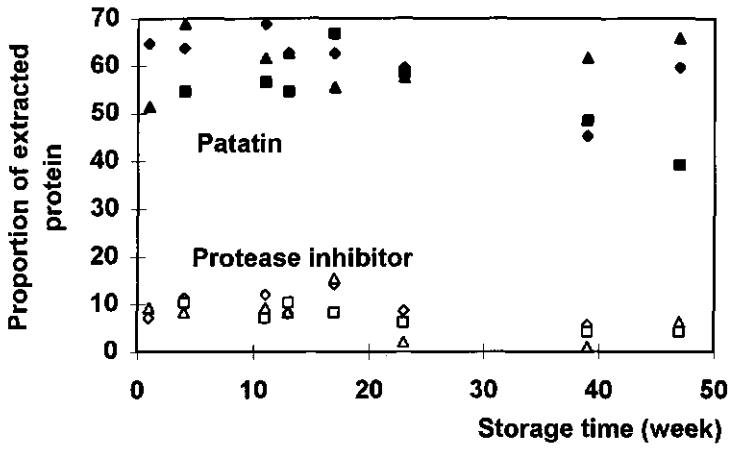

Figure 2 *Patatin (closed symbols) and PP_{20-22} proteins (open symbols) in Bintje (◆), Desiree (■) and Elkana (▲) during storage of whole potatoes expressed as proportion of the amount of extracted protein. The error in the measurements is about 15%.*

The proportion of PP_{20-22} was about 10% from 0 up to 16-18 weeks of storage, decreasing to 0-5% after week 20 (Fig. 2). These proteins decreased more than proportionally with the decreasing amount of total extractable protein. Since the patatin proportion remained virtually constant, the contribution of proteins other than the ones examined here has become bigger. This could be due to a better extractability or a lower extent of degradation in the tuber of these unknown proteins.

In order to further characterise patatin and PP_{20-22} proteins present in the potato tuber, the pooled fractions after gel filtration were subjected to MALDI-TOF MS and CE analysis. Typical MALDI-TOF spectra of patatin and PP_{20-22} protein are shown in Figures 3A and 3B, respectively. Spectra from PP_{20-22} protein were generally of better quality than the patatin spectra (Fig. 3A,B). Results of MALDI-TOF MS analysis of patatin and PP_{20-22} as a function of storage time are presented in Table 1. The spectrum obtained from Bintje patatin showed two peaks, at 40390 and 41690 Da. Furthermore, the corresponding double charged ions (Bahr *et al.*, 1994) can be seen at 20195 and 20845 Da.

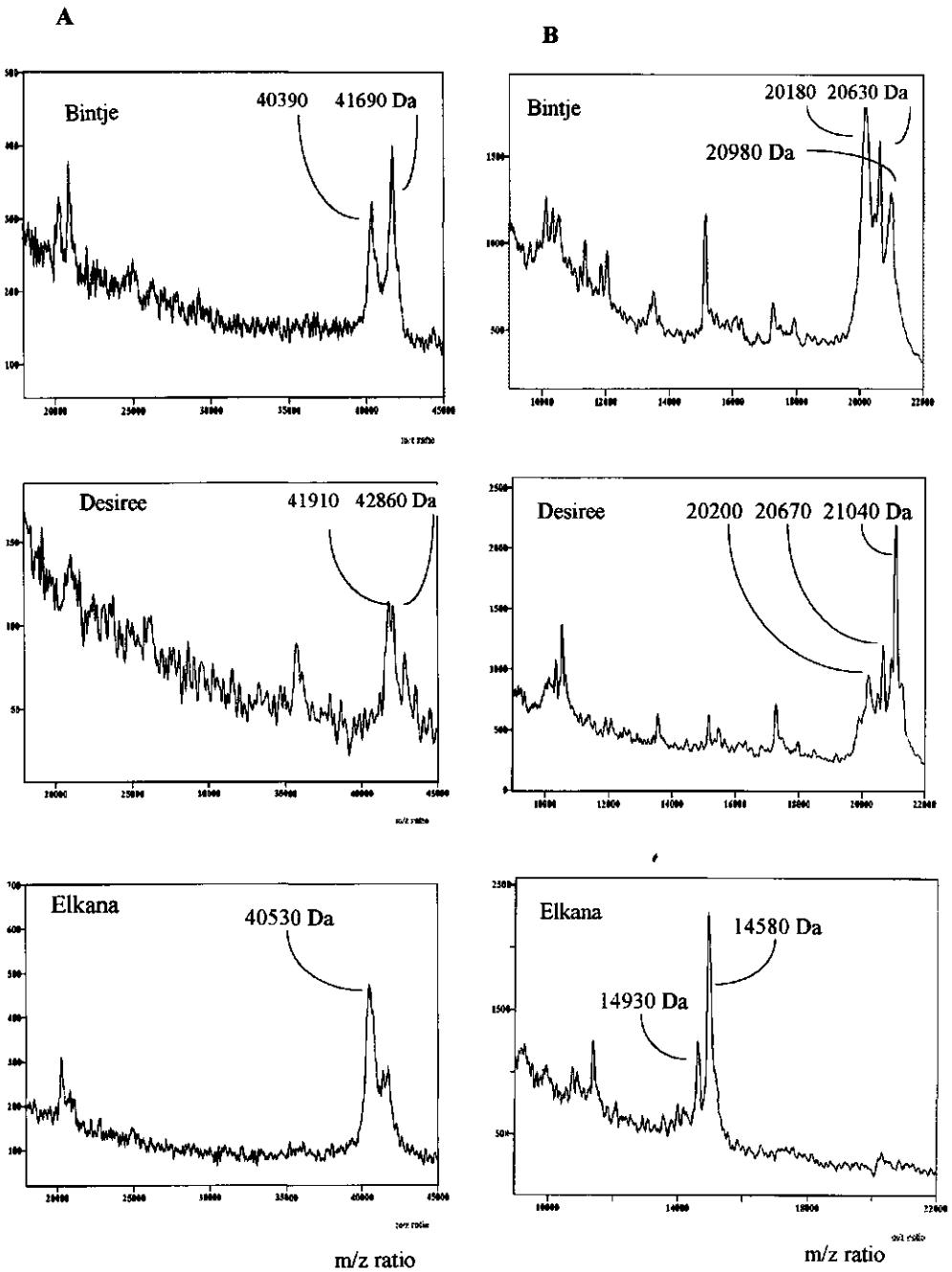

Figure 3 MALDI-TOF mass spectra of Bintje, Desiree and Elkana patatin (A) and PP_{20-22} proteins (B), all obtained from proteins isolated from tubers after one week of storage.

Table 1 Molar masses of patatin isoforms and PP_{20-22} proteins isolated from whole potatoes of Bintje, Desiree and Elkana as a function of storage time

	Masses patatin isoforms (Da ± 0.1%)					
	1 Week		15 weeks		47 weeks	
Bintje	40390	41690	40390	41680	40460	41790[2]
Desiree	41900	42900[2]	-[1]		-	
Elkana	40530		40520		40530	

	Masses PP_{20-22} proteins (Da ± 0.1%)								
	1 Week			15 weeks			47 weeks		
Bintje	20180	20630	20980	20160[2]			20220	20680	20990
Desiree	20200	20670	21040	20190	20670	21070	20180	20700	21100
Elkana	14580		14930	14590		14920	14600		14930

1 no peaks could be obtained

2 poor spectrum, error up to 0.5%

The molecular weights obtained after 47 weeks of storage differed by approximately 70 and 100 Da from the weights of week 1 and 15. This difference was not considered significant since the spectra obtained at 47 weeks were of poor quality. Elkana patatin exhibited one major peak at 40530 Da and two smaller shoulder peaks which could not be assigned. In this spectra the double charged ions are visible at 20265 Da. The spectra of Desiree patatin were of very poor quality, nevertheless an estimation of two masses was made at week one: 41900 and 42900 Da. An unknown peak was observed at approximately 36 kDa. Despite sample preparation and conditions being identical to those for the other cultivars, no responses on MALDI-TOF MS were obtained after prolonged storage of Desiree tubers. Patatin from Desiree is apparently more difficult to ionise as compared to that from Bintje or Elkana. This difference in properties between patatin from Desiree and that from Bintje or Elkana coincides with its CE pattern deviating from those of Bintje and Elkana (Fig. 4, see further discussion below) as well as with its substrate specificity deviating from those of the cultivars Bintje, Elkana and Kennebec (Table 2; Racusen, 1985).

MALDI-TOF MS analysis of the PP_{20-22} protein fraction showed that, in week 1 and 47, three molecular masses could be distinguished for Bintje and Desiree, whereas Elkana showed two masses at all times (Fig. 3B, Table 1). It is remarkable that the masses of the PP_{20-22} proteins of Elkana are clearly different from those of Bintje and Desiree. After 1 and 47 weeks of storage the PP_{20-22} proteins isolated from Bintje showed three peaks around 20180, 20630 and 20980 Da, respectively, from which the 20180 Da peak gave by far the highest response (Fig. 3B). Apart from the double charged ions at the half m/z ratios various other small peaks were observed. These minor components are not discussed further. After 15 weeks of storage only one peak could be observed, at 20160 Da. This peak had shoulders

with similar masses as the samples obtained after 1 and 47 weeks, but these shoulder-peaks could not be assigned exactly. The accuracy of the MALDI-TOF MS data was 0.1% based on measurements using standard proteins (thioredoxin, myoglobulin, cytochrome C). Some of the potato proteins did not exhibit ms spectra of high quality, for unknown reasons. In those cases an error of 0.5% was assumed, based on the standard devation of repetitions. Desiree exhibited three peaks, having comparable masses to those obtained from Bintje. Elkana showed peaks of approximately 14580 and 14930 Da at all storage times.

SDS-PAGE suggested a 43 kDa band for each patatin fraction, which is in reasonable accordance with the results obtained with MALDI-TOF MS. The SDS-PAGE results of the PP_{20-22} proteins, however, are not consistent with the molar masses as obtained with MALDI-TOF MS. MALDI-TOF reveals, due to its better resolution, not only one peak more for Bintje and Desiree, but also a relatively large difference in molecular mass of Elkana PP_{20-22} proteins as compared with SDS-PAGE (18 and 22 kDa on SDS-PAGE and 14.5 and 14.9 kDa with MALDI-TOF). It is possible that the apparent molecular masses of the Elkana PP_{20-22} proteins as obtained with SDS-PAGE are overestimated. Generally, no differences of this size are observed between molecular masses determined with MALDI-TOF MS and SDS-PAGE, nevertheless, in a few papers significant differences have been mentioned (Hedrich *et al.*, 1993; Saito and Shimoda, 1997).

In order to further analyse the different patatin isoforms, patatin samples were examined using free zone capillary electrophoresis (CE). In Fig. 4 electropherograms of patatin isolated from Bintje, Desiree and Elkana are shown.

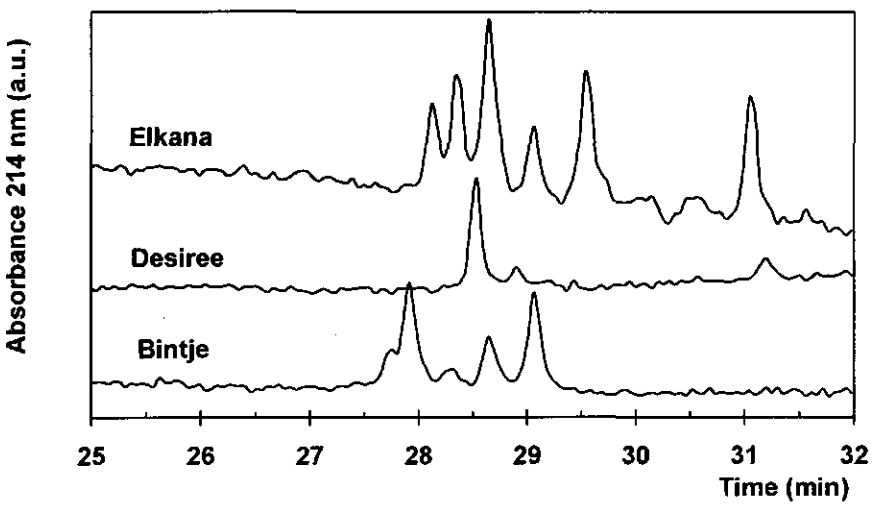

Figure 4 Electropherograms of patatin (0.5-1 mg·ml^{-1}) isolated from Bintje, Desiree and Elkana after one week of storage.

The patatin components of each cultivar had retention times between 27 and 32 min. The electropherogram of patatin isolated from Bintje showed three major and two minor components from 27.5 to 29.5 min. Desiree patatin showed a major and two minor peaks, after 28.5, 29 and 31 min, respectively. Patatin isolated from Elkana could be separated in six components.

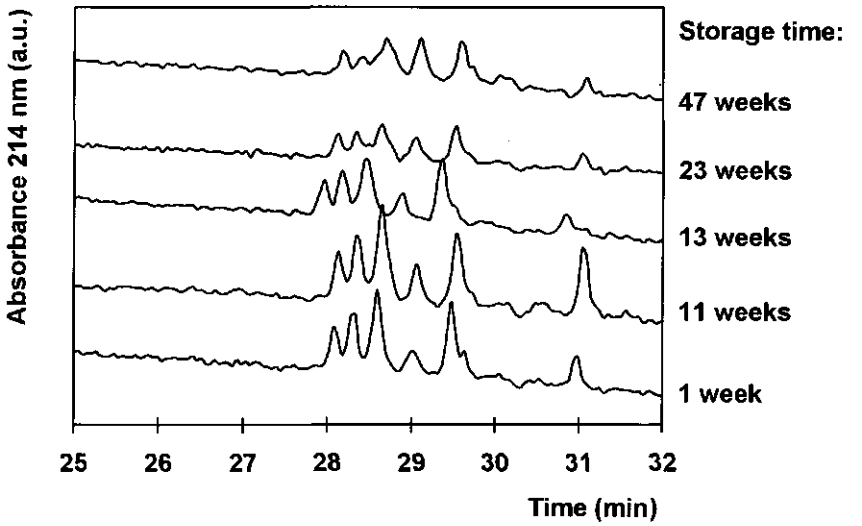

Figure 5 Electropherograms of patatin isolated from Elkana as a function of storage time of whole potatoes.

It is possible that the patatin isoforms exhibit differing stabilities towards enzymic degradation upon storage, similar to for example, genetic variants of milk proteins (Schmidt and van Markwijk, 1993). Therefore, the stability of patatin isoforms was studied. Electropherograms of patatin from Elkana as a function of storage time are presented in Fig. 5. Six peaks were observed at all storage times. The peak at 31 min from the sample after 11 weeks was relatively high, whereas the peak at 28.2 min seemed to decrease after longer storage times. Overall, the electropherograms of Elkana patatin showed only a minor decrease of four of the six peaks present as a function of the storage time of whole potatoes. In addition, when looking at changes between peak area ratios of the different peaks within each cultivar as a function of storage time no significant differences could be observed. The electropherograms of Bintje and Desiree showed even smaller changes in peak area ratios as a function of storage time than Elkana (no further results shown). Therefore, it was concluded that no differences in the stability of patatin isoforms from the three examined cultivars occurred as a function of storage time.

MALDI-TOF MS measurements revealed two masses with a difference between the proteins of about 1300 and 1000 Da for Bintje and Desiree, respectively. Patatin has three possible glycosylation sites, where the isoform examined by Sonnewald *et al.* (1989; cultivar

Berolina) showed 2 glycosylations. The difference observed with MALDI-TOF is of the order of the mass of one carbohydrate-chain as reported for Bintje (Sonnewald *et al.*, 1989). It is possible that, in addition to mass differences caused by point mutations in the primary sequence (Stiekema *et al.*, 1988), one population has two glycosylation sites, whereas the other is glycosylated at one or three positions (Park *et al.*, 1983; Sonnewald *et al.*, 1989). As CE analysis showed more than two peaks for both cultivars, at least one of the two mass populations isolated from Bintje will consist of isoforms. Since patatin of the cultivar Elkana showed one major mass as measured by MALDI-TOF MS (Table 1) and 5-6 peaks using CE (Fig. 4), it is possible that the majority of the patatin isoforms of Elkana has the same type and degree of glycosylation, whereas the point mutations cause the different peaks on CE (Fig. 4).

Bioactivity of patatin and PP_{20-22} fractions

Lipid acyl hydrolase-activity The LAH-activity of patatin ($\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$) and the trypsin inhibiting activity (mg trypsin inhibited·mg $protein^{-1}$) of the PP_{20-22} proteins are presented in Table 2 and expressed as their specific activity.

Table 2 Specific lipid acyl hydrolase (LAH) activity ($\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$) of isolated patatin and trypsin inhibiting activity of PP_{20-22} proteins (mg trypsin inhibited·mg $protein^{-1}$) from whole potatoes of Bintje, Desiree and Elkana as a function of storage time.

	LAH patatin specific activity ($\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$)		
	1 week	15 weeks	47 weeks
Bintje	8.4 ± 0.8	10.2 ± 1.0	0.1 ± 0.05
Desiree	0.7 ± 0.1	0.4 ± 0.1	0.1 ± 0.03
Elkana	5.4 ± 0.8	4.8 ± 0.7	7.2 ± 1.1

	Trypsin inhibition specific activity (mg trypsin inhibited per mg protein)		
	1 week	15 weeks	47 weeks
Bintje	32.5 ± 2.1	37.2 ± 3.5	1.3 ± 0.5
Desiree	30.8 ± 2.9	24.6 ± 2.3	27.3 ± 1.6
Elkana	21.1 ± 1.4	12.4 ± 0.4	10.6 ± 0.9

Directly after harvest, patatin isolated from Bintje exhibited a specific activity about 1.5 times higher than that of Elkana, whereas Desiree LAH activity was about 10 times lower. Differences of this order between cultivars have been reported before (Racusen, 1985). Upon

storage of whole potatoes the LAH-activity of Bintje patatin did not change significantly up to 15 weeks, whereas after 47 weeks, the LAH-activity had decreased dramatically.
Gel filtration in combination with silver stained SDS-PAGE gels (results not shown for both cases) and MALDI-TOF MS analysis (Table 1) indicated that patatin was present at week 47. Apparently, the level of active patatin was low in Bintje after 47 weeks of storage when the tubers were heavily sprouted. Remarkably, the absence of LAH-activity at week 47, coincided with the lack of CE-detectable patatin peaks (results not shown).
The LAH-activity of patatin isolated from Desiree showed a gradual decrease, from a specific activity of 0.7 to 0.1 $\mu mol \cdot min^{-1} \cdot mg^{-1}$protein. No other indications (Figs. 3, 5; Table 1) for denaturation or inactivation could be found, which was similar for Bintje. The activity exhibited by Elkana patatin remained at approximately the same level as a function of storage time.

Trypsin inhibitor activity As a function of storage time, the three cultivars showed different patterns. The specific trypsin inhibiting activity of $PP_{20\text{-}22}$ protein isolated from Bintje showed a decrease to about 4% at week 47, a comparable behavior as was observed for the LAH-activity of patatin (Table 2). Bintje showed a markedly decreased activity after 47 weeks of storage. Desiree did not show a significant decrease as a function of storage time. The trypsin inhibiting activity of Elkana $PP_{20\text{-}22}$ proteins decreased by 40% after 15 weeks, whereas a further decrease was not observed. Cultivar-dependent differences in trypsin inhibiting activity were observed directly after harvesting (Table 2). No studies are reported comparing the trypsin inhibiting activity of proteins from various cultivars.

Summarising, it was seen that, during storage of whole potatoes, a general decrease of the amount of extractable protein occurred. The proportion of patatin revealed no significant differences, whereas that of the $PP_{20\text{-}22}$ proteins decreased gradually during the storage of whole potatoes. All cultivars contained several different patatin isoforms. Bintje and Desiree showed patatin populations with two masses, whereas for Elkana only one molecular mass was found. No inactivation of patatin or protease inhibitors by partial degradation of these proteins was observed, whereas activity assays suggested that the biological activity of especially the cultivar Bintje decreased markedly at the break of dormancy.

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Chapter 3

Isolation and characterization of patatin isoforms*

Abstract

Patatin has so far been considered a homogenous group of proteins. A comparison of the isoforms in terms of structural properties or stability has not been reported. A method to obtain various isoform fractions as well as a comparison of the physico-chemical properties of these pools is presented. Patatin could be separated in four isoform-pools, denoted A, B, C, and D, representing 62, 26, 5, and 7% of the total amount of patatin, respectively. These isoforms differed in surface charge, resulting in different behavior on anion exchange chromatography, iso electric focusing, native poly acrylamide gel - and capillary electrophoresis. All isoforms of the the patatin family contained proteins with two molecular masses, of approximately 40.3 and 41.6 kDa, respectively. This difference in molar mass (1300 Da) is of the size of order of one carbohydrate moiety. Despite the above given biochemical differences no variations in structural properties nor in thermal conformational stability could be observed using far ultraviolet circular dichroism, infrared - and fluorescence spectroscopy.

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Isolation and characterization of patatin isoforms
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Introduction

Patatin accounts for 40-50% of the soluble proteins (Racusen and Foote, 1980). When present in potato fruit juice patatin, like all other proteins, is prone to an irreversible heat precipitation. The mechanism leading to the irreversible precipitation in the applied industrial process is unknown. Knowledge on the structural properties and stability of patatin should help to establish the link between the observed precipitation and possible heat-induced structural changes. This could lead to the design of alternative processes of isolation, resulting in soluble potato protein that can be used in food or alternative applications. Patatin consists of a group of proteins encoded by two multigene families of which the Class I genes are expressed in relatively high amounts, exclusively in the tuber; Class II genes are expressed at a low level throughout the whole plant (Pikaard et al., 1987). Due to its high accumulation in the tuber patatin is considered as a storage protein (Racusen and Foote, 1980; Rosahl et al., 1986). It has a lipid acyl hydrolase activity for both wax ester formation and lipid deacylation (Dennis and Galliard, 1974). It has been suggested that this activity may have a role in the plant defense mechanism (Racusen, 1983B).

Based on the high degree of homology within the gene families and the identical immunological responses (Park et al., 1983; Mignery et al., 1984) patatin was considered as a group of single protein species. A few papers have been published covering the genes encoding patatin, which present also some information on the individual isoforms (Park et al., 1983; Twell and Ooms, 1988; Stiekema et al., 1988; Höfgen and Willmitzer, 1990), but a comparison in terms of structural properties or stability has not been reported. Furthermore, no papers describe the purification and subsequent characterization of patatin isoforms from the whole patatin family.

This paper presents a method to obtain various isoform pools as well as a comparison of the biochemical and the physico-chemical properties of these isoform pools in relation to those of the patatin family. Our study adresses the question whether patatin can be studied as a whole, or that isolated isoforms need to be examined individually.

Materials and Methods

Purification of the patatin family

The patatin family was isolated from the variety Bintje (Solanum tuberosum, harvest 1995) as described in Chapter 4 applying consecutively DEAE, concanavaline-A-Sepharose (con-A) and gelfiltration chromatography,.

Purification of patatin isoforms

Patatin isoforms were isolated using the procedure applied for the patatin family, which was modified after the con-A affinity chromatography step (see Chapter 4). The patatin containing fraction which eluted from the con-A column (denoted Pool II, 216 mL, 1.1 mg

protein/mL in a 30 mM Tris-HCl buffer pH 8 containing 0.5 M NaCl and 2 mM sodium azide) was diluted to 800 mL with a 30 mM Tris-HCl buffer pH 8 containing 2 mM sodium azide. Next, the diluted Pool II was applied to a Source Q column (d x h: 6 x 10 cm; Pharmacia Biotech, Uppsala, Sweden), equilibrated and run at 60 mL/min at 20°C with the above described buffer using an Äkta explorer 100 (Pharmacia Biotech, Uppsala, Sweden). All protein from Pool II bound to the column material and was eluted using a NaCl gradient in the buffer described above applying the following gradient: 0-560 mL: 0 M; 560-1960 mL: 0.16 M; 1960-2240 mL: 0.16 to 0.18 M; 2240-3080 mL: 0.18 to 0.3 M; 3080-3500 mL: 0.3 to 0.5 M; 3500-4200 mL: 1 M NaCl. Detection was at 280 nm and fractions of 22 mL were collected. Appropriate fractions were combined in 4 pools, A-D. The fractions A, B and D were diluted to a NaCl concentration of 0.15 M using the equilibration buffer and re-applied to the column. Fraction C represented a very small proportion of the total protein and was neither purified nor characterized further. After re-chromatography fraction B was eluted as one peak with a NaCl gradient of 0.16 to 0.25 M in 1400 mL. Fraction D was re-eluted as two peaks with a NaCl gradient of 0.16 to 0.3 M in 1400 mL which were separately re-applied to the column and eluted, using the same NaCl-gradient as in the previous run, resulting in single peaks (D1, D2) for each protein. Pool D1 was denoted as Pool D or isoform D from now on, since it was shown that pool D2 contained a 22 kDa protein (MALDI-TOF MS) and was not characterized further. This 22 kDa protein was removed by gel filtration chromatography in the standard isolation procedure for the patatin family (Chapter 4). The protein in Pool A was re-eluted as a single peak using a NaCl-gradient of 0.16 to 0.3 M in 840 mL.

Biochemical analysis

Protein content was determined with the Bradford assay (Bradford, 1976) using bovine serum albumin (Sigma, A-4503) as a standard.

SDS-PAGE and IEF were performed with a Pharmacia PhastSystem according to the instructions of the manufacturer using Gradient 8-25 and IEF 4.5-6 Phastgels, respectively. Gels were stained using Coomassie Brilliant Blue.

Lipid acyl hydrolase activity of the patatin family and isoforms A, B and D were determined as described in Chapter 4 using *p*-nitrophenyl laurate as a substrate.

The N-terminal sequence of the first 15 amino acids of the protein was determined according to the Edman procedure using an Applied Biosystems Protein sequencing system.

Matrix assisted laser desorption-ionisation-time of flight (MALDI-TOF MS) analysis was performed on a Vision 2000 instrument (Thermo Bioanalysis, Hemel Hampstead, UK). The matrix used was a 9:1 (v/v) mixture of 10 mg/mL 2,5 dihydroxybenzoic acid in 0.1% TFA and 10 mg/mL 2-methoxy 5-hydroxybenzoic acid in ethanol. Detection was performed of 1 µl of a 10 µmol patatin/mL solution in 10 µl matrix solution in the linear mode.

Capillary electrophoresis (CE) was performed of the patatin family and the isoforms A, B and D as described in Chapter 2 using a coated capillary (57 cm x 50 µm, Celect p150,

Supelco, Bellefonte, USA) in a 150 mM sodium citrate buffer pH 3 containing 6 M urea and 0.05 % methyl-hydroxyethyl cellulose (MHEC, E111-10248; Hoechst, Frankfurt am Main, Germany). The injection time was 10 s.
Reversed phase HPLC (Thermo Separation Products, Fremont, Ca, USA) was performed using a Hi-pore RP 3-18 column (2.4-250 mm; cat. no. 125-12551, Biorad, Hercules, Ca, USA). Protein was injected via a 200 µl loop and eluted at 0.8 mL/min using a acetonitril gradient in millipore water as obtained by mixing solvent A (90% v/v millipore water, 10% v/v acetonitril and 0.1% v/v trifluoro-acetic acid) and B (10% v/v millipore water, 90% v/v acetonitril and 0.07% v/v trifluoro-acetic acid) in the following linear gradient steps: 0-45 min: 58 to 50% A; 45-50 min: 0 % A; 50-55 min: 58% A. Detection was at 220 and at 280 nm. Samples containing protein were collected manually in fractions of approximately one mL and analyzed by MALDI-TOF MS.

Structural analysis

Far-ultra violet circular dichroism (far-UV CD) -, tryptophan fluorescence - and Fourier transform infrared (FT-IR) spectra were recorded in 30 mM sodium phosphate pH 8 solutions containing the patatin family and isoforms A, B and D (about 0.1 mg/mL protein) at 20°C as described in Chapter 4.

Results

Fractionation into isoform pools

In Figure 1 the elution profile of Pool II on a Source Q column is shown. It can be seen that Pool II is fractionated into four major pools, A-D. Fraction A was eluted as a very broad peak during isocratic elution with 0.16 M NaCl. Fraction B eluted from the column 0.16-0.18 M NaCl. The fractions C and D (two major sub-peaks) eluted at 0.18 and 0.25 M of NaCl. The Pools A, B and D were subsequently re-applied to the column and eluted as single peaks (results not shown) A, B, D1 and D2, respectively. As stated before, Pool C represented a very small proportion of the total protein and was not studied further and pool D2 was not patatin. Pools A, B and D1 showed a 43 kDa band on SDS-PAGE and exhibited the lipid acyl hydrolase activity characteristic for patatin (Table 1; Galliard and Dennis, 1974; Racusen and Foote, 1980). Based on their contributions to the absorbance at 280 nm the composition of the patatin family was 62% of pool A, 26% of pool B, and 5% and 7% for the isoforms C and D1, respectively. The pools A, B and D1 will be denoted from now on as isoform A, B and D, respectively.
Isoelectric focusing (IEF) of the patatin family showed six bands ranging from pH 4.6 to 5.2, where the isoform A showed two bands, at pH 5.0 and 5.2, respectively (Table 1). Isoform B contained two bands (pH 4.6 and 4.7) and isoform D gave one band at pH 4.7. Native PAGE of the patatin family, containing all isoforms, revealed two bands, whereas the isoforms A, B

and D showed a single band (Table 1). Isoform A exhibited the upper band of the two observed in the patatin family, whereas isoforms B and D contained the lower band.

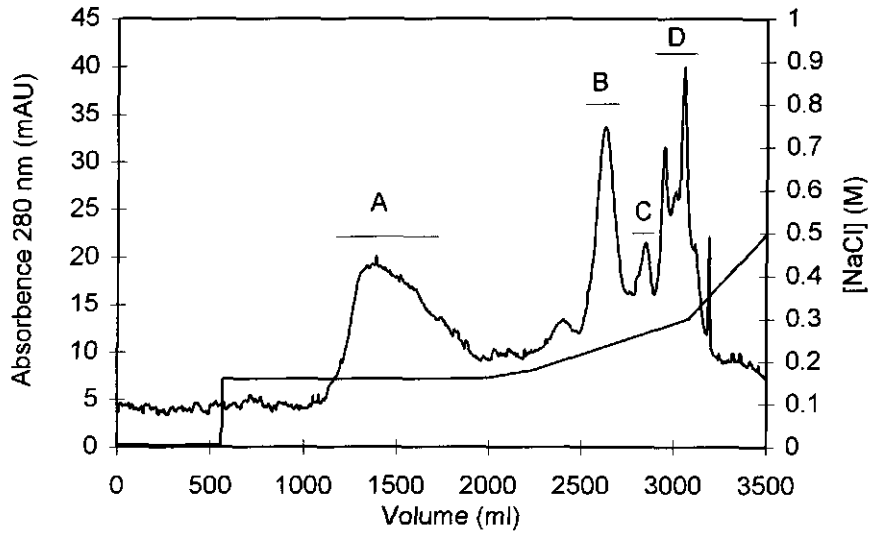

Figure 1 Elution profile of patatin isoforms on a Source-Q anion exchange column using a NaCl-gradient in a 30 mM Tris-HCl buffer pH 8 containing 2 mM sodium azide.

Table 1 Biochemical properties of the patatin family and isoforms A, B and D.

	Patatin family	Isoform A	Isoform B	Isoform D
SDS-PAGE	43 kDa	43 kDa	43 kDa	43 kDa
IEF-PAGE	6 bands	2 bands	2 bands	1 band
	pH 4.6-5.2	pH 5.0; 5.2	pH 4.6; 4.7	pH 4.7
Native PAGE	2 bands	1 band	1 band	1 band
		(upper)	(lower)	(lower)
MALDI-TOF MS	40345 Da	40405 Da	40330 Da	40473 Da
	41590 Da	41631 Da	41599 Da	41703 Da
LAH-activity[1]	3.72 ± 0.14	3.66 ± 0.08	3.55 ± 0.12	3.80 ± 0.12

[1] Specific activity in $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein ± standard deviation

Biochemical properties

A number of analytical techniques were applied to examine the differences between the isoforms. All isoforms and the patatin family showed two distinct molecular masses with MALDI-TOF MS, of approximately m/z 40390 (std. error ± 65) Da and 41630 (std. error ± 50) Da, respectively (Table 1). The intensities of the peaks as observed with MALDI-TOF MS for these molecular masses were essentially the same (results not shown). No significant

differences in lipid acyl hydrolase activity between the patatin family and the patatin isoforms were observed (Table 1). The N-terminal sequence of the first 15 amino acids was Thr Leu Glu Glu Met Val Thr Val Leu Ser Ile Asp Gly Gly Gly and both the patatin family and the isoforms showed mutations at the first (Thr/Lys) and the third (Glu/Gly) position.
In Figure 2 the electropherograms are presented of the patatin family and the isoforms A, B and D, normalized for the protein content of the samples. It can be seen that the patatin family exhibits three major peaks (Rt: 30.7; 31.5; 32.1 min) and at least five minor peaks (Rt: 30.0; 30.4; 31.2; 32.2 [shoulder of 32.1 min major peak]; 33.7 min).

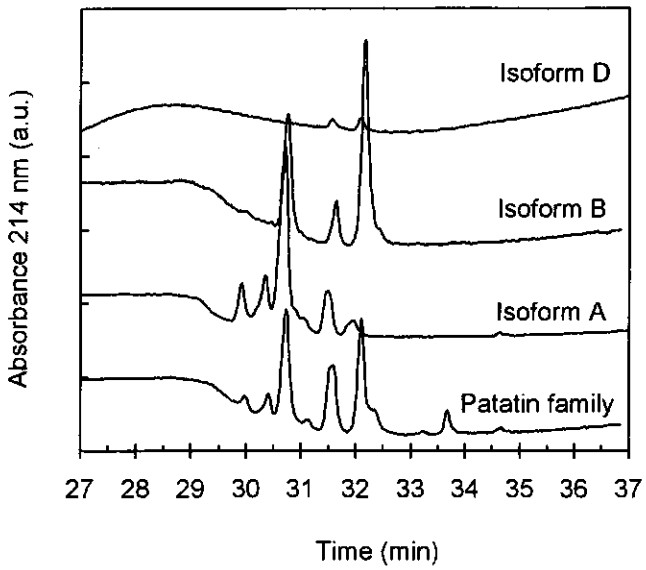

Figure 2 Electropherograms normalized for the protein concentration of the patatin family and the isoforms A, B and D. Samples were separated using a coated capillary (57 cm x 50 μm) in a 150 mM sodium citrate buffer pH 3 containing 6 M urea and 0.05% MHEC.

Isoform A contains one major peak (Rt: 30.7 min), and four minor peaks (Rt: 30.0; 30.4; 31.5; and 32.0 min). Isoform B exhibits all three major peaks which are observed in the patatin family, whereas the minor peaks are virtually absent. Isoform D shows only two minor peaks at 31.5 and 32.1 min, the limited contribution of this protein fraction to the total amount of protein in the patatin family (Fig. 1) obstructed further analysis of this isoform.
The patatin family could be separated into about 10 peaks using reversed phase HPLC (RP-HPLC). Major peaks were observed around 15, 19 and 39 min (Figure 3), minor peaks eluted after 12 and 23-28 min. Isoform A exhibited two major peaks, at 15 and 19 min. It showed minor peaks that were significantly lower as compared to those of the patatin family and the peak at 38 min as observed in the patatin family is absent. Isoform B showed two major peaks around 15 and 19 min. The peaks at 9-12, 22-28 and especially the one at 39 min were significantly lower as compared to those of the patatin family, as was observed for isoform A

as well. The major peak at 19 min of isoform B seems to consist of two proteins, as can be seen from the shoulder peak. The broad peaks around 19 min of the patatin family and isoform A allow to speculate about the presence of at least two proteins in these peaks also. The top of the major peak at 19 min of isoform A seems to be at shorter retention times than those of the patatin family and isoform B, it could be possible that isoform A contained relatively more of the proteins causing the shoulder peaks at 19 min in the patatin family. To investigate whether the isoform pools could be separated into mass-isomers, the major peaks of the patatin family (15, 19 and 38 min) and the isoforms B and A (15 and 19 min) were collected and the molecular mass of the corresponding protein was determined with MALDI-TOF MS.

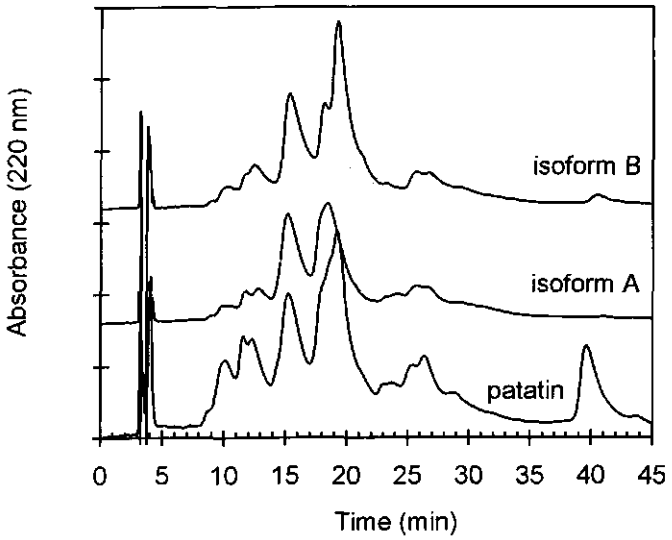

Figure 3 RP-HPLC chromatograms of the patatin family and patatin isoforms A and B on a Hi-pore 3-18 reversed phase column.

Table 2 MALDI-TOF MS analysis of RP-HPLC fractionated patatin family and isoforms

Sample	Retention time (min)	Molecular mass (Da)
Patatin family unfractionated	-	40345 41590
Patatin family fractionated	15 min	40444 41576[1]
	19 min	n.d.
	38 min	n.d.
Isoform A	15 min	n.d.
	19 min	40489 41607
Isoform B	15 min	40377 41532
	19 min	40425 41589

1: error 0.5%
nd: no peaks could be detected.

In Table 2 molecular masses are presented of the unfractionated patatin family, the RP-HPLC fractionated patatin family and isoforms A and B. All RP-HPLC fractions giving peaks using MALDI-TOF MS exhibited the two mass isomers as observed for the unfractionated patatin family. The reason for the difference in molecular masses apparently does not influence the interactions of the protein with the column material, i.e. the mass isoforms are not separated using RP-HPLC. The molecular mass of the corresponding proteins of all major RP-HPLC peaks could not be established and could possibly be due to aggregation of the proteins. This aggregation could be due to polarity and pH of the solvent (acetonitril in water containing TFA), since the protein unfolds at acidic pH-values (Chapter 5). This could allow aggregation and resulted in very low responses in the MALDI-TOF mass spectrometer. These low responses caused the relatively high error in the measured molecular mass.

Structural properties and thermal stability

To study possible differences in conformation and structural stability between the isoforms far-UV CD, FT-IR and fluorescence spectra were recorded at ambient temperature and as a function of temperature. In Figure 4 far-UV CD spectra are shown of the patatin family and the isoforms A, B and D.

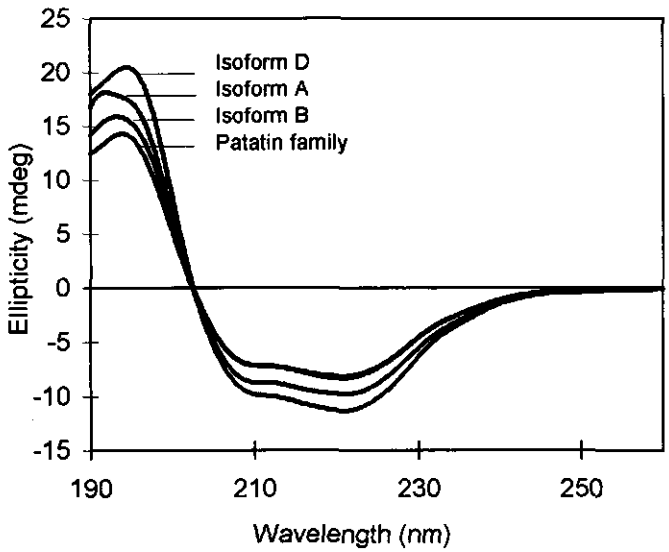

Figure 4 The secondary structure of the patatin family and isoforms A, B and D as determined using far-UV CD.

All spectra exhibit the same extrema at 195, 210 and 222 nm, as well as a zero crossing at 203 nm. This indicates that all isoforms have a highly identical secondary structure (Johnson, 1988). These results were confirmed with FT-IR spectroscopy. The amide I region of all

isoforms showed similar shape and a maximum at about 1645 cm^{-1} (results not shown). Also, the wavelength of the maximum intensity (335 nm) and the shape of the tryptophan fluorescence spectra were similar for all isoforms and the patatin family (results not shown). This implies that there are no conformational differences between the isoforms at a tertiary level of folding of the tryptophan environment (Pace et al., 1988) at ambient temperatures.
In Figure 5 the ellipticity is shown of the patatin family and the patatin isoforms A, B and D at 222 nm as a function of temperature. No significant differences in the thermal structural stability were observed between the isoforms, since all isoforms showed a similar unfolding pattern resulting in a midpoint of unfolding of 49°C, as previously observed for the patatin family at pH 8 also (Chapter 5). The same unfolding temperatures were obtained with tryptophan fluorescence as a function of temperature (results not shown). Despite the charge differences between the isoforms their conformation and structural stability appears to be similar. These mutations have no influence on the catalytic activity of patatin and neither a conformational nor an effect on the structural stability.

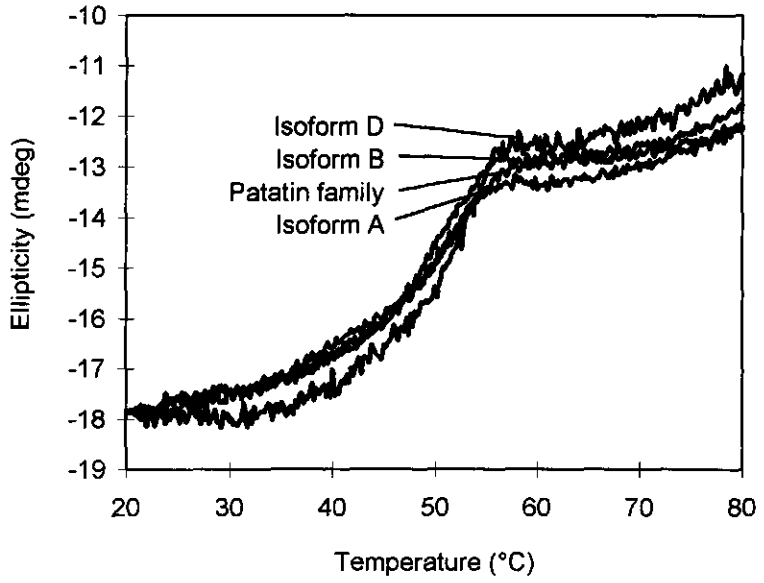

Figure 5 The thermal unfolding of the patatin family and the isoforms A, B and A as determined using far-UV CD. The ellipticity at 222 nm was measured upon heating from 20 to 80°C at a rate of 20°C per hour.

Discussion

The aim of this study was to obtain a procedure to isolate patatin isoforms, in order to answer the question whether the individual isoforms have the same structural properties and stability as the patatin family. If so, it would be sufficient to study the physico-chemical properties of

the whole patatin family instead of those of the individual isoforms. The two major isoforms presented 88% of the protein in the patatin family.

Charge differences between the isoforms

The differences between the patatin isoforms (Figs. 1, 2; Table 1) can be explained by charge differences between the proteins. Isoform A has, for example, at pH 8 the weakest interaction with the anion exchange column (Fig. 1). Furthermore, it exhibits the shortest running distance on native PAGE at pH 8, at similar molecular masses as the other isoforms (Table 1). Apparently, isoform A has the lowest amount of surface charge of all isoforms. Furthermore, isoform A has the highest IEP (Table 1) and the shortest retention time on CE (at pH 3; Fig. 2). This suggests that isoform A either contains relatively more residues that are positively charged at pH 3 (His, Lys) than isoforms B and D, or relatively more residues that are negatively charged (Asp, Glu, His) at pH 8 than isoforms B and D, or both. The presence of patatin isoforms with mutated charged amino acids has been described in literature. The primary sequences of two patatin isoforms from the cultivar Bintje as obtained from the cDNA sequence show one mutation which can have effect on the net charge of the protein (mutation Asp/Asn_{355}; Stiekema et al., 1988). The isoforms from cultivar Superior showed four mutations involving charged residues (Gly/Glu_3, Val/Glu_{215}, Asn/Asp_{226}, Glu/Gln_{250}; Mignery et al., 1984). The first mutation (Gly/Glu_3) of charged amino acids was also observed in this research. So, it occurs not only in the cultivar Superior but also in the variety Bintje. Sequencing of the complete primary structure of the isoforms could establish the presence of these charge differences due to mutations. That was not performed, however, since it would be extremely time consuming, and the behavior, structure and the conformational stability apparently is not influenced by these charge differences. The error in the accuracy of the MALDI-TOF MS measurements was 0.5%, which did not allow assignment of the measured differences between isoforms (Table 2).

Molar mass differences between isoforms

All isoform pools were still a mixture of two mass-isomers (approximately 40.4 and 41.8 kDa; Tables 1 and 2). The differences in molecular masses observed within the patatin family and the isoforms can not likely be explained by mutations in the primary sequence. For example, the two examined isoforms of the variety Superior vary in 21 of the 362 amino acids (94% homology; Mignery et al., 1984). When the differences in the molar massess of the mutated amino acids are summed, a difference of maximal 663 Da can be obtained. The actual calculated difference between two isoforms was found to be 100 Da (Mignery et al., 1984). The same calculations can be performed for isoforms of Bintje. These isoforms show a homology of 97.5% and can have a mass difference due to point mutations of maximal 198 Da (Stiekema et al., 1988). Even if one isoform contains all the amino acids with the lowest

molecular mass, the mass difference can not explain the mass difference observed with the MALDI-TOF MS experiments. This difference (approximately 1.2 kDa) has the size of order of the molecular mass of the carbohydrate antenna of the protein (Man(α1-3)[Man(α1-6)][Xyl(β1-2)]Man(β1-4)GlcNAc(β1-4)[Fuc(α1-3)]GlcNAc, Mw: 1169 Da; Sonnewald et al., 1989). The latter study revealed that patatin has three possible glycosylation sites (Asn at position 37, 67 and 181) of which two were actually glycosylated in the examined isoform (Sonnewald et al., 1989). In analogy to the results found by Sonnewald et al. (1989) it could be possible that one fraction of the protein in the potato is glycosylated at one position and that the other fraction is glycosylated at two positions, whereas the third is not used. The molecular mass of a patatin isoform without the carbohydrate contribution, as calculated from its primary sequence derived from the cDNA, is 39745 Da (Stiekema et al., 1988) or 39564 and 39664 (Mignery et al., 1984). Adding the mass of one, two or three carbohydrate chains would increase the total mass to 40914, 42083 and 43252 Da as observed by Stiekema et al., (1988), or 40733, 41902 or 43071 and 40833, 42002 or 43171 (Mignery et al., 1984), respectively. Comparing the calculated with the measured molecular masses in this study, differences are obtained of 300-500 Da and 100-200 Da for the low and high molecular mass isomer, respectively. These differences could be due to point mutations in the primary sequence, but also to an incorrect presentation of the carbohydrate moiety in literature (Sonnewald et al., 1989) or inaccuracy of the MALDI-TOF measurements.

Structural properties of isoforms

From literature it is known that for other proteins consisting of various genetic variants (for example β-lactoglobulin) differences in behavior between genetic variants exist, like susceptibility to proteolysis or denaturation temperature (Schmidt and van Markwijk, 1993). Patatin does not show a comparable behavior, in this research no differences between the isoform pools in terms of conformation or structural stability were observed. It has also been reported that patatin isoforms show no differences towards proteolytic breakdown in the whole potato during storage (Chapter 2). Furthermore, only minor differences are observed in the substrate specificity of Class I and II patatin (Höfgen and Willmitzer, 1990).

In conclusion, the patatin family, showing 10 peaks with RP-HPLC, can be separated into four pools (A-D) based on surface charge differences. The two major pools, A and C, represent 62 and 26 % of the patatin family, respectively. Within all isoform pools 2 molecular masses were found. Despite differences between the isoforms in surface charge and mass differences within the pools, the latter most likely due to differences in degree of glycosylation, neither differences in biochemical and structural properties nor conformational stability could be observed. The behaviour of patatin can be studied without he need to examine the isoforms individually.

Chapter 4

Heat-induced conformational changes of patatin, the major potato tuber protein*

Abstract

This paper presents the first structural characterization of isolated patatin, the major potato tuber protein, at ambient and elevated temperatures. Isolated patatin at room temperature is a highly structured molecule at both a secondary and a tertiary level. It is estimated from far-ultra violet circular dichroism data that about 33% of the residues adopts an α-helical and 46% a β-stranded structure. Patatin is thermally destabilized at temperatures exceeding 28°C, as was indicated by near-ultra violet circular dichroism. It was shown that parts of the α-helical contributions unfold in the 45 to 55°C region, whereas the β-stranded parts unfold more gradually at temperatures from 50 to 90°C. This was confirmed with Fourier transform infrared spectroscopy. Differential scanning calorimetry indicated a cooperative transition between 50 and 60°C, most likely reflecting the unfolding of α-helical parts of the molecule. Furthermore, fluorescence spectroscopy confirmed a global unfolding of the protein between 45 and 55°C. The observed unfolding of the protein coincides with the inactivation of the patatin enzyme activity and with the precipitation as occurs in the potato fruit juice upon heating. At high temperatures, patatin still contains some helical and stranded structures. Upon cooling the protein refolds partly, it was observed that mainly α-helical structures were formed.

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European Journal of Biochemistry, 1998, vol. 252, p. 66-72.

Introduction

Patatin accounts for about 40% of the soluble potato tuber proteins (Racusen and Foote, 1980). It has a lipid acyl esterase activity for both wax ester formation and lipid deacylation (Dennis and Galliard, 1974). This might play a role in the plant defense mechanisms, although at present its physiological function is not known. Patatin is encoded by two multigene families, mainly expressed in the tuber and throughout the whole plant, respectively. Due to its high accumulation in the tuber it is considered to be a storage protein (Racusen and Foote, 1980; Rosahl *et al.*, 1986).

The amino acid sequence of patatin (362 amino acids) shows neither extended hydrophilic nor hydrophobic clusters (Stiekema *et al.*, 1988). The positive and negative charges of the side-chains are randomly distributed over the sequence. The protein contains 17 tyrosines and 2 tryptophans, the latter being positioned closely together in the primary sequence (residues 254 and 258). It is glycosylated at the 37 and 67 asparagine residues for about 4% (w/w) according to Sonnewald and co-workers (Sonnewald *et al.*, 1989). Patatin has an estimated molecular mass on SDS-PAGE of 43 000 whereas in media without SDS or urea it appears as a dimer with an apparent molecular mass of about 80 000 (Racusen and Weller, 1984).

In the literature, limited information on the structural properties of patatin on a molecular level is present. Lindner and co-workers (Lindner *et al.*, 1980) recorded a far-ultra violet CD spectrum of a patatin-rich fraction and concluded just qualitatively that patatin has a significant amount of α-helical and β-stranded contributions. In two studies the more general effect of heat on potato juice proteins and the ultrastructure of denatured potato proteins has been investigated (Nuss and Hadziyev, 1980; Shomer *et al.*, 1982). These papers describe the effect of heat on the whole potato juice studied by SDS-PAGE, IEF, ultra-centrifugation and transmission- and scanning electron microscopy. Aggregation of the proteins in network-like aggregates was observed.

In industrial processes potato proteins are recovered as by-product of potato starch (Knorr *et al.*, 1977). This is done by an acidic heat-treatment of the so called potato juice and results in irreversibly precipitated proteins which have lost all functionality (Knorr *et al.*, 1977). Consequently, the proteins can only be applied as low-value feed. The mechanism of the irreversible heat-precipitation in the potato juice is unknown. Knowledge on temperature induced conformational changes of the proteins will help to establish the link between the observed precipitation and possible heat-induced structural properties. This could lead to the design of alternative procedures of isolation, resulting in soluble protein that is applicable in food, comparable to for example storage proteins from legumes and oats. Therefore, the aim of this research is to describe the structural properties of native patatin at both ambient and elevated temperatures.

Although numerous proteins have been characterized upon thermal denaturation (Haltia *et al.*, 1994; Taneva *et al.*, 1995; van Stokkum *et al.*, 1995; Conejero-Lara *et al.*, 1996) this

case is of special interest since in literature there is a lack of information on structural properties and thermal stability of plant proteins, as such an under-exposed class of proteins.

Experimental procedures

Purification of patatin

Patatin was isolated according to the procedure of Racusen and Foote (1980) with the modifications as described below. Potatoes used were *Solanum tuberosum* cultivar Bintje (1995 harvest, stored at -40°C). After removal of peel and eyes 10 potatoes were chopped (1x1x5 cm approximately) and mixed. 176 g was transferred to 100 ml of a 30 mM Tris-NaOH buffer (pH 8.0) containing 56.8 mM sodium ascorbate (Buffer A) in a water-cooled vessel at 4°C. The potatoes were homogenized for 5 minutes using an Ultra Turrax (Janke & Kunkel, 170 W) at maximum speed. The headspace of the filled vessel was flushed with nitrogen gas for 5 minutes before and during grinding. After filtration (Schleicher and Schüll; 301310) the potato homogenate was centrifuged at 10 000g for 30 minutes at 4°C, resulting in a clear yellowish extract (2.3 mg protein/ml, 214 ml).

A 300 ml diethylaminoethyl Sepharose CL-6B (Pharmacia Biotech, Uppsala, Sweden) anion exchange column was run at 0.32 cm/min and equilibrated at 4°C with 600 ml Buffer A before 200 ml of the extract was loaded. The column was subsequently rinsed with 530 ml Buffer A and 1400 ml Buffer A without sodium ascorbate. Next, the bound protein was eluted with a 30 mM Tris-HCl buffer (pH 8.0) containing 1 M NaCl. The proteins were detected at 280 nm. NaCl-eluted protein was collected in fractions of 5 ml and pooled (Pool I).

Pool I (80 ml 1.85 mg protein/ml) was loaded on an 150 ml Concanavalin A Sepharose 4B column (Pharmacia Biotech, Uppsala, Sweden). The column was run at 4°C at 0.32 cm/min and equilibrated using 600 ml of a 0.45 μm filtrated 30 mM Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl and 20 mM sodium azide. It was rinsed with 600 ml of this buffer and the proteins were eluted by a linear α-D-methyl glucopyranoside gradient (0 to 1 M in 300 ml) in the same buffer. Detection of the proteins was at 280 nm. Fractions of 5 ml were collected and pooled if their protein concentration exceeded 2 mg/ml (Pool II).

Finally, 80 ml of Pool II (2.85 mg protein/ml) was separated at 4°C on a Sephacryl S-100 HR (Pharmacia Biotech, Uppsala, Sweden) gel filtration column (100 x 2.6 cm) at 0.38 cm/min using a 0.45 μm filtrated buffer containing 30 mM Tris-HCl, 200 mM NaCl and 20 mM sodium azide (pH 8.0). Detection was at 280 nm. Fractions of 10 ml were collected. Fractions containing patatin were pooled, dialyzed at 4°C (Visking V20) against a 30 mM sodium phosphate buffer (pH 8.0) and stored at -20°C.

Protein purity

Protein contents were determined using the Bradford assay (Bradford, 1976) using bovine serum albumin (Sigma, A-4503) as a standard. SDS-PAGE and IEF were performed with a

Pharmacia PhastSystem according to the instructions of the manufacturer using Gradient 8-25 and IEF 4.5-6 Phastgels, respectively. The N-terminal sequence of the protein was determined according to the Edman procedure using an Applied Biosystems Protein sequencing system. Matrix assisted laser desorption-ionisation-time of flight analysis was performed on a Vision 2000 instrument (Thermo Bioanalysis, Hemel Hampstead, UK). The matrix used was a 9:1 (v/v) mixture of 10 mg/ml 2,5 dihydroxybenzoic acid in 0.1% TFA and 10 mg/ml 2-methoxy 5-hydroxybenzoic acid in ethanol. Detection of 10 μmol patatin/ml was performed in the reflector mode.

Spectroscopic measurements

All spectroscopic measurements were performed in a 0.22 μm filtrated 30 mM sodium phosphate buffer (pH 8.0). To investigate the kinetics of the temperature induced conformational changes, CD at 222 nm was measured during heating to 40, 60 and 90°C and for 60 minutes at those temperatures, respectively. It was observed that the signal changed only during heating, but remained constant for the time-scale of the experiments as soon as the temperature became constant (results not shown). In the following experiments the samples were allowed to equilibrate to assure a steady state for 20 minutes at each temperature before measurements were performed.

Far-ultra violet CD Far-ultra violet CD spectra of 0.08 mg patatin/ml were recorded as averages of 10 spectra on a Jasco J-715 spectropolarimeter (Jasco Corp., Japan) at temperatures ranging from 20 to 94°C ± 0.5°C at intervals of approximately 6 degrees. The temperature was measured in the sample using a thermocouple wire. Quartz cells with an optical path length of 0.1 cm were used. The scan range was 260-185 nm, the scan speed was 50 nm/min, the data interval was 0.2 nm, band width was 1.0 nm, the sensitivity was 20 mdeg and the response time was 0.125 seconds. Spectra were corrected for a protein-free spectrum obtained under identical conditions and noise reduction was applied according to the Jasco software. To check the reversibility the samples were cooled to 20°C with 6°C per min and allowed to equillibrate for 20 minutes prior to the measurement. The spectra were analyzed from 240 to 190 nm to calculate the secondary structure content of the protein. Analysis was performed using a non-linear regression procedure (de Jongh *et al.*, 1994) with two prerequisites: (i) the summed square of the differences between the fitted and the recorded curve (RMS) did not exceed 8 and (ii) the calculated protein concentration remained within 5% of the actual protein concentration. Spectra were fitted from 190-250 nm with 1 nm resolution using the reference spectra of poly-lysine in the α-helix, β-strand and random coiled conformation (Greenfield and Fasman, 1969) and the spectrum of β-turn structures, extracted from spectra of 24 proteins with known X-ray structure (Chang *et al.*, 1978). Such a fitting procedure gives the relative contributions of the reference spectra that make up the best fit of the measured spectrum and from which the secondary structure can be calculated. A problem with the interpretation of the far-ultra violet CD data at different temperatures could be the unknown effect of elevated temperatures on the spectra of

secondary structure types used as a reference in the analysis. However, far-ultra violet CD measurements of poly-lysine in the random coil conformation showed no spectral changes at elevated temperatures and the RMS of the fits remained below eight over the whole temperature range (no results shown). The intensity of the CD signal decreases, but the shape of the spectrum remained unchanged.

Near-ultra violet CD Near-ultra violet CD spectra of 0.8 mg patatin/ml were recorded as averages of 25 spectra at temperatures ranging from 20 to 98°C ± 0.5°C at intervals of approximately 6 degrees. Quartz cells with an optical path length of 1.0 cm were used. The scan interval was 350-250 nm, the scan speed was 50 nm/min, the data interval was 0.5 nm, the band width was 1.0 nm, the sensitivity was 20 mdeg and the response time was 0.25 seconds. Noise reduction was applied according to the software supplied by Jasco Ltd. To check the reversibility the samples were cooled to 20°C with 6°C per min and allowed to equillibrate for 20 minutes prior to the measurement.

Fluorescence spectra Fluorescence spectra of 0.08 mg patatin/ml were recorded as averages of three spectra on a Perkin Elmer Luminescence Spectrometer LS 50 B with pulsed Xenon source. Spectra were recorded at temperatures ranging from 22 to 91°C ± 0.5°C at intervals of approximately 6 degrees. Excitation was at 295 nm and the resulting emission was measured from 305 to 405 nm with a scan speed of 120 nm/min. Both the excitation and emission slit were set at 3.5 nm. Spectra were corrected for a protein-free spectrum obtained under identical conditions and smoothed using the software supplied by Perkin Elmer. To check the reversibility the samples were cooled to 20°C with 6°C per min and allowed to equillibrate for 20 minutes prior to the measurement.

FTIR Prior to FTIR measurements, patatin was lyophilized and redissolved in 30 mM sodium phosphate buffer pH 8 in ^2H_2O to a final concentration of 0.8 mg protein/ml and incubated for 3 days at room temperature. Measurement of the enzyme activity of patatin in aqeous environment under the same conditions showed that the protein was stable during this time. Spectra were recorded as averages of 16 scans on a Biorad FTS 6000 Spectrometer equipped with a KBr beamsplitter, a deuterated tri-glycine sulfate (DTGS) detector and an Eurotherm automatic temperature controller. A Graceby Specac heated transmission CaF_2 cell with a 15 μm path length was used to measure at temperatures ranging from 20 to 95°C ± 0.5° C at intervals of 5°C. Spectra were recorded from 400 to 6000 cm^{-1} and stored from 1200 to 2000 cm^{-1}. The nominal resolution was 2 cm^{-1}, enhanced to 1 cm^{-1} by zero filling prior to Fourier transformation. The interferograms were symmetrized and corrected by subtraction of an atmospheric water spectrum and a corresponding protein-free sample, subsequently.

Differential scanning calorimetry

DSC was performed on a Micro-DSC III (Setaram, Caluire, France) using 0.9 ml vessels and a detection limit for transitions of minimal 84 $\mu J \cdot g^{-1} \cdot C^{-1}$. A 0.8 mg/ml patatin solution in a 30 mM sodium phosphate buffer (pH 8.0) was heated from 20 to 100°C with a scan rate of

0.5°C/min, cooled to 20°C with 3°C/min and subsequently re-heated to 100°C with 0.5° C/min.

Lipid acyl esterase activity measurements

Typically, 0.5 ml of 7.3 μg patatin/ml in a 30 mM sodium phosphate buffer pH 8.0 was heated for 15 minutes at temperatures ranging from 30 to 100°C ± 0.5°C with 10°C intervals. After cooling the samples to 30°C, 0.5 ml 5.26 mM para-nitrophenol butyrate (Fluka) in distilled water at 30 °C was added and the absorbance at 410 nm was monitored for 120 s on a Hitachi U3000 Spectrophotometer (Racusen, 1985). The lipid acyl esterase specific activity is determined from the initial conversion rate. The experiments were performed in duplicate.

Results

Characteristics and structural properties of isolated patatin at room temperature

From 176 g of potatoes 94 mg patatin was purified. Gelfiltration chromatography and SDS-PAGE showed only one peak or band, whereas IEF showed multiple bands which could be related to patatin isoforms (Racusen and Foote, 1980; Park *et al.*, 1983). Furthermore, mass spectrometry resulted in two peaks, of molecular mass 40,518 and 41,800 respectively (accuracy better than 0.1%), most probably representing isoforms. According to the literature (Park *et al.*, 1983; Mignery *et al.*, 1984) these isoforms are probably differing in type and degree of glycosylation and in a few mutations in the amino acid sequence (Sonnewald *et al.*, 1989) but behave immunologically and chromatographically identically. At present we are studying isolated isoforms and so far no differences in thermal stability could be observed (see Chapter 5). Therefore, the patatin isoforms will be dealt with as a structural homogenous protein and structural changes during heating will be interpreted as gross differences. The purified patatin-isoforms have the N-terminal sequence Lys/Thr-Leu-Gly/Glu-Glu-Met-Val which demonstrated that the 43 kDa protein was patatin that did not contain the 23 residue pre-sequence other researchers found (Park *et al.*, 1983; Stiekema *et al.*, 1988).

In Figure 1 it can be seen that the far-ultra violet CD spectrum at 20°C (curve a) shows a positive extreme at 195 nm and negative extremes around 210 and 220 nm, respectively. The extreme around 220 nm is larger than the one around 210 nm, suggesting that patatin is a protein with a relative high level of both β-stranded and α-helical regions (Hennessey and Johnson, 1981). An estimation of the secondary structure content of patatin, obtained by curve-fitting analysis, shows that native patatin contains about 45% β-stranded, 33 % α-helical and 15% random coiled structures at 20°C. The amount of β-turn is negligible. At room temperature isolated patatin exhibits an emission maximum at 335 nm, indicating that the tryptophan residues are buried in the hydrophobic core of the protein (see Discussion and Figure 6; Berkhout, 1987).

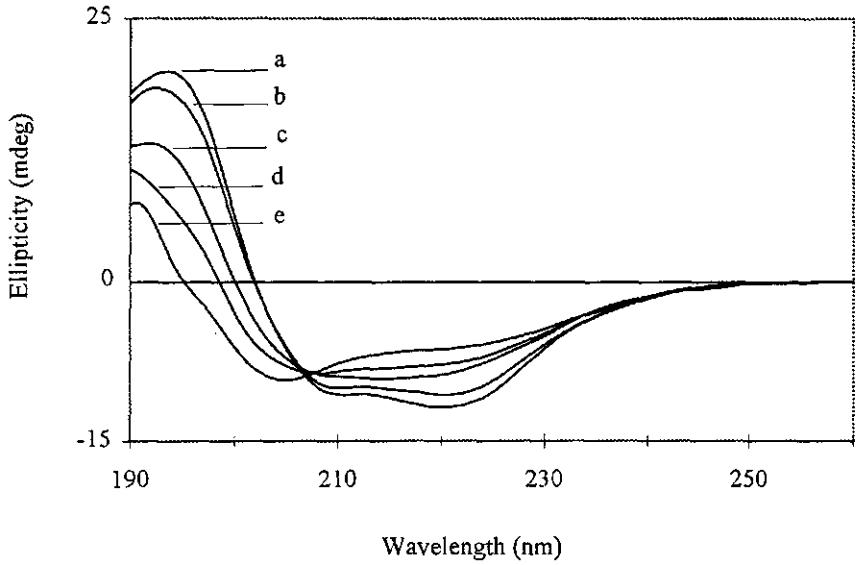

Figure 1 Temperature-induced changes in far-UV circular dichroism of patatin. Spectra recorded at 20 (a), 42 (b), 57 (c), 76 (d) and 94°C (e).

Heat-induced unfolding of the secondary structure

Far-ultra violet CD To analyze heat-induced changes in the secondary structure content of patatin far-ultra violet CD spectra were recorded at various temperatures. A selection of typical spectra is displayed in Figure 1. At temperatures above 20°C the net intensity of the spectra decreases in the 195 and 220 nm regions and the zero crossing shifts to lower wavelengths. This suggests a transition from a structured to a less structured molecule (Johnson, 1988). Curve-fitting analysis of these spectra, as presented in Figure 2, shows that up to 50°C the amount of β-strand does not change significantly, whereas above 50°C a gradual decrease in the β-strand content is observed. The α-helical content of the protein is stable up to about 45°C (33%), but decreases to 23% when the temperature is raised to approximately 55°C. Above 55°C the amount of α-helical contributions does not change significantly. Consequently, the extent of random coiled conformation is constant up to 45°C, increases rapidly from 45 to 55°C, and more gradually above 60°C.

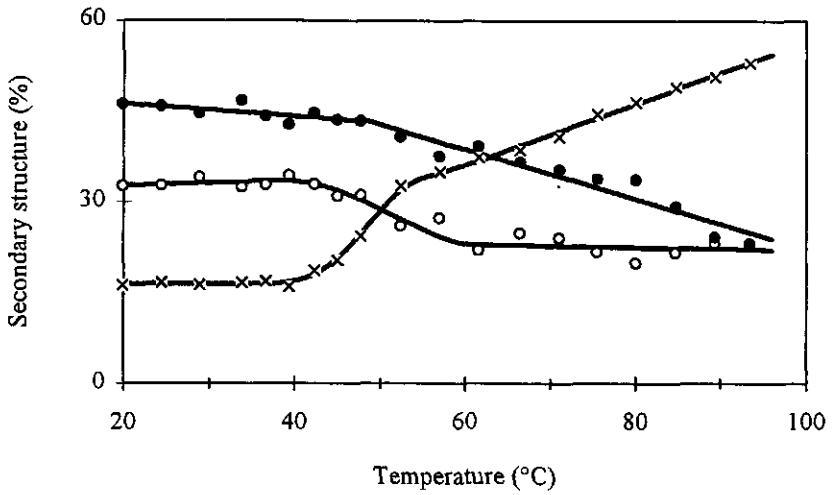

Figure 2 Analysis of far UV-CD spectra shows the secondary structure content of patatin at temperatures ranging from 20 to 94°C, presented as the percentages α-helical contributions (o), β-stranded (•) parts and random coil (×).

FTIR Additional measurements to study conformational changes at a secondary level were performed using FTIR (Susi *et al.*, 1967; Timasheff *et al.*, 1967). Figure 3 shows the amide I region of patatin at various temperatures ranging from 20 to 90°C with 5°C intervals. Generally, random coiled and α-helical parts of the protein exhibit absorptions in the region around 1650 cm^{-1}, whereas the β-strand absorbs in the 1615 to 1635 cm^{-1} region (Goormaghtigh *et al.*, 1994). Up to 50°C the shape of the amide I band remains unchanged, which implies that the secondary structure of the protein is not affected. However, heating from 50 to 55°C results in a decreased intensity in the 1670-1630 cm^{-1} region, whereas in the 1700-1670 and 1630-1600 cm^{-1} regions no significant changes in intensity are observed. This could be explained by an unfolding of helical structures to random coiled structures since the molar absorptivity of a random coil structure is lower than that of the α-helix (De Jongh *et al.*, 1996). At 55°C a shoulder around 1620 cm^{-1} appears, assigned to the contribution of the β-strand which becomes more pronounced with decreasing absorption in the 1670-1630 cm^{-1} region. Upon increasing the temperature to 90°C a gradual decline of this β-strand absorption can be observed. The structural changes observed with FTIR were not quantified in detail since the exact side-chain contributions in this spectral region are unknown. Nevertheless, no changes in the side-chain contributions are expected as a function of temperature, since no change in neither the absorption nor the frequency of the tyrosine ring at 1515 cm^{-1} could be observed. Therefore, the spectral changes can validly be interpreted as conformational changes of the polypeptide backbone.

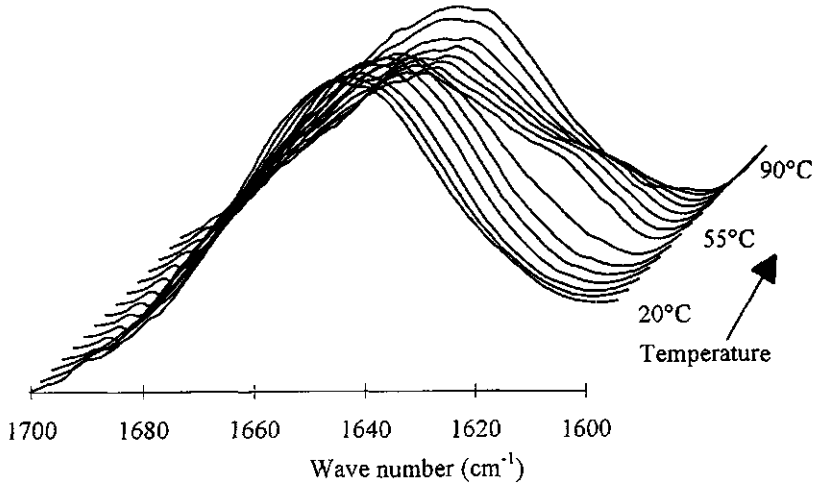

Figure 3 FT-IR spectra of ^2H_2O-buffer dissolved patatin recorded in a transmission cell. The amide I band of patatin is shown at temperatures ranging from 20 to 95°C.

Differential scanning calorimetry DSC measurements were performed to determine the energy content of the heat-induced conformational transitions of patatin (Donovan and Ross, 1975). Patatin exhibits an endothermic transition of approximately 20 kJ/mol between 50 and 60°C (Fig. 4). This temperature coincides with that observed for the unfolding of the protein as observed with CD and FTIR. Although, the observed transition is small, as can be seen from the relatively high level of noise, it is reproducible (results not shown). The slope of the thermogram below 50 and above 60°C is different indicating that heat-capacity before and after unfolding is different (Fig. 4). This suggests a different conformational packing of the protein before and after the transition.

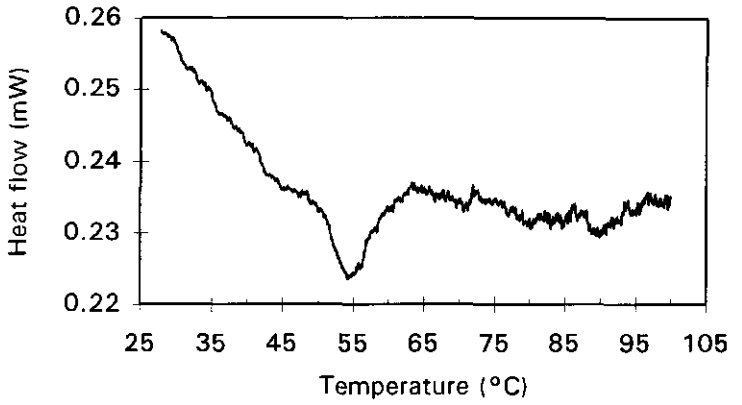

Figure 4 Differential scanning calorimetry of a 0.8 mg/ml patatin solution heated with 0.5°C/min.

Heat-induced unfolding at tertiary level

Near-ultra violet CD To study the effects of temperature on the tertiary level of folding of patatin near-ultra violet CD spectra were recorded. Near-ultra violet CD is a measure for interactions of aromatic rings with other types of functional groups, such as side-chain amide and carboxylate groups and main chain peptide bonds (Hennessey and Johnson, 1981). Phenylalanine shows absorptions in the region of 260 to 270 nm whereas tyrosine and tryptophan absorb around 270-280 and 280-290 nm, respectively. Typically, a decrease of intensity is indicative for a destabilization of the protein conformation (Vuilleumier *et al.*, 1993).

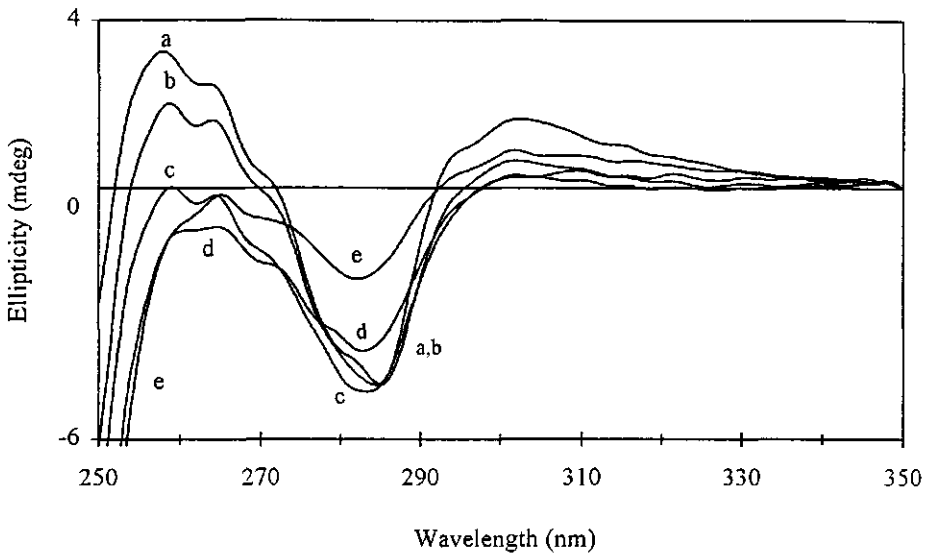

Figure 5 Near UV-CD spectra recorded at 20 (a), 44 (b), 52 (c), 78 (d), 98°C (e).

In Figure 5 a selection of typical near-ultra violet CD spectra is shown. At 20°C the spectrum (spectrum a) shows a positive extreme around 260 nm, a negative extreme about 280 nm and a second positive extreme in the 305 nm region. From temperatures exceeding 28°C (spectrum not shown) up to 52°C the shape of the spectrum is changed compared to the spectrum at 20°C. The intensities in the 260 and 305 nm regions are decreased, whereas the negative extreme around 280 nm remains comparable. This suggests that tertiary interactions are significantly affected from 28°C on. The level of tertiary interactions decreases further at elevated temperatures as can be concluded from the reduced intensities of the spectra in the 280 nm region. Comparison of spectra obtained at 44 and 52°C (spectrum b and c) shows a blue-shift of 3 nm at the minimum about 280 nm, indicating a change of the local environments of the tyrosine and tryptophan residues to a more polar character (Donovan, 1970).

Tryptophan fluorescence An alternative way to study the local environment of the aromatic residues is given by fluorescence spectroscopy (Fig. 6). With increasing temperature the tryptophan fluorescence of patatin displays a decreasing intensity up to 46°C, as expected since this is an intrinsic property of fluorescence (Chang, 1981). As visualized in Figure 6 the spectrum recorded at 55°C shows a red-shift of the emission maximum of 8 nm, from 334 to 342 nm, compared to that recorded at 46°C. Furthermore, the intensity of the signal increases with 23%, despite the opposite intrinsic temperature effect. Above 55°C the emission maximum of the tryptophan fluorescence remains unchanged, but the intensity decreases with increasing temperature, as expected (Chang, 1981).

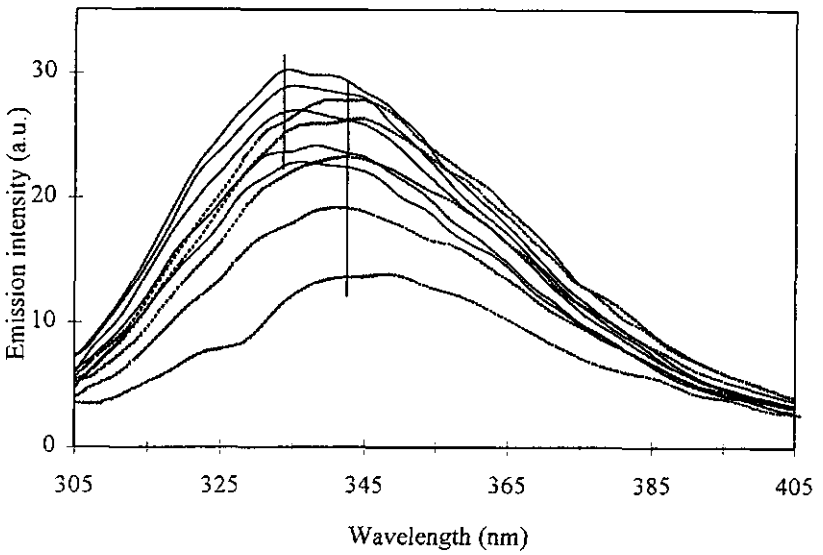

Figure 6 Tryptophan fluorescence of patatin recorded at temperatures ranging from 22 to 91°C. The left vertical bar crosses the emission maxima of spectra recorded at 22, 26, 33, 40 and 46°C, respectively (from top down, solid lines). The right vertical bar indicates the emission maxima of spectra recorded at 55, 63, 71, 76 and 91°C, respectively (from top down, dashed lines).

Reversibility of unfolding

Far-ultra violet CD In order to measure the reversibility of the heat-induced conformational changes the structure of patatin was studied after cooling a sample that had been heated for 30 minutes at 98°C. The secondary structure content was determined by curve fitting analysis of far-ultra violet CD spectra recorded before and after heat-treatment (not shown). After cooling to 20°C, the α-helix content increases to 30%, whereas the extent of β-strand only slightly recuperates (to 26%). Hence, the unfolding is only partial reversible where especially the α-helical parts are able to refold.

Near-ultra violet CD Near-ultra violet CD (results not shown) shows that the spectrum of heat-treated patatin and the spectrum recorded at 20°C both show a comparable intensity and shape in the 350-290 nm region. Furthermore, the shape of the two spectra in the 250-275 nm region is comparable, although the intensity slightly differs. Upon cooling the negative extreme around 280 nm (Fig. 5) remained shifted towards lower wavelengths, implying an irreversible change of the packing of the aromatic side-chains. Apparently, also the near-ultra violet CD spectra demonstrate a partial refolding of the protein after a heat-treatment.

Fluorescence Tryptophan fluorescence was used to study in more detail the repacking of the tryptophan side-chains after cooling of a heat-treated sample of patatin. This repacking was observed for the aromatic residues using near-ultra violet CD. As can be seen in Figure 7 the emission maximum shifts from circa 342 to about 338 nm upon cooling to 20°C. Furthermore, the absolute emission intensity increases about 25%, suggesting a refolding of the local tryptophan environment, but not to its original state at 20°C.

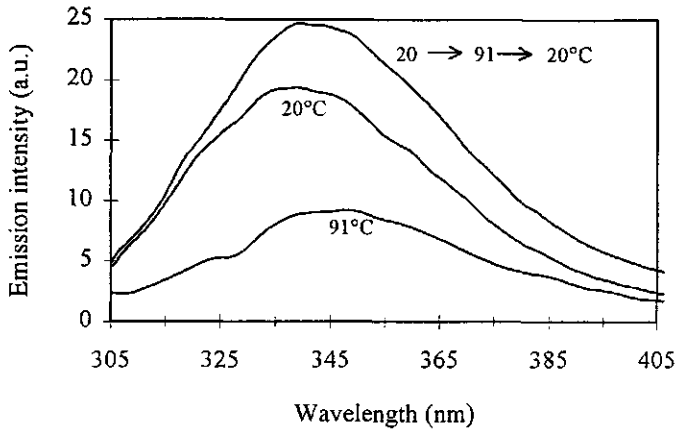

Figure 7 Tryptophan fluorescence of patatin at 20 and 91°C and after cooling to 20°C.

Table 1. Lipid acyl esterase activity of patatin on p-nitrophenyl butyrate measured at to 30°C after heating the protein samples for 15 minutes at indicated temperatures. In brackets the percentage residual activity is presented.

Temperature (°C)	Specific activity (μmol pnp·min^{-1}·mg^{-1} protein)
30	4.54 ± 0.02 (100%)
40	3.74 ± 0.17 (82%)
50	2.13 ± 0.05 (47%)
60	0.17 ± 0.02 (4%)
70	0

Enzyme activity The enzyme activity after heat-treatment was determined as a probe for conformational changes irreversibly interfering with the enzyme activity. The enzyme activity was defined as 100% at 30°C for a non-heated sample of patatin. Heating to 50°C results in a loss of 50% of the activity and incubations at temperatures exceeding 60°C completely destroys the enzyme activity (Table 1).

Discussion

The aim of this research was to describe the thermal unfolding of patatin, to get a better understanding of the heat-induced precipitation of patatin as observed in the potato fruit juice. Isolated patatin does not precipitate when heated, and precipitation in the potato fruit juice only occurs at temperatures exceeding the observed unfolding temperature of patatin (unpublished results; Knorr *et al.*, 1977). Baxter and co-workers suggest that polyphenols and (proline-rich) proteins (patatin: 16 proline residues (Stiekema *et al.*, 1988)) can interact cooperatively to achieve precipitation (Baxter *et al.*, 1997). Since the potato fruit juice contains numerous (poly)phenolic compounds (Lisinska and Leszcynski, 1989) it might be possible that this phenomenon occurs in the potato juice upon heating.

Thermally induced structural changes of patatin

Patatin was found to be a tertiary stabilized (Fig. 5) globular protein and is highly structured, containing an estimated 45% β-stranded and 33% α-helical structures (Fig. 2). The tryptophan fluorescence emission maximum suggests a buried, not solvent exposed, character of the local (tryptophan)-environment at ambient temperatures (Fig. 6).

The behavior of patatin conformation towards higher temperatures can be divided in three phases, which are described subsequently in the next sections. First, at temperatures exceeding 28°C tertiary interactions of patatin decrease (spectra not shown), whereas the polarity of the local tryptophan environment remains virtually constant up to 45°C (Fig. 6). Between 25 and 35°C the enzyme activity on fatty acid-esters of PNP is maximal (Racusen, 1985). It could be possible that the enhanced freedom of mobility of patatin at higher temperatures increases the enzyme activity. The secondary structure content, studied using far-ultra violet CD, remains unchanged up to approximately 45°C (Fig. 2).

Second, at temperatures above 45°C a progressive decrease of tertiary stabilization is observed (Fig. 5) and an unfolding at a secondary level occurs (Fig. 2). From about 45 to 55°C a rapid unfolding of parts of the α-helical structures is observed (Fig. 2), as qualitatively confirmed by FTIR (Fig. 3). The unfolding of α-helical parts could be cooperative since DSC experiments show an endothermic peak around 55°C (Fig. 4). Unfolding of about one third of the α-helices (Fig. 3) results in a transition enthalpy of circa 20 kJ/mol. Globular proteins having a molecular mass of the same order of magnitude as patatin exhibit generally a transition enthalpy of 300-800 kJ/mol (Sen *et al.*, 1992; Muga *et*

al., 1993B). The value of 20 kJ/mol resulting from an unfolding of approximately 10% of the residues as observed for patatin around 55°C is in the same size of order. At temperatures from 46 to 55°C the local tryptophan-environment unfolds and the side-chains become exposed to water (Fig. 6). The fluorescence intensity increases between 46 and 55°C, most probably caused by a lower level of quenching resulting from the (local) unfolding of the tryptophan environment. All techniques used indicate an unfolding of patatin between about 45 and 55°C, which coincides with the loss of lipid acyl esterase activity of patatin (Table 1). Third, at temperatures above 55°C the polarity of the local tryptophan environment remains unchanged (Fig. 6). Since the emission maximum of tryptophan residues directly exposed to water is expected around 350 nm (Schmid, 1989), the emission maximum of patatin above 55°C (342 nm) indicates that the tryptophan side-chains are not fully accessible for water. This is in accordance with the observation that up to 93°C patatin contains a significant amount of helical and stranded structures (Fig. 2). It is assumed that no interference caused by self-association of patatin is apparent with the measurements, since (i) the patatin solution remained visually clear over the whole temperature trajectory, (ii) no protein was adsorbed to the cuvet wall after the measurements, and (iii) no spectral indications for aggregation were observed (CD, IR, fluorescence) (Haltia *et al.*, 1994; Van Stokkum *et al.*, 1995).
No literature is available on structural properties of potato proteins, but a similar unfolding behavior has been observed for other proteins. Van Stokkum and co-workers (1995), for example, described the heat-induced unfolding of human serum albumin and lysozyme. They found a tertiary destabilization of both proteins before a decrease of the intermolecular secondary structure was observed. Heat-induced unfolding of ribonuclease showed at temperatures exceeding 70°C a significant amount of residual secondary structure (Lustig and Fink, 1992).

Reversibility of unfolding

The reversibility of unfolding was studied with far- and near-ultra violet CD, fluorescence spectroscopy (Fig. 7), DSC (Fig. 4) and enzyme activity measurements (Table 1), which results lead to the same conclusion: patatin refolds only partly upon cooling. The polarity of the local tryptophan environment in the native state and after a heat-treatment is comparable. The emission intensity, however, has increased significantly (25%) due to a lower level of quenching of the two tryptophans. This could indicate that upon refolding water is excluded when the protein partly regains its tertiary and secondary structure, but the tryptophan-aromatic side-chains do not refold to their native stacked conformation. This is also indicated by DSC, showing that despite the cooperative unfolding and subsequent partial refolding of particularly the α-helical contributions, no transition could be observed in the second cycle of the DSC measurements. Parts of the protein that are essential for its enzyme activity do not refold to its native conformation.

In conclusion, it was firstly demonstrated that patatin is a highly structured protein at ambient temperatures. Furthermore, we showed that the protein exhibits a tertiary destabilization before it unfolds at a secondary level between 45 and 55°C. This unfolding coincides with the inactivation of its enzyme activity and the precipitation of proteins as observed in the potato fruit juice (unpublished results; Knorr *et al.*, 1977). Further studies need to clarify whether patatin-unfolding is related to precipitation in the potato fruit juice where (poly)phenolic compounds could interact with hydrophobic sites of the partly unfolded protein. We believe that a more detailed understanding of this mechanism is of great importance to food technological purposes, in general since many raw plant materials contain a large variety of phenolic compounds, which might hinder the application.

Acknowledgements

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Chapter 5

The pH-dependence of the structural stability of patatin*

Abstract

This paper presents the structural stability of patatin, the major potato (*Solanum tuberosum* cultivar Bintje) tuber protein. Using far and near-ultraviolet circular dichroism and fluorescence spectroscopy the conformation of patatin was studied under various conditions as a function of temperature. Patatin is a protein which unfolds partly due to either heat- or acid-treatments. When the protein is highly structured at the start of a heat-treatment (near-neutral pH) an apparent two-state thermal unfolding is observed. At low pH, when the starting conformation is already irreversibly unfolded to a certain extent, only minor changes occur upon heating. The residual structures could be part of one or more relatively stable domains. The acidic and the thermal unfolding appear to be similar, but are not identical. These results could contribute to an improved method of isolation, enabling novel food applications of potato proteins.

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Introduction

Patatin accounts for about 40% of the soluble potato tuber proteins (Racusen and Foote, 1980) and demonstrates a lipid acyl esterase activity for both wax ester formation and lipid deacylation (Dennis and Galliard, 1974). Due to its high accumulation in the tuber it is generally considered as a storage protein (Racusen and Foote, 1980; Rosahl *et al.*, 1986; Stiekema *et al.*, 1988).

The amino acid sequence of patatin (362 amino acids) shows neither extended hydrophilic nor hydrophobic clusters (Stiekema *et al.*, 1988). The positive and negative charges of the side-chains are randomly distributed over the sequence. The protein contains 17 tyrosines and 2 tryptophans, the latter being positioned closely together in the primary sequence (residues 254 and 258). It is glycosylated at the 37 and 67 asparagine residues for about 4% (w/w) according to Sonnewald and co-workers (1989). Patatin has an estimated MW of 43 kDa on SDS-PAGE whereas in media without SDS or urea it forms a dimer with an apparent MW of about 80 kDa (Racusen and Weller, 1984).

In Chapter 4 it was shown that patatin at pH 8 at room temperature is a highly structured molecule both at the secondary and the tertiary structure level. It is estimated from far-UV CD data that about 33% of the residues adopt an α-helical and 46% a β-stranded structure. Upon heating at pH 8 parts of the α-helices unfold cooperatively between 45 to 55 °C, whereas the β-stranded parts unfold more gradually at temperatures from 50 to 90 °C. The observed unfolding of the protein coincides with the inactivation of its enzymatic activity and with the precipitation that occurs in the so called potato fruit juice upon heating (Knorr *et al.*, 1977). At elevated temperatures (80-90 °C), patatin still contains some helical and β-stranded structures. Upon cooling the protein refolds partly, where mainly the α-helical structures were able to refold (Chapter 4).

In industrial processes potato proteins are recovered as a by-product of potato starch production (Knorr *et al.*, 1977) and are commonly obtained by a combined acid and heat-treatment of the potato fruit juice resulting in irreversibly precipitated proteins. These precipitates have lost functional properties and, consequently, they can only be applied as a low-value feed. The mechanism of the irreversible heat-precipitation in the potato fruit juice is unknown. Knowledge of temperature induced conformational changes of the proteins under various conditions, such as low pH and high ionic strength, might help to establish the link between the observed precipitation in the potato fruit juice and possible heat-induced structural properties. It could lead to the design of alternative procedures of isolation of potato proteins, which allow the protein to resolubilize and thus enable novel food applications comparable to for example those of storage proteins of oats and legumes. Furthermore, in the literature there is a general lack of knowledge on thermal stability and structural properties of plant storage proteins, which are an under-characterized class of proteins. In this study the pH and ionic strength dependency of the structural stability of

patatin was investigated. Future work will deal with the thermal aggregation and precipitation of patatin.

Materials and methods

Preparation of patatin solutions

Patatin from Solanum tuberosum cultivar Bintje was purified and characterized as described elsewhere (Racusen and Foote, 1980; Chapter 4). After purification, the protein solutions were dialyzed (Visking V20, Carl Roth GmbH + Co, Karlsruhe, Germany) at 4 °C against sodium phosphate buffers at different pH (2.0, 3.0, 4.0, 6.0, 7.0 and 8.0) with a calculated ionic strength of 0.035 M, that was kept constant by using phosphate concentrations of 82, 39, 35, 30, 22, and 13 mM, respectively. These buffers will be denoted as Buffer pH 2, pH 3, etc. In addition, patatin solutions dialyzed as described above against similar sodium phosphate buffers pH 3, 6 and 8 containing 0.165 M NaCl (I=0.2 M) were also used. After dialysis, samples were frozen to - 20 °C in small aliquots till use.

Between pH 4.3 and 5.7 no representative sample of patatin could be obtained since iso-electric focusing and solubility experiments showed that the iso-electric point of patatin is about pH 4.7 (unpublished results).

Spectroscopic measurements

All spectroscopic measurements were performed in 0.22 μm filtered sodium phosphate buffers. Between two measurements the cuvette was cleaned with a 0.18 M potassium dichromate solution in 47% sulfuric acid and subsequently rinsed extensively with distilled water.

Far-ultraviolet circular dichroism Far-UV CD wavelength-scan spectra of 0.1 mg patatin/mL in Buffers pH 2, 3, 4, 6, 7 and 8 were recorded as averages of 8 spectra on a Jasco J-715 spectropolarimeter equipped with a thermostatted cell holder (Jasco Corp., Japan) at 20 °C. Furthermore, 1.0 mL of pH 3 and pH 8 (denoted as: pH 8 heated sample) samples were heated for 15 min at 80 °C in closed glass tubes (13x100 mm Kimax culture tube, Kimble glass Inc., USA) and cooled to 0 °C by placing the tube on ice. Next, the pH 3 sample was brought by dialysis as described above to pH 8 (denoted as: pH 3 heated sample). Quartz cells with an optical path length of 0.1 cm were used. The scan range was 260-190 nm, the scan speed 100 nm/min, the data interval 0.2 nm, the band width 1.0 nm, the sensitivity 20 mdeg and the response time was 0.125 s. Spectra were corrected for a protein-free spectrum obtained under identical conditions and noise reduction was applied subsequently using the Jasco software. Analysis of far-UV wavelength-scan spectra to determine the secondary structure content of the protein was performed using a non-linear regression procedure, as described in Chapter 4.

Far-UV CD temperature-scans were monitored as the ellipticity at 222 nm (for Buffers pH 3, 6, 7 and 8) or 208 nm (for Buffers pH 2, 3, 4) from 20 to 80 °C at a heating-rate of 20 °C per

hour and a step resolution of 0.2 °C. The response time was 16 s, the band width 1.0 nm and the sensitivity was 20 mdeg.

Near-UV CD Near-UV CD spectra of 0.4 mg patatin/mL Buffer at pH 2,4 6 and 8 were recorded as averages of 24 spectra at various temperatures ranging from 20 to 80 °C. Starting from 20 °C the protein solution was heated to the desired temperature and equilibrated for 6 min at that temperature before the wavelength-scans were recorded. A quartz cell with an optical path length of 1.0 cm was used. The scan interval was 250-350 nm, with further similar conditions as described above for far-UV CD wavelength scans.

Fluorescence spectra Fluorescence spectra of 0.1 mg patatin/mL were recorded as averages of three spectra on a Perkin Elmer Luminescence Spectrometer LS 50 B with a pulsed Xenon source and equipped with a thermostatted cell-holder (accuracy ± 0.5 °C). The dimensions of the cuvette were 1.0 x 0.4 cm, with the longer path parallel to the incident excitation light. Spectra were recorded at temperatures ranging from 20 to 80 °C. Excitation was at 295 nm and the emission was measured from 325 to 365 nm (305 to 450 nm for patatin samples at 20 °C) at a scan speed of 120 nm/min. Both the spectral band width of the excitation and emission were set at 3.5 nm. Spectra were corrected for a protein-free spectrum obtained under identical conditions and subsequently smoothed using the software supplied by Perkin Elmer.

Fluorescence of ANS (8-anilino-1-naphtalene-sulfonic acid; Sigma, art. no. A-5144) was measured at 20 °C using the above described apparatus. To 0.5 mL sample in Buffers pH 2-8 (0.2 mg patatin/mL) 10 µl of a 0.722 mg ANS/mL dissolved in the same buffer was added repetitively until the fluorescence intensity became constant. Excitation was at 385 nm, emission was measured from 440 to 520 nm at a scan speed of 120 nm/min. The excitation and the emission slit were 10.0 and 5.0 nm, respectively. After the addition of ANS the sample was equilibrated for two minutes before measurement. The fluorescence emission was expressed as a function of the molar ratio ANS/protein.

Lipid acyl hydrolase (LAH) activity

Patatin solutions were diluted to a final concentration of 0.65 µM in a 30 mM Tris-HCl buffer pH 8.2.

In a microtiter plate well 200 µL of these solutions were equilibrated at 30 °C before 50 µL of a 5.26 µM para-nitrophenyl laurate in the same buffer were added (Racusen, 1985). After incubation at 30 °C for 5 min the absorbance at 410 nm was measured. The experiment was performed four times and the LAH-activity was expressed as the specific activity.

Results

In this study the structural and physico-chemical properties of patatin at 20 °C in phosphate buffers with pH ranging from 2 to 8 were determined. Next, the pH-dependency of the thermal stability of patatin is presented. Since the structure and structural stability of proteins

can be affected by both the type and the ionic strength of the buffer (Brouilette *et al.*, 1987; Griko *et al.*, 1994; Tan *et al.*, 1995; Boye *et al.*, 1996) all sodium phosphate buffers were set to have a constant ionic strength (I=0.035 or in some cases 0.2 M) .

Structural properties of patatin

In Figure 1 it can be seen that far-UV CD spectra of patatin obtained at pH 6, 7 and 8 show a zero-crossing about 203 nm and negative extrema at 208 and, more intense, at 220 nm, which point at a high level of structured conformations (Johnson, 1988; Hennessey and Johnson, 1981). Based on the spectral similarity, the spectra indicate a virtual identical secondary folding of the protein in this pH range (I=0.035M). Results of curve fitting analysis (de Jongh *et al.*, 1994; Chapter 4), as presented in Table 1, show indeed a highly structured protein, containing about 30% and 45-50% helical and stranded structures, respectively.
Spectra recorded at pH 3 and 4 show a smaller negative extreme around 220 nm as compared to those at pH 6 or 8 and furthermore, the zero-crossing of the curves has shifted to 200 nm. This suggests a lower level of secondary structured domains of patatin at pH 3 and 4 compared to the pH range 6 to 8. Indeed, spectral analysis illustrated a decreased level of the α-helix and β-strand content at pH 3 and 4 compared to near-neutral pH (Table 1).

Table 1 Structural stability of patatin as a function of pH and temperature

	20°C					80°C
secondary struct[1)]	pH 2	pH 3-4		pH 6-8		pH 8
α-helix	20	20-25		30		20-25
β-strand	30	35-40		45-50		35-40
random	45	35		15-20		35
	Midpoint of unfolding (°C)					
	pH 2	pH 3	pH 4	pH 6	pH 7	pH 8
secondary[2)]	-	-	-	58	55	49
tertiary	-	-	-	precip.	n.d.	48

1) : Percentage of secondary structured elements

2) : Structure level of the protein

\- : Only minor transitions could be observed

precip. : precipitation occurred

n.d : Not determined

At pH 2 a small decrease in the ellipticity can be observed as compared to the spectrum obtained at pH 3 especially in the 190-200 nm region (Fig. 1). Furthermore, the zero-crossing of the spectrum at pH 2 is located around 197 nm, both indicating a further unfolded protein as compared to pH 3 and 4. Curve fitting analysis as presented in Table 1 supports this conclusion. The presence of 0.165 M NaCl, resulting in a total ionic strength of 0.2 M, had

no influence on the CD spectra at the various pH, hence, on the secondary structure of patatin (results not shown).

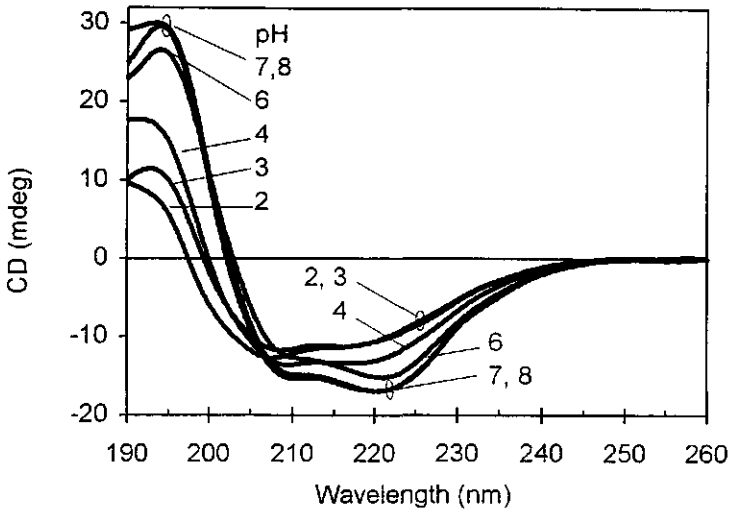

Figure 1 Far-UV CD of 0.1 mg patatin at 20 °C per mL phosphate buffer pH 2-8 (I=0.035M).

Near-UV CD spectra provide a measure for interactions of side-chain aromatic rings with other groups such as side-chain amide and carboxylate groups and main chain peptide bonds and, therefore, are applicable as a measure for the tertiary structure of a protein (Vuilleumier *et al.*, 1993). The near-UV CD spectra obtained at pH 6 and 8, as presented in Figure 2, show similar positive extremes at 292, 270, 263 and 258 nm and negative ellipticities at 277 and 86 nm, suggesting a comparable tertiary fold.

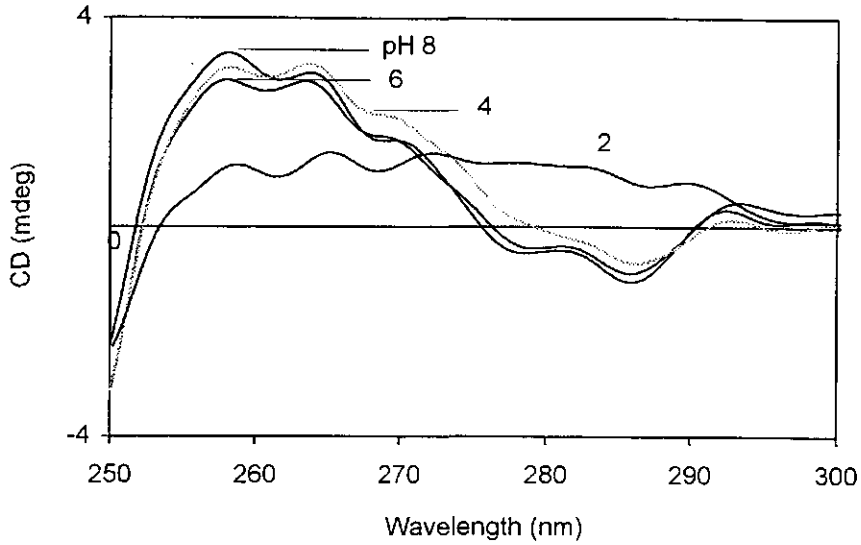

Figure 2 Near-UV CD spectra of 0.4 mg patatin per mL Buffer pH 2, 4, 6, and 8 (I=0.035M) at 20 °C.

The spectrum obtained of patatin at pH 4 diverges in the 270-286 nm region from those recorded at pH 6 or 8. This indicates a different packing of tyrosine and tryptophan residues at pH 4, since CD-bands in this region are typical for these residues (Woody and Dunker, 1996).

Patatin at pH 2 exhibits a completely different spectrum, showing a positive signal from 255 to 290 nm, instead of the negative extreme in the 280-290 nm region observed at pH 4, 6 and 8. A protein with a negligible tertiary structure can still exhibit some ellipticity in the 250-350 nm region (Das *et al.*, 1995; Sarkar and DasGupta, 1996), therefore, it is suggested that patatin at pH 2 is significantly more unfolded than at pH 4, despite the observed ellipticity.

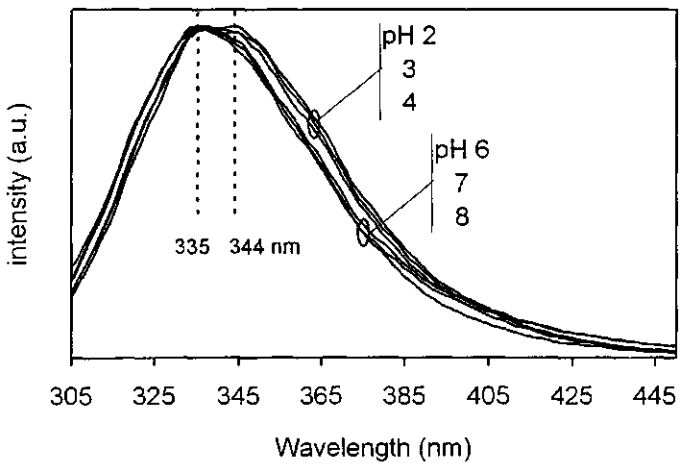

Figure 3 Fluorescence spectra of 0.1 mg patatin per mL Buffer pH 2, 3, 4, 6, 7 and 8 at 20 °C.

Fluorescence spectroscopy can provide information about the polarity of the tryptophan environment, hence, about the solvent-accessibility of the chromophore, being sensitive to local conformational changes at a tertiary level of folding (Pace *et al.*, 1988). Fluorescence spectra of patatin at pH 6, 7 and 8 are identical; both the shape of the emission spectra and the wavelength of their maxima indicate no differences in the tryptophan packing (Fig. 3). Spectra of patatin recorded at pH 2, 3 and 4 also show an identical shape; however, the emission maximum has shifted 9 nm towards higher wavelengths compared to the spectra obtained at pH 6, 7 or 8. This indicates that the polarity of the two tryptophan residues of patatin has changed to a more polar, i.e. more unfolded character. The difference between pH 4 and pH 6 or 8 in the 270-286 nm region, as observed with near-UV CD, is therefore expected to be due to altered interactions of side-chain aromatic groups. No effect on the conformation of patatin by 0.165 M NaCl could be observed at the various pH values (no further data shown).

Fluorescent probes like ANS are widely used as a measure for changes in the exposure of hydrophobic sites of proteins due to heat-treatment or at acidic or alkaline pH values (Andley and Chakrabarti, 1981; Goto and Fink, 1989; Vanderheeren and Hanssens, 1994). Interpreted carefully, they can provide useful additional information on the conformational state of a

protein (Shi *et al.*, 1994). In Figure 4 it can be seen that no differences in the normalized fluorescence intensity of the ANS-protein complex could be observed in the range pH 2 to 8. Apparently, no significant changes in the accessibility of solvent-exposed hydrophobic sites occur in this pH-range. The absolute amount of ANS-fluorescence increased with decreasing pH at identical ANS/protein ratios, whereas an ANS solution in a 50% (v/v) methanol/buffer mixture did not show pH-dependent differences in its fluorescence emission intensity (no results shown). This could be caused by a different interaction of the hydrophobic sites of the protein surface with the fluorescent probe due to a decreased level of electrostatic repulsion.

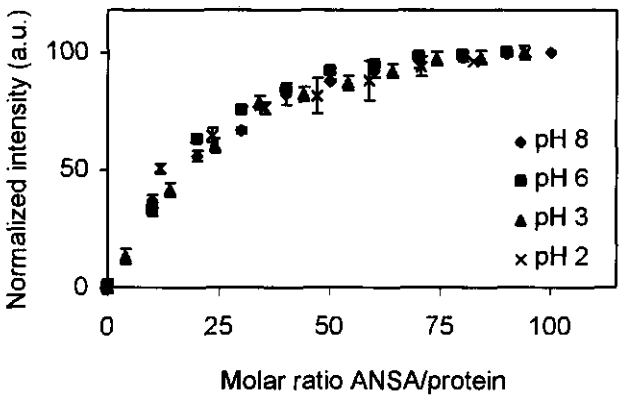

Figure 4 The pH-dependence of the exposed hydrophobic sites of patatin determined as ANS fluorescence. The fluorescence intensity is plotted as function of the ANS-protein ratio at pH 8 (◆), pH 6 (■), pH 3 (▲) and pH 2 (x).

Thermal unfolding

To investigate thermal unfolding of the secondary structure of patatin at various pH, the ellipticity of the protein at 222 nm was measured during heating from 20 to 80 °C (Fig. 5). The protein structure was monitored at that wavelength since at 222 nm the combination of α-helical and β-stranded structures is measured, whereas the influence of random coil conformations is negligible (Hennessey and Johnson, 1981). The CD curve of patatin at pH 8 shows a small linear decrease in the absolute ellipticity up to 35 °C and above 55 °C (Fig. 5A). Between 35 and 55 °C a decrease of about 4 mdeg is observed, suggesting a transition towards a less structured conformation, which is in agreement with previous data (Chapter 4). The first derivative of the unfolding curve, as obtained using the Jasco software, shows a symmetric band with a maximum at 49 °C (Fig. 5B). The symmetry suggests that the unfolding possibly follows a two-state mechanism, since the integral of a symmetric peak forms a sigmoidal curve which can be indicative for a two-state unfolding (Pace et al., 1988). Patatin at pH 7 shows a comparable unfolding behavior as at pH 8, however, the midpoint of unfolding is shifted about 6 °C to higher temperatures (55.5 °C; Figure 5B).

To obtain further evidence that the unfolding could be a two-state process as well as an estimate of the enthalpy of unfolding (ΔH_u), the temperature-curves were fitted with a procedure based on thermodynamic equations (Elwell and Schellman, 1977; Becktel and Schellman, 1987; Pace *et al.*, 1988; Stokkum, van *et al.*, 1995). Identical unfolding curves at both half and twice the above mentioned heating-rate indicated that the unfolding was in equilibrium, being a prerequisite to apply such a fitting procedure (results not shown). In Table 2 it can be seen that the midpoint of unfolding at pH 8 and 7 is at 48.8 °C and 55.4 °C, respectively. This thermodynamic fit of the thermal unfolding curves of patatin at pH 7 and 8 has a small error (χ^2) implying that the conformational change indeed follows a two-step mechanism. The calculated ΔH_u is about 89 and 74 kJ/mol for pH 7 and 8, respectively (Table 2). Interestingly, DSC-results revealed an ΔH_{cal} of only 20 kJ/mol for the cooperative transition between 50 and 55 °C (Chapter 4). This indicates that a restricted part of the protein unfolds in a small temperature range. The wavelength-scan spectra at 80 °C of patatin at pH 7 and 8 indicate a similarly structured protein (results not shown). In both cases the protein is soluble up to at least 80 °C. At pH 8 a continued decrease in the ellipticity is observed above the transition temperature range, which is not exhibited by the protein at pH 7.

Table 2 Parameters related to the denaturation of patatin determined with a thermodynamic fit of the far-UV CD unfolding curves as described in the text.

pH	Tm (°C)	ΔH_u (kJ/mol)	χ^2
8	48.8 (±0.1°C)	73.7 (± 0.97)	2.05
7	55.4 (±0.1°C)	88.7 (± 0.505)	2.95

At pH 6 the behavior of patatin upon heating is clearly different from that at pH 7 or 8. From about 50 °C on, a decrease in ellipticity is observed, just preceding the precipitation of the protein which starts between 52 and 55 °C. A midpoint of unfolding at pH 6 could not be obtained directly from the first derivative of the ellipticity since the decrease in the CD-signal is a combined effect of unfolding and precipitation. However, the photomultiplier voltage recorded simultaneously during the measurement can be converted into an optical density-curve which can be used as a measure for the formation of insoluble light scattering aggregates. When the normalized first derivative of the ellipticity during a thermal experiment is corrected with the normalized first derivative of the corresponding photomultiplier voltage, the resulting derivative can be interpreted as the unfolding curve at pH 6. The result is depicted in Figure 5B where it can be seen that at pH 6, prior to precipitation the protein unfolds with a midpoint of 58 ± 0.2 °C.

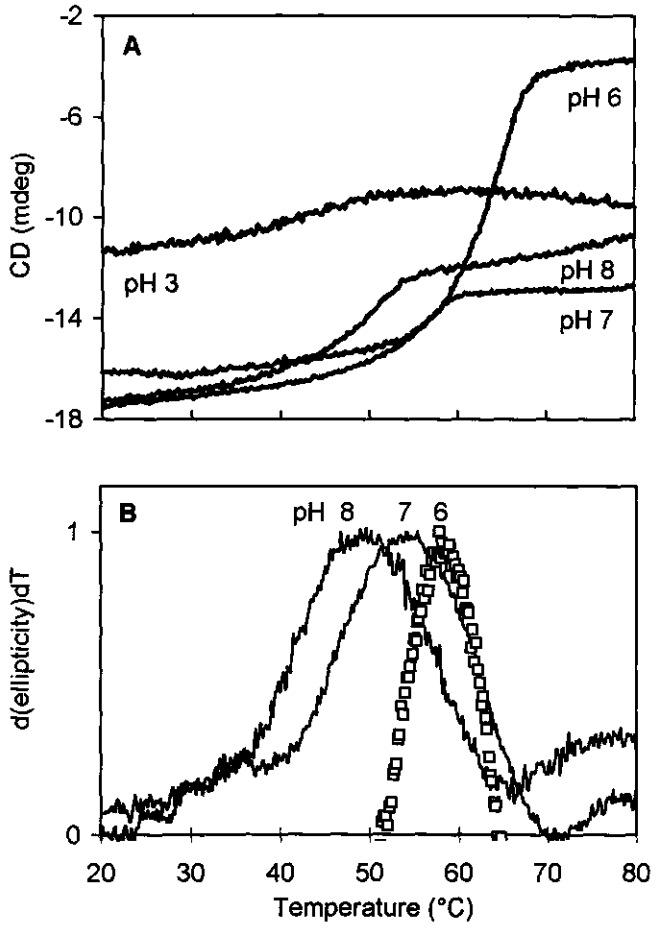

Figure 5 A: Thermal unfolding of patatin in phosphate buffer (I=0.035 M) pH 6, 7 and 8 measured as CD at 222 nm and at pH 3 measured at 208 m. **B:** The first derivative of the pH 7 and 8 unfolding curves and the calculated (see description below) first derivative the unfolding at pH 6 (□)

Heating from 20 to 80 °C has a negligible effect on the CD signal at 222 nm when the protein is in buffer pH 3 (no results presented), whereas around 208 nm a small transition can be seen between 30 and 50 °C (Fig. 5A). The zero-crossing of the wavelength-scan at 80 °C shifts approximately 2 nm to higher wavelengths and in the 190-200 nm region no significant differences are observed when compared to the spectrum obtained at 20 °C (no further data shown). These changes as well as the observation that the transition was the most pronounced at 208 nm could be indicative for a helix-to-strand transition (Johnson, 1988; Hennessey and Johnson, 1981). Curve fitting analysis indeed indicated that the protein at 80 °C still contains about 10% of helical and 50% of stranded structures (Table 1). The thermal unfolding behavior as was observed for pH 3 was also seen for patatin at pH 2 and 4 (no further data

shown). No effect of 0.165 M NaCl on the thermal stability of patatin in Buffer pH 3, 6 and 8 could be observed (data not shown).

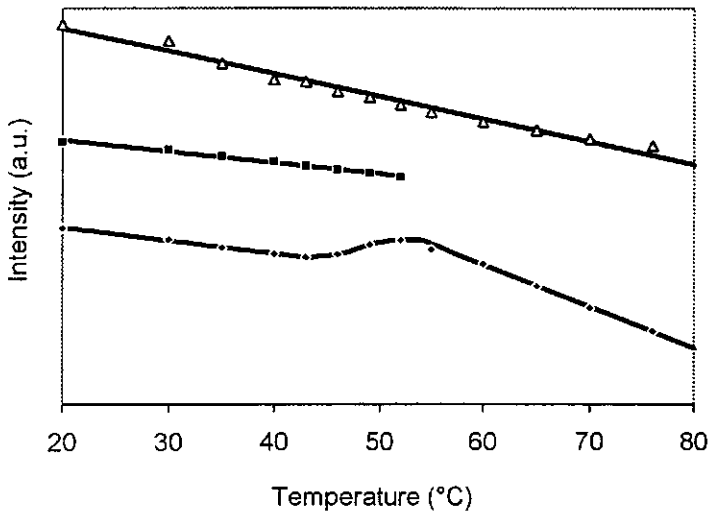

Figure 6 Thermal unfolding of patatin in phosphate buffer (I=0.035 M) pH 3 (▲), 6 (■) and 8 (◆) measured as fluorescence at 343 nm.

Fluorescence spectroscopy as a function of temperature offers a sensitive method to study unfolding of the tryptophan environment of a protein and, thereby, tertiary interactions of that area (Pace *et al.*, 1988). In Figure 6 examples of the behavior of the tryptophan residues at near-neutral pH (pH 6 and 8) and at acidic pH (pH 3) can be seen. The almost linear decrease of the fluorescence intensity as can be observed for patatin at pH 3 and pH 6 and for pH 8 outside the transition region with increasing temperature is an intrinsic property of fluorescence and as such not an effect of conformational changes (Chang, 1981).

The curve obtained of patatin at pH 8 shows a transition between 45 and 55 °C as indicated by an increase in its fluorescence emission due to a lower level of quenching of the two tryptophan residues upon unfolding (Chapter 4). As was already observed with far-UV CD, patatin at pH 6 precipitates between 52 and 55 °C. Patatin at pH 3 shows no transition upon heating but only the linear decrease of the fluorescence intensity. This linear decrease is expected because the tryptophan environment of patatin at acidic pH appears to be already maximally solvent exposed (Fig. 3) and because a heat-treatment did not result in a significant change in tertiary structure (Fig. 5). The different slopes of the fluorescence decay with increasing temperature may be due to different packing of the tryptophan-residues in the protein at various pH.

To study the conformational changes of patatin at the tertiary level, the CD-signal at 263 nm was monitored as a function of temperature for samples at various pH (Fig. 7). This wavelength was chosen arbitrarily to present the typical trend indicative for the potential

tertiary unfolding of the protein. Patatin at pH 8 shows a distinctive change of the CD-signal upon heating from 42 to 60 °C, in agreement with results as presented in Chapter 4. In this temperature range the tertiary structure of the protein appears to change significantly.

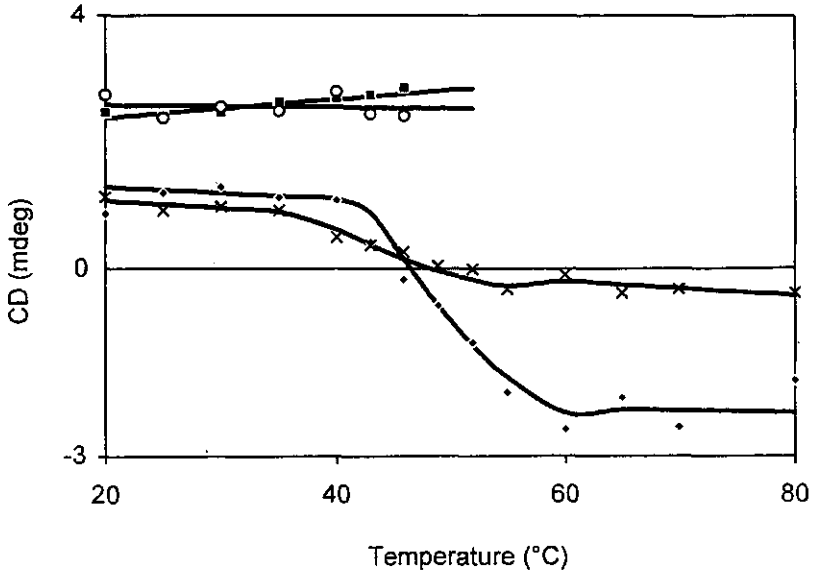

Figure 7 Near-UV CD at 263 nm of patatin showing thermal tertiary destabilisation in phosphate buffers (I=0.035M) pH 2 (x), 4 (o), 6 (■) and 8 (◆) measured as near-UV CD.

The behavior of patatin at pH 4 and 6 is similar, though different from that at pH 8. At pH 4 and 6 up to about 46 °C no changes in tertiary interactions are observed, but between 52 and 55 °C the protein precipitates. Patatin in Buffer pH 2 shows upon heating a minor change of about 1 millidegree. This is to be expected since patatin at pH 2 has already at 20 °C a low level of tertiary structure (Figs. 2; 7)

Thermal versus acidic unfolding

Figures 1, 2, and 3 and 5A suggest that particular parts of the protein exhibit a relatively high tendency to unfold upon an acid or heat-treatment, whereas other areas have a more stable character. To study the thermal and the acidic unfolding of patatin, firstly far-UV CD spectra were recorded of patatin that had been dialyzed to pH 3 and subsequently to pH 8. Second, to establish the stability of the residual secondary structure after acidic unfolding, the effect of a heat-treatment (15 min 80 °C) on patatin at pH 3 was studied. The effects of these treatments on the secondary structure of patatin are compared to that of the untreated pH 8 sample. In Figure 8 far-UV CD spectra are shown of these pH 3 and pH 8 samples.

When patatin has been dialyzed to pH 3 and subsequently to pH 8, its secondary structure has been irreversibly unfolded as compared to the untreated protein-structure at pH 8. The

spectrum obtained from the pH 8 heated sample indicates a highly similar secondary structure as compared to both samples that have been at pH 3, irrespective of the heat-treatment at pH 3. Interestingly, LAH-measurements of patatin that has been at pH 3 -but not heated- showed a residual specific activity of 1.16 ± 0.04 μmol/min/mg protein (64%) compared to 1.82 ± 0.1 μmol/min/mg (100%) for the unheated pH 8 sample that has not been dialyzed to pH 3. Furthermore, the unheated pH 3 sample showed, after dialysis to pH 8, upon heating from 20 to 80 °C a small transition in the ellipticity at 222 nm. This transition had a midpoint of unfolding of 52.1 ± 0.2 °C (ΔH_u 123±10.8 kJ/mol; χ^2=1.74; unfolding curve not shown) as determined with the fitting procedure as described above. After heat-treatment of samples at any pH no residual LAH-activity could be detected.

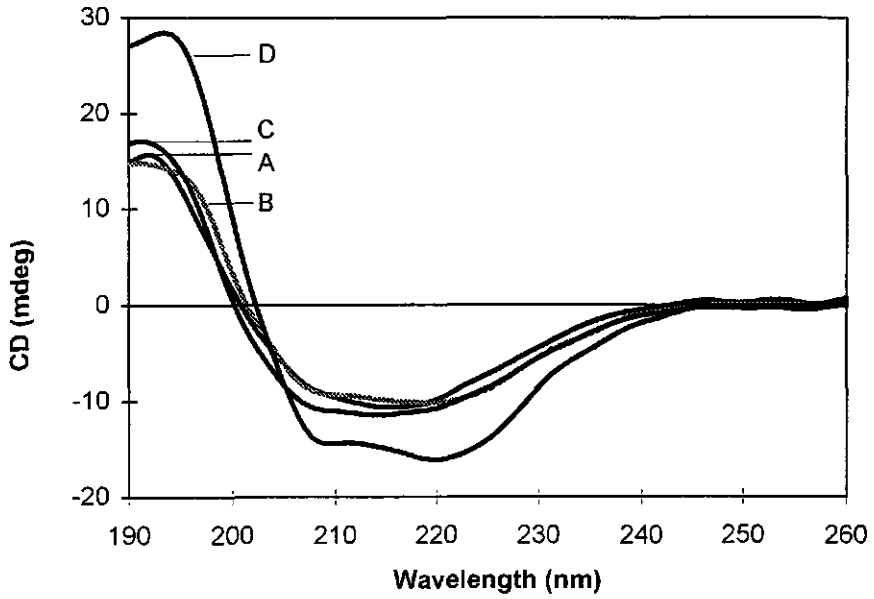

Figure 8 Reversibility of the unfolding of patatin at acidic pH at a secondary level of folding measured using far-UV CD at 20 °C. All samples contained 0.1 mg patatin per mL buffer. Samples at pH 3 were dialyzed to pH 8 prior to the measurement. Samples were: A) heated at pH 3 (15 minutes 80 °C); B) unheated at pH 3; C): heated at pH 8 (15 minutes 80 °C) and D): unheated at pH 8.

Discussion

To establish a possible link between the observed precipitation in the potato fruit juice and heat-induced structural changes of patatin, the pH-dependence of the structural stability of patatin at ambient and elevated temperatures is presented in this study. Since the conformational stability of patatin is shown to depend strongly on the pH, first the structural stability of the protein at near-neutral pH will be discussed, and next, this will be done for acidic pH.

Structural stability of patatin at near-neutral pH

Patatin at near-neutral pH at ambient temperatures is a highly structured protein at both a secondary and a tertiary level of folding (Figs. I, 2 and 3; Table 1). Restricted parts of patatin exhibit a relatively high tendency to unfold, whereas at elevated temperatures the protein still contains a significant amount of helical and β-stranded structures (Fig. 5A). Apparently, patatin has one or more relatively thermo-stable domains. In Chapter 4 it was shown that at pH 8 mainly the α-helical parts of patatin appear to unfold in the 45 to 55 °C region, whereas the β-stranded parts of the molecule unfold more gradually at temperatures from 50 to 90 °C. This could imply that particularly the β-stranded areas of the protein are part of these stable domains. The linear decrease of the ellipticity above the transition temperature at pH 8 which could not be observed at pH 7 (Fig. 5A), could be due to differing structural stabilities of the stable domains (Creighton, 1996). At near-neutral pH the protein shows a thermal unfolding (Fig. 5) which can be described using a fit based on thermodynamic parameters. The midpoint of unfolding can be deduced precisely with such a method and particularly the fact that the observed unfolding can be fitted accurately with this procedure (Table 2) suggests that the unfolding could follow a two-state transition (Pace *et al.*, 1988). Formally the application of the thermodynamic fit is only allowed for a two-state fully reversible transition. These conditions are not entirely fulfilled here because the thermal unfolding is not completely reversible. Nevertheless, the unfolding was in equilibrium and the obtained fit of good quality (Table 2). The midpoints of unfolding at pH 6, 7 or 8 (58, 55 and 49 °C, respectively; Table 2; Fig. 5) indicate a decreased stability of the protein with increasing pH (Fig. 5B). This is expected since proteins tend to have higher stability at pH values closer to their iso-electric point (Creighton, 1996). pH-Induced changes in electrostatic interactions would be the major forces influencing the protein conformation and stability in this pH range. Minor changes in the protein structure are expected, since only the six histidine residues of patatin can be protonated in this pH-range (pKa of histidine: 6.5-7; Creighton, 1996). A protein like patatin, having its iso-electric point around 4.7, contains at pH 8 more negative net charge as compared to pH 6 or 7, possibly influencing the structural stability of the protein. Interestingly, at pH 6 where patatin has the thermally most stable conformation (Fig. 5), it precipitates between 52 and 55 °C. This difference in behavior as compared to pH 7 or 8 was shown to be not an effect of pH-dependent differences in this pH-range since in the Figures 1, 2 and 3 it can be seen that there are no significant differences in the structure of the protein. Upon heating at near-neutral pH the protein unfolds (Fig. 5B), generally resulting in an increased exposure of hydrophobic sites. At a pH further away from its iso-electric point the solubility of a protein is generally higher (Damodaran, 1997). The effect of the increased exposure of hydrophobic sites is smaller at higher pH, which can explain the precipitation of patatin at pH 6 whereas at pH 7 or 8 the protein remains soluble up to at least 80 °C.

Structural stability of patatin at acidic pH

Patatin contains significantly less secondary and tertiary structure elements at acidic than at near-neutral pH (Figs. 1, 2, 3, Table 1). Furthermore, it exhibits only a minor thermal unfolding at acidic pH values (Figs. 5A, 6, 7). The latter suggests that patatin possibly contains one or more stable domains. Below pH 4, the tertiary stabilization of patatin decreases (Fig. 3). Due to this destabilization the secondary structure can unfold further. This partial unfolding of patatin at acidic pH is probably caused by altered electrostatic interactions. Below the iso-electric pH the net charge of the protein becomes net positive since the aspartic- and glutamic acid residues protonate (pKa 3.9-4.0 and 4.3-4.5, respectively; Creighton, 1996). The pH dependence of the conformation and the structural stability observed for patatin is not unusual, but it varies among proteins (Puett, 1973; Anderson *et al.*, 1990; Welfle *et al.*, 1992; Ward *et al.*, 1993; Creighton, 1996; Folawiyo and Owusu Apenten, 1996; Chen *et al.*, 1997). Despite the changes in conformation of patatin in the acidic pH range as observed with CD (Figs 1, 2), no significant differences using fluorescence spectroscopy were observed in this pH-region (Fig. 3). The solvent accessibility of the tryptophan environment is already maximal at pH 4 at ambient temperatures. A further increase in temperature does not result in a prolonged unfolding (Fig. 3), whereas the emission maximum (344 nm, Fig. 3), compared to that of free tryptophan in water (about 352 nm, Schmid, 1989), suggests that the tryptophan residues are not maximally exposed to water. They could be part of or located nearby a stable domain of patatin.
Precipitation of patatin at pH 4 occurred during the near-UV CD measurements and is most likely caused by its higher concentration as compared to the far-UV CD measurements (Fig. 7; Boye *et al.*, 1997; Jiménez, *et al.*, 1995).

Comparison of the thermal and the acidic unfolding

To answer the question whether remaining structured elements or domains of patatin after acidic and thermal unfolding are different, the reversibility of the acidic unfolding as well as the effect of a heat-treatment in acidic environment as compared to near-neutral circumstances, were studied (Fig. 8). The heated pH 8 sample and the unheated pH 3 sample show a highly similar secondary structure, possibly suggesting that the same parts of the molecule do unfold, at acid and heat-treatments. After dialysis of the unheated pH 3 sample to pH 8 a residual LAH-activity was observed, indicating that the acidic and thermal unfolding are not fully similar, despite the resemblance of the secondary structure of the so called stable domain. This was confirmed with a thermal unfolding experiment at pH 8 with the unheated pH 3 sample which showed a small transition. This transition is not understood in terms of its position and magnitude, but could possibly be related to unfolding of parts of the protein that are involved in its enzyme activity, which are apparently not unfolded at acidic pH.

The pH-dependence of protein stability and conformation has been described in many studies, although the reversibility of these conformational changes received far less attention. Nevertheless, for food applications this is of great importance. In this study it was shown that patatin is already destabilized at low pH at ambient temperatures. This acid-induced unfolding is largely irreversible, which is of importance for both novel food applications and for research concerning an alternative method to isolate potato proteins from the industrial potato fruit juice.

In conclusion, patatin is a protein which unfolds partly due to either heat- or acid-treatments. When the protein is highly structured at the start of the heat-treatment (near-neutral pH) an apparent two-state thermal unfolding is observed. At low pH, when the starting conformation is already unfolded to a certain extent, only minor changes occur upon heating. The residual structures could be part of one or more relatively stable domains of patatin. The acidic and the thermal unfolding appear to be very similar, but are not fully the same.

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Chapter 6

Kinetic modeling of the thermal aggregation of patatin*

Abstract

A kinetic model of the thermal aggregation is presented based on chromatographic analysis of the proportions of non-aggregated and aggregated patatin. It was observed that the decrease of the amount of non-aggregated patatin proceeded initially fast and was followed by slower aggregation at longer incubation times. It was shown that this behavior was not due to heterogeneity of the starting material.

It was noted that overestimation of the amount of native molecules after a heat-treatment, caused by refolding of the unfolded protein during the cooling step prior to the analysis, was significant and could not be neglected. Hence, corrections were applied, based on information on the structural properties of patatin. Taking this into account, a model was proposed consisting of a first order formation of reactive particles, followed by a second order aggregation reaction. This model described the thermal aggregation of patatin rather accurately and was confirmed by experiments at various protein concentrations.

* This Chapter has been submitted as:
Kinetic modeling of the thermal aggregation of patatin
André M. Pots, Harry Gruppen, Harmen H.J. de Jongh, Martinus A.J.S. van Boekel, Pieter Walstra, Alphons G.J. Voragen

Introduction

Potato proteins are of interest as ingredients for fabricated foods, since they exhibit promising functional properties in the undenatured state, such as foam and emulsion forming and stabilizing capacity (Holm and Eriksen, 1980; Wojnowska *et al.*, 1981; Jackman and Yada, 1988) and have a high nutritional value (Kapoor *et al.*, 1975; Liedl *et al.*, 1987). Despite work performed to obtain undenatured potato proteins, an industrially method has not been developed so far (Lindner *et al.*, 1980; Ahldén and Trägårdh, 1992). Therefore, no potato protein preparation applicable in foods is available. If undenatured potato proteins would become available as a food ingredient, they could be applied in the same way as, for example, wheat, soy and other legume proteins. These proteins are used in the food industry for their functional properties, such as their capacity to form and stabilize foams and emulsions and to form gels (Damodaran, 1997; Oakenfull *et al.*, 1997). A gel, i.e. a continuous protein network, can be formed upon prolonged aggregation under suitable conditions (Damodaran, 1997). Aggregation occurs usually upon (thermal) denaturation of proteins (Damodaran, 1997; Catsimpoolas and Meyer, 1970) and the mechanism of aggregation determines the type, hence, the properties, of the gel. To establish the aggregation mechanisms for various classes of (food) proteins, fundamental knowledge on the physico-chemical properties of these proteins must be combined with understanding of the molecular aggregation mechanism. This would provide means to optimize the aggregation and subsequent gelation, leading to gels that better meet the desired functionality in food systems.

Patatin is the most abundant potato tuber protein, accounting for about 40% of the soluble tuber proteins (Racusen and Foote, 1980). The apparent molar mass of patatin determined by SDS-PAGE is 43 kDa, whereas in media without SDS or urea it appears as a dimer with an apparent molar mass of about 80 kDa (Racusen and Weller, 1984). Patatin, in fact, consists of glycoproteins encoded by two multigene families, one of which is expressed only in the tuber in relatively large quantities (Park *et al.*, 1983; Pikaard *et al.*, 1987; Sonnewald *et al.*, 1989). In Chapter 3, it was shown that these isoforms behave uniformly under various conditions; hence, they can be studied as a single protein species. Patatin does not contain extended clusters of hydrophobic nor of charged amino acids, and only one cysteine residue is present (Mignery *et al.*, 1984; Stiekema *et al.*, 1988). The molecular properties of patatin during heat-treatment under various conditions have been described in the Chapters 4 and 5.

In the present work chromatographic analysis of the amounts of non-aggregated and aggregated patatin as a function of incubation time and temperature is presented. This enabled kinetic modeling of the aggregation rate of the protein.

Experimental procedures

Preparation of patatin solutions

Patatin was purified from *Solanum tuberosum* cultivar Bintje as described in Chapter 4. After isolation, the protein solution was dialyzed (Visking V20, Carl Roth GmbH + Co, Karlsruhe, Germany) at 4°C against a 22 mM sodium phosphate buffer pH 7 (ionic strength 35 mM). Samples were frozen in small aliquots and stored at -20°C till use. Protein contents were determined by the Bradford assay (Bradford, 1976) using bovine serum albumin (Sigma, A-4511) as standard.

Heating experiments

In closed glass tubes (13 x 100 mm Kimax culture tube, Kimble glass Inc., USA) 0.2 mL solutions containing 0.1 to 1.5 mg patatin per mL buffer pH 7, were incubated in a waterbath at temperatures ranging from 40 to 65°C (± 0.5°C; temperature is reached within 30 s). The samples were heated for times ranging from 2 minutes up to 24 h and stored on ice prior to and immediately after heating (temperature of the sample below 5°C within 30 s). After heat-treatment, the samples were analyzed at 20°C to determine the proportions of non-aggregated and aggregated protein as a function of incubation time using gel filtration chromatography. No corrections were made for the times required to reach the incubation temperature and to cool the samples.

Analytical gel filtration chromatography

Chromatographic analysis of heat-treated samples, loaded via a 50 µL loop, was performed using a Smart System (Pharmacia Biotech, Uppsala, Sweden). Columns used were Superose 6 and Superdex 75, but generally a Superdex 200 column (all 3.2 x 300 mm), equilibrated and run at 20°C and 80 µL/min in the above described pH 7 sodium phosphate buffers. The Superdex 200 and Superose 6 columns were calibrated using blue dextran (MW $2 \cdot 10^3$ kDa), thyroglobulin (667 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (BSA; 67 kDa) and sodium ascorbate (176 Da). The Superdex 75 column was characterized using blue dextran, BSA and sodium ascorbate. Detection of protein was at 280 nm. Changes in the amounts of aggregated and non-aggregated protein after a heat-treatment were negligible for at least 24 h of storage. Therefore, analysis of the heated samples was performed on the day of the heat-treatment.

Preparative gel filtration chromatography

A patatin sample (0.5 mg/mL) that had been incubated for 30 min at 55°C in a 22 mM sodium phosphate buffer pH 7 was subsequently injected at 20°C via a 2 mL loop and fractionated using a Superose 6 column (1 x 30 cm) attached to an FPLC-system (Pharmacia Biotech, Uppsala, Sweden). The column was equilibrated and run at 20°C with a flow rate of

0.5 mL/min using a 22 mM sodium phosphate buffer pH 7. Detection of protein was at 280 nm and fractions of 1 mL were collected. Fractions containing non-aggregated and aggregated protein were pooled and analyzed by capillary electrophoresis (CE) as described in Chapter 2. The heated, non-aggregated protein fraction was subjected to a second heating experiment at 55°C.

Kinetics of heat-induced aggregation

The amount of protein present in the aggregated and non-aggregated fraction was determined by integrating the corresponding peak-area after gel filtration. The peak areas of non-aggregated and aggregated patatin were expressed as proportion of the peak area of unheated patatin (the latter denoted as N(0)). To determine the rates of the changes in non-aggregated and aggregated protein kinetic models were fitted to the data applying the mathematical program Matlab for Windows (version 4.2c1; The Mathworks, Inc., Natick, Massachusetts, USA), making use of a Levenberg-Marquardt procedure (Press *et al.*, 1986). A Gear algorithm was applied to numerically solve differential equations describing the kinetic processes (Stoer and Bulirsch, 1980). Monte Carlo simulations were performed assuming an experimental error of on average 10%. To estimate the necessary number of simulations 100, 200 and 400 repetitions were performed and the sizes of the resulting 90% confidence intervals were compared (Alper and Gelb, 1990).

Results and discussion

The aim of this research was to describe the thermal aggregation of patatin in terms of a kinetic model. Although numerous papers describe the thermal denaturation and subsequent aggregation and gelation of proteins of various origins, a description of the underlying mechanism is usually not presented (Hohlberg and Stanley, 1986; Kella, 1988; Bacon *et al.*, 1989; Boyer *et al.*, 1996; Elofsson *et al.*, 1996; Sathe and Sze, 1997). Only a few papers propose a kinetic model based on the occurring reactions (e.g: Roefs and De Kruif, 1994). To establish general rules for the thermal denaturation and aggregation behavior of various classes of (food) proteins, a description of the physical and chemical mechanism of unfolding and aggregation combined with knowledge on physico-chemical properties of the proteins, is essential. In that respect, patatin could, as a supposed multi-domain glycoprotein (Chapter 5) with only one cystein residue (Mignery *et al.*, 1984), provide an example of a class of proteins exhibiting a typical denaturation and aggregation behavior.

Temperature dependence of the aggregation

Representative gel filtration elution profiles of patatin that had been heated for various times at 50°C are shown in Figure 1. Non-aggregated protein eluted as a dimer of approximately 72 kDa, as expected (Racusen and Weller, 1984). Upon incubation for a defined period of time

at 50°C or higher, a second peak appeared at shorter retention times, as an aggregate with an apparent molar mass of at least 1300 kDa. In addition, the area of the non-aggregated patatin peak decreased concomitantly. Interestingly, no distinct peaks were observed between 1.0 and 1.4 mL elution volume, indicating that hardly any particles with apparent masses between about 80 and 1300 kDa were present. It may be that during the incubation relatively small aggregates were formed, but that these particles are highly reactive and aggregated rapidly to larger and more stable particles that were detected after cooling.

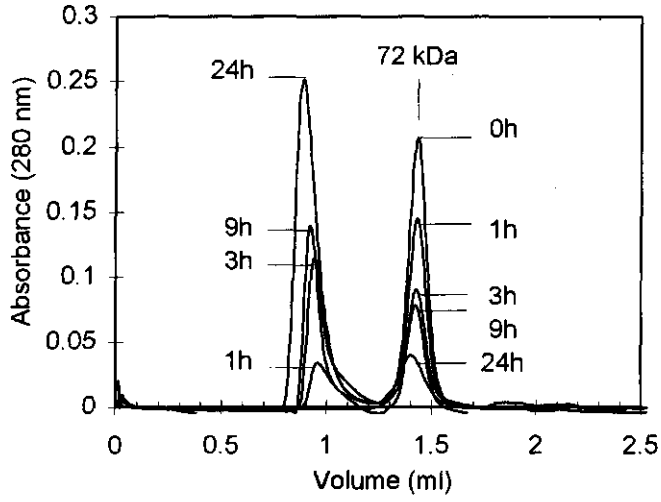

Figure 1 Gel filtration (Superdex 200) chromatograms as measured at 20°C after incubation for various times at 50°C of 0.33 mg patatin per mL 22 mM sodium phosphate buffer pH 7. Indicated are the apparent molar mass of the non-aggregated protein and the heating times. The included and excluded volumes of the column were 0.9 and 2.1 mL, respectively.

After 24 h at 50°C (Fig. 1) the peak area of the aggregated protein was 1.5 times that of the corresponding amount of unheated native patatin. A similar effect could be observed after 3 h at 55°C (no further results shown) where the peak area of aggregated protein was 1.1 times that of the native protein. This may be due to an increase in the absorbance coefficient of the protein. Patatin contains 2 tryptophan residues that are stacked in the native state. This configuration is lost after unfolding (see Chapters 4 and 5) which may result in an increased absorbance coefficient (Schmid, 1989). This increase in optical density could also be caused by an increased level of scattering of the incident light. Contrary to these effects the peak area of the aggregated protein decreased and it virtually disappeared after longer incubation times (6-24 h) at 55°C. Since visually no precipitation was observed, this may have been caused by adsorption of particles to the column material or by soluble particles that were too large to elute from the column. Dynamic light scattering measurements of patatin under comparable conditions indicated that the maximum radius of the particles present (measured both at 65°C and after cooling) after 1 h was approximately 24 nm (no further results

shown), which should allow elution in the excluded volume of the Superdex 200 column. Therefore, any discrepancy in the observations was neglected for incubation times up to 1 h. Similar aggregation experiments were performed at temperatures from 40 to 65°C. The resulting amounts of aggregated and non-aggregated patatin are depicted in Figure 2, where the quantities of protein are expressed as proportion of the peak area of the unheated patatin.

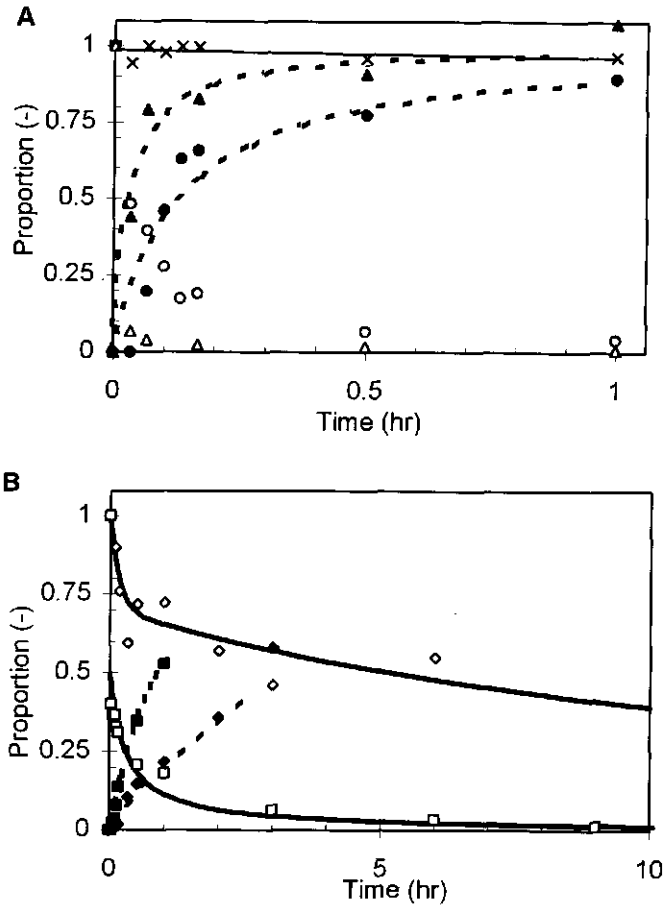

Figure 2 Proportion of non-aggregated and aggregated patatin at temperatures ranging from 40 to 65 °C as a function of time. A: Temperatures 40, 60 and 65°C, depicted up to one h. B: Temperature 50 and 55°C, depicted up to 10 h. Non-aggregated and aggregated protein is represented by open and closed symbols, respectively: 40°C : x, aggregate not present; 50°C : ◊,◆; 55°C : □,■; 60°C : ○,●; 65°C : △,▲. Fits of these points are depicted by full lines (decrease non-aggregated patatin) and dashed lines (increase of aggregated protein).

It can be seen in Figure 2 that patatin at 40°C aggregated at a negligible rate at the indicated time-scales and conditions, whereas at temperatures exceeding 50°C the protein coagulated at increasing rates. The initial fast decrease of non-aggregated patatin was followed by a slower decrease at longer incubation times. Samples heated at 50 and 55°C (Fig. 2B) were measured

for 24 h, but for clarity only shown up to 10 h. When heated at 50°C the residual amount of non-aggregated protein decreased to approximately 10% after 24 h, whereas the sample heated at 55°C contained virtually only aggregated protein after 10 h.

Homogeneous behavior of the patatin sample

The fast initial decrease and the slower subsequent decrease suggest either a heterogeneity of the starting material or multiple rate limiting steps in the unfolding and subsequent reactions (Creighton, 1990). The former seemed the most obvious option to be examined, since it is known that patatin consists of various isoforms (Park *et al.*, 1983; Pikaard *et al.*, 1987). To test for heterogeneity in the patatin sample, non-aggregated protein was separated from the aggregated protein after heating the sample for 30 min at 55°C. Next, the non-aggregated protein was heated again and the aggregation rate of this heated, non-aggregated patatin fraction was determined and compared to the behavior of non-preheated protein. The aggregation kinetics of the heated, non-aggregated protein were similar to those observed at 55°C for the non preheated protein, showing again an initial fast phase followed by a slower one (no further results shown). Furthermore, in Chapter 3 revealed comparison of electropherograms (CE) of heated, non-aggregated protein and non-heated protein that all isoforms were present in the same peak ratios in both samples (results not shown). All these results indicate that the patatins behave as a homogeneous system, and that the behavior as observed in Figure 2 is due to a more complex reaction mechanism rather than to heterogeneity of the starting material.

Modeling of the aggregation

Assuming a single step reaction and first, second or n-th order kinetics (Van Boekel, 1996), fitting of the decrease of the amount of non-aggregated patatin gave poor (unacceptable) fits for the data at 50 and 55°C: In the models tested, the distribution of the residuals was not random (results not shown). Therefore, more complex reaction mechanisms were examined (Pyun, 1971; Sadana, 1991; Schokker and Van Boekel, 1997). The simplest plausible mechanism consisting of two consecutive reactions, is presented in Scheme 1, where, besides the reactions, also the corresponding differential equations are given. This model describes the formation of reactive particles (1), followed by an aggregation reaction of reactive particles (2). The formation of the reactive particles itself is most likely also a process that consists of two steps; first a rapid equilibrium unfolding, that is followed by a slower conformational change that results in the formation of the reactive particle. This was thought since modeling of the data assuming a reversible reaction 1 resulted in reaction rates that were too slow to account for unfolding only (reaction half times up to 396 s, no further results shown). Furthermore, generally unfolding of proteins under these conditions occurs within (parts of) seconds (Kuwajima, 1996). In addition, from the Chapters 4 and 5 it is

known that the unfolding of patatin is a rapid process indeed. Therefore, it is proposed that the observed reaction rate, k_1 in Scheme 1, is the overall resulting reaction rate for the formation of reactive particles that takes the proportion of unfolded and native patatin into account by the equilibrium constant K (Scheme 1), the ratio of unfolded-to-native protein. The unfolded state U consists most probably of a mixture of unfolded conformations (Chapter 4; Creighton, 1990). The second reaction (2), as an aggregation reaction typically of second order, presents an averaged ensemble of simultaneously occurring reactions.

$$N \xrightarrow{k_1} U \qquad (1)$$

$$U_p + U_q \xrightarrow{k_2} U_{(p+q)} \qquad (2)$$

$p,q \in \{1,2,3...\}$

$$\frac{d[N]}{dt} = -\frac{k_1}{1+1/K}\{[N]+[U]\}$$

$$\frac{d[U]_{(p+q)}}{dt} = k_2[U]^2$$

Scheme 1 Proposed mechanism of the aggregation of patatin with its corresponding mathematical model.

The correctness of a kinetic model can be tested by fitting it to data describing both the decrease of reactants and the increase of products as a function of time. Moreover, the temperature and the concentration dependence must be consistent with theory and the prerequisites of the model. Furthermore, the lowest possible number of parameters should be used and the residuals must be randomly distributed over the whole time range (Van Boekel, 1996).

Using a least squares fit procedure the data were fitted to the differential equations. It should be noted, however, that the proportion of non-aggregated protein (N) at any time will be an overestimate since it was measured as the sum of the fraction of native protein plus that fraction of unfolded protein (U) that had refolded to N upon cooling. Similarly, the amount of aggregated protein ($U_{(p+q)}$; Scheme 1) is overestimated since it is based on the sum of aggregated protein plus the fraction of U that aggregates to $U_{(p+q)}$ during cooling. Most of the studies in the literature neglect or merely note the overestimation of the amount of native protein due to the reversible character of the unfolding (e.g: Manji and Kakuda, 1987; Levieux *et al.*, 1995; Verheul *et al.*, 1998). When we applied the model without the assumptions correcting for the overestimation of native protein, an adequate fit of the data and a consistent dependence on temperature and concentration was obtained (χ^2: ranging from 0.01 to 0.015; further results not shown). Nevertheless, this is known to be systematically wrong because the native protein content determined at room temperature is

not equal to the native protein content at the reaction temperature. In this work, therefore, the overestimation was corrected by using the knowledge presented in the Chapters 4 and 5 on the thermal stability of patatin. The following assumptions were made: The amount of U at 50°C was estimated as 5% of the non-aggregated protein present, since in Chapter 5 it was shown that approximately 5% of the protein was unfolded at that temperature. This results in a *K*-value of 20 (Scheme 1). The midpoint of unfolding of patatin at pH 7 is at 55°C, therefore, at this temperature the amount of U must equal that of N , hence, *K* should equal 1. To obtain the correct measure for the proportion of N during the heat-treatment at 55°C, the measured amount of N was multiplied by 0.5. These corrected data are presented in Figure 2. Finally, it was assumed that at 60 and 65°C virtually all protein was in the U form (Chapter 5). This would imply that the increase of $U_{(p+q)}$ at these temperatures can be fitted accurately with a single step second order reaction (reaction 2 in Scheme 1). At all temperatures the amount of U aggregating to $U_{(p+q)}$ during cooling is neglected. This is allowed, since it was shown that virtually no aggregation occurred at 40°C or below (Fig. 2A) and since the samples were cooled to below 5°C within 30 s. As illustrated by the full and dashed lines in Figure 2B, this model describes sufficiently (χ^2 ranging from 0.01 to 0.035) the temperature dependent decrease of the primary particle and the growth of the aggregates at 50 and 55°C. Due to the assumption that all protein was in the reactive state at 60 and 65°C, only the growth of the aggregates had to be modeled at these temperatures (Fig. 2A). Therefore, only k_2 was obtained at 60 and 65°C. The fit of the aggregation at 60°C seemed not as good as the fit at 65°C. The confidence intervals as obtained with this procedure are greatly overestimated, due to a large standard deviation and a small number of degrees of freedom (Arabshahi and Lund, 1985; Cohen and Saguy, 1985). A better estimate of the confidence intervals can be obtained by a Monte Carlo simulation procedure (Alper and Gelb, 1990). Assuming an experimental error of 10%, 200 and 400 repetitions gave essentially the same confidence intervals, therefore, 200 simulations were performed. In Table 1 the so obtained reaction rates, with their 90% confidence intervals, are given. The obtained correlations between the parameters at 50 and 55°C were 0.977 between k_1 and k_2. These correlations were not too strong (Bates and Watts, 1988), a problem that often occurs in kinetic analysis since the experimental range of temperatures is small as compared to the absolute temperature range (Van Boekel, 1996).

Table 1 Reaction constants with their 90% confidence intervals as a function of the temperature.

Temperature	k_1 (s^{-1}) [90% c.i.]	k_2 ($l \cdot mol^{-1} \cdot s^{-1}$) [90% c.i.]
50 °C	$1.75 \cdot 10^{-3}$ [$1.6 \cdot 10^{-3}$-$3.3 \cdot 10^{-3}$]	149.3 [76.6-253.2]
55 °C	$3.5 \cdot 10^{-2}$ [$2.1 \cdot 10^{-2}$-$5.0 \cdot 10^{-2}$]	144.5 [133.8-195.9]
60 °C	-[1]	274.7 [249.6-296.2]
65 °C	-[1]	1205.2 [1016.5-1379.6]

1) Not determined

It was observed that a conformational change of the protein is essential to allow aggregation (Chapter 5; Fig. 2). As a result of unfolding, hydrophobic or reactive parts of the protein molecule will become available to interact with other molecules. It is known from work presented in Chapter 4 that patatin unfolds partly but rapidly during an increase in temperature, and that the secondary structure content remains constant when the temperature is kept constant. The model presented in Scheme 1 implies, however, a continuing decrease of the amount of native particles N, by reaction of U to aggregates at a constant temperature. This suggests that the reactivity in the unfolded state U would not primarly depend on the change in the amount of secondary structure, but on other structural changes of the protein.

In Figure 2 it was shown that the model fits the data mathematically reasonably well. Since only at 50 and 55°C a value for the temperature dependence of k_1 could be obtained it is difficult to interpret the values for the reaction constants. According to Table 1 k_1 would strongly increase with increasing temperature, which suggests a single step unfolding-type reaction. On the other hand, K may be overestimated at 50°C, resulting in an underestimation of k_1. The reaction constant k_2 exhibits no significant differences up to 60°C, to show a strong increase at 65°C. From step 2 in Scheme 1, one would expect a gradual increase of k_2 with rising temperatures. The fact that this behavior is not observed may suggest that the proposed model does not represent the entire mechanism. It may be possible, for example, that the polydispersity of the system increases with temperature, resulting in an increased aggregation rate (Overbeek, 1952). Step 2 in Scheme 1 neglects differences between aggregation rates of, for example, primary particles with multiple ones and the multiple ones amongst themselves. The theory of Smoluchowski-Fuchs does take these reactions into account (Overbeek, 1952), but did not provide a better fit of the measured data than our model (no results shown). Therefore, we did not use the Smoluchowski-Fuchs approach and applied the reaction as presented in step 2 of Scheme 1. Nevertheless, it should be noted that the aggregation mechanism is of a more complex and not fully understood nature.

Concentration dependence of the aggregation

The reaction rate of the aggregation (step 2, Scheme 1) should exhibit a quadratic dependence of the reactant concentration as implied by its second order kinetics, whereas the reaction constant of aggregation should, by definition, be independent of the protein concentration (Sadana, 1991). To test this, the concentration dependence of the aggregation was examined at 55°C. In Figure 3A the decrease of non-aggregated and the increase of aggregated patatin are shown at protein concentrations ranging from 0.1 to 1.5 mg/mL (2.3-34.9 μM). Peak areas of both fractions were normalized for each concentration using the peak-area of the amount of unheated native protein. The peak area of non-aggregated protein was corrected by a factor 0.5, as discussed above. The values of the reaction constants k_1 and k_2, as obtained at 55°C in the experiments dealing with the temperature dependence of the

reactions, were used here, and only k_2 was allowed to vary. It can be seen that the model fits the results and that the curves of the decrease of native patatin coincide (Fig. 3A). Therefore, it can be concluded that the relative decrease of native particles was independent of the protein concentration, as expected for a first order reaction. Furthermore, it was observed that the aggregation constant was not strongly dependent of the protein concentration (Table 2), whereas the aggregation rate, as determined from the initial tangent, increases with increasing protein concentration.

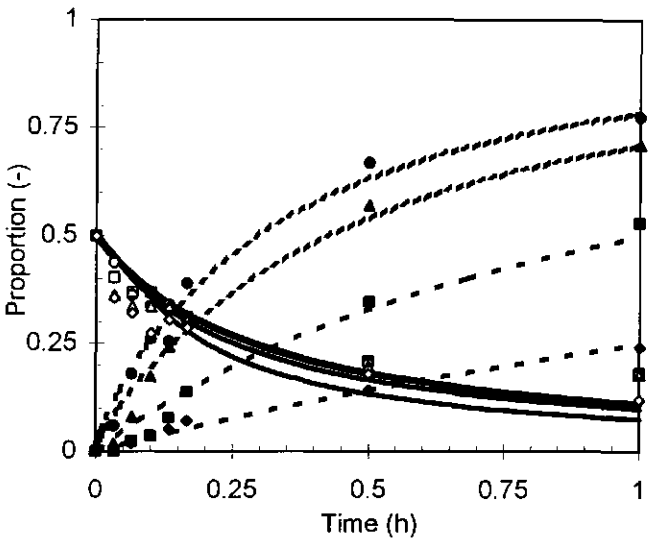

Figure 3 The concentration dependence of the decrease of non-aggregated and the increase of aggregated patatin, determined at 55°C. Protein concentrations (non-aggregated and aggregated protein is represented by open and closed symbols, respectively): 0.1 mg/mL (2.3 μM) : ◇; 0.33 mg/mL (7.7 μM) : □; 1.0 mg/mL (23.2 μM) : △; 1.5 mg/mL (34.9 μM) : o. Fits are represented by lines (aggregated protein: dashed lines; non-aggregated protein: solid lines).

Table 2 Reaction constants with their 90% confidence intervals as a function of protein concentration.

Concentration	k_2 ($l \cdot mol^{-1} \cdot s^{-1}$) [90% c.i.]
2.3 μM	155.3 [143.8-167.2]
7.7 μM	159.0 [147.3-175.1]
23.2 μM	115.9 [105.1-126.6]
34.9 μM	113.8 [103.8-125.0]

Summarizing, a model was proposed for the thermal aggregation of patatin at pH 7, that consisted of two steps. First, the formation of reactive particles, that is followed by a second order aggregation reaction. It was observed that the overestimation of the amount of native molecules after a heat-treatment, due to refolding of the unfolded protein during the cooling

step prior to the analysis, was significant and could not be neglected. This overestimation was corrected by using the information on the structural information on patatin, as reported in the Chapters 4 and 5. The resulting model described the thermal aggregation of patatin reasonably well.

Acknowledgements

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Chapter 7

Thermal aggregation of patatin studied *in situ*[*]

Abstract

In this work dynamic lightscattering was used to study the thermal aggregation of patatin *in situ*, in order to elucidate the physical aggregation mechanism of the protein and to be able to relate the aggregation behavior to its structural properties. The dependence of the aggregation rates on the temperature and the ionic strength suggested a mechanism of slow coagulation, being both diffusion and chemically limited. The aggregation rate dependence on the protein concentration was in accordance with the mechanism proposed. The aggregation rates as obtained at temperatures ranging from 40-65°C correlated well with unfolding of the protein at a secondary level.

Small angle neutron scattering and dynamic lightscattering results were in good accordance, they revealed that native patatin has a cylindrical shape with a diameter and length of 5 and 9.8 nm, respectively.

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Thermal aggregation of patatin studied *in situ*
André M. Pots, Erik ten Grotenhuis, Harry Gruppen, Alphons G.J. Voragen, Kees G. de Kruif

Introduction

The rationale of research concerning aggregation phenomena of globular food proteins is the fact that aggregation governs gelation and that the mechanism of gelation determines the properties of the formed particle gel (Damodaran, 1997). Potato proteins are of interest in this perspective since they exhibit promising functional properties in the undenatured state such as the capacity to form and stabilize foams and emulsions (Wojnowska *et al.*, 1981; Jackman and Yada, 1988).

Patatin is the most abundant potato tuber protein (Racusen and Foote, 1980). It consists of 362 amino acids and shows neither extended hydrophilic nor hydrophobic sequences. It is a glycoprotein of 43 kDa (observed with SDS-PAGE), appearing in media without SDS or urea as a dimer with an apparent molar mass of about 80 kDa (Racusen and Weller, 1984). The positive and negative charges of the side chains are randomly distributed over the sequence and the protein contains one cysteine residue (Mignery *et al.*, 1984; Stiekema *et al.*, 1988). In Chapter 6 the aggregation kinetics of patatin were studied by analyzing the proportions of non-aggregated and aggregated protein after a heat treatment.

Based on a high or low sticking probability two main aggregation mechanisms are distinguished; so called chemically limited (CLA) and diffusion limited aggregation (DLA), respectively (Lin *et al.*, 1989). Computer models simulating these mechanisms have been developed and applied to validate the assumption concerning a specific type of coagulation (Meakin, 1988; Einarson and Berg, 1993; Elofsson *et al.*, 1996; Kyriakidis *et al.*, 1997).

Aggregation behavior in terms of aggregate size or form can be studied by dynamic lightscattering (DLS) and small-angle neutron scattering (SANS). DLS has shown to be of great value to study polymerization and thermal aggregation of proteins (Ware, 1984; Schurtenberger and Augusteyn, 1991; Griffin *et al.*, 1993). In addition, it provided information that was used to develop and validate models for the thermal denaturation and aggregation of β-lactoglobulin (Roefs and de Kruif, 1994; Elofsson *et al.*, 1996; Hoffmann *et al.*, 1996; Verheul *et al.*, 1998). SANS enables the determination of the size, form and structure of particles as well as the packing density of particles in agglomerates (Timmins and Zaccai, 1988; Verheul, 1998).

In this research the aggregation of patatin is studied *in situ*. One of the problems in defining protein aggregation is the difficulty to distinguish between aggregation, precipitation and gel formation, because these events tend to occur simultaneously (Boye *et al.*, 1997). Therefore, the aggregation of patatin was studied under conditions where no precipitation occurred, i.e. at sufficiently low protein concentrations. This should lead to a physical mechanism that describes the aggregation kinetics.

Experimental procedures

Preparation of patatin solutions

Patatin from *Solanum tuberosum* cultivar Bintje was purified as described in Chapter 4. After isolation, the protein solution was dialyzed (Visking V20, Carl Roth GmbH + Co, Karlsruhe, Germany) at 4°C against a 22 mM sodium phosphate buffer pH 7 (ionic strength 35 mM). Samples were frozen in small aliquots and stored at -20°C till use. Protein contents were determined using the Bradford assay (Bradford, 1976) using bovine serum albumin (Sigma, A-4503) as a standard.

Dynamic light scattering

Dynamic light scattering (DLS) measures the auto-correlation function of the fluctuating intensity resulting from the diffusional properties of particles (Berne and Pecora, 1976). For a polydisperse sample the auto-correlation function can be fitted with the method of cumulants (Koppel, 1972). The decay rate of the auto-correlation can be related to a translational diffusion constant (Koppel, 1972). From this the hydrodynamic diameter can be obtained using the Stokes-Einstein equation (Ware, 1984). Experiments were performed at NIZO food research (Ede, The Netherlands) using a self-constructed apparatus consisting of a Spinnaker 1161 laser (514.4 nm) and a detector (Thorn EMI 9863/100B, Middlesex, England) mounted on the arm of a goniometer. The output of the photomultiplier was fed into an ALV5000 autocorrelator (ALV-laser GmbH, Langen, Germany). The equipment was validated using latex standard particles with a radius of 51 ± 3.8 nm (Duke Scientific Co. Palo Alto, Ca, USA). Laser output power was set at an approximate value of 275 mW and the intensity of the scattered light was measured at angles varying from 35 to 135°, but in this paper always at 90°. The temperature of the samples was maintained constant within 0.5°C by immersing the sample cuvets in a toluene bath which temperature was controlled with a water bath.

Protein solutions (see below) were filtered over 0.1 µm low-protein binding filters (Millipore Millex-VV, SLVV025LS; Bedford, MA, USA) into a cylindrical glass cuvet (radius 9 mm). The filled cuvets were centrifuged for 5 min at 500g at room temperature prior to the measurements.

Samples containing 0.3 mg/mL patatin in the above described buffer were measured *in situ* at temperatures from 40-65°C for times ranging from 1 to 68 h. Experiments dealing with the concentration dependence of the aggregation were performed at 55°C with protein concentrations ranging from 0.45 to 2.0 mg per mL. The ionic strength dependence of the aggregation was investigated at 55°C, with 0.33 mg protein per mL. Ionic strength values over 35 mM (42, 50, 57, 65, 72 and 80 mM, respectively) were obtained by the addition of sodium chloride to the buffer solutions. Experiments of 0.33 mg patatin per mL were also performed in the presence of N-ethylmaleimide (NEM), applying molar ratios NEM : patatin

of 2:1 and 1:1. The aggregation rates were determined by taking the tangent of the initial increase in particle size.

Small-angle neutron scattering

Small-angle neutron scattering (SANS) was performed using the D22 diffractometer at the Institut Laue-Langevin in Grenoble, France. The spectra were recorded for each sample using two different spectrophotometer configurations by changing the sample to detector distance from 3.00 to 18.00 m. The wavelength of the incident neutrons was 1 nm. In this way a range for the wave vector q was covered from 0.05 to 2 nm. The data were collected by means of a two-dimensional detector, converted in intensity versus q and normalized for transmission and sample path length. The data were normalized to a pure water sample.
Samples contained 0.5 mg patatin per mL sodium phosphate buffer pH 7 in D_2O and were unheated and heated for 1 and 10 h at 55°C prior to the measurement.

SDS-PAGE

SDS-PAGE was performed with a Pharmacia PhastSystem according to the instructions of the manufacturer using Gradient 8-25 Phastgels. Samples at pH 7 heated for various times in the presence and absence of NEM were analyzed in the presence and absence of 20 mM β-mercaptoethanol and 6 M urea in the sample buffer and combinations thereof.

Results and Discussion

The aim of this research was to elucidate the mechanism of aggregation, by describing the thermal coagulation kinetics of patatin. In addition, combining the observations on the thermal aggregation of patatin (as presented in Chapter 6) with that on its thermal stability (as presented in the Chapters 4 and 5) may lead to a better understanding of the structure-function relation of the protein.

Temperature dependent aggregation

Aggregation of patatin in buffer at pH 7 was studied *in situ* at temperatures ranging from 40 to 65°C using dynamic light scattering (DLS). At these temperatures the intensity auto-correlation functions were measured. In Figure 1 representative auto-correlation functions are shown as obtained at 55°C. It can be seen that the decay became slower with longer heating times, hence, the calculated diffusion coefficient was larger as a function of heating time. From the diffusion coefficient the apparent particle size was calculated with the Stokes-Einstein equation (Berne and Pecora, 1976).
The scattering intensities measured at an angle of 90° and the corresponding apparent Stokes-Einstein radii are shown in Figure 2. It can be seen that patatin at pH 7 did not aggregate at 40°C whereas at 50°C and at higher temperatures the protein coagulated with increasing rate.

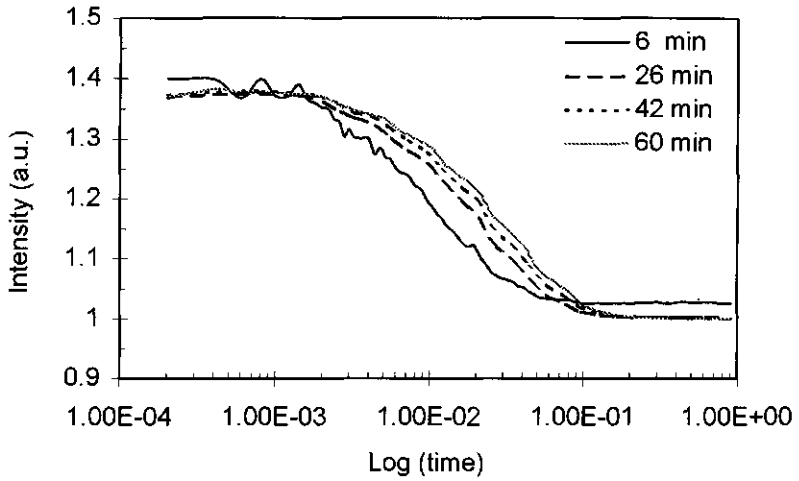

Figure 1 The time-dependent decay of the intensity auto-correlation function as obtained after various incubation times of 0.33 mg patatin per mL buffer at pH 7 at 55°C.

At 55, 60 and 65°C a fast initial aggregation was observed. Incubations up to 68 h at 55°C showed that the aggregates under these conditions remained soluble and that they continued to increase in size at these long time scales (no further results shown). A relatively small difference, of approximately 1 nm on a particle radius of 23 nm, was observed between the radii obtained at 60 and 65°C. Measurement of the size of the aggregates after rapid cooling to 0°C after one h for each temperature revealed no changes in particle size as compared to the size prior to cooling (no further results shown). This observation indicates that the assumption that the particles did not aggregate further during cooling, as stated in Chapter 6, was allowed indeed. The observed increase in aggregation rate coincides with the measured loss of secondary structure of the protein as observed in Chapter 5.

Mechanism of the aggregation

An indication for the mechanism of flocculation can be obtained from the dependence of the aggregation rate on the ionic strength of the medium (Overbeek, 1952). The increase in particle size of patatin during incubation at 55°C was therefore determined at various ionic strength values at pH 7. In Figure 3 it is shown that the effect of increasing ionic strength is relatively small up to 50 mM, and that above 50 mM the aggregation rate increases considerably with the ionic strength.

The Smoluchowski-Fuchs mechanism for slow coagulation of particles presents a dependence of the aggregation rate on the ionic strength (Von Smoluchowski, 1917; Overbeek, 1952). This mechanism describes coagulation limited by both diffusion and reaction. It accounts by a delay or retardation factor W for the fact that not all collisions of particles result in permanent contact. Various experiments with colloidal particles other than

proteins and model calculations have shown that a logarithmic plot of W as a function of the ionic strength can exhibit a linear decrease with the ionic strength and becomes constant at higher ionic strengths (e.g. Reerink and Overbeek, 1954; Amal *et al.*, 1990). If the Smoluchowski-Fuchs mechanism applies, the aggregation rate is proportional to the reciprocal value of W (Reerink and Overbeek, 1954). Hence, a plot of the reciprocal aggregation rate as a function of the ionic strength could show the same dependence on the ionic strength as W. In Figure 4 it can be seen that the logarithm of the reciprocal aggregation rate remains constant up to an ionic strength of 50 mM and exhibits the expected linear decrease with increasing ionic strengths. The constant part of the reciprocal aggregation rate at high ionic strengths was not observed. It is possible that the level of ionic strength where W could become independent was not reached. The horizontal line in Figure 4 below 50 mM shows that the aggregation rate is independent of the ionic strength in this range. The decrease of W with increasing values of ionic strength, however, is typical for both diffusion and reaction limited aggregation.

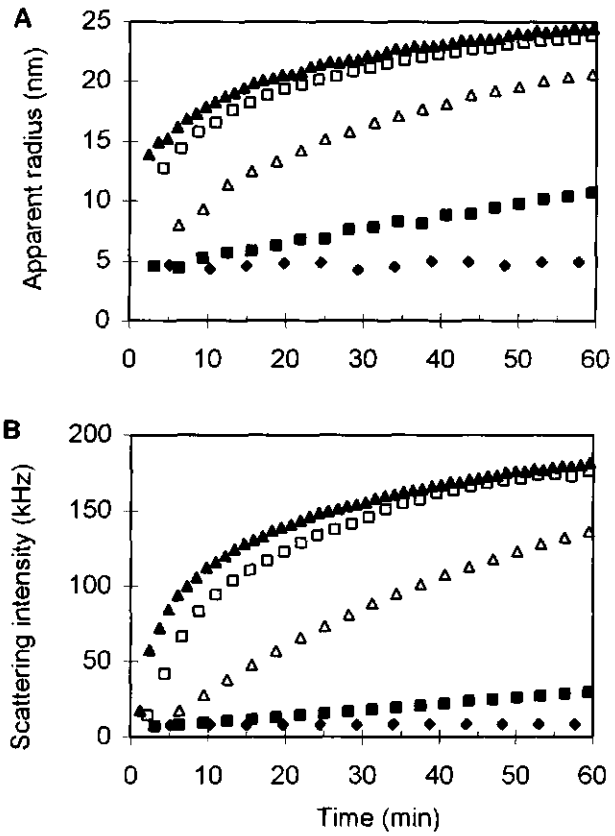

Figure 2 A: Apparent Stokes-Einstein radii of 0.33 mg patatin per mL at pH 7 at temperatures ranging from 40 to 65°C. B: Scattering intensity measured at an angle of 90°. Symbols: 40°C:◆ ; 50°C : ■ ; 55°C : Δ ; 60°C : □ ; 65°C : ▲.

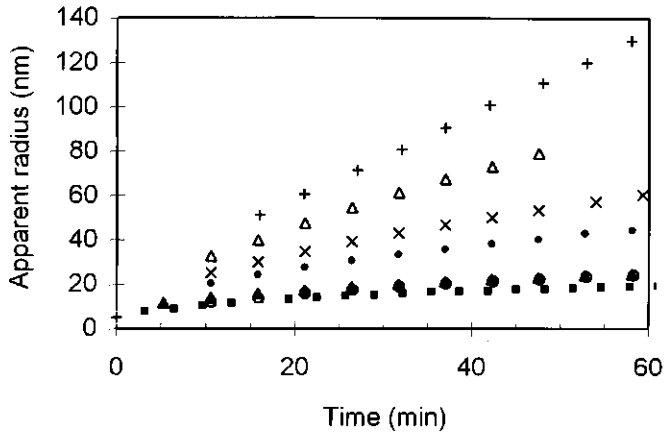

Figure 3 Apparent Stokes-Einstein radii of of 0.33 mg patatin per mL at 55°C at pH 7 at ionic strengths ranging from 35 to 80 mM. 35 mM : ■ ; 42 mM : o ; 50 mM : ▲ ; 57 mM : ●; 65 mM : x ; 72 mM : Δ ; 80 mM : +.

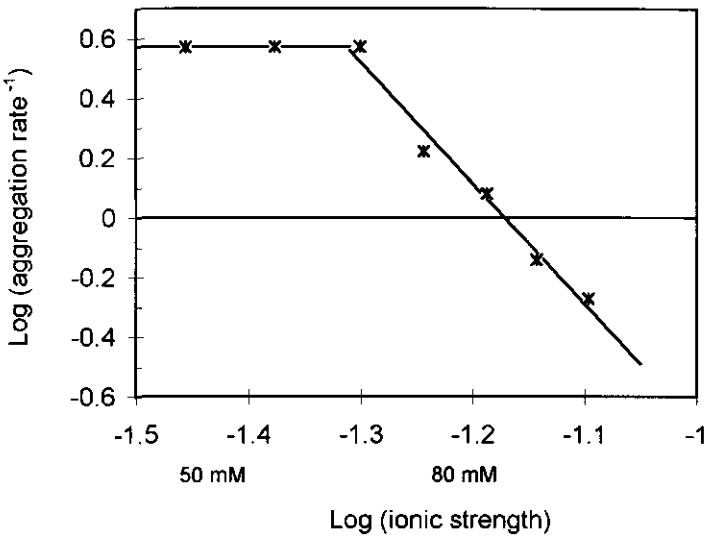

Figure 4 Logarithmic plot of the aggregation rate as a function of ionic strength.

If the Smoluchowski-Fuchs mechanism is valid, the kinetics of aggregation should be of second order. To test this, the aggregation rate of patatin at pH 7 at 55°C was measured at various protein concentrations. Figure 5A shows the apparent Stokes-Einstein radii obtained at patatin concentrations ranging from 0.45 to 2.0 mg patatin per mL (10.5-46.5 μM). It can be seen that the aggregation rate increased with increasing protein concentration. A plot of the aggregation rate should reveal a linear dependence of the squared protein concentration and it should cross the origin, if the kinetics were of second order. The solid line in Figure

5B is the best linear fit that includes the origin. It can be seen that its course is in reasonable accordance with the Smoluchowski-Fuchs model. In Chapter 6 second order kinetics were observed for the aggregation reaction, which was confirmed independently in this paper.

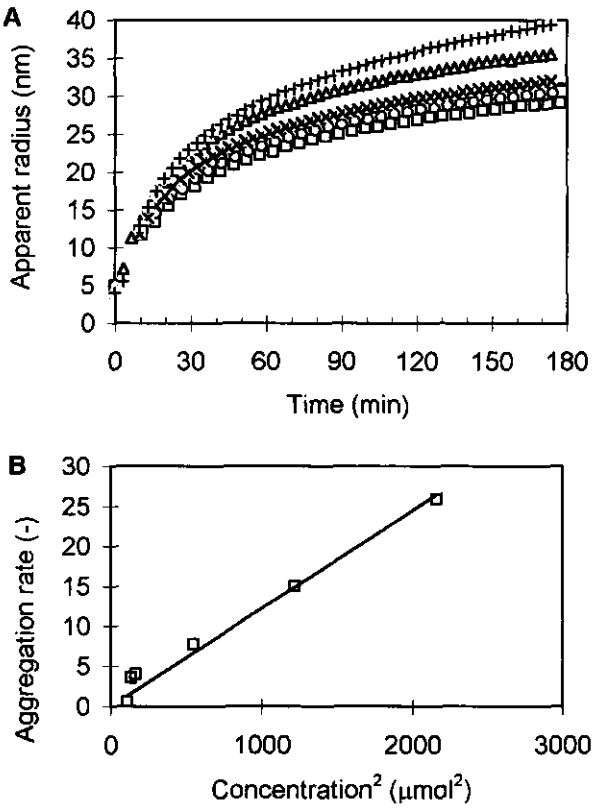

Figure 5 A: Apparent Stokes-Einstein radii of patatin at 55°C at pH 7 at protein concentrations ranging from 0.45 to 2.0 mg/mL. 0.45 mg/mL: □; 0.5 mg/mL : -; 0.55 mg/mL: o; 1.0 mg/mL: x; 1.3 mg/mL: Δ; 2.0 mg/mL: +. B: Plot of the inital aggregation rate as a function of the squared protein concentration. Solid line is the best linear fit of the data that passes trough the origin.

Chemical interactions during aggregation

In order to elucidate the nature of the bonds between aggregated patatin molecules SDS-PAGE analysis was performed under various conditions. In Table 1 the resulting molecular masses are shown of patatin that was heated under various conditions. Analysis of unheated patatin under non-reducing conditions revealed only a 43 kDa band. SDS-PAGE of heated patatin samples, however, showed under non-reducing conditions three bands, at 43, 82 and 108 kDa, respectively (Table 1). The presence of β-mercaptoethanol during SDS-PAGE analysis resulted in the dissociation of the 82 and 108 kDa bands into the patatin monomer

(43 kDa, Table 1). This suggests that the proteins are connected by sulfur bridges. The bands at 82 and 43 kDa represent most probably dimer and monomer patatin, respectively. The band at 108 kDa could consist of an aggregate, constituted of 3 or 4 patatin monomers, that does not migrate in accordance with its molecular mass. Although SDS-PAGE results sometimes can exhibit considerable errors in the estimation of molar masses (Hedrich *et al.*, 1993; Saito and Shimoda, 1997), it seems unlikely that the 108 kDa band originates from a tetramer, since 108 kDa deviates largely from the expected mass of a tetramer (172 kDa). Therefore, it is denoted as a trimer from now on. According to the literature (Mignery *et al.*, 1984; Stiekema *et al.*, 1988) patatin contains only one cysteine residue per molecule, therefore, the formation of sulfur-bridge linked dimers is comprehensible. The formation of the trimer, however, is more difficult to understand. It could be possible that in this trimer the third protein is entrapped by the dimer, and that breakage of the dimer using β-mercaptoethanol results in the release of the third molecule.

Table 1 Molar masses of unheated patatin and of particles present after heating under different circumstances, that are analyzed under several dissociating conditions.

	Native	Heated pH 7	Heated pH 7 NEM[3]	Heated pH 3
Buffer[1]	Dimer	Aggregate	Aggregate	Aggregate
SDS[2]	Monomer (43 kDa)	43, 82, 108 kDa	43, 82, 108 kDa	43, 82, 108 kDa
SDS + β-ME[2]	Monomer (43 kDa)	43 kDa	43 kDa	43 kDa

[1]: Data from size exclusion chromatography

[2]: In the presence of 6 M urea, data from SDS-PAGE

[3]: NEM: N-ethyl maleimide

The sensitivity to β-mercaptoethanol suggested that the dimers and trimers are disulfide linked. However, heating in the presence of N-ethyl maleimide (NEM) did not prevent the formation of the di- and trimers. In addition, patatin that was heated at pH 3 did show the 82 and 108 kDa band on SDS-PAGE analysis, and only a 43 kDa band in the presence of β-mercaptoethanol (Table 1). The intensities of the aggregate bands were similar under all conditions. Therefore, it was thought that the formation of sulfur bridges is not the determining mechanism of the aggregation. Moreover, the sulfur bridges apparently are formed when the proteins have coagulated already and are present in specific configurations. This aggregation mechanism is clearly different from that of the whey protein β-lactoglobulin where sulfur bridge formation plays a major role during the initial stage of aggregation (Roefs and de Kruif, 1994; Elofsson *et al.*, 1996; Hoffmann and Van Mil, 1997). They both could be representative of typical classes of proteins, one where sulfhydryl groups and sulfur bonds play an important role in the aggregation and one where other interactions are rate determining.

The packing density of the aggregates and the form of the native particle

If the aggregation of patatin obeys Smoluchowski-Fuchs kinetics, it must be possible to describe its aggregation behavior with that model (Meakin, 1988; Amal *et al.*, 1990). From the model data the apparent radius of the aggregates can be calculated if the fractal dimension or the packing density is known. Furthermore, the fractal dimension can provide information on the mechanism of the aggregation (Meakin, 1988; Lin *et al.*, 1989). The fractal concept can be used provided that the size of the aggregate and that of the monomeric unit are separated by several orders of magnitude (Rouw and de Kruif, 1989). This condition is not met here, therefore, in this study the term packing density will be used and not fractal dimension.

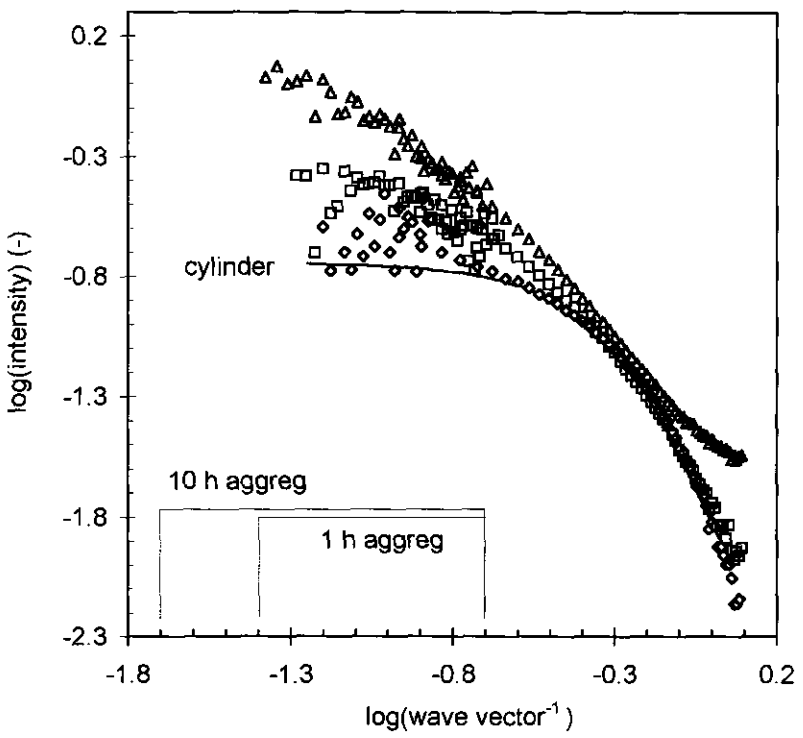

Figure 6 SANS spectra measured at ambient temperature of unheated patatin (○) and patatin at pH 7 heated at 55°C for 1 (□) and 10 h (Δ), respectively. Indicated are the limits of wave vector region where the packing density of the aggregates after 1 and 10 h can be obtained as wel as the modeled scattering of a cylindrical particle (solid line).

Using small-angle neutron scattering (SANS) it is possible to measure scattering intensities in the wave vector range of 0.05 to 2 nm^{-1}, theoretically enabling the determination of the packing density of patatin aggregates. SANS was performed since the wave vector range as

covered using the DLS equipment as described above did not allow the determination of the packing density of the patatin aggregates (Rouw and de Kruif, 1989). Figure 6 shows the SANS spectra of samples containing 0.5 mg patatin/mL at pH 7 that were unheated and heated for 1 and 10 h at 55°C, respectively. It can be seen that the scattering data in Figure 6 are rather noisy, nevertheless, the data contain useful information. First, the scattering behavior is highly typical for an aggregating system (Renard *et al.*, 1996; Verheul, 1998), since the sample heated for 10 h showed the highest scattering intensity and that the sample heated for one h and native patatin induced a lower amount of scattering. The sample heated for 10 h exhibited between -0.1 and 0.1 (logarithm of wave vector values) a behavior deviating from the unheated and the sample heated for one h, which is not understood. In Figure 6 are indicated the regions of the wave vector range from which the packing density of the aggregates can be derived for the samples heated for 1 and 10 h. The samples heated for 1 and 10 h both showed in this area a non-linear change in scattering intensity, therefore, no accurate measure for the packing density could be obtained.
Second, from SANS-measurements information on the shape of the particles can be obtained, by fitting the scattering intensity simulated from a particle with known shape, to the experimental data (Pedersen, 1997). He presented expressions to simulate scattering for differently shaped particles. We found that the scattering of native patatin could be modeled satisfactory (χ^2 :1.3) assuming a cylindrical particle having a radius of 2.5 nm and a length of 9.8 nm (Fig. 6). It can be seen that the model accurately describes the scattering from 0.1 to -0.7, whereas below this value the predicted intensity is too low. From DLS measurements a spherical particle with a radius of 4 to 5 nm was derived (Fig. 2), which correlates well with the size of the cylinder as obtained from SANS (Fig. 6).

In conclusion, this work showed that the aggregation of patatin could follow the mechanism of slow coagulation of the Smoluchowski-Fuchs theory. The aggregation rate dependence on the protein concentration was in accordance with that mechanism. The aggregation rates as obtained at temperatures ranging from 40-65°C correlated well with unfolding of the protein at a secondary level. SDS-PAGE analysis suggested that the formation of sulfur bridges is not the determining process for the aggregation of patatin. DLS measurements, assuming a spherical shape, indicated that native patatin has a radius of 4-5 nm, which was in good accordance with the results of SANS, which suggested a cylindrical particle with a radius and length of 2.5 and 9.8 nm, respectively.

Acknowledgments
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Chapter 8

General discussion

Introduction

Aim and outline

Potato proteins are a promising source of plant proteins for human consumption. Therefore, a considerable amount of work has been performed to obtain undenatured resoluble potato proteins that can be applied in human consumption (Knorr *et al.*, 1977; Knorr, 1977, 1980; Lindner *et al.*, 1980, 1981; Shomer *et al.*, 1982; Boruch *et al.*, 1989; Gonzalez *et al.*, 1991). Despite this, no industrial method for the production of a resoluble potato protein preparation has been developed, hence, no potato protein product applicable in foods is available so far. A reason for this could be the fact that this work generally not was performed on a well defined system of isolated proteins, but on protein mixtures. Moreover, this was generally done in a complex matrix containing unknown potato compounds of non-protein origin.
Therefore, the goal of this work was, first, to extend the knowledge on the presence and characteristics of soluble potato tuber proteins by biochemical analysis of the various (classes of) proteins. Next, the objective was to examine the thermal structural stability of isolated patatin and its thermal aggregation mechanism in a well defined system. The information on the structural stability may be of help to elucidate the mechanism of the irreversible precipitation as occurring in the industrial process. Furthermore, when combined with the mechanism of aggregation, it would allow us to relate its functional behavior to the structural properties of the protein.

Biochemical analysis of potato proteins

Identification and classification of potato cultivars

A rapid identification of potato cultivars from the tubers is of importance for both the trade and the processing of potatoes (Stegemann and Loeschcke, 1977). Up to about 30 years ago the most reliable method to identify potato cultivars from the tubers was by visual examination of their sprouts (Zwartz, 1967). This method required several weeks before an answer was obtained. Therefore, it was tried to develop rapid methods for the identification of cultivars from the tubers. By the end of the 1960's electrophoretic methods had been developed that replaced the time consuming sprouting procedure (Zwartz, 1967; Stegemann and Loeschcke, 1977). At present, the identification of cultivars from the tubers is increasingly performed using molecular biological techniques such as amplified fragment

length polymorphism (AFPL) or the polymerase chain reaction (Schneider and Douches, 1997; Kim *et al.*, 1998). By molecular biology it will eventually be possible to asses all genes encoding for proteins in potatoes, as well as the expression levels of those genes. This will not only facilitate the identification of potato cultivars, it will also enable categorization of cultivars in genetically closely related groups. These cultivars can be classified based on similar characteristics of their proteins such as protease inhibiting activity or LAH-activity. The advantage of such a classification may be, for example, that heat-treatments required to destroy enzyme activity could be optimized for each class.

Although the developments in molecular biology proceed rapidly, it will take a considerable time to reach the described level of knowledge. Until then, the biochemical analysis of proteins and protein mixtures are of importance for the analysis and the study of possible applications of (potato) proteins. With regards to biochemical characterization, (free zone) capillary electrophoresis (CE) and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) have become available as tools to analyze biopolymers (Bahr *et al.*, 1994; Kaufmann 1995; Wehr *et al.*, 1997). CE can be of use for the analysis of potato proteins, for example to study variation among individual patatin isoforms in processes like thermal aggregation (Chapter 6), or degradation of proteins during storage (Chapters 2). MALDI-TOF MS can be used as an additional tool to classify proteins based on their molar masses. As an example, in Chapter 2 both the MALDI-TOF MS spectra and trypsin inhibitor activity analysis (Fig. 3B, Table 2) indicate that Bintje and Desiree exhibit a similar trypsin inhibitor activity as well as a comparable spectrum with MALDI-TOF MS. Elkana however, revealed both a significantly deviating trypsin inhibitor activity and MALDI-TOF peak pattern. To modulate the protease inhibitor activity of proteins, Bintje and Desiree may be processed similarly, whereas Elkana requires a different treatment. This kind of knowledge provides information on the protein quality and may result in a more economical way of processing, since it is fine-tuned at the specific cultivars, hence, at the properties of its proteins

Molar masses of patatin

MALDI-TOF MS revealed that patatin of Bintje (40.4 and 41.7 kDa) and Desiree (41.9 and 42.9 kDa) consists of two mass isomers. The difference between the masses is approximately 1.3 and 1 kDa for Bintje and Desiree, respectively. In Elkana one molar mass is predominantly present, of 40.5 kDa (Table 1). The differences in molar masses between cultivars as calculated from the primary sequences are at most 200 Da (Table 2; Mignery *et al.*, 1984; Stiekema *et al.*, 1988). Patatin can have one, two or three possible glycosylation sites (Stiekema *et al.*, 1988, Sonnewald *et al.*, 1989), based on the consensus sequence for N-linked glycosylation: Asn-X-Ser/Thr, where X may be any amino acid other then proline or asparagine (Katsube *et al.*, 1998).

The variation in the measurements of the molar masses of patatin within each mass isomer is about 0.25%. A single carbohydrate chain (approximately 1.2 kDa) contributes 2.9% (w/w) to the mass of patatin (Sonnewald *et al.*, 1989). Two carbohydrate moieties would result in 5.5% (w/w) of glycosylation, which is in reasonable accordance with the result of Sonnewald *et al.* (1989) who found about 4% (w/w) of glycosylation.

This implies that presence of one, two or three carbohydrate moieties has a significantly larger influence on the molar mass of the protein than the cultivar specific differences in the amino acid sequence. Therefore, it may be possible to establish the degree of glycosylation from a comparison of MALDI-TOF MS results with the molar masses as calculated from the amino acid sequence. This approach can provide information on the question whether one or two, or two or three of the possible glycosylation sites of patatin are used (Sonnewald *et al.*, 1989).

Table 1 Molar masses of patatin (kDa) as obtained with MALDI-TOF MS

Cultivar	Chapter 2		Chapter 3		Chapter 4	
Bintje	40.4	41.7	40.3	41.6	40.5	41.8
Desiree	41.9	42.9	n.d.	n.d.	n.d.	n.d.
Elkana	40.5	-	n.d.	n.d.	n.d.	n.d.

-: could not be obtained

n.d: not determined

In Chapter 3 it is stated that differences between the measured and the calculated masses including the carbohydrate moieties, could be caused by point mutations in the primary sequence and by differences in the carbohydrate groups. Comparing the calculated and the measured masses (Tables 1 and 2), it seems likely that patatin from Bintje is glycosylated at one or two positions, whereas the predominant isoform from Elkana patatin is glycosylated once. It is possible that the number of carbohydrate chains is limited by the presence of glycosylation sites (see also Chapter 1), or that not all of these sites are used. The patatin from Desiree may be glycosylated 2 or 3 times; however, the MALDI-TOF MS spectra were relatively noisy (Chapter 2, Fig. 3A) and these data should be interpreted with caution.

Table 2 Molar masses (kDa) calculated from the primary sequence of patatin (Mignery *et al.*, 1984; Stiekema *et al.*, 1988), without carbohydrate chain and with the masses added of 1, 2 or 3 carbohydrate chains of approximately 1200 Da (Sonnewald *et al.*, 1989).

Reference	no glycosylation	1 glycosylation	2 glycosylations	3 glycosylations
Stiekema *et al.*, 1988	39.8	40.9	42.1	43.3
Mignery *et al.*, 1984	39.5	40.7	41.9	43.0
	39.6	40.8	42.0	43.2

Genetic variants of proteins

Differences between the isoform pools were shown to be all related to variation in the surface charge of the proteins (Chapter 3). The presence of molar mass differences had no influence on the fractionation, both mass-isomers of patatin were observed in all isoform pools. The isolation was carried out at 4°C in the absence of protein modifying chemicals. Therefore, the explanation for the surface charge differences between the patatin isoforms is more likely to be found in the genetic diversity of patatin (Mignery *et al.*, 1984; Stiekema *et al.*, 1988). In the tuber, patatin (and the protease inhibitor I) is encoded by various genes, which results in proteins with point mutations in their primary sequence. In the literature, mutations in the primary sequence of patatin resulting in an altered charge of patatin are described, as noted in Chapter 3 (Pikaard *et al.*, 1987; Twell and Ooms, 1988; Graham *et al.*, 1985; Cleveland *et al.*, 1987).

The presence of genetic variants of proteins is not unique for potatoes, it is widely observed in other organisms also. For example glycinin, a storage protein of soy, consists of various highly homologous families (Nielsen *et al.*, 1989) and also in milk proteins genetic variants are commonly observed (Swaisgood, 1992). From an evolutionary point of view it is not surprising that proteins exist in various isoforms, or genetic variants. In the case of a storage protein like patatin that also has an enzyme activity, it can be argued that as long as spontaneous mutations do not result in loss of lipid acyl hydrolase (LAH) activity (or any other biologically relevant function in case of other proteins), there is no additional selection pressure on plants producing patatins with a changed primary sequence. Since LAH-activity is found in all varieties and isoform pools (Chapter 2; Table 2; Chapter 3, Table 1; Racusen, 1985; Galliard and Dennis, 1974), it is apparently of benefit for the plant, and, therefore, it is conserved.

In conclusion, patatin exists in two mass-isomers that can be divided into various patatin isoforms with slightly differing primary sequences (Figure 1). It is possible that the mass-isomers have similar point mutations.

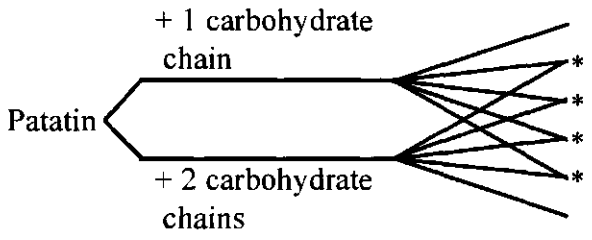

Figure 1 Symbolic representation of the patatin family
*These isoforms show similarities on charge level

Behavior of patatin in potato fruit juice, as compared to that in a model system

Unfolding and precipitation

The general problem obstructing the recovery of potato proteins in a form that makes them applicable in foods is the irreversibility of the precipitation as induced in the industrial process. This precipitation is generally done at pH values of 3.5-5.2 and at temperatures from 80 to 120°C (Knorr, 1977). To be able to relate the precipitation of patatin in the potato fruit juice to their heat-induced structural changes, the thermal structural stability of isolated patatin was studied in a well defined system.

Figure 2 illustrates an interpretation of the findings on the temperature and pH dependence of the structural properties of patatin. In Chapters 4 and 5 it was shown that patatin is a highly structured protein, at room temperature and at near-neutral pH. Upon lowering the pH or increasing the temperature, the protein unfolds partly. A heat-treatment at low pH, however, does not result in a further unfolding of the protein.

It was suggested that patatin unfolds partly upon lowering the pH or upon a heat-treatment (Chapters 4 and 5). This is in accordance with the relatively low value for the molar denaturation enthalpy of 20 $kJ \cdot mol^{-1}$ as observed with DSC in Chapter 4. Assuming a cooperative transition, which is supported by the temperature dependence of the thermal unfolding (Chapters 4 and 5), a higher transition energy per unity of mass is to be expected if the protein would unfold completely (Boye *et al.*, 1997). This is an additional indication that the protein unfolds only partly upon denaturation. The denaturation enthalpy calculated from the unfolding curves measured with CD is approximately 75 $kJ \cdot mol^{-1}$ (Chapter 4), which is still relatively low for a complete unfolding of a protein.

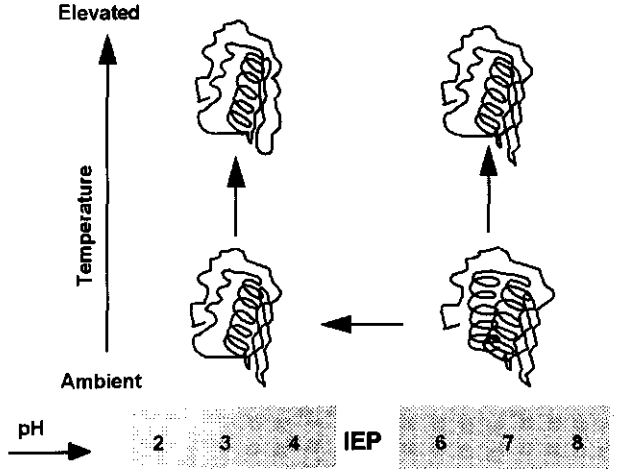

Figure 2 Schematic representation of the structural stability of patatin.

Isolated patatin in buffer solution did not precipitate upon heating, and precipitated reversibly at its isoelectric pH (4.8-5.2). At pH values below the isoelectric point, the protein remained soluble. The protein precipitated irreversibly, however, at the same pH and temperature when present in the potato fruit juice (Van Koningsveld *et al.*, 1999). From this, it was concluded that in the potato fruit juice unknown compounds could be present that may mediate the precipitation making it irreversible. In Chapter 4 it was suggested that these unknown components could be of phenolic origin. The potato contains a relatively high amount of phenolic compounds, such as chlorogenic acid and cafeic acid (Lisinska and Leszcynski, 1989), of which it is known that they tend to interact with proteins (Gupta and Haslam, 1980). This interaction can lead to the formation of insoluble complexes (Shahidi and Naczk, 1995). Furthermore, in various studies the protein solubility in potato fruit juice was observed to be minimal below pH 3, whereas the isoelectric point of the proteins is between pH 5 and 8 (Ahldén and Trägård, 1992; Lindner *et al.*, 1980; Van Koningsveld *et al.*, 1999). The optimum pH for the precipitation of various proteins in the presence of phenolic compounds was found to be up to 3.1 pH units below their isoelectric points (Naczk *et al.*, 1996). These observations could be an indication that phenolic compounds play an important role during the acid-heat precipitation of potato proteins. In our laboratory a Ph.D. project is devoted to the identification of the unknown compounds and the prevention of their interactions with the proteins.

Soluble potato protein from industry

Lindner *et al.* (1980) provide a method to separate potato proteins into an acid-soluble and an acid-coagulable fraction. They dialyzed the potato fruit juice against a phosphate buffer pH 6.35 and precipitated at pH 3 all proteins with a molar mass above 23 kDa (mainly patatin). The other proteins remained soluble. This provides a way to separate, for example, the protease inhibitors from patatin. However, the authors did not investigate the resolubility of the patatin. In the literature, furthermore, many attempts are described to obtain undenatured potato proteins by an industrial process. This has not been successful, which is most likely related to fact that the importance of non-protein potato fruit juice components, was insufficiently recognized. All methods described, involved either an acidic environment or heat-treatment, or both. These treatments inevitably result in the unfolding of patatin (Chapters 4 and 5), and possibly of the other proteins also. Unfolding of the proteins may enhance the interactions that make the precipitation of the proteins irreversible.

Aggregation

Aggregate sizes after cooling

From the gel filtration elution patterns of patatin after heat-treatment it can be seen that the patatin aggregates all elute in the excluded volume and that no aggregates elute in the separation range of the column (between 80 and 1300 kDa, Chapter 6, Figure 1). Apparently, no patatin aggregates with molar masses between 80 and 1300 kDa are present after cooling. This was explained by assuming that small aggregates coagulate rapidly to bigger ones upon cooling (Chapter 6), since DLS-experiments at elevated temperatures showed that the aggregation proceeded continuously towards larger particles (Chapter 7, Figure 2). DLS experiments, however, showed no changes in the aggregate size upon cooling (Chapter 7). This seems contradictory to the explanation on the lack of aggregates eluting in the separation range of the column on gel filtration chromatography after cooling. It is possible, however, that small aggregates represent only a minor proportion of all particles during the aggregation and that only this small fraction coagulates further upon cooling. Moreover, it is known that DLS is poorly sensitive for the presence of relatively small particles, the measured radius is strongly influenced by large particles (Berne and Pecora, 1976). Therefore, it is possible that the aggregation of the small particles upon cooling did occur, but was not observed with DLS.

Structural aspects of refolding or aggregation

The reversibility of the thermal unfolding of patatin was described in Chapter 4. It is observed that upon cooling the α-helical proportion increased the most. At a tertiary level partial refolding is concluded from a shift in the emission maximum of the tryptophan fluorescence (from 342 to 338 nm upon cooling) and from a change in the near-UV CD spectra around 280 nm. An additional indication that the protein refolds only partly, was the irreversible loss of enzyme activity at temperatures exceeding 60°C. Such a partial refolding of proteins after a heat-treatment is also observed for other proteins (Van Mierlo *et al.*, 1998). In Chapters 6 and 7 it was seen that the protein aggregates at temperatures exceeding 50°C. From the observations on unfolding and aggregation at temperatures exceeding 50°C the question may rise whether the phenomena observed with fluorescence and CD spectroscopy actually may be interpreted as a refolding of the proteins, or that they are in fact partly the result of aggregation.

The effects on a tertiary level of folding as observed with fluorescence and near-UV CD upon cooling could also be induced by interactions occurring between several proteins in an aggregate (Ben-Naim, 1995). This may imply that the observed changes in the far-UV CD and fluorescence spectra upon cooling could be due to both aggregation and refolding. From far-UV CD and aggregation measurements, however, it can be concluded that aggregation of

the proteins does not affect their secondary structure content. This is based on the observations that, first, the aggregation proceeds at constant temperatures above 50°C (Chapters 6 and 7), whereas the secondary structure content of the protein changes during the increase of temperature only (Chapter 4). Second, spectra of samples that were heated for different times exhibited after cooling a similar secondary structure. If aggregation had significantly affected refolding, then different levels of aggregation would be expected to result in different levels of refolding. This was not observed and it is concluded that the changes at the secondary level are due to partial refolding of the protein, and not to aggregation.

It should be noted that the absence of increasing intensities at 1686 and 1620 cm^{-1} in the infra-red spectra (Chapter 4, Figure 4), which are claimed to be generally assigned to aggregation of proteins (Haltia *et al.*, 1994; Muga *et al.*, 1993B), is no proof of the absence of aggregation. In this work, spectral changes at these wave numbers were not observed, whereas aggregation did occur.

Formation of reactive particles

Patatin in its native state does not aggregate, whereas upon unfolding at temperatures exceeding 50°C reactive particles are formed that can aggregate. The formation of reactive particles is presented as a single step reaction in the aggregation model. This model (Scheme 1) describes the formation of reactive particles (1), followed by an aggregation reaction of reactive particles (2).

$$N \xrightarrow{k_1} U \qquad (1)$$

$$U_p + U_q \xrightarrow{k_2} U_{(p+q)} \qquad (2)$$

$p,q \in \{1,2,3...\}$

N	: Native patatin
U	: Reactive patatin
U_p, U_q	: Aggregates consisting of p or q patatin molecules, respectively
$U_{(p+q)}$	: Aggregates consisting of p+q patatin molecules

Scheme 1 Proposed mechanism of the aggregation of patatin

With this model, the thermal aggregation of patatin can be described in an acceptable way as a function of temperature and protein concentration (Chapter 6, Figures 2 and 3). The mechanism underlying the model of the thermal aggregation of patatin as presented in Chapter 6 is assumed not to change with temperature. It is possible, however, that interactions

between protein molecules in an aggregate are temperature dependent. The model is based on the consequences for the aggregation rates of the sum of all the interactions. It does not take into account what force is predominant, or rate-determining at each temperature or stage of the aggregation.

As a concluding remark on the modeling of kinetics, it can be said that the models do not explain all observations. Nevertheless, modeling is of great value for the understanding of the thermal aggregation of proteins, and it can facilitate the design of new experiments.

Schematic representation of the aggregation of patatin at pH 7

The rate of slow coagulation, according to the Smoluchowski-Fuchs mechanism (Von Smoluchowski, 1917; Overbeek, 1952), is limited by both diffusion and reaction. This mechanism does not present information on the origin of the interactions. The quantitative contribution of, for example, hydrophobic or electrostatic interactions, can not be determined, though they are often expected to be the major forces in the aggregation (Spassov *et al.*, 1995).

In Figure 3 a schematic representation of the aggregation mechanism of patatin is presented. The aggregation starts with native patatin, which is in the form of a non-covalently bound dimer (Racusen and Weller, 1984; Chapter 6, Figure 1). Patatin contains one sulfhydryl group (Mignery *et al.*, 1984; Stiekema *et al.*, 1988) which can not be determined by Ellman's procedure (Ellman, 1959). It is assumed to be buried in the molecule in its native state. Upon heat-treatment an activated reactive particle is formed (Chapters 6 and 7). Next, the reactive patches of the molecules interact and aggregates are formed.

SDS-PAGE analysis in the absence of β-mercaptoethanol showed that the aggregates dissociated in the presence of SDS into the 43 kDa monomer, an 82 kDa dimer and a 108 kDa trimer (Chapter 7, Table 1). In the presence of β-mercaptoethanol/SDS the aggregates dissociated completely into 43 kDa monomer. The interaction that forms the dimer could be a sulfur bridge, the structure of the trimer, however, is not understood. Remarkably, heating in the presence of N-ethyl maleimide (NEM) or at pH 3 does not prevent the formation of the sulfur bridge linked 82 and 108 kDa dimers and trimers. They are found in the same proportions as when patatin is heated at pH 7 without NEM. The NEM is apparently not able to react with free thiol groups and also at low pH the SDS non-dissociable dimers and trimers are observed. Therefore, it is thought that the formation of sulfur bridges is possibly not the determining factor for the aggregation, and would merely occur when patatin molecules are close to eachother in a specific orientation.

In the literature only limited information is available on aggregation mechanisms of proteins. The only extensively studied food-protein in this respect is β-lactoglobulin (e.g. Roefs and De Kruif, 1994; Elofsson *et al.*, 1996; Hoffmann *et al.*, 1996; Verheul *et al.*, 1998). Comparing the aggregation mechanism of β-lactoglobulin with that of patatin, it can be said that where

the formation of sulfur bridges is the initial step in aggregation of β-lactoglobulin, this seems of secondary importance in patatin aggregation. Interestingly, when β-lactoglobulin was heated in the presence of NEM, it seemed to aggregate via an different mechanism, with comparable reaction rates. After heating, aggregates were observed that dif not dissociate in the presence of SDS only. Therefore, even in the presence of NEM the formation of sulfur bridges during heating could not be excluded (Hoffmann *et al.*, 1997). Also after aggregation of patatin in the presence of NEM sulfur bridges are present, despite the fact that they may not be rate determining.

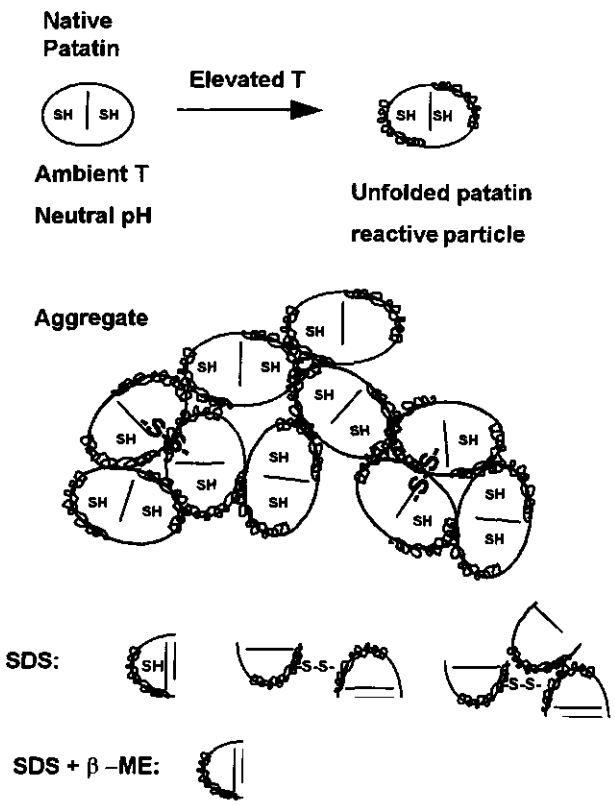

Figure 3 Symbolic representation of the aggregation of patatin

Concluding Remarks

Patatin can be divided into two mass isomers, that each can be divided into various proteins with a slightly differing primary sequence (genetic variants). The isoforms appeared to be of highly homogenous character and can be studied as a single protein species.

Native patatin at room temperature appeared to be a cylindrical particle, with a length and diameter of approximately 10 and 5 nm, respectively. At ambient temperatures it is a highly

structured protein that unfolds partly in a small temperature trajectory. Upon lowering the pH below its isoelectric pH patatin unfolds irreversibly, whereas a further heat-treatment at these pH values has only a minor effect on its secondary structure. The unfolding of patatin coincides with its precipitation in the potato fruit juice. The acid or heat precipitation of patatin present in this juice may be enhanced by so far unknown components, possibly of phenolic origin.

The aggregation of patatin requires the unfolding of the protein and can be described quantitatively with a model consisting of two steps. The first step involves the formation of reactive particles whereas in the second step these particles aggregate via second order kinetics. The formation of sulfur bridges is probably not the rate determining factor during aggregation. The course of aggregation suggested mechanism of slow coagulation, the rate of which would be limited by both reaction and diffusion.

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Summary

Potato proteins are present in the effluent of the potato starch industry, the so called potato fruit juice. They are recovered by an acidic heat-treatment of the potato fruit juice. This results in irreversibly precipitated proteins, which have lost all functionality and can only be applied as low value cattle-fodder. As potato proteins are a promising source of plant proteins, a considerable amount of work has been performed to obtain undenatured resoluble potato proteins that can be applied for human consumption. Despite this, no industrial method for the production of such a protein preparation has been developed, hence, no potato protein product applicable in foods is available so far. A reason for this could be the fact that this work generally was performed with protein mixtures. Moreover, this was generally done in a complex matrix containing potato compounds of non-protein origin.

The aim of this work was, first, to extend the knowledge on the presence and characteristics of soluble potato tuber proteins. Next, the objective was to examine the thermal structural stability of isolated patatin, and its thermal aggregation mechanism in a well defined system. The information on the structural stability may be of help to elucidate the mechanism of the irreversible precipitation as occurring in the industrial process. This could lead to alternative procedures of isolation, resulting in a resoluble protein preparation that can be applied in food.

Patatin is the most abundant potato tuber protein, it is a 43 kDa glycoprotein with a lipid acyl hydrolase (LAH) activity. In Chapter 2 the content and biological activity of patatin and the protease inhibitors of molecular size 20-22 kDa were investigated as a function of storage time of whole potato tubers. It was observed that the amount of buffer-extractable protein decreased gradually during 47 weeks of storage of whole potatoes of Bintje and Desiree whereas, for Elkana, it increased after approximately 25 weeks. The patatin proportion of the extractable protein did not decrease significantly during storage, whereas the proportion of $PP_{20\text{-}22}$ protease inhibitors decreased. Each cultivar contained several different patatin isoforms. In Bintje and Desiree these isoforms could be divided into two populations with different masses, whereas in Elkana one molar mass was predominant. The isoforms of the three cultivars showed no significant differences in stability towards degradation during storage, as was concluded from capillary electrophoresis analysis and matrix assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS). Also, no degradation of patatin or protease inhibitors was observed using MALDI-TOF MS, whereas enzyme-activity assays suggested that the biological activity, especially that of cultivar Bintje, decreased markedly at the break of dormancy.

In Chapter 3 a method is presented to obtain various patatin isoform pools as well as a comparison of the physico-chemical properties of these fractions. Patatin could be separated in four isoform-pools, of which two together represented approximately 90% of the total amount of patatin. These isoforms differed in surface charge, resulting in different behavior on anion exchange chromatography, isoelectric focusing, native poly acrylamide gel - and

capillary electrophoresis. Despite the biochemical differences, no variations in structural properties nor in thermal conformational stability could be observed. All isoforms of the patatin family contained proteins with two molar masses, of approximately 40.3 and 41.6 kDa, respectively. This difference in molar mass (1300 Da) is of the size of order of one carbohydrate moiety.

Chapter 4 presents a structural characterization of isolated patatin at ambient and elevated temperatures. Isolated patatin at room temperature is a highly structured molecule at both a secondary and a tertiary level. It is estimated from far-ultra violet circular dichroism data that about 33% of the residues adopts an α-helical and 46% a β-stranded structure. Patatin is thermally destabilized at temperatures exceeding 28°C, as was indicated by near-ultra violet circular dichroism. It was shown that parts of the α-helical contributions unfold in the 45 to 55°C region, whereas the β-stranded parts unfold more gradually at temperatures from 50 to 90°C. This was confirmed with Fourier transform infrared spectroscopy. Differential scanning calorimetry indicated a cooperative transition between 50 and 60°C, most likely reflecting the unfolding of α-helical parts of the molecule. Furthermore, fluorescence spectroscopy confirmed a global unfolding of the protein between 45 and 55°C. The observed unfolding of the protein coincides with the inactivation of the patatin enzyme activity and with the precipitation as occurs in the potato fruit juice upon heating. At elevated temperatures, patatin still contains some helical and stranded structures.

Chapter 5 presents the conformation of patatin under various conditions as a function of temperature. Patatin unfolds partly due to either heat- or acid-treatments. When it is highly structured at the start of a heat-treatment (near-neutral pH) an apparent two-state thermal unfolding is observed. At low pH, when the starting conformation is already irreversibly unfolded to a certain extent, only minor changes occur upon heating. The residual structures could be part of one or more relatively stable domains. The acidic and the thermal unfolding appear to be similar, but are not identical. In Chapters 4 and 5 it was shown that the isolated protein in buffer did not precipitate upon heating, and precipitated reversibly at its isoelectric pH (4.8-5.2). At pH values below the isoelectric point, the protein remained soluble. The protein precipitated irreversibly, however, at the same pH and temperature in the potato fruit juice. From this, it was concluded that in the potato fruit juice unknown compounds may be present that could mediate the precipitation and make it irreversible.

In Chapter 6 chromatographic analysis of the proportions of non-aggregated and aggregated patatin as a function of incubation time and temperature is performed. It was observed that the decrease of the amount of non-aggregated patatin proceeded initially fast and was followed by a slower reaction at longer incubation times. This behavior was shown not to be due to heterogeneity of the starting material. It was noted that overestimation of the amount of native molecules after a heat-treatment, caused by refolding of the unfolded protein during the cooling step prior to the analysis, was significant and could not be neglected. Hence, corrections were applied, based on information on the structural properties of patatin as described in the Chapters 4 and 5. Taking this into account, a model consisting of a first

order reversible formation of reactive particles, followed by a second order aggregation reaction was proposed. This model described the thermal aggregation of patatin accurately and was confirmed by experiments at various protein concentrations.

In Chapter 7 dynamic lightscattering was used to study the thermal aggregation of patatin *in situ*, in order to elucidate the physical aggregation mechanism of the protein. The dependence of the aggregation rates on the temperature and the ionic strength suggested slow coagulation kinetics, a both diffusion and chemically limited mechanism. The aggregation rate dependence on the protein concentration was in accordance with that mechanism. The aggregation rates as obtained at temperatures ranging from 40-65°C correlated well with unfolding of the protein at a secondary level. Small angle neutron scattering and dynamic light scattering results were in good accordance, they revealed that native patatin has a cylindrical shape with a diameter and length of 5 and 9.8 nm, respectively.

In Chapter 8 the main results of the thesis are briefly discussed. Knowledge on patatin from chromatographic analysis, MALDI-TOF MS measurements and genetic studies is combined to establish the biochemical composition the patatin family. Besides this, a schematic representation the thermal aggregation mechanism of patatin is presented.

Samenvatting

Aardappeleiwit is een bijproduct van de aardappelzetmeelindustrie, waar het aanwezig is in het zogenaamde aardappelsap. Het eiwit wordt gewonnen uit dit sap door het neer te laten slaan wanneer het verhit wordt onder zure omstandigheden. Als gevolg hiervan is het zeer slecht oplosbaar en kan het alleen gebruikt worden in veevoer, een economisch laagwaardige toepassing. Gebruik in menselijke voeding zou dan ook een aanzienlijke verhoging van de waarde van het eiwit kunnen betekenen. Aardappeleiwit bezit behalve een uitstekende voedingswaarde ook andere voor de levensmiddelenindustrie gunstige eigenschappen, zoals schuim- en emulsievormende en stabiliserende capaciteiten. Dit is de reden dat er al veel onderzoek is verricht naar het verkrijgen van een in levensmiddelen toepasbaar eiwitpreparaat. Dit is tot dusverre niet succesvol gebleken, wat veroorzaakt zou kunnen zijn doordat er altijd aan een mengsel van aardappeleiwitten is gewerkt. Daarnaast zijn die onderzoeken uitgevoerd met eiwitten in het aardappelsap, dat nog vele andere componenten bevat.

Het onderzoek beschreven in dit proefschrift is allereerst gericht op het verkrijgen van een beter inzicht in het voorkomen en in de biochemische eigenschappen van aardappeleiwitten. Daarnaast is het effect bestudeerd van temperatuur verhoging bij een neutrale of juist lage zuurgraad (pH) op de structuur van patatine, het meest voorkomende aardappeleiwit. Tenslotte is de neiging van patatine tot aggregatie bij verhitten onderzocht. Kennis over de structurele eigenschappen van dit patatine onder goed gecontroleerde omstandigheden zou kunnen helpen bij het oplossen van het probleem zoals dat nu speelt in de aardappelzetmeel industrie.

Patatine is een 43 kDa glycoproteine met opslagfunctie dat ook nog een lipide acyl hydrolase (LAH) activiteit heeft. Een andere belangrijke groep van aardappeleiwitten zijn de aardappel protease remmers. In hoofdstuk 2 is van drie aardappelrassen het gehalte en de biologische activiteit van patatine en een groep van 20-22 kDa protease remmers beschreven. Het is gebleken dat bij Bintje en Desiree de hoeveelheid extraheerbaar eiwit afneemt naarmate de opslagduur van de hele aardappels langer wordt (tot 47 weken), terwijl het bij Elkana na ongeveer 25 weken toenam. Het patatinegehalte daalt evenredig met de totale hoeveelheid geëxtraheerd eiwit, terwijl het protease remmer gehalte wat sneller afneemt. Patatine afkomstig van de aardappelsoorten Bintje en Desiree bestaat uit twee molecuul massa's, terwijl in Elkana vooral één massa is gevonden. Alle rassen bevatten patatine dat bestaat uit meerdere isovormen, eiwitten met kleine onderlinge verschillen in hun aminozuur volgorde. De isovormen vertonen geen verschillen in hoeveelheid van voorkomen tijdens de opslag, hetgeen is aangetoond met capillaire electroforese. Ook werd bij patatine en de protease remmers geen afbraak waargenomen met matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS), terwijl enzymactiviteitsmetingen aantonen dat de biologische activiteit van deze eiwitten merkbaar afneemt bij het begin van het spruiten van de aardappelen, en dan met name bij het ras Bintje.

In hoofdstuk 3 is een methode beschreven waarmee de scheiding van patatine in 4 groepen kan worden verkregen. De twee belangrijkste groepen vertegenwoordigen samen 90% van alle patatines. Deze groepen zijn op diverse manieren met elkaar vergeleken. De structurele eigenschappen en stabiliteit van de isovormen vertoonden geen verschillen. De verschillen die wel werden waargenomen, met natieve- en SDS-PAGE, capillaire electroforese en isoelectrische focussering, waren alle te herleiden tot ladingsverschillen op het oppervlak van de eiwitten. Deze ladingsverschillen worden hoogstwaarschijnlijk veroorzaakt door de puntmutaties in de aminozuurvolgorde van patatine. Alle isovormen van de patatine familie uit Bintje bevatten eiwitten met twee molaire massa's, van respectievelijk 40.3 en 41.6 kDa. Dit verschil is ongeveer even groot als de massabijdrage van één suikergroep (1300 Da). De reden van het massaverschil is vermoedelijk dan ook een verschil in het aantal suikergroepen dat aan het eiwit gekoppeld is.

In de hoofdstukken 4 en 5 wordt, onder verschillende condities, de structurele stabiliteit van patatine beschreven. Met behulp van spectroscopische technieken als circulair dichroisme, fluorescentie spectroscopie en Fourier transform infrarood spectroscopie bleek dat patatine bij kamertemperatuur en neutrale pH (pH 6-8) een hoog gestructureerd eiwit is. Het bevat ongeveer 33% helix en 46% stranded structuren. Bij verhogen van de temperatuur ontvouwt het eiwit deels, afhankelijk van de pH, tussen 49 en 58 °C. Differential scanning calorimetrie toonde aan dat het eiwit coöperatieve ontvouwt tussen 50 en 60°C. Vooral de helix-structuren ontvouwen in dit temperatuur gebied, terwijl bij een verdere verhoging van de temperatuur de stranded-structuren meer geleidelijk ontvouwen. De enzymactiviteit gaat verloren bij de ontvouwing tussen 50 en 60°C. Wanneer bij kamertemperatuur de zuurgraad verlaagd wordt tot onder de isoelectrische pH van patatine (pH 4,8-5,2) ontvouwt het eiwit op vergelijkbare manier als bij het verhogen van de temperatuur bij neutrale pH. Een temperatuurverhoging bij lage pH leidt slechts tot een geringe verdere ontvouwing van het eiwit. In alle gevallen lijkt slechts een deel van het eiwit te ontvouwen, mogelijk bestaat patatine uit enkele domeinen, waarvan er één minder stabiel zou moeten zijn dan de andere. Als het gedrag van geïsoleerd eiwit vergeleken wordt met dat in het aardappelsap blijkt dat er in het aardappelsap onbekende componenten aanwezig zijn die ervoor zouden kunnen zorgen dat het eiwit niet meer kan heroplossen nadat het neergeslagen is.

In de hoofdstukken 6 en 7 wordt het aggregatie gedrag van patatine bij temperaturen van 40 tot 65°C beschreven. Het blijkt dat de ontvouwing van patatine noodzakelijk is voor aggregatie. In hoofdstuk 6 worden de hoeveelheden van geaggregeerd en niet geaggregeerd patatine bepaald na hittebehandelingen van verschillende duur. Op deze manier wordt de snelheid van het aggregeren bepaald, hetgeen informatie geeft over de processen die optreden. Patatine reageert als één enkel eiwit, ondanks dat het uit verschillende isovormen bestaat. De aggregatie van patatine verloopt aanvankelijk snel en wordt langzamer bij langere incubatietijden. In het onderzoek blijkt dat ontvouwen eiwitmoleculen, die aanwezig zijn bij verhoogde temperaturen, hervouwen tijdens het afkoelen van het monster, hetgeen een overschatting van de hoeveelheid natieve moleculen tot gevolg heeft. Deze overschatting is

significant en mag niet worden genegeerd. Om hiervoor te corrigeren wordt de informatie toegepast over de structurele eigenschappen van patatine, die is beschreven in de hoofdstukken 4 en 5. Dit in beschouwing nemend is een model opgesteld dat bestaat uit twee stappen. In de eerste stap ontvouwt patatine en wordt het reactief. In de tweede stap reageren de eiwitmoleculen met elkaar tot aggregaten. Het model was in staat bij verschillende temperaturen en eiwitconcentraties het gedrag van patatine redelijk accuraat te beschrijven.
In hoofdstuk 7 wordt met dynamische lichtverstrooiing de aggregatie bestudeerd terwijl het verloopt, dus niet pas na het afkoelen. Met dynamische lichtverstrooiing kan de grootte van deeltjes in een oplossing bepaald worden, door dit gedurende de tijd te doen kan de toename van de deeltjesgrootte door aggregatie gemeten worden. De toename van de grootte van de eiwitclusters suggereert een zogenaamd langzaam aggregatiemechanisme. Dit houdt in dat de reactie beperkt wordt door zowel de kans dat twee deeltjes op elkaar botsen als door de kans dat ze bij een botsing blijvend aan elkaar plakken. De afhankelijkheid van de eiwitconcentratie is in overeenstemming met de theorie en met de observaties in hoofdstuk 6. Metingen met behulp van small angle neutron scattering gaven resultaten te zien die goed in overeenstemming waren met die van dynamische lichtverstrooiing. Natief patatine is een cilindervormig molecuul met een lengte van 19.8 nm en een diameter van 5 nm.
In hoofdstuk 8 worden de belangrijkste resultaten uit het proefschrift bediscussieerd. Kennis over patatine, afkomstig van chromatografische analysis, massa spectrometrie en genetische studies is gecombineerd om een model voor de samenstelling van de patatines te maken. Daarnaast is een symbolisch model voor de aggregatie van patatine beschreven.

Nawoord

Zo, dit is het dus, een proefschrift vol aardappeleiwit. Het moge duidelijk zijn dat een aantal mensen een fikse bijdrage heeft geleverd aan de totstandkoming van het geheel.

Allereerst wil ik mijn beide promotoren, Fons Voragen en Pieter Walstra, bedanken voor de begeleiding en ondersteuning die ik van hen, ieder op hun eigen wijze, kreeg. Verder heeft Harry Gruppen als co-promotor een zeer belangrijke rol gespeeld. Harry, je nooit ophoudende streven naar verbetering is indrukwekkend en dat heb ik, zeker in de schrijffase, enorm gewaardeerd. Harmen de Jongh verdient eveneens veel dank, niet alleen voor het overdragen van zijn kennis over spectroscopie, maar ook voor zijn bijdrage aan het aanscherpen van het onderzoek en het verbeteren van de manuscripten. De afkorting rp (rephrase) zal ik niet licht vergeten.

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Van het NIZO *food research* maakten Kees de Kruif, Jan Klok, Erik ten Grotenhuis en Remco Tuinier mogelijk dat het proefschrift kon worden uitgebreid met een stuk lichtverstrooiing. Daarnaast had de neutronenverstrooiing tijdens de gezellige meet-week in Grenoble zonder hen niet kunnen plaatsvinden.

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Curriculum vitae

André Marcel Pots werd geboren op 8 maart 1971 te Estcourt, Zuid Afrika. In 1989 behaalde hij het VWO-diploma aan het Thijcollege te Oldenzaal. In datzelfde jaar begon hij met de studie levensmiddelentechnologie aan de Landbouwuniversiteit Wageningen. In het kader van deze studie deed hij afstudeervakken bij de leerstoelen Organische Chemie en Levensmiddelenchemie, waarbij het laatste afstudeervak werd uitgevoerd bij TNO-Voeding te Zeist. De studie rondde hij af met een stage bij het Institute of Food Research te Norwich, Engeland. In januari 1995 studeerde hij af.

Van 1995 tot en met 1998 werkte hij als Assistent in Opleiding aan een promotieonderzoek binnen het Centrum voor Eiwittechnologie TNO-LUW, een samenwerkingsverband van TNO-Voeding en de Landbouwuniversiteit Wageningen. Het onderzoek uitgevoerd in deze periode staat beschreven in dit proefschrift. Vanaf 15 februari 1999 is hij werkzaam als Process Manager bij Unox te Oss.

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