Physico-chemical and functional properties of potato proteins

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Stellingen

1. Het feit dat Knorr *et al.* (1977) het verhoogde ijzergehalte in met $FeCl_3$ geprecipiteerd aardappeleiwit opmerkelijk vinden, is pas echt opmerkelijk.
Knorr, D.; Kohler , G.O.; Betschart, A.E.; *J. Food Technol.,* **1977**, *12*, 235-247

2. Oplosbaarheid is één van de belangrijkste en meest complexe, maar desondanks één van de minst bestudeerde functionele eigenschappen van eiwitten.

3. De flexibiliteit van de tertiaire structuur van een eiwit is van groot belang tijdens de schuimvorming.
Dit proefschrift, hoofstuk 5.

4. De lipase activiteit van patatine is tot nu toe sterk onderschat.
Dit proefschrift, hoofdstuk 6.

5. Vanuit het oogpunt van de plantenmorfologie zou het beter zijn het woord aardappel**vrucht**water te vervangen door aardappel**knol**water.

6. Per 1 januari 2001 is de tempobeurs voor AIO's ingevoerd.

7. Een goede manier om ervoor te zorgen dat rondgestuurde stukken ook daadwerkelijk worden gelezen, is om op de voorzijde het woord "VERTROUWELIJK" te vermelden.

8. Naast het verplicht stellen van een vaardigheidsbewijs voor besturen van een auto, boot, bromfiets, etc., zou er ook een verplicht vaardigheidsbewijs moeten komen voor dragen/vervoeren van een rugzak binnen de bebouwde kom en in het openbaar vervoer.

Stellingen behorende bij het proefschrift

Physico-chemical and functional properties of potato proteins

Gerrit van Koningsveld
Wageningen, 27 juni 2001

G.A. van Koningsveld

Physico-chemical and functional properties of potato proteins

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. ir. L. Speelman
in het openbaar te verdedigen
op woensdag 27 juni 2001
des namiddags om vier uur in de aula

ISBN: 90-5808-444-2

Abstract

Koningsveld van, G.A. Physico-chemical and functional properties of potato proteins

Ph.D. thesis Wageningen University, Wageningen, The Netherlands, 2001

Key words potato proteins, patatin, protease inhibitors, solubility, structure, pH, temperature, ethanol, ionic strength, phenolic compounds, foams, emulsions

Potato proteins were isolated from potato fruit juice (PFJ) by different precipitation methods. The use of various strong and weak acids resulted in a maximum protein precipitation of 60 % of total protein at pH 3. The best results with respect to precipitation (91 %) and resolubility (91 %) were obtained using organic solvents.

Potato proteins unfold between 55°C and 75°C. The monomers of dimeric patatin were shown to thermally unfold independently at 60°C. Thermal unfolding of the protease inhibitors, between 60°C and 70°C, resulted in a decrease in inhibitor activities and protein solubility. Potato proteins were soluble at neutral and strongly acidic pH. At mildly acidic pH potato protein solubility was dependent on ionic strength and the presence of unfolded patatin. The presence of ethanol significantly reduced the unfolding temperature of potato proteins. In the presence of ethanol the thermal unfolding of the tertiary and the secondary structure of patatin were shown to be almost completely decoupled. The different mechanisms by which phenolic compounds may affect protein solubility are discussed in relation to the solubility and resolubility behavior of potato proteins in PFJ and when separated.

Less foam could be formed from untreated patatin than from the protease inhibitors. The foam forming properties of patatin could be strongly improved by partial unfolding of the protein. Whipping tests indicated that foams made with an ethanol precipitated protein isolate (PPI) were more stable against Ostwald ripening and drainage than those made with β-casein and β-lactoglobulin. The average droplet size of emulsions made with potato proteins was found to be presumably determined by the release due to the lipolytic activity of patatin of surface active fatty acids and monoglycerides from the tricaprylin oil phase during the emulsification process.

Γ	surface excess (mg/m^2)
γ	interfacial tension (N/m)
ρ	density of liquid phase (kg/m^3)
η_{SD}	surface dilational viscosity (= dγ/(d ln A/dt))
A	interfacial area (m^2)
ASP	ammonium sulfate precipitate
CD	circular dichroism
c_S	relative width of the droplet size distribution
d_{32}	volume-surface average droplet size (µm)
DSC	differential scanning calorimetry
E_{SD}	surface dilational modulus ≡ dγ/dln$A$
Δh_{cal}	calorimetric enthalpy of unfolding per unit mass (J/g)
ΔH_{cal}	molar calorimetric enthalpy of unfolding (kJ/mole)
ΔH_{vH}	van't Hoff enthalpy = $4RT_m^2C_p^{max}/\Delta H_{cal}$ (kJ/mole)
HP-SEC	high performance size-exclusion chromatography
I	ionic strength (M)
L	distance between pins on beater (m)
LAH	lipid acyl hydrolase
n_D	refractive index
PAT-5	patatin resolubilized at pH 7 after precipitation at pH 5
PAT-5E	patatin resolubilized at pH 7 after precipitation at pH 5 in the presence of 20 % (v/v) ethanol
PFJ	potato fruit juice
PIP	protease inhibitor pool, obtained from PFJ
PIP-5E	PIP after precipitation at pH 5 in the presence of 20 % (v/v) ethanol
p_L	Laplace pressure = $2\cdot\gamma/R$ (Pa)
PPI (20 % EtOH)	potato protein isolate prepared from PFJ by precipitation at pH 5 in the presence of 20 % (v/v) ethanol
PPI or PPI (15 % EtOH):	potato protein isolate prepared from PFJ by precipitation at pH 5 in the presence of 15 % (v/v) ethanol
R:	bubble radius (m)
SD:	standard deviation
SDS-PAGE:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
T_d:	denaturation temperature (°C)
v:	whipping speed (m/s)

Chapter 1

General Introduction

POTATO (*Solanum tuberosum* L.)

Potato belongs to the family of *Solanaceae*, which contains about 90 genera and 2800 species. The genus *Solanum*, to which potato and all wild relatives belong, consists of about 2000 species. Within this genus, the section *Tuberarium* includes the tuber-bearing members, of which the cultivated potato is best known (Correl, 1962). The potato is a herbaceous plant, 0.5 – 1 meter high, which only in some cases bears fruits in the form of berries (Burbank, 1921). Tubers form underground out of rhizomes (Burton, 1969).

The potato is a major world crop, of which 300 million tons are produced world-wide annually (FAO, 2000). It is exceeded only by wheat, rice and maize in world production for human consumption (Ross, 1986). Potato tubers give an exceptionally high yield per area, about 42 tons per hectare, many times that of any grain crop (CBS, 2000), and are used in a wide variety of uses (Feustel, 1987;Talburt, 1987). Potato is a major crop in the Netherlands also. It contributes about 5 % to the total value of the Dutch agricultural production and resulted in 1999 in a total production of 8.2 million tons of potatoes. Some 2.8 million tons (28 %) were used for the production of potato starch in 1999 in the Netherlands (CBS, 1999). The potatoes used in the starch industry are of special varieties and may contain up to 22 % starch as dry matter (ISI, 1999) and generally contain 1.0 – 1.5 % protein (Plieger, 1986;Lisinska and Leszczynski, 1989).

POTATO STARCH MANUFACTURE

Potato starch was first produced in Germany in the late 1700s. Potato starch has many applications and is e.g. used in the paper and textile industry, as raw material for noodles, as an ingredient in soups and sauces and for the production of glucose (CBS, 1999). The process for the manufacture of starch from potatoes (Figure 1) is relatively simple and yields 160-180 kg of starch per ton of potatoes (Swinkels, 1990). The process starts by washing the potatoes, followed by grinding in a rasping machine. Sodium bisulfite is added during the process to prevent excessive formation of brown polymers resulting from the oxidation of phenolic compounds. The rasped potato is then passed through rotating sieves. The fibers are retained and are discharged as potato pulp (potato fibers). The remaining starch slurry contains soluble compounds (sugars, proteins, acids, salts) and fine fibers. These are separated by further treatment through continuous centrifugal separators or hydrocyclones and fine sieves. The purified starch slurry is used for the production of potato starch derivatives or is dewatered and dried (Swinkels, 1990). The by-products that remain after starch manufacture are fibres and potato fruit juice, which are both used as animal feed.

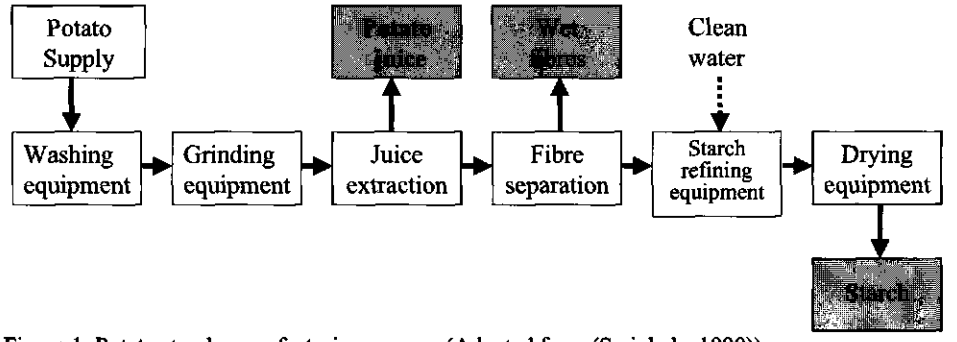

Figure 1. Potato starch manufacturing process (Adapted from (Swinkels, 1990))

COMPOSITION OF INDUSTRIAL POTATO FRUIT JUICE (PFJ)

The potato fruit juice (PFJ) resulting from the starch manufacturing process (Fig. 1) contains about 5 % dry matter (Plieger, 1986) and has a pH between 5.6 and 6.0. Per ton of potatoes usually 650 – 750 liter of PFJ are produced (De Noord, 1975;Strætkvern et al., 1999). The composition of the dry matter of PFJ is presented in Table 1. The free amino acids in PFJ are mainly composed of glutamine, glutamic acid, aspargine and γ-amino butyric acid, of which the latter is not incorporated in proteins, and are supposed to serve as nitrogen storage in the potato (Plieger, 1986). The other low molecular mass nitrogen containing compounds in PFJ include, amongst others, the glycoalkaloids, of which α-solanine and α-chaconine are the most abundant (Lisinska and Leszczynski, 1989).

The sugars in PFJ are composed of the reducing sugars D-glucose and D-fructose and the non-reducing disaccharide saccharose (Lisinska and Leszczynski, 1989). The lipids in PFJ are mainly composed of free fatty acids (30 %), fats (30 %) and phospholipids (40 %) (Lisinska and Leszczynski, 1989). Also the presence of phenolic compounds present in PFJ is important as will be discussed in this thesis.

Proteins account for approximately 25-30 % of the dry matter in PFJ. The proteins in PFJ will be discussed in the next section.

PROTEINS IN PFJ

Since this thesis discusses the properties of the soluble potato proteins present in PFJ, the knowledge already present about these proteins will be extensively described. This knowledge is of great importance to understand the behavior of potato proteins in PFJ and in a more purified fractions.

The potato proteins present in PFJ have been tentatively classified into three groups (Pots, 1999). No overall quantitative data on the protein composition of potato varieties are available, except those for PFJ from cultivar *Elkana* (Pouvreau et al., 2001). Patatin, the major potato tuber protein, comprises 38 % of the protein in PFJ from cultivar *Elkana*. The protease inhibitors make up about 50 % and other proteins up to 12 % of total protein in PFJ from cultivar *Elkana* (Pouvreau et al., 2001).

Table 1: Average composition of potato fruit juice (dry matter)

Component	*Concentration in PFJ* (g/l) (min – max)	% of dry matter
Protein (N x 6.25)	13.4 (8.5 – 22.2)	26.8
Peptides (N x 6.25)	2.2 (1.5 – 3.1)	4.4
Amino acids + amides (N x 5.13)	4.8 (3.3 – 7.8)	9.6
Other N-containing compounds	0.9	1.8
Sugars	7.9 (3.0 – 24.9)	15.8
Lipids	1.1	2.2
Citric acid	5.0 (2.0 – 12.0)	10.0
Ascorbic acid	0.3 (0.1 –0.6)	0.6
Other organic acids (e.g. malic and pyrolidone carboxylic acid)	1.3 (0.7 – 5.4)	2.6
Chlorogenic acid	0.2 (0.1 – 0.5)	0.4
Caffeic acid	0.07 (0.03 – 0.3)	0.1
Potassium	5.6 (3.9 – 7.3)	11.2
Phosphorus	0.5 (0.2 – 0.9)	1.0
Other components	5.0	10.1

Data calculated from own data, Plieger (Plieger, 1986), ISI (ISI, 1999) and Knorr and coworkers (Knorr et al., 1977;Knorr, 1980a)

Patatin

Patatin was given its trivial name by Racusen and Foote (Racusen and Foote, 1980), but the isolation and partial characterization of the 45 kDa glycoprotein, as it was denoted, had been performed in the early 70's already (Galliard, 1971;Galliard and Dennis, 1974;Dennis and Galliard, 1974). Patatin consists of a family of 40-42 kDa glycoproteins (Pots et al., 1999a) with pI's between pH 4.5 and 5.2 (Racusen and Foote, 1980;Park et al., 1983;Strætkvern et al., 1999). It is considered to be a storage protein because of its high accumulation in the tuber (Rosahl et al., 1986).

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). In aqueous solution, it is active on phospholipids, monoacylglycerols and *p*-nitrophenylesters, moderately active on galactolipids but less active on di-acyl and tri-acyl glycerols (Andrews et al., 1988). It has been suggested that this LAH-activity may play a role in the plant defense mechanism (Pots, 1999). The enzymatic activity of patatin has also gained interest in industry because of its specificity towards mono-acyl glycerols. This unusual specificity makes the enzyme very suitable for the production, in organic solvents, of mono-acyl glycerols with a high purity (> 95 %), from glycerol and fatty acids, which are an important group of emulsifiers (Macrae et

al., 1998).

The primary sequence of patatin (362 amino acids) shows neither extended hydrophilic nor hydrophobic amino acid sequences and the positive and negative charges of the side-chains are randomly distributed over the sequence (Pots, 1999). The patatin family consists of a number of isoforms but these do not seem to differ significantly in their structural properties and thermal conformational stability (Pots et al., 1999b). The apparent molecular weight of patatin, of 43 kDa, as determined using SDS-PAGE, differs from its apparent molecular weight in non-dissociating media where it appears as a dimeric protein of 80 kDa (Racusen and Weller, 1984).

Protease inhibitors

Although protease inhibitors have long been considered only as antinutritional factors, they have regained interest in recent years because of their possible anti-carcinogenic (Kennedy, 1998) and positive dietary effects (Hill et al., 1990). In contrast to patatin, the major potato tuber protein (Racusen and Foote, 1980), the protease inhibitors are a more heterogeneous group of proteins. They differ with respect to molecular mass, amino acid sequence and inhibitory activity (Pouvreau et al., 2001). A general characteristic of protease inhibitors is that they are small, cysteine-rich and heat-resistant proteins of 3-23 kDa (excluding tandemly repeated inhibitor domains resulting in inhibitors of 36-85 kDa) and are expected to play a major role in plant defence (Jongsma, 1995). The large number of cysteine residues present in protease inhibitors results in the formation of large number of disulphide bridges, which are necessary to uphold the original peptide conformation of the inhibitor upon hydrolysis by a protease (Jongsma, 1995). The potato protease inhibitors can be classified in seven different families (Pouvreau et al., 2001).

Potato Inhibitor I (PI-1) is a pentameric serine protease inhibitor composed of five 7.7 –7.9 kDa isoinhibitor protomers (Richardson and Cossins, 1974;Ralet and Guéguen, 1999). It represents 4.5 % of total protein in PFJ from cultivar *Elkana* and 2 % and 19 % of the total trypsin and chymotrypsin inhibiting activity in PFJ from cultivar *Elkana*, respectively (Pouvreau et al., 2001). The eight different forms of PI-1 found, have pI's between pH 5.1 and pH 7.8 (Pouvreau et al., 2001).

Potato Inhibitor II (PI-2) is a dimeric serine protease inhibitor composed of two 10.2 kDa subunits (Bryant et al., 1976) linked by a disulfide bridge (Lee et al., 1999). In PFJ from cultivar *Elkana* 7 isoforms of PI-2 are present, making up 22 % of the total protein and 82 % and 50 % of the total chymotrypsin and trypsin inhibiting activity, respectively. The isoforms of PI-2 have pI's in the range 5.5-6.9 (Pouvreau et al., 2001).

Potato Cysteinyl Protease Inhibitors (PCPI) are reported to be represented by at least 9 different inhibitors in PFJ from cultivar *Elkana* (Pouvreau et al., 2001). They differ in molecular weight, ranging from 20.1 to 22.8 kDa, and pI, ranging from pH 5.8 to >9. They constitute about 12 % of total protein. Apart from their inhibiting activity against cysteinyl proteases like e.g. papain (Brzin et al., 1988), they also constitute 16 % and 10 % of the total chymotrypsin and trypsin inhibiting activity present in PFJ, respectively (Pouvreau et al., 2001). Besides the cysteinyl protease inhibitors described above, the 85 kDa potato

multicystatin (Waldron et al., 1993;Walsh and Strickland, 1993) is also expected to be present in PFJ.

Potato Aspartyl Protease Inhibitors (PAPI) consist of 6 different inhibitors representing in total 6 % of total protein in PFJ. Their molecular weights are in the range 19.9 – 22.0 kDa, while their pI's are in the range pH 6.2 – 8.7. Apart from their activity against the aspartyl protease cathepsin D (Mares et al., 1989), they also comprise 9 % and 2 % of the total chymotrypsin and trypsin inhibiting activity present in PFJ, respectively (Pouvreau et al., 2001).

Potato Kunitz Protease Inhibitors (PKPI) consist of two members, both having a molecular weight of 20.2 kDa (Walsh and Twitchell, 1991). They have pI's of 8.0 and > 9.0, respectively, and constitute about 4 % of total protein in PFJ. They represent 2 % and 3 % of the total chymotrypsin and trypsin inhibiting activity present in PFJ, respectively (Pouvreau et al., 2001).

Other Serine Protease Inhibitors (OSPI) are represented by two members in PFJ and they represent 1.5 % of total protein. Their molecular weights are 21.0 (Valueva et al., 1999) and 21.8 kDa (Suh et al., 1990), respectively, and their pI's are 7.5 and 8.8, respectively. They comprise 2 % and 3 % of the total chymotrypsin and trypsin inhibiting activity present in PFJ, respectively (Pouvreau et al., 2001).

Potato Carboxypeptidase Inhibitors (PCPI) are present in only one form in PFJ. PCPI has a molecular weight of 4.3 kDa and represents about 1 % of total protein in PFJ (Pouvreau et al., 2001). PCPI is known to be remarkably heat stable (Hass et al., 1975;Huang et al., 1981;Olivia et al., 1991).

Other proteins

All proteins not belonging to the patatin family or not showing protease inhibitor activity are classified in this group. It was shown that this group accounts for about 12 % of the proteins present in PFJ from cultivar *Elkana* (Pouvreau et al., 2001). This group mainly consists of proteins with higher molecular weights such as the 65.5 kDa lectin (Allen et al., 1996), polyphenoloxidases of 60 and 69 kDa (Partington and Bolwell, 1996), protein kinases (Man et al., 1997;Subramaniam et al., 1997), enzymes involved in starch synthesis (140 kDa) (Marshall et al., 1996) and phosphorylase isozymes (180-600 kDa) (Gerbrandy and Doorgeest, 1972;Shivaram, 1976)

PROTEIN RECOVERY FROM PFJ

Potato proteins have a high nutritional value, as can be derived from amino acid composition, bio-assays and human feeding trials (Kapoor et al., 1975;Meister and Thompson, 1976b;Liedl et al., 1987;Friedman, 1996); it is comparable to that of whole egg (Knorr, 1978). The lysine content (7.5 %) is higher than in most plant proteins, and potato proteins would therefore be an excellent supplement for lysine-poor proteins, such as cereal proteins in bread (Knorr, 1978).In the few reports that have been published on the functionality of undenatured potato protein, they are claimed to have good "foam properties" (Wojnowska et al., 1981;Jackman and Yada, 1988;Edens et al., 1997) and "emulsion properties" (Holm and Eriksen,

1980;Wojnowska et al., 1981;Jackman and Yada, 1988;Edens et al., 1997). Also, potato protein concentrates that can be expected to contain at least partly denatured proteins are reported to show good functionality in foams (Knorr et al., 1977;Knorr, 1980a;Knorr, 1980b;Wojnowska et al., 1981) and emulsions (Wojnowska et al., 1981;Ralet and Guéguen, 2000). Because of their promising nutritional and functional properties, many efforts have been made to recover undenatured potato proteins from dilute potato processing waste streams.

Protein recovery methods

Heat treatment is the only method that has been used to commercially recover potato proteins from waste streams up to now. Heat precipitation results in very high yields, but the precipitate can not be resolubilized at neutral pH (Meister and Thompson, 1976a;Knorr et al., 1977;Rosenau et al., 1978;Knorr, 1980a;Boruch et al., 1989). Since the largest mass of the potato proteins have a pI at acidic pH (Seibles, 1979), precipitation of potato proteins at low pH has frequently been described using various acids. Precipitation at pH 3, which results in optimal precipitation yields, resulted in precipitates with a decreased solubility at neutral pH (Meister and Thompson, 1976a;Knorr et al., 1977;Knorr, 1980a). The use of citric acid has been described by Knorr (Knorr, 1982) and was reported to result in high precipitation yields and a high solubility of the protein precipitate at neutral pH. The use of the metal salts $AlCl_3$ (Knorr, 1982) and $FeCl_3$ (Meister and Thompson, 1976a;Knorr et al., 1977;Knorr, 1980b;Knorr, 1982) has also been reported to result in high precipitation yields and a high solubility of the precipitate at neutral pH. Lindner and coworkers (Lindner et al., 1981) have used bentonite at pH 4.5 to efficiently precipitate potato protein from starch factory waste, but the difficulty of desorbing the protein again poses a major problem.

Several authors have failed to notice the discrepancy between the pI's of potato proteins (pI 5 - >9) (Ahldén and Trägårdh, 1992;Pouvreau et al., 2001) and the pH for optimum protein precipitation yield (Meister and Thompson, 1976a;Knorr et al., 1977;Knorr, 1980b). The mechanism causing this remarkable difference therefore remained unclear (Pots, 1999).

Other methods than precipitation have also been used. Ion-exchange on carboxymethyl cellulose has been reported to be successful in recovering potato proteins (Gonzalez et al., 1991) but since the binding takes place at low pH (1.5-4.0) the resulting product can be expected to be at least partially denatured (Pots et al., 1998b).Ion-exchange, using the Expanded Bed Adsorption technique, has also been used for the preparative isolation of patatin from industrial PFJ (Strætkvern et al., 1999), but the technique used will be too expensive for industrial application. Ultrafiltration has been used by several authors (Boruch et al., 1989;Wojnowska et al., 1981). However, in large-scale practice considerable membrane fouling may occur and the resulting protein concentrates have been reported to contain elevated levels of glycoalkloids (Wojnowska et al., 1981). Treating potato fruit juice before ultrafiltration by flocculation through addition of $CaHPO_4$, at pH 7.5, (Edens et al., 1997) is reported to prevent excessive membrane fouling and seems the most promising method described in this section.

PROPERTIES OF PROTEINS

From the above it is clear that the solubility and structural stability of potato proteins at various conditions are of paramount importance for the recovery of useful potato protein preparations.

Protein structure and stability

Proteins are complex macromolecules whose primary structure is given by their amino acid sequence. The peptide chain of globular proteins in their native state is folded in a defined manner resulting in a secondary and a tertiary structure. The secondary structure is the local conformation of the polypeptide backbone, and three main structure types can be distinguished. The random coiled conformation is the natural unordered state of a polymer. The α-helix is the structure type that consists of a helical winding of the backbone resulting in a rod-like structure. The β-sheet is an extended structure consisting of closely interacting β-strands. The tertiary structure is the overall arrangement of the elements of secondary structure into a globular structure, while the quaternary structure is the association of the separate polypeptides into multimeric proteins (Creighton, 1996). The secondary and higher structure of a protein results from physical forces including hydrophobic interactions, van der Waals interactions, hydrogen bonds, electrostatic interactions and the solvation of polar groups and from chemical forces in covalent cross links (Alber, 1989). The stabilizing and the destabilizing interactions within the protein and between the protein the solvent, together with the loss of conformational entropy upon folding, almost equalize. The stability of the protein structure is, therefore, dependent on a small difference in free energy between a relatively small ensemble of folded conformations and an infinite ensemble of unfolded states. Unfolding of the protein structure, or denaturation, is characterized by a rearrangement of the polypeptide chain, resulting in a loss of the native folded conformation and may result in the exposure of hydrophobic groups to the solvent (Tanford, 1961). Loss of the folded structure of proteins can be readily followed by observing changes in the absorption spectra, circular dichroism, fluorescence spectra or heat capacity (DSC) of proteins (Darby and Creighton, 1993). The structure of the protein can be disrupted by relatively minor alterations in pH or temperature and the addition of denaturing agents (Darby and Creighton, 1993).

Unfolding at extremes of pH usually occurs by ionization of non-ionized groups buried inside the protein. Also electrostatic repulsion between charged groups at the surface and effects on salt bridges may contribute to pH induced unfolding (Darby and Creighton, 1993;Creighton, 1996). Heat induced unfolding is related to the temperature dependence of the different stabilizing and destabilizing interactions, the gain in conformational entropy upon unfolding and the change of solvent structure with temperature. Also, the action of denaturants is based on their ability to change solvent structure.

Isolated patatin at room temperature was shown to be a highly structured protein at both a secondary and tertiary level. Its structure at neutral pH was estimated using CD spectroscopy to consist of 33 % α-helical and of 46 % β-stranded structures (Pots et al., 1998a). Patatin becomes thermally destabilized at temperatures > 28°C and its α-helical parts unfold cooperatively between 45 and 55°C. Its β-stranded parts unfold more gradually between 50

and 90°C (Pots et al., 1998a). The thermal unfolding of patatin is accompanied by a loss of enzyme activity and was shown to coincide with the temperature at which protein precipitation occurs in PFJ. Thermal unfolding of patatin was shown to be only partly reversible upon cooling (Pots et al., 1998a). Also, at pH values below 4.5 patatin was shown to irreversibly unfold resulting in a partial loss of its enzymatic activity (Pots et al., 1998b;Strætkvern et al., 1999).
Little is presently known about the structure and structural stability of potato proteins other than patatin.

Protein solubility

The solubility of a compound and, therefore of a protein, is strictly defined as the amount of that compound that can be dissolved in a given amount of solvent at a given temperature, where the solute is in equilibrium with its crystalline form. The amount of protein dissolved should thus be independent of the procedure used to dissolve it. Because most proteins discussed in this thesis are either a mixture of proteins or contain proteins in various (un)folding states, a more practical definition of solubility is needed. Operationally, solubility denotes the proportion of a given amount of protein that goes into solution (or into colloidal dispersion) under specified conditions and is not sedimented by moderate centrifugal forces (Kinsella, 1984).
The solubility of a protein in water or an aqueous solvent is determined by its free energy when surrounded by aqueous solvent relative to its free energy when interacting in an amorphous or ordered solid state (Creighton, 1996). The interactions of a protein molecule with solvent or with other molecules are determined primarily by its surface. The most favorable interactions with water are provided by charged and polar groups of hydrophilic amino acid side chains (Creighton, 1996). At the isoelectric pH (pI) the net charge on the protein is zero and the solubility of a protein is lowest. This is especially so at low ionic strength. Away from the isoelectric pH the net charge of the protein increases and therefore, even at low ionic strength, provides electrostatic repulsion between the proteins, which keeps them in solution (Creighton, 1996). This argument is of course restricted to the pH range in which the protein remains in its native conformation, as unfolding would alter its surface and thus solubility properties. The solubility of proteins is also affected by the presence of salts, especially near the isoelectric pH. Low concentrations of salt generally tend to increase protein solubility as they decrease the thickness of the electric double layer around the charges on the protein, which causes these charges to be felt as local individual charges rather than as the net charge of the protein and thus provide local electrostatic repulsion between protein molecules, which is called salting-in. At higher salt concentrations the solubility of a protein tends to decrease. The magnitude of this salting-out effect depends on the nature of the salt and generally follows the Hoffmeister series (Creighton, 1996). On the basis of this series a distinction can be made between lyotropic and chaotropic salts. Lyotropic salts tend to alter water structure in such a way that the solvation of the protein surface becomes energetically less favorable thereby decreasing protein solubility. Chaotropic salts do the opposite. Generally, lyotropic salts tend to increase protein stability, while chaotropic salts

tend to decrease protein stability by favoring the solvation of hydrophobic residues that are normally buried inside the protein.

The solubility of proteins can also be decreased at much lower salt concentrations when specific complexation occurs. Metal ions such as Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+} can form strong complexes with carboxylic acid groups and also histidine, and can non-covalently cross link proteins into large aggregates that may become insoluble (Bell et al., 1983). The effect of metal ions such as, e.g., Fe^{3+} and Al^{3+}, is more complex; apart from forming stable complexes with protein groups they form strong complexes with water and hydroxide, which, depending on the pH, may result in the formation of hydroxyl gels (Lovrien and Matulis, 1997).

Water-miscible organic solvents can also be used to lower protein solubility. Their ability to lower the solubility of proteins, especially at their pI, was believed to be due to a decrease in the dielectric constant of the solvent, which resulted in an increase of electrostatic interactions between proteins thus lowering their solubility (Foster, 1994). This mechanism has been questioned, since at the low temperature at which organic solvents are usually applied, there is only a negligible difference with the dielectric constant of water at 20°C (Van Oss, 1989;Rothstein, 1994). Van Oss (Van Oss, 1989) demonstrated that ethanol may act in the same way as a lyotropic salt, i.e. increasing protein stability and decreasing protein solubility. Ethanol and other water-miscible organic solvents may, however, destabilize proteins. Whether an organic solvent acts as stabilizing agent or as a destabilizing agent depends on its concentration and the temperature at which it is applied (Brandts and Hunt, 1967;Velicelibi and Sturtevant, 1979).

FORMATION AND STABILITY OF FOAMS AND EMULSIONS

As stated above potato proteins are claimed to possess good foaming and emulsifying properties.

Foams and emulsions are both systems in which one phase (air for foam, oil for oil-in-water emulsions) is dispersed in another phase. Also, the processes that occur during their formation and stabilization are similar. The differences between foams and emulsions include the particle diameter, i.e. about 0.1-1 mm and 0.2-10 μm, respectively, the solubility of the dispersed phase in the continuous phase, the interfacial tension, and others (Walstra, 1987;Prins, 1988). The difference in particle size between foams and emulsions also means that the specific surface area is much higher in an emulsion than in a foam and thus much more surfactant is needed to make and stabilize an emulsion.

When discussing foams and emulsions, formation and stabilization should be considered separately, since different mechanisms and time-scales play a role in these processes.

In foams formation and stability can often not be distinguished, while in emulsions these processes are clearly distinct (Walstra and Smulders, 1997). The formation of a foam and an emulsion involves the break-up of large particles in to smaller ones. This process requires the input of energy because the deformation of the droplet is opposed by the Laplace pressure (p_L) inside the particle, which is given by:

$$p_L = 2\gamma/R$$

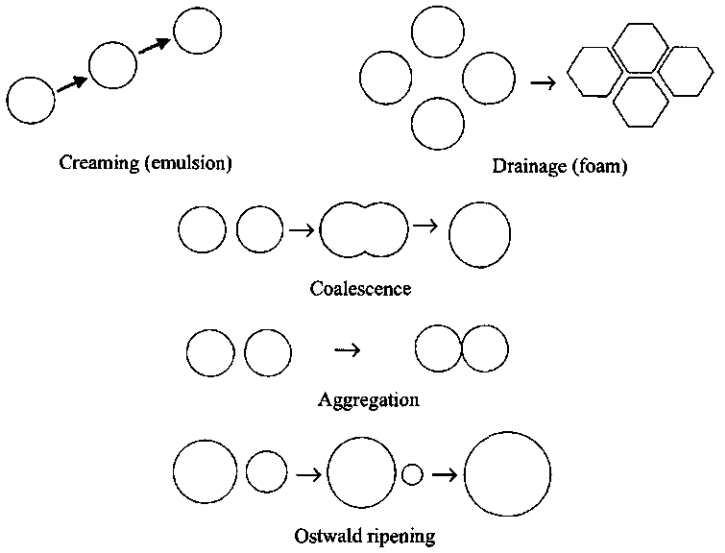

Figure 2. Types of instability in foam and oil-in-water emulsions

where γ is the surface tension and R is the radius of the particle. The role of the protein, or any other surfactant, in this process is that by adsorbing to the particle interface it may lower the surface tension and thereby make droplet break-up easier, although in this process also the rheology of the particle surface, i.e. how the surface reacts on deformation, is important. Another role of the surfactant during this process is to prevent the particles from immediately coalescing again by its ability to form γ-gradients.

The extent to which a γ-gradient develops increases with increasing surface dilational modulus E_{SD} (Lucassen-Reijnders, 1981), which is given by:

$$E_{SD} = d\gamma/d\ln A$$

in which E_{SD} is the surface dilational modulus, γ is the interfacial tension, and A is the surface area. Various other aspects are also important during the formation and stabilization of the newly formed particle, such as the adsorption rate of the surfactant (Walstra, 1993).

Also after formation, foams and emulsions are exposed to different types of instabilities, as indicated in Figure 2. Creaming and drainage are caused by the density difference between the dispersed phase and the continuous phase. Apart from the density difference, also the particle size, the rheological properties of the particle surface and the viscosity of the continuous phase influence the rate of creaming and drainage.

Ostwald ripening can occur when the dispersed phase is soluble in the continuous phase, which is uncommon in oil-in water emulsions but common in foams. Due to a higher Laplace pressure the solubility of the dispersed phase in the continuous phase is higher around smaller bubbles than around larger bubbles, resulting in the growing of larger bubbles at the cost of smaller bubbles. This process can be retarded or stopped if the surfactant stays adsorbed at the interface of the shrinking bubble, because then the surface tension will decrease when the surface area is reduced. The relation between the surface tension and change in surface area is

given by E_{SD}. It has been shown that Ostwald ripening in a foam will completely stop if E_{SD} becomes equal to or larger than $\gamma/2$ (Lucassen, 1981).
Aggregation is the process in which two or more particles stick together. Aggregation is governed by the balance of the attractive and repulsive forces between the droplets. In emulsions specific mechanisms of aggregation may be observed that are called bridging and depletion flocculation. Bridging flocculation occurs at low concentrations of polymeric surfactants. In this situation one polymer molecule adsorbs at two interfaces thus forming a bridge between the droplets. Depletion flocculation may occur when high concentrations of non-adsorbing polymers are present, because then there is zone near the particle interface that is, because of steric hindrance, depleted of this polymer. The concentration difference between this layer and the bulk forces the droplets together in order to minimize the volume devoid of polymer. Coalescence occurs when the film between two particles becomes thin for prolonged time and is eventually ruptured, causing them to flow together.
All the mentioned instability processes affect each other. The rate of coalescence e.g., depends on the time the particles stay together and is therefore, amongst others, affected by drainage/creaming and aggregation.

The role of proteins in foam and emulsion formation and stabilization

In many food products proteins are used for their foam and emulsion forming and stabilizing properties since they can adsorb on to oil/water and air/water interfaces, thereby lowering the surface tension. Proteins are much more surface active than small-molecule surfactants, i.e. they adsorb at much lower molar concentration (Walstra and De Roos, 1993). Proteins predominantly adsorb at interfaces via their hydrophobic segments and once adsorbed they rearrange their conformation to expose these segments to the interface, thus attaining an energetically most favorable conformation (Smulders, 2000). Molecular properties, e.g. conformational stability/flexibility, of proteins govern the ability of the proteins to lower the interfacial tension during foam formation and emulsification, hence the formation of small particles: they also stabilize the particles against immediate coalescence by affecting the rheological properties of the particle surface (Smulders, 2000). After formation proteins also determine the properties of the adsorbed layer by affecting its rheological properties and also by providing steric and electrostatic repulsion thereby stabilizing the particles against aggregation, and thus against creaming and coalescence.
The absorption behavior of proteins depends on their net charge, hence pH. Also the conformation and conformational stability of proteins depend on pH. At the isoelectric pH the net charge on the protein is zero and the electrostatic repulsion between the proteins is minimal. This may result in a low protein solubility and often leads to poor emulsion forming and stabilizing properties due to a decrease in effective molar protein concentration and a lack of electrostatic repulsion between the emulsion droplets (Halling, 1981). In contrast, foam formation and stability are often observed to be highest near the isoelectric pH (German and Phillips, 1991;Graham and Phillips, 1976). In the stability of protein foams electrostatic repulsion between the bubbles is not important and the favorable effect on foam stability of increased interfacial protein-protein interactions, reflected in a higher E_{SD}, predominates.

Away from the isoelectric pH the foam and emulsion properties of proteins are generally less affected by pH, as long as no unfolding occurs. Ionic strength affects the functionality of proteins by reducing the electric double layer around the charges on the proteins and thus reduces the distance at which electrostatic repulsion acts between the proteins in solution, as well as in the interface and between interfaces. Unfolding of proteins by heat-treatment, or other treatments, may in some cases improve foam and emulsion properties of proteins. However, unfolding often results in protein aggregation and concomitant loss of solubility, which is one of the most important properties determining the performance of proteins in foam and emulsions (Halling, 1981).

AIM OF THE STUDY

Only little is known about the protein and non-protein composition of PFJ and the behavior of these components under various conditions. The most important question which resulted in the research described in this thesis is: Can potato proteins be recovered from PFJ, in such a way that they retain their functional properties, most importantly their solubility? This recovery method should be applicable at a large scale and result in a high yield. Potato protein recovery was expected to be complicated by the presence of and the interactions with non-protein components in PFJ. The aim of this thesis was to examine how extrinsic factors like pH, ionic strength and temperature would influence the structure of potato proteins, this in relation to the functionality of the proteins in making and stabilizing foams and emulsions.

OUTLINE OF THE THESIS

Chapter 2 describes the influence of pH and additives, such as metal salts and water-miscible organic solvents, on the precipitation yield of potato proteins from PFJ and the solubility of these precipitates at neutral pH. Chapters 3 and 4 describe the effects of pH, temperature, ionic strength and ethanol precipitation on the structure and solubility of potato proteins in various fractions. Chapter 5 discusses the foam properties of the protein fractions described in chapters 3 and 4, while in Chapter 6 the emulsion properties of these fractions are described. Finally, chapter 7 combines the information described in Chapter 2-6 into a more general overview.

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Chapter 2

The solubility of potato proteins from industrial potato fruit juice as influenced by pH and various additives

ABSTRACT

The effects of pH and various additives on the precipitation and (re)solubility at pH 7 of potato proteins from industrial potato fruit juice (PFJ) were studied. Various strong and weak acids did not result in differences in protein precipitation, which occurred to a maximum of 60 % of total protein at pH 3. Weak acids did, however, increase the resolubility of the precipitates at pH 7. At pH 5 addition of $FeCl_3$ or $ZnCl_2$ increased both precipitation and resolubility. The largest increase in precipitation and resolubility was achieved using organic solvents, resulting in maximum precipitation (pH 5) of 91 % of total protein and a maximum resolubility of 83 % of total protein.

The results described in this study lead to the hypothesis that precipitation and resolubilization of potato proteins from PFJ is not determined by their isoelectric pH but by their interactions with low molecular weight components.

This chapter has been submitted as:

The solubility of potato proteins from industrial potato fruit juice as influenced by pH and various additives.

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INTRODUCTION

In potato starch manufacture an aqueous byproduct remains, after recovery of potato starch, which is called potato fruit juice (PFJ). On a dry matter basis PFJ contains about 35% protein and amino acids, 35% sugars, 20% minerals, 4% organic acids and 6% other components (Knorr et al., 1977). Potato protein has a relatively high nutritional quality, comparable to that of whole egg (Kapoor et al., 1975;Knorr, 1978), and it therefore has high potential for utilization in food applications. Protein recovery from industrial PFJ is presently achieved through heat coagulation by steam injection after pH adjustment. This method is very efficient in removing protein from solution. However, it leads to protein precipitates that exhibit a low solubility (Knorr et al., 1977;Knorr, 1978), which hampers potential food applications (Kinsella, 1976). An economic method to recover soluble potato protein efficiently would considerably increase its possibilities for use in food and add to its commercial value.

Several efforts have been made in the past to recover potato protein from industrial PFJ. Precipitation with bentonite (Lindner et al., 1981) and carboxymethyl cellulose (Gonzalez et al., 1991) offer alternatives for heat coagulation, but suffer from drawbacks such as removal of adsorbent and high concentrations of anti-nutritional factors (protease inhibitors) in the precipitate. The use of membrane techniques like ultrafiltration gives promising results in laboratory studies (Wojnowska et al., 1981;Boruch et al., 1989). However, in large scale practice considerable membrane fouling may occur and it also yields protein concentrates containing anti-nutritional factors, like protease inhibitors and glycoalkaloids (Wojnowska et al., 1981).

Several methods of precipitation at acidic pH have been used for the recovery of potato proteins (Meister and Thompson, 1976;Knorr et al., 1977;Knorr, 1980;Knorr, 1982). The most promising results with respect to the (re)solubility of protein precipitates were obtained with citric acid (Knorr, 1980;Knorr, 1982) and ferric chloride (Knorr et al., 1977;Knorr, 1980;Knorr, 1982).

The purpose of this study was to examine how precipitation of potato proteins can be optimized in terms of recovery of soluble potato proteins. Experiments were done in the presence of various additives, such as acids, (metal) salts or organic solvents, in order to optimize both protein precipitation and solubility of the resulting precipitates. The results of these experiments should not only give information about how both precipitation and resolubility of potato proteins from industrial PFJ can be optimized, but also provide insight into what processes and interactions govern the precipitation and resolubilization of potato proteins.

MATERIALS AND METHODS

Preparation of PFJ

Potatoes (cv. Elkana) were washed thoroughly with water and cut into large pieces (max. 8 x 2.5 cm) which were immediately dipped in a 20 mg/ml solution of sodium bisulfite to prevent enzymatic browning. The potato pieces were ground in a domestic type juice extractor (AEG). The remaining turbid juice was allowed to settle for 15 minutes. Next, the liquid was decanted and centrifuged (15 minutes, 19000 × *g*, 10°C) and the supernatant filtered through a paper filter (Schleicher & Schuell, ref.no. 311653). The resulting clear yellowish filtrate, which has a pH of 5.7 - 6.0, is known to be a good simulant for industrial PFJ (AVEBE B.A., Foxhol, The Netherlands) and is further denoted as PFJ.

Protein precipitation and resolubilization

Protein precipitation experiments were performed by adjusting the pH of stirred duplicate PFJ samples in 15 ml Kimax tubes at room temperature, or on ice when organic solvents were used. The acidified samples were left to settle for 1 hour at room temperature, or on ice when organic solvents were used. The settled samples were centrifuged for at least 15 minutes (3600 × *g*) until clear supernatants were obtained, at 22°C or at 0°C when organic solvents were used. Supernatants were analyzed in duplicate for nitrogen content. Precipitated nitrogen was calculated as nitrogen in PFJ minus nitrogen in the supernatant and was assumed to be of protein origin and expressed as proportion of the TCA-precipitable nitrogen present in PFJ.

Precipitates formed using different acids were washed twice by suspending them in 5 ml of either 0.1 M sodium acetate buffer (pH 3.5 - 5.5) or 0.1 M sodium citrate buffer (pH 2.5 and 3). Precipitates obtained with metal salts or organic solvents were washed twice with 5 ml of a 0.1 M sodium acetate buffer (pH 5) that contained the appropriate amount of the metal salt or the appropriate amount of the organic solvent used during precipitation. After each washing step the samples were centrifuged (3600 × *g*, 15 min, 22 or 0°C).

Washed precipitates were suspended as described by Betschart (1974) in the original Kimax tube in 10 ml of 113 mM sodium phosphate buffer (pH 7.0) with an ionic strength of 0.2 M. When metal salts were used during precipitation, the buffer contained 30 mM of disodium EDTA. After adjusting the pH to 7.0 the tubes were completely filled with buffer, capped and incubated for 60 minutes at 30°C in a test-tube rotator. Next, the tubes were centrifuged (3600 × *g*, 22°C, 15 min) and supernatants were sampled for determination of soluble nitrogen. Protein resolubility was calculated as the amount of soluble nitrogen and expressed as proportion of total protein (i.e. TCA precipitable) nitrogen present in untreated PFJ.

Effect on precipitation and resolubility of:

1. Various acids

The effect of pH and different acids (sulfuric (0.5 M), hydrochloric (1 M), citric (25 % w/v) or acetic acid) was studied by lowering the pH of samples of 8 ml PFJ to pH's ranging from 2.5

to 5.5 with intervals of 0.5 pH units. When citric or acetic acid was used pH values lower than 3.0 were not applied.

The relation between the volume of resolubilization buffer and the amount of protein resolubilized was examined by resolubilizing precipitates obtained from four 8 ml samples of PFJ adjusted to pH 5 using 0.5 M H_2SO_4 in 12.5, 25, 62.5 or 125 ml resolubilization buffer.

2. Dialysis and ionic strength

PFJ was dialyzed at 4°C in cellulose ester dialysis tubes with a molecular weight cut-off of 8000 Dalton (Spectrum Medical Industries, Laguna Hills ,CA., USA) against 50 volumes of 0.5 g/l of sodium bisulfite. The bisulfite solution was changed regularly until no further decrease in conductivity of the retentate was observed. The resulting turbid retentate (pH 5.2) was clarified by adjusting the pH to 7 with 0.5 M NaOH.

The influence of pH on protein precipitation in dialyzed PFJ was examined by adjusting the pH of samples (10 ml) of the clarified retentate to the desired pH in the range of pH 2.5 - 6.5 with intervals of 0.5 using 0.5 M H_2SO_4.

The influence of ionic strength was examined by adding different amounts of 5.0 M NaCl to dialyzed PFJ samples (9 ml) before the pH was adjusted to pH 5.0 with 0.5 M sulfuric acid. The total volume of all samples was kept the same (10 ml) by adding water. The final NaCl concentrations used in the samples ranged from 0 to 0.5 M.

3. Metal salts

The effect of the addition of metals salts was studied by adding solutions of 0.1 M of either $FeCl_3$, $FeSO_4$ or $ZnCl_2$, which were adjusted to a final ionic strength of 0.6 M with NaCl, to PFJ. Different amounts of these solutions were added to PFJ samples (6.8 ml), prior to adjustment to pH 5 with 0.5 M H_2SO_4, while the ionic strength and the final volume of all samples was kept the same by adding different amounts of 0.6 M NaCl and water. Final metal ion concentrations in the PFJ samples ranged from 0 to 15 mM. EDTA was used during resolubilization to remove metal ions from the protein and thereby exclude their possible charge effect on protein resolubility.

4. Organic solvents

The effect of organic solvents was studied by adding precooled (-20°C) aqueous solutions of methanol, ethanol, isopropanol or acetone to stirred 5 ml PFJ samples kept on ice. After addition of organic solvents the samples were kept on ice and brought to pH 5.0 with 0.5 M sulfuric acid. Final concentrations of organic solvents used in the samples ranged from 0 to 40 % (v/v).

The relation between the volume of resolubilization buffer and the amount of protein resolubilized was examined by simultaneously obtaining precipitates from five samples of PFJ that contained 5 % (v/v) (0.65 M) isopropanol and were adjusted to pH 5 using H_2SO_4. The precipitates obtained were resolubilized in 1, 2, 3, 5 or 13 ml of resolubilization buffer.

Protein analysis

Nitrogen (N) content was determined by the micro-Kjeldahl method (AOAC, 1980). Protein-N of PFJ was determined as N recovered by centrifugation (15,000 x g, 22°C, 20 min) 1 hour after addition of trichloroacetic acid (TCA) to PFJ to a final concentration of 12.5 % (w/v) TCA. Protein content was calculated as 6.25 x (N_{total} - $N_{supernatant\ (12.5\ \%\ TCA)}$). PFJ, on average, contained 3.59 ± 0.09 (SD) mg of nitrogen per ml. Of this nitrogen 60 ± 5.6 (SD) % could be precipitated with 12.5 % (w/v) TCA and was therefore assumed to be of protein origin, which leads to an average protein (N x 6.25) concentration of 13.4 ± 0.9 (SD) mg protein per ml of PFJ. Dialyzed PFJ contained 4.7 ± 0.2 mg protein per ml. The determination of precipitated protein had an average absolute standard deviation of 3 % of total protein. The determination of resolubilized protein had an average absolute standard deviation of 4 % of total protein.

SDS-PAGE

SDS-PAGE and isoelectric focussing (IEF) were performed with a Pharmacia Phastsystem according to the instructions from the manufacturer, using Gradient 8-25 or IEF 3-9 gels, which were stained with either Coomassie brilliant blue R-250 or silver staining. The intensity of the protein bands was estimated visually and "normalized" to the intensity of the respective band in PFJ.

Circular dichroism spectroscopy

In order to investigate the effect of precipitation and subsequent resolubilization on patatin, purified patatin Pots et al. (1998) was precipitated from a 1 mg/ml solution in 10 mM sodium phosphate buffer pH 7.0, by adjusting 1.5 ml of this solution to pH 5 with 0.1 M H_2SO_4 at room temperature. After 1 hour the turbid patatin solution was centrifuged (15,000 x *g*, 20 min, 22°C.) and the supernatant was used for protein analysis. The precipitate, containing 90 % of the patatin originally present, was completely resolubilized by adding 1.5 ml of water and adjusting the pH to 7.0 with 0.1 M NaOH. The effect of precipitation and resolubilization on the secondary structure of patatin was studied by far-UV CD spectroscopy. Far-UV CD spectra of 0.3 mg/ml solutions of both untreated and resolubilized patatin were recorded 10-fold on a Jasco J-715 spectropolarimeter (Jasco Corp., Japan) at ambient temperature in quartz cells with an optical path length of 0.02 cm. Instrument parameters and analysis of recorded spectra were performed as described by Pots et al. (1998).

RESULTS

The experiments described in this study aimed at establishing conditions to precipitate maximum amounts of resoluble protein from industrial PFJ. This means that not only protein precipitation had to be optimized but also the resolubility of the precipitated proteins. The protein precipitates obtained should also retain other functional properties, that are not described here.

Quantitative precipitation yield and resolubility

1. Various acids

Most of the proteins in PFJ have isoelectric points at acidic pH; between pH 4.5 and pH 6.5 (Seibles, 1979). Therefore, recovery of potato proteins by precipitation at acidic pH seems obvious. Different acids, more specifically their conjugated anions, may in practice cause profound differences in conformational stability of proteins and thus affect their solubility (Salt et al., 1982;Hoare and Dunill, 1983).

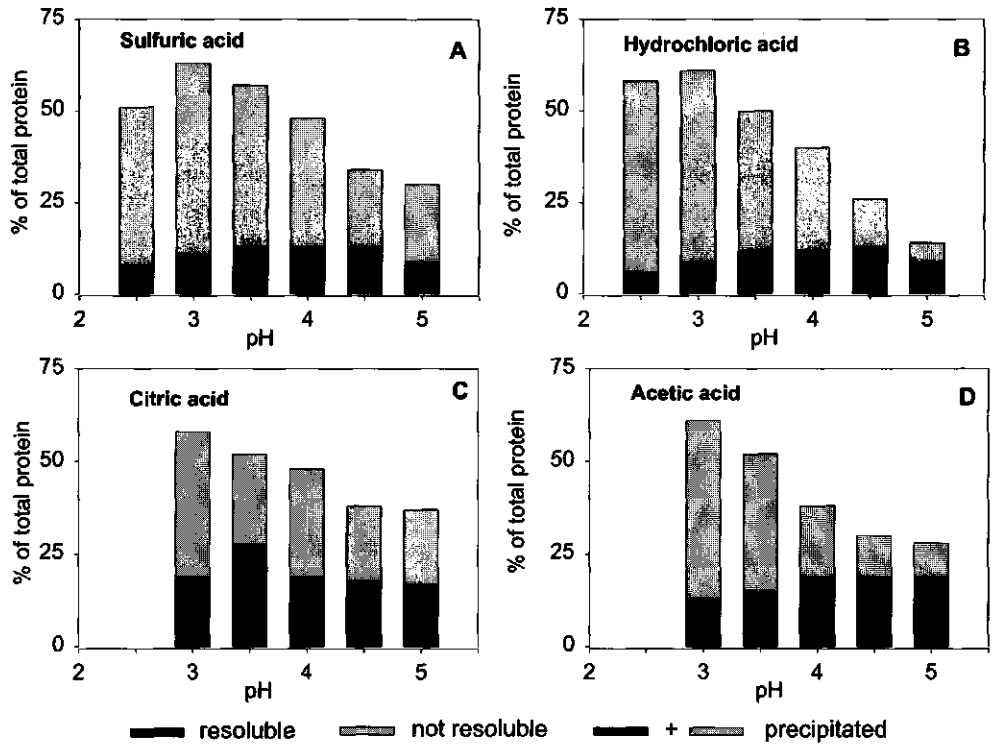

Figure 1: Amount of protein precipitated and amount of precipitated protein resoluble (pH7, $I = 0.2$ M) expressed as proportion of total protein originally present as a function of pH for various acids.

As can be seen in Figure 1, the amount of precipitated protein increased with decreasing pH for pH values ≥ 3. When using HCl and H_2SO_4 precipitation of potato protein was maximal at pH 3, causing 61 % and 63 % of total protein to precipitate, respectively. Using citric acid and acetic acid similar amounts of protein could be precipitated at pH 3. Figure 1 also shows that the acid used for acidification of PFJ did not have a major effect on the precipitation behavior observed.

The amount of precipitated protein that was resoluble at pH 7, expressed as proportion of total protein originally present, is also shown in Figure 1 as a function of the pH of precipitation for

the different acids used. The resolubilities of the precipitates obtained with the strong acids (H_2SO_4, HCl) were significantly lower than those obtained with the weak acids (citric acid, acetic acid), especially if the pH of precipitation was higher than pH 3.5.

Since in this study different amounts of protein precipitated under different conditions and the precipitates were resolubilized in similar volumes, the relation between the resolubilization volume and the amount of protein resolubilized was examined for a precipitate obtained from PFJ at pH 5 using H_2SO_4 (Fig. 2). As can be seen from Figure 2 a four times larger resolubilization volume, from 12.5 ml to 50 ml, only caused an additional 4 % of total protein to resolubilize, which was only twice the standard deviation of the total analysis.

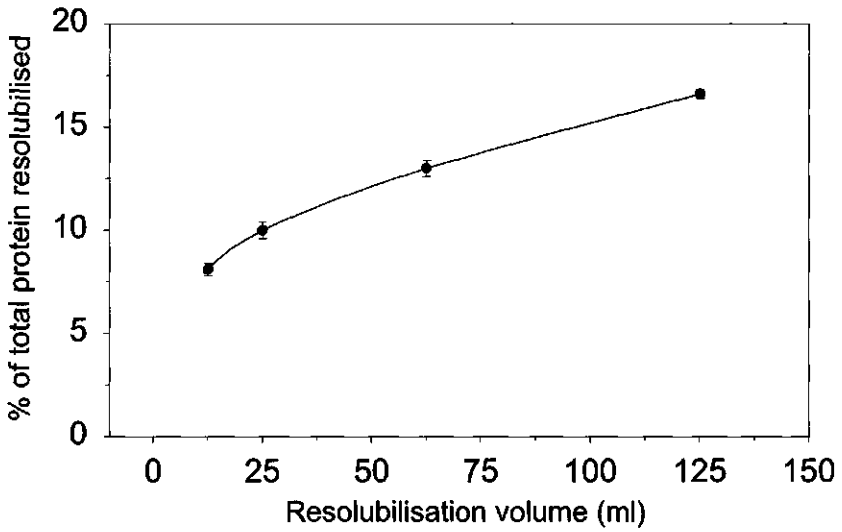

Figure 2: Amount of precipitated protein resoluble (pH7, I = 0.2 M) of potato protein precipitate (pH 5, H_2SO_4) expressed as proportion of total protein originally present as a function of resolubilisation volume.

The amounts of precipitate formed within one series generally differed a factor four (see Fig. 1). The resolubilities of the precipitates generally differed far more than 4 % and thus showed significantly different properties (Fig. 1). Therefore, the error introduced by using the same resolubilization volume is acceptable, because it leads to a standardized resolubilization procedure, whereas resolublization volumes proportional to the amounts of precipitate formed will not.

2. Dialysis and ionic strength

The precipitation curve obtained after lowering the pH of dialyzed PFJ with sulfuric acid is shown in Figure 3. The optimal pH for protein precipitation had shifted from pH 3 in untreated PFJ (Fig. 1A) to pH 3.5 - 4 for dialyzed PFJ (Fig. 3), resulting in the precipitation of 90 % of total protein.

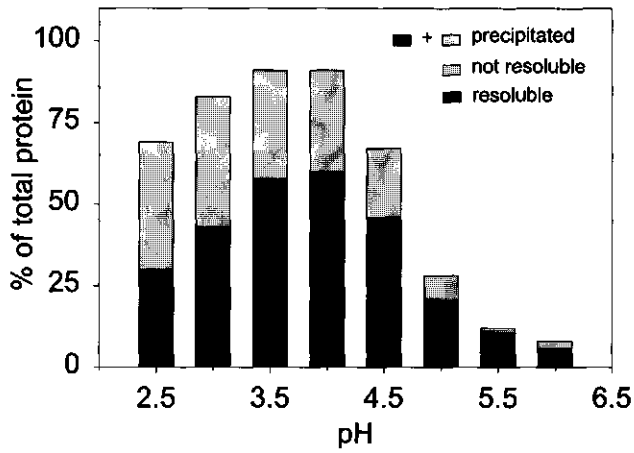

Figure 3: Amount of protein precipitated (H_2SO_4) and amount of precipitated protein resoluble (pH7, I = 0.2 M) expressed as proportion of total protein originally present as a function of pH for dialysed PFJ.

The resolubility of the precipitates showed more or less the same trend (Figure 3) as observed with non-dialyzed PFJ (Figure 1A). Precipitates from dialyzed PFJ resulted in increased amounts of resolubilized protein when increased amounts of precipitate were formed, unlike the results for untreated PFJ (Fig. 1A).

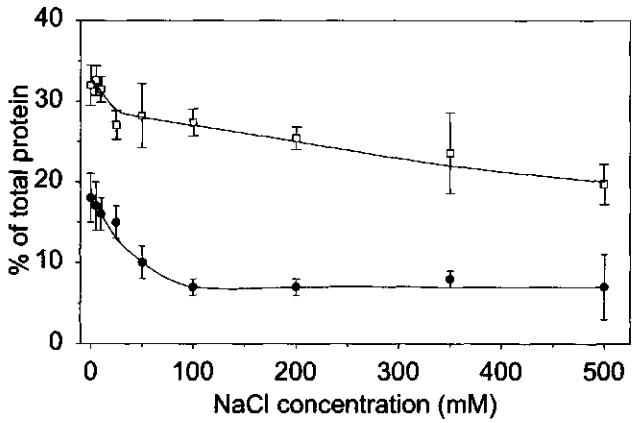

Figure 4: Amount of protein precipitated (□, pH 5, H_2SO_4) and amount of precipitated protein resoluble (●, pH7, I = 0.2 M) expressed as proportion of total protein originally present as a function of the added NaCl concentration for dialyzed PFJ

The effects of ionic strength on precipitation and resolubility of potato proteins from dialyzed PFJ were studied by addition of various amounts of NaCl and subsequent acidification to pH 5 with H_2SO_4. This pH was chosen because purified patatin did not irreversibly unfold at pH 5, as analyzed by far-UV CD (results not shown), in contrast to pH's ≤ 4.5 (Pots et al., 1998b). Increasing the ionic strength lead to decreasing amounts of precipitate being formed (Figure 4), as is commonly seen with proteins (Creighton, 1996).

Figure 4 shows that with increasing ionic strength the proportion of the precipitate that was resoluble decreased to a minimum at about 100 mM of salt during precipitation. A further increase in the salt concentration during precipitation had no significant effect on the resolubility.

3. Metal salts

Di- and trivalent metal cations have since long been used to precipitate proteins. Their action is believed to be mainly based on their ability to form stable complexes with histidine, cysteine and tryptophan residues (Zachariou and Hearn, 1996).

The effects on precipitation caused by adding various metal salts to PFJ and subsequent adjustment of the pH to 5 are shown in Figure 5. Of the metal salts used only addition of $FeCl_3$ and $ZnCl_2$ gave increased protein precipitation at pH 5.

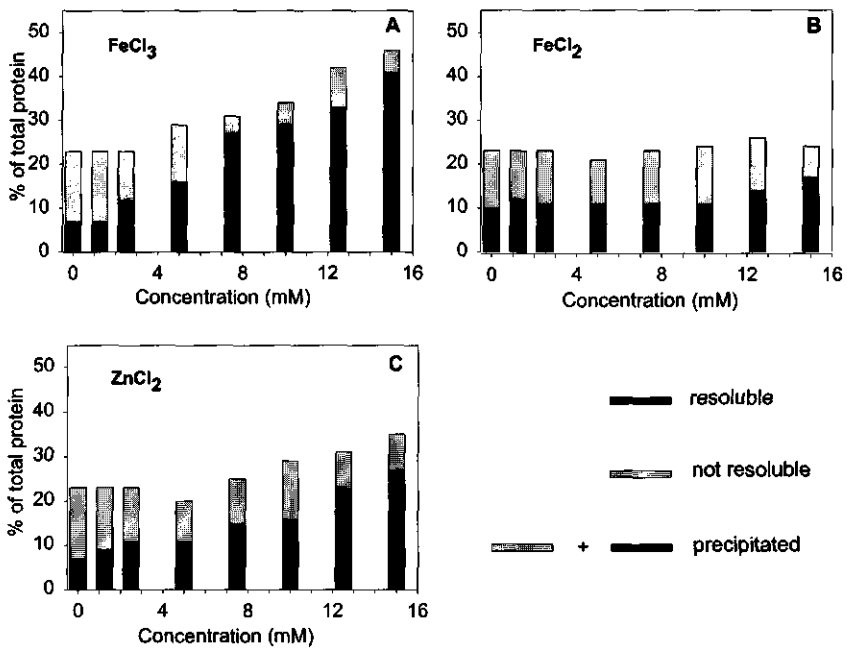

Figure 5: Amount of protein precipitated (pH 5, H_2SO_4) and amount of precipitated protein resoluble (pH7, I = 0.2 M) expressed as proportion of total protein originally present as a function of metal salt concentration for various metal salts.

$FeCl_3$, at a concentration of 15 mM, caused a 100 % increase in precipitation, whereas $ZnCl_2$, at the same concentration, caused a 52 % increase in precipitation. The resolubility at pH 7 of the precipitates obtained after addition of metal salts was influenced in a similar way as the precipitation (Fig. 5).

The precipitates that were best resoluble were obtained when $FeCl_3$ was added. Of these protein precipitates 88 % could be resolubilized. The resolubility of precipitates with $ZnCl_2$ increased with increasing $ZnCl_2$ concentration during precipitation. 77 % of the precipitated protein could be resolubilized, when 15 mM of $ZnCl_2$ was added. Addition of $FeCl_2$ during precipitation had no significant effect on resolubility.

4. Organic solvents

Organic solvents induced a marked increase in precipitation of potato proteins at pH 5, especially at concentrations between 1 and 4 M (Fig. 6). Using methanol and ethanol, maximum precipitation was reached at concentrations of about 5 M causing 90 % of total protein to precipitate. No maximum in the precipitation was found with acetone, in the concentration range studied. At a concentration of 3 M, isopropanol already caused maximum precipitation (86 % of total protein).

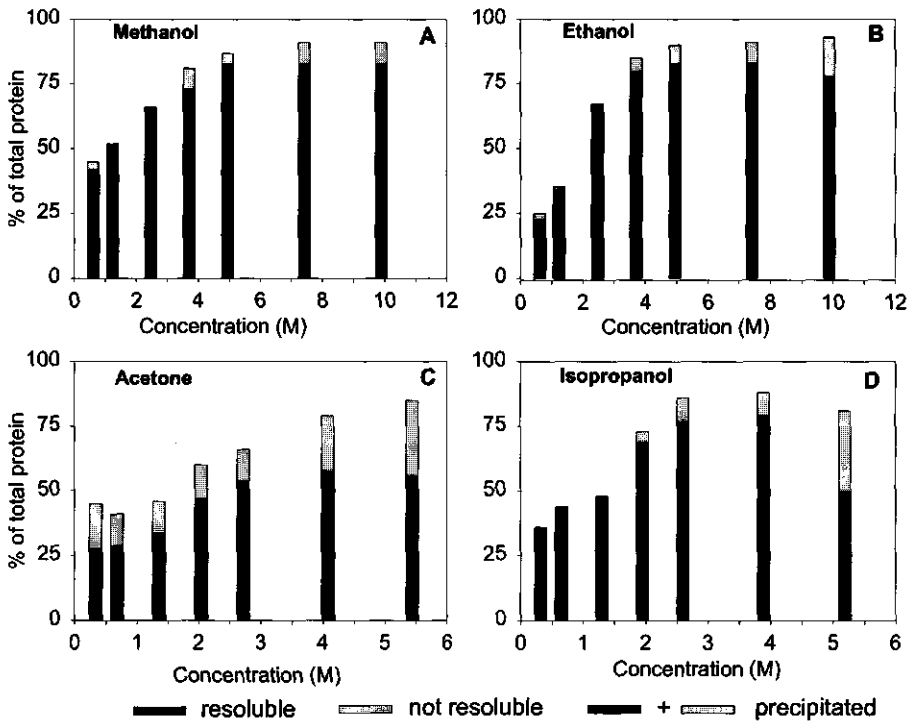

Figure 6: Amount of protein precipitated (pH 5, H_2SO_4) and amount of precipitated protein resoluble (pH7, I = 0.2 M) expressed as proportion of total protein originally present as a function of concentration for various organic solvents.

The resolubilities of the precipitates obtained using organic solvents were high, even at low concentrations of organic solvents, as compared to precipitates obtained in the absence of organic solvents (Fig. 1). In some cases complete resolubilization could be achieved.
The resolublization volume had no significant effect on the amount of protein resolubilized, when taking into account the differences in amounts of precipitate formed using organic solvents (Figure 7).

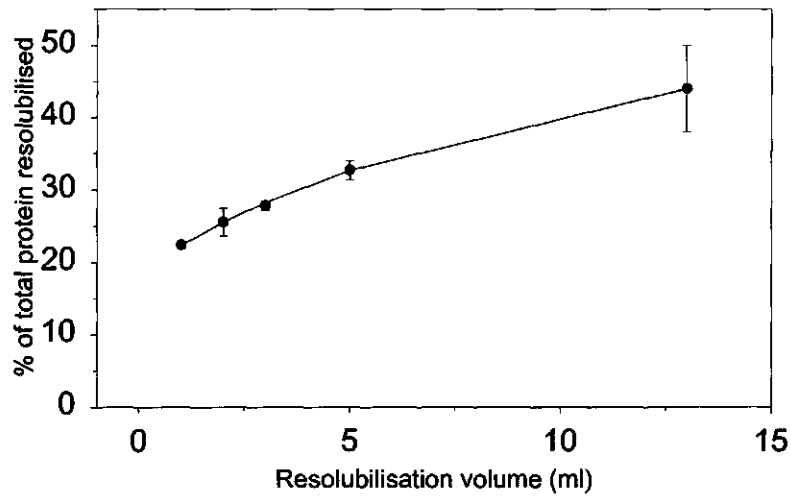

Figure 7: Amount of precipitated protein resoluble (pH7, I = 0.2 M) of potato protein precipitate (pH 5, 1.3 M isopropanol) expressed as proportion of total protein originally present as a function of resolubilization volume.

Protein compositions of precipitated and resolubilized protein fractions

In Table 1 an overview of the quantitative yields of individual potato protein classes in precipitates and resoluble parts thereof as obtained via SDS-PAGE is presented together with a summary of the results on precipitation yield and resolubility. The protein composition of untreated PFJ shows various strong protein bands, which can be subdivided in 5 molecular weight classes. The most abundant protein was patatin (43 kDa), which comprises 40 % of soluble potato protein (Racusen and Foote, 1980). Another class of proteins, with molecular weights between 21 and 25 kDa, was present in somewhat lower amounts. Two other classes of proteins, the 20 kDa proteins and proteins with molecular weights between 14 and 20 kDa, were present in lower amounts. Various protein bands with a molecular weight below 14 kDa showed lower intensities. Table 1 also shows the quantitative yield of the individual protein classes of precipitates (pH 3, pH 5) obtained with various precipitants and the resoluble part thereof.
Visual estimation of the intensity of different bands on SDS-PAGE gels showed that lowering the pH of PFJ with strong acids resulted mainly in the precipitation of patatin (43 kDa) and

small proteins (< 14 kDa). Precipitation at pH 5 using weak acids resulted in precipitation of a large part of the patatin and the proteins with molecular weights between 14 and 20 kDa. Decreasing the pH to 4 increased the solubility of patatin but decreased the solubility of the other proteins, while further lowering of the pH to 3 lead to poor solubility of all the potato proteins present.

Resolubilization of precipitates obtained using strong acids resulted mainly in resolubilization of patatin (43 kDa) and smaller proteins (< 14 kDa). Precipitation below pH 4 resulted in less patatin to resolubilize. The resoluble part of precipitates obtained using weaker acids showed the same trend as the strong acids, although more patatin and 20 kDa protein was resolubilized.

In contrast to what might be expected, only a small amount of the protein precipitated at pH 5 from dialyzed PFJ consisted of patatin (43 kDa); it mainly consisted of proteins with molecular weights between 14 and 20 kDa. At pH 4 almost all protein had disappeared from the supernatant, while at pH values below pH 3.5 the solubilities of all proteins, except patatin and the smaller proteins (< 14 kDa), increased again (no further results shown). The resoluble part of the precipitates obtained from dialyzed PFJ had the same protein composition as the precipitate was expected to have, as deduced from the composition of the supernatants, except when precipitation took place at pH < 3.5. Precipitates obtained below this pH showed decreased resolubility of both patatin and proteins with molecular weights between 14 and 20 kDa. Increasing the ionic strength of dialyzed PFJ did not change the protein composition of the precipitate and the resoluble part of the precipitate. Increasing the salt concentration resulted in precipitates with decreased resolubilities of both patatin and proteins with molecular weights between 21 and 25 kDa (no further results shown).

Addition of $FeCl_3$ increased precipitation of all proteins and addition of $ZnCl_2$ lead to a small increase in the precipitation of proteins with a molecular weight ≥ 20 kDa. Addition of $FeCl_2$ did not change the protein composition of the precipitate. The increased resolubility of precipitates obtained in the presence of $FeCl_3$ was mainly caused by improved resolubilities of smaller proteins (< 14 kDa) and 20 kDa proteins. Addition of $ZnCl_2$ concentrations above 7.5 mM resulted in precipitates with increased resolubilities of all proteins except proteins with molecular weights between 20 and 25 kDa. $FeCl_2$ did not change the protein composition of the resoluble part of the precipitate.

Methanol and ethanol at low concentrations caused precipitation of mainly low molecular weight proteins (≤20 kDa). Acetone and isopropanol already at low concentrations caused extensive precipitation of patatin (43 kDa). Higher concentrations of organic solvents caused precipitation of all proteins. Precipitates obtained with low concentrations of methanol, ethanol and isopropanol showed a lower than expected resolubility of proteins with a molecular weight between 14 and 20 kDa. From the precipitates obtained using acetone a large part of the patatin (43 kDa) remained insoluble after precipitation.

Table 1: Maximum precipitation yield and resolubility values and quantitative yields of individual potato protein classes in precipitates and resoluble parts thereof obtained via SDS-PAGE [1].

Precipitant class				Acids				Metal salts		Organic solvents					
Compound				Strong		Weak		$FeCl_3$	$ZnCl_2$	Methanol Ethanol		Isopropanol		Acetone	
Precipitation	Maximum (% of total protein)			62 % (pH 3)		60 % (pH 3)		46 %	35 %	91 %		88 %		83 %	
	Protein classes	Mw (kDa)	*PFJ*	pH 3	pH 5	pH 3	pH 5	15 mM		Low Conc.	High Conc.	Low Conc.	High Conc.	Low Conc.	High Conc.
		43	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+ +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +
		21-25	+	+	-	+ +	+	+	+	+ +	+	+ +	+	+	+
		20	+ + +	+	-	+	+ +	+	+	+	+ + +	+	+ + +	+	+ +
		14-20	+ + +	+	+	+ +	+	+ +	+	+	+ + +	+ +	+ +	-	+ +
		< 14	+ +	+	-	+ +	+	+	+	+	+ + +	+	+ + +	+	+ +
											+ +		+ +	-	+
Resolubilization	Maximum (% of total protein)			13 %		28 %		41 %	27 %	83 %		79 %		58 %	
	Protein classes	Mw (kDa)		pH 3		pH 5		15 mM		Low	High	Low	High	Low	High
		43		+ +	+	+	+ + +	+ +	+ +	+ +	+ + +	+ +	+ + +	+	+
		21-25		+	+ +	+	+	+	+	+	+ +	+	+ + +	+	+ + +
		20		+	+ +	-	+ +	+ +	+	+	+ +	+	+ +	+	+ +
		14-20		+	+	+	+	+	+	+	+ +	+	-	+	+ +
		< 14		+	+	+	+	+ +	+ +	+	+ +	+	+	+	+ +

Protein bands: not detectable [-], detectable [+, ++, +++, ++++] very strong; [1] Normalized towards intensity of protein bands in PFJ

DISCUSSION

Protein "solubility"

In this study the term solubility is used frequently. The authors do realize that the data presented are not really solubility data, since these should be expressed as amount per unit volume. The solubility of a component is a well defined property and is the amount of that component remaining in solution when it is in equilibrium with the crystals (or a liquid or gas phase), under well defined conditions. Most proteins have well defined solubilities, which depend on factors like solvent quality, temperature, pH and ionic strength. Moreover, proteins can change their conformation (e.g. denaturation) in such a way that their solubility greatly alters (mostly decreases). In the case of a single protein solubility data, e.g. at various pH, can be readily obtained if the protein is available in crystalline form. For protein mixtures this requires more effort, because the solution has to be saturated with every single protein present in the mixture and this would thus require large amounts of protein. The potato protein mixture in PFJ can not be worked up in such a way that a solution becomes saturated with every single protein present. Instead the proportion of total protein that becomes "insoluble" is used. This property is not well defined because it does not give information about the changes in the solubilities of the proteins of which the saturation concentration is not reached. When precipitates are resolubilized, the situation is even more complex because, as was mentioned before, the resolubilization volume was kept constant, regardless of the amount of precipitate, and the resolubility was again expressed as proportion of total protein originally present in PFJ. This means that, although a precipitate was always present after resolubilization the solution need not have been saturated with all proteins, since some may have a high solubility and others may be present in small amounts. This means that both precipitation and resolubility data are not real solubility data; they indicate how average protein solubility differs from that at the starting conditions.

In Figures 2 and Figure 7 it can be observed that the precipitate obtained from PFJ at pH 5 does not show normal solubility behavior, since the amount of protein resolubilized is not proportional to the resolubilization volume. The precipitate seems to consist of at least two fractions, of which one would be quite soluble, the part until 25 ml (Fig. 2) and 3 ml (Fig. 7). The other fraction then would have a rather low solubility and does not dissolve when a resolubilization volume of 125 ml (Fig. 2) or 13 ml (Fig. 7) is used. SDS-PAGE of the resoluble part of the precipitates at various resolubilization volumes (results not shown) did not show resolubilization of specific proteins or protein fractions. It appears as if potato proteins precipitate by a mechanism that results in a more soluble and a less soluble fraction having the same gross protein composition. Although real solubilities could be determined in this study the solubility data presented are quite sufficient to recognize trends in both precipitation and resolubility data.

Comparison of the effectiveness of precipitants

A maximum in protein precipitation in PFJ at pH 3 (60 % of total protein) has also been found by others (Meister and Thompson, 1976;Knorr et al., 1977;Knorr, 1980;Knorr, 1982). The precipitation curves obtained are also similar to those previously found (Meister and Thompson, 1976;Knorr et al., 1977;Knorr, 1980;Knorr, 1982;Lindner et al., 1980;Gonzalez et al., 1991), although none of these, except Meister and Thompson (1976), found significant precipitation at pH values higher than 4.5. Precipitation at pH values above pH 4.5 was expected to take place because the major potato protein, patatin, having an isoelectric pH of 4.9, is known to precipitate at the latter pH (Pots et al., 1998a). When using citric acid at pH 3 we found 58 % of the total protein to precipitate. In contrast, Knorr (Knorr, 1980;Knorr, 1982), using the same conditions, was able to precipitate all of the protein present. The resolubility, of 39 %, of the citric acid precipitate obtained at pH 4, was much less than the resolubility of 91 % of precipitated protein obtained by Knorr (Knorr, 1980;Knorr, 1982). Also the precipitate obtained after treatment with HCl at pH 3, of which 17 % could be resolubilized, had a markedly lower solubility than the precipitate Knorr obtained, of which 56 % was resoluble. Therefore, the differences in resolubility observed are probably due to differences in the PFJ used.

SDS-PAGE showed that using strong acids more patatin precipitates as the pH is lowered, whereas in the presence of weak acids less patatin precipitates at pH 4 than at pH 5 or pH 3. In general it can be concluded that the acid used does not have a large effect on the precipitation of potato proteins from PFJ. The best acid, in terms of resolubility, was citric acid, which resulted in a resolubility of 28 % of total protein at pH 3.5. Although the precipitate obtained with citric acid at pH 3.5 consisted mainly of patatin the resoluble part consisted mainly of proteins other than patatin.

The results of the experiments with dialyzed PFJ show that, although the solubility behavior of potato proteins is changed by dialysis, the solubility behavior is still not comparable to that of isolated patatin, because the precipitation of potato protein at pH's ≥ 5 was not reversible. SDS-PAGE showed that the diminished precipitation at pH$\leq$ 3.5 (Fig. 3) was due to increased solubilities of proteins other than patatin and small proteins (< 14 kDa).

Addition of metal salts at pH 5 causes less precipitation than does lowering the pH to 3 with acid. $FeCl_3$ and $ZnCl_2$ cause 46 % and 35 % of total protein to precipitate, respectively, resulting in especially proteins with a higher molecular weight ($\geq$ 20 kDa) to precipitate. $FeCl_3$ and $ZnCl_2$ also do not lead to the recovery of a larger amount of resoluble protein, 41 % and 27 % of total protein, respectively. These metal salts do, however, result in precipitates that contain a much higher proportion of resoluble protein, which consists mainly of patatin and small proteins (< 14 kDa). The metal salt resulting in the highest resolubility (41 % of total protein at 15 mM) was $FeCl_3$. The precipitate obtained in the presence of 15 mM $FeCl_3$ had the same protein composition as untreated PFJ, as did the resoluble part of the precipitate (Table 1).

From the organic solvents used ethanol and isopropanol seem to be the most promising for industrial application. They lead to much more precipitation than did the other precipitants,

about 90 % of total protein, which concerns all proteins present in PFJ in significant amounts. Precipitation with organic solvents also resulted in much higher resolubilities than with other precipitants; about 80 % of total protein is recovered as (re)soluble protein comprising all proteins except those with molecular weights between 14 and 20 kDa.
Wilhelm and Kempf (1977) used the same organic solvents to precipitate potato protein at ambient temperature, but without lowering the pH. At concentrations comparable to the highest concentration used in this study, they also found isopropanol to be the most effective precipitant and acetone to be one of the least effective, causing 25 % and 21 % of total protein to precipitate, respectively. These results clearly show that lowering the pH is an essential step in effective potato protein precipitation when organic solvents are used. The best organic solvents, in terms of resolubility, were ethanol and methanol, yielding 83 % of total protein as resoluble protein at a concentration of 4.9 M. Both the precipitate and the resoluble part thereof had, at this concentration, the same protein composition as PFJ.

Interactions involved in protein precipitation

The observation that maximum precipitation occurred at pH's lower than 4 is quite unexpected, since isoelectric focussing showed (results not shown) that none of the proteins present in PFJ in significant amounts has, as expected (Seibles, 1979), an isoelectric pH below pH 4.5. Furthermore, the solubility curve of pure patatin as a function of pH shows that patatin is almost completely soluble at pH $\leq$ 4 (Pots et al., 1998b), whereas in PFJ maximum precipitation took place at pH's below 4 (Fig. 1). Precipitates formed at pH 5 from purified patatin were also completely resoluble at pH $\geq$ 6, whereas precipitates formed at pH 5 in PFJ were only partly resoluble at pH 7 (Fig. 1). From these results it can be concluded that the solubility behavior of patatin, and probably that of potato proteins in general, is in purified form different from that in PFJ. The precipitation of potato proteins in PFJ seems not to be determined by their isoelectric pH. Dialysis changed the precipitation of potato proteins as a function of pH in a way that can not be ascribed to the removal of salts. The results suggest that constituents of PFJ, other than simple salts, interact with potato proteins at low pH and thereby cause these proteins to become insoluble at low pH and cause their precipitation to be partly irreversible. These compounds can be partly removed by dialysis, causing their concentration to become limiting, as can be concluded from the observation that the amount of protein resolubilized increases with the amount of precipitate formed (Fig. 3). The interactions that play a role during precipitation of potato proteins, at pH 5, are strengthened with increasing ionic strength, until they reach a maximum, and thereby render precipitation less reversible (Fig. 4).
Both Fe^{3+}-ions and Zn^{2+}-ions, and to a lesser extent Fe^{2+}-ions, may act by inducing another mechanism of precipitation via the formation of complexes with specific amino acid residues on several proteins. These non-covalent bonds are easily broken at neutral pH during resolubilization. Alternatively, metal ions (Haslam, 1989;Mila and Scalbert, 1996;McDonald et al., 1996;Ferrali et al., 1997) and proteins (McManus et al., 1985;Beart et al., 1985) are known to form stable soluble or insoluble complexes with plant phenolic compounds, which

are known to be present in potato (Friedman, 1997;Lewis et al., 1998). Addition of metal ions may therefore also prevent the formation of complexes between polyphenols and proteins and thereby shift protein precipitation towards another mechanism. The importance of protein polyphenol interactions in potato protein precipitation would also explain the higher resolubilities of protein precipitates obtained after dialysis of PFJ, because dialysis will remove a large part of the phenolics present. Both mechanisms described here do, however, not explain why Fe^{2+} does not affect protein precipitation and resolubility.

The high resolubility obtained using organic solvents may be partly caused by the use of a moderately low pH, which tends to prevent the unfolding of patatin. Alternatively, the use of aqueous organic solvent mixtures improves the solvent quality for polyphenols (Haslam, 1989), which would lower their activity, hence their affinity for proteins, and could thus prevent protein precipitation from becoming irreversible.

ACKNOWLEDGEMENT

We thank André Pots for purifying the patatin and useful comments on the manuscript. Potatoes were kindly provided by AVEBE B.A. (Foxhol, The Netherlands). The research described in this paper is part of a project supported by IOP-Industrial Proteins and AVEBE B.A.

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Chapter 3

The effects of pH and heat treatments on the structure and solubility of potato proteins in different preparations

ABSTRACT

The soluble potato proteins are mainly composed of patatin and protease inhibitors. Using DSC and both far-UV and near-UV CD spectroscopy, it was shown that potato proteins unfold between 55°C and 75°C. Increasing the ionic strength from 15 to 200 mM generally caused an increase in denaturation temperature.

It was concluded that the dimeric protein patatin unfolds either in its monomeric state or its monomers are loosely associated and unfold independently. Thermal unfolding of the protease inhibitors was correlated with a decrease in protease inhibitor activities and resulted in an ionic strength dependent loss of protein solubility. Potato proteins were soluble at neutral and strongly acidic pH. The tertiary structure of patatin was irreversibly altered by precipitation at pH 5. At mildly acidic pH the overall potato protein solubility was dependent on ionic strength and the presence of unfolded patatin.

This chapter has been submitted as:

The effects of pH and heat treatments on the structure and solubility of potato proteins in different fractions

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INTRODUCTION

Potato protein has a relatively high nutritional quality, comparable to that of whole egg (Kapoor et al., 1975;Knorr, 1978), and it, therefore, has high potential for utilization in food applications. The soluble proteins can be tentatively classified into three classes: Patatin (41 kDa), protease inhibitors (5-25 kDa) and others (mostly high Mw) (Pots et al., 1999b). After industrial potato starch manufacture, potato proteins are present in an aqueous solution called potato fruit juice (PFJ). After they have been heat-coagulated to collect them, potato proteins are considered a byproduct of low value.

Since a more profitable utilization of potato proteins would be of economical interest for potato starch manufacturers several efforts have been made to recover potato proteins from PFJ that have retained their native properties, of which solubility is most important. Since most of the proteins in PFJ have isoelectric points at acidic pH (Seibles, 1979), most research has been devoted to precipitation of potato protein at low pH (Meister and Thompson, 1976;Knorr et al., 1977;Knorr, 1980;Knorr, 1982; Chapter 2). Precipitation at low pH may, however, severely affect the molecular structure of potato proteins (Pots et al., 1998b).

The effects of heat treatments on potential food proteins need also be known, because heat treatment is often a necessary processing step in food manufacture. In addition, heat treatment may prove to be unavoidable to diminish the activity of potato proteinase inhibitors. During these necessary treatments the molecular structure of the potato proteins is likely to be affected (Pots et al., 1998a).

The purpose of this study was to examine the effects of pH and heat treatment on the conformation and the conformational stability of various potato protein fractions. Since protein solubility is a prerequisite for functional application of proteins in foods (Kinsella, 1976), the effects of structural changes on the solubility characteristics of potato proteins were also studied,

MATERIALS AND METHODS

Preparation of protein fractions

1. Potato fruit juice (PFJ)

Potatoes (cv. *Elkana*) were washed thoroughly with water and cut into pieces (max. 8 x 2.5 cm) which were immediately dipped into a 20 mg/ml solution of sodium bisulfite to prevent enzymatic browning. The potato pieces were ground in a domestic type juice extractor (AEG). The resulting turbid juice was allowed to settle for 15 min. Next, the liquid was decanted and centrifuged (15 min, 19000 × *g*, 10°C) and the supernatant filtered through a paper filter (Schleicher & Schuell, ref.no. 311653). The resulting clear yellowish filtrate, which has a pH of 5.7 - 6.0, is known to be comparable to industrial PFJ (AVEBE B.A., Foxhol, The Netherlands) and is further denoted as PFJ. PFJ, on average, contained 3.59 ± 0.09 (SD) mg of nitrogen per ml. Of this nitrogen 60 ± 5.6 (SD) % could be precipitated with 12.5 % (w/v) TCA and was therefore assumed to be of protein origin, which leads to an average protein (N x 6.25) concentration of 13.4 ± 0.9 (SD) mg protein per ml of PFJ (Chapter 2).

2. Ammonium sulfate precipitate (ASP)

Ammonium sulfate precipitate (ASP) was prepared from PFJ by adding $(NH_4)_2SO_4$ to 60 % saturation as proposed by Seppälä et al. (1999) while keeping the pH at 5.7 by addition of small volumes of 0.5 M H_2SO_4. After 1 hour at 4°C the suspension was centrifuged (30 min, 19000 × *g*, 4°C) and the resulting precipitate was washed twice with half the starting volume of 50 mM sodium phosphate buffer (pH 7) that contained $(NH_4)_2SO_4$ up to 60 % saturation. Subsequently, the precipitate was suspended in distilled water and dialyzed (MWCO 3.5 kDa, Spectrum Medical Industries, Laguna Hills, CA., USA) against distilled water until the conductivity of the retentate remained constant. The retentate was subsequently freeze-dried and stored at -20°C.

3. Patatin

Patatin was purified by applying PFJ, diluted ten times with water and adjusted to pH 8, on a Source 15 Q column (10 x 15 cm) (Amersham Pharmacia Biotech AB, Uppsala, Sweden) equilibrated with a 25 mM Tris-HCl buffer (pH 8) containing 0.5 g/l $NaHSO_3$. After washing out the unbound compounds the bound fraction was eluted with the above mentioned buffer containing 0.35 M NaCl. Further purification of the bound fraction was realized by gel filtration on a Superdex 75 column (63 × 10 cm) (Amersham Pharmacia Biotech AB, Uppsala, Sweden) eluted with a 30 mM sodium phosphate buffer (pH 7) containing 0.5 g/l $NaHSO_3$ at a linear flow rate of 30 cm/h. The first peak, as observed from the absorbance at 280 nm, containing patatin of > 95 % purity (SDS-PAGE), was collected and concentrated 10 times using Xampler UFP-3-C cross-flow hollow fiber laboratory cartridges with a molecular weight cut-off of 10 kDa (A/G Technology Corp., Needham, USA) at 4°C. The patatin was subsequently diafiltered with 5 volumes of a 9 mM sodium phosphate buffer (pH 7) and stored at –20°C.

4. Protease inhibitor pool (PIP)

Protease inhibitor pool (PIP) proteins were prepared by gel filtration of PFJ on a Superdex 75 column (63 × 10 cm) (Amersham Pharmacia Biotech AB, Uppsala, Sweden) eluted with a 30 mM sodium phosphate buffer (pH 7) containing 0.5 g/l $NaHSO_3$ with a linear flow rate of 30 cm/h. The second peak eluting, as observed from the absorbance at 280 nm, was collected and concentrated 10 times using Xampler UFP-3-C cross-flow hollow fiber laboratory cartridges with a molecular weight cut-off of 5 kDa (A/G Technology Corp., Needham, USA) at 4°C. The concentrated PIP was subsequently diafiltered with distilled water until no further decrease of the conductivity of the permeate could be observed. The final PIP was subsequently freeze-dried and stored at -20°C.

Protein composition

Protein composition of PFJ, ASP, PIP and patatin was estimated by electrophoresis and subsequent densitometric analysis of Phastgel IEF 3-9 gels, or IEF 2.5-6 for patatin, after separation on a Phast System (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and staining with Coomassie Brilliant Blue. The relative intensity of the separated bands was

measured using a Molecular Dynamics 300 computing densitometer (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Circular dichroism (CD) spectroscopy

Sample preparation

CD experiments were performed to investigate the effect of various treatments on the conformation and the thermal stability of patatin and PIP. Conformational characteristics and thermal stability of both patatin and PIP were estimated at pH 7 using a 9 mM sodium phosphate buffer adjusted to ionic strengths of 15 or 200 mM by addition of NaCl.

The conformation and the thermal stability of patatin were also determined after precipitation at pH 5 and subsequent resolubilization at pH 7 (PAT-5) in order to investigate the effect of precipitating patatin at its isoelectric pH. For these experiments patatin was precipitated from 4 mg/ml solutions of patatin in 9 mM sodium phosphate buffer pH 7.0 by adjusting 8 ml of this solution to pH 5 with 0.1 M H_2SO_4 at room temperature. After 1 hour the turbid solutions were centrifuged (15000 x *g*, 20 min). The precipitates were resolubilized by adding 3 ml of water, adjusting the pH to 7.0 with 0.1 M NaOH, and extensively dialyzing the samples against a 9 mM sodium phosphate buffer (pH 7). These samples are further denoted as PAT-5.

The effect of pH on the conformation of proteins in PIP was studied at pH 7, pH 5 and pH 3 at ionic strengths of 15 and 200 mM. For these experiments PIP (0.2 mg/ml) was solubilized either in a 9 mM sodium phosphate buffer (pH 7) containing 0 or 185 mM NaF, in a 24 mM sodium acetate buffer (pH 5) containing 0 or 185 mM NaF or in a 16 mM sodium phosphate buffer (pH 3) containing 0 or 185 mM NaF.

Studies on the effect of ionic strength on the temperature induced unfolding of PIP were conducted with 0.2 mg/ml solutions of PIP in a 9 mM sodium phosphate buffer (pH 7) that contained no NaF ($I \approx 15$ mM) or 185 mM NaF ($I = 200$ mM).

Far-UV CD

Far-UV CD spectroscopy was used to monitor changes in the secondary structure of patatin (0.1 mg/ml) and PIP (0.2 mg/ml) caused by various treatments. Far-UV CD spectra (190 – 260 nm) were recorded 10-fold and averaged on a Jasco J-715 spectropolarimeter (Jasco Corp., Japan) at ambient temperature in quartz cells with an optical path length of 1 mm. Far-UV CD spectra were also recorded at various temperatures after heating for 6 min at a specified temperature prior to recording the CD spectrum. The scan speed was 100 nm/min, the data interval 0.2 nm, the bandwidth 1.0 nm, the sensitivity 20 mdeg and the response time 0.125 s. All recorded spectra were corrected by subtracting the spectrum of a protein free sample.

Changes in the thermal stability of the secondary structure of patatin and PIP, after various treatments, were also monitored. For patatin samples this was done by measuring the ellipticity at 222 nm as a function of temperature at a heating rate of 20 K/h. PIP samples were heated at the same rate but in this case the ellipticity was monitored at 228 nm.

Near-UV CD

Near-UV CD was used to monitor changes in the structure of patatin (4 mg/ml) at a tertiary level. Near-UV CD spectra (250-350 nm) were recorded 25 fold and averaged at temperatures in the range from 20°C to 80°C with a heating rate of 20 K/h and heated for 15 min at specified temperatures before spectra were recorded. Samples were tested in a cuvet with an optical path length of 1.0 cm. The scan speed used was 50 nm/min, the data interval 0.5 nm, the bandwidth 1.0 nm, the sensitivity 10 mdeg and the response time was 0.25 s. Recorded spectra were corrected by subtraction of the spectrum of a protein free sample.

Differential scanning calorimetry (DSC)

DSC experiments were performed on a VP-DSC MicroCalorimeter (MicroCal Inc., Northampton (MA), USA). Thermograms were recorded from 20°C to 90°C with a heating rate of 20 K/h. DSC experiments were conducted with untreated PFJ (13.5 mg protein/ml). ASP, PIP and patatin were used at concentrations of 10, 6 and 4 mg/ml, respectively. Patatin was also analyzed at lower concentrations. All fractions except PFJ were analyzed in 9 mM sodium phosphate buffer (pH 7) that contained no NaCl ($I \approx 15$ mM) or 185 mM of NaCl ($I =$ 200 mM).

High performance size-exclusion chromatography (HP-SEC)

HP-SEC experiments at various temperatures (30°C – 80°C) were performed with a Spectra Physics P1000 solvent delivery system equipped with an AS3000 auto-sampler (Thermo Separations Products, Fremont CA, USA) and a SpH 99 column oven (Spark Holland, Emmen, The Netherlands). Patatin (0.85 mg/ml) samples were preheated during 10 min in a thermostated waterbath. Quantities of 100 µl of preheated sample were injected on a TSKgel G2500PWXL column (7.8 (ID) × 300 mm) (TosoHaas, Montgomeryville PA, USA) equilibrated and eluted with a thermostatted 9 mM sodium phosphate buffer (pH 7) with a flow rate of 0.8 ml/min. Dextran standard solutions (27 mg/ml) with molecular weights of 40 and 70 kDa were used as external calibration standards throughout the temperature range. Proteins and dextrans were detected at 280 nm with a Spectra Physics UV2000 absorbance detector (Thermo Seperations Products, Fremont CA, USA) and a Viscotek Model 250 refractometer (Viscotek Benelux B.V., Oss, The Netherlands), respectively.

Protein solubility

Protein solubility experiments were performed with undiluted PFJ. ASP and PIP were dispersed to final concentrations of 10 and 6 mg/ml, respectively, in a 9 mM sodium phosphate buffer (pH 7) that contained no NaCl ($I \approx 15$ mM) or 185 mM of NaCl ($I = 200$ mM). Solutions of patatin were adjusted to a final concentration of 4 mg/ml in the same buffers as mentioned above.

Effect of pH

Protein precipitation experiments as a function of pH were performed in duplicate by adjusting the pH of stirred samples with 0.5 M H_2SO_4 in 15 ml Kimax tubes at room

temperature to set values. The acidified samples were left to bed down for 1 hour at room temperature. The samples were then centrifuged for at least 15 min (3600 × *g*, 20°C) until clear supernatants were obtained. Supernatants were analyzed in duplicate for nitrogen content using the micro-Kjeldahl method (AOAC, 1980) when PFJ samples were used. When other protein fractions than PFJ were used supernatants were analyzed for protein content using the method of Bradford (Bradford, 1976) with bovine serum albumin (Sigma A-7511) (Lot 92H93131) as a standard. In the case of PFJ protein nitrogen was calculated as total nitrogen minus 12.5 % (w/v) TCA soluble nitrogen. In treated PFJ samples soluble protein nitrogen was calculated as protein nitrogen in PFJ minus precipitated nitrogen of the sample and expressed as proportion of protein nitrogen present in PFJ. Precipitated nitrogen was assumed to be of protein origin. Soluble protein was expressed as the proportion of the protein originally present in solution at pH 7 and was corrected for the volume of liquid added during acidification.

Effect of heat treatment

Protein precipitation as a function of temperature was determined by heating 1.5 ml samples for 15 min in a closed Kimax tube in a thermostatted waterbath (accuracy: ± 0.5 °C). After heating the samples were immediately cooled on ice. After a cooling time of 15 min the heated samples were centrifuged for at least 15 min (3600 × *g*, 20°C) until clear supernatants were obtained. Protein analysis of the supernatants was performed as described above.

Lipolytic acyl hydrolase (LAH) activity

LAH activity of samples containing patatin was measured using *p*-nitrophenyl butyrate as a substrate. Further conditions were as described by Pots et al. (Pots et al., 1998b). The substrate concentration used was 0.41 mM. The specific LAH-activity was determined from the initial rate of PNP release and expressed as $\mu mol \cdot min^{-1}$ per mg protein.

Protease inhibitor activity

Trypsin from bovine pancreas (T-0134) (lot 100H0685), Type II α-chymotrypsin from bovine pancreas (C-4129) (lot 58H7001), papain from papaya latex (P-9886) (lot 66H7130), carboxypeptidase A from bovine pancreas (C-0261) (lot 116H8020) and cathepsin D from bovine spleen (C3138) (lot 103H8005) were obtained from Sigma Chemical Co. Trypsin inhibitor activity was estimated by the Kakade method as modified by Smith et al. (Smith et al., 1980) with 0.36 mM benzoyl-DL-Arg-*p*-nitroanilide (Merck) as a chromogenic substrate. Chymotrypsin inhibiting activity was estimated according to Geiger (Geiger, 1984) with 0.88 mM N-succinyl-L-Phe-*p*-nitroanilide (Sigma) as a substrate.

Inhibition of papain was estimated by the method of Mole and Horton (Mole and Horton, 1973) with 2.53 mM of benzoyl-L-Arg-*p*-nitroanilide (Merck) as a substrate. Cathepsin D inhibiting activity was estimated according to Van Jaarsveld et al (Van Jaarsveld et al., 1997) with 0.67 % (w/v) of acid denatured hemoglobin as a substrate. Carboxypeptidase A inhibiting activity was measured as described by Riordan and Holmquist (Riordan and

Holmquist, 1984) with 0.1 mM of N-(furanacryloyl)-L-Phe-L-Phe (Bachem) as a substrate. For all protease inhibition assays the degree of inhibition was measured as a function of protein concentration (not shown). Subsequent inhibition measurements were conducted in the concentration range where inhibition was linear with protein concentration. Residual inhibitor activity after heat treatment was measured in supernatants of heated PIP solutions and expressed as % activity remaining per volume of supernatant relative to the activity present in an unheated solution.

RESULTS

Protein composition

The IEF gels (not shown) of the various potato protein fractions all showed several protein bands in the pI-range from pH 4.5 to pH >9. The protein distributions based on the isoelectric pH of the constituent proteins of the fractions used in this paper are shown in Table 1. The data in Table 1 are estimated from the optical density of protein bands on IEF gels within a chosen pI-range and expressed as proportion of the total density of the protein bands within one sample. The number of bands indicated between parentheses in Table 1 should be taken as an indication rather than as an absolute number because of the large differences in the concentrations of the various proteins in the different fractions.

Table 1: Relative composition of various potato protein fractions on the basis of pI

Sample	pI < 5.2	5.2 < pI < 6	6 < pI < 7	7 < pI < 8	pI > 8
PFJ	38 %[a] (11)[b]	11 % (4)	15 % (5)	20 % (4)	17 % (2)
ASP	51 % (11)	6 % (1)	11 % (2)	5 % (3)	27 % (4)
PIP	10 % (4)	18 % (5)	22 % (6)	20 % (4)	30 % (8)
patatin	100 % (11)				

[a]: expressed as proportion of the totalized density of the protein bands within one lane

[b]: the number between parentheses presents the number of protein bands detected

Table 1 shows that the industrial potato juice (PFJ) contained about 38 % patatin, consisting of 11 protein bands with pI's between 4.5 and 5.2 representing different isoforms of patatin (Pots et al., 1999a). About half of the total amount of protein in PFJ had an isoelectric pH below pH 6 (Table 1). The ammonium sulfate precipitate (ASP), which represents about 75 % of total potato protein, contained relatively more patatin and proteins with a high isoelectric pH (pI > 8) as compared to PFJ. The protease inhibitor pool (PIP), of which SDS-PAGE analysis showed that it did not contain patatin, but mainly proteins with a molecular weight between 20 and 25 kDa, consisted of proteins which proportions were evenly distributed over

a wide range of isoelectric pH values. The distribution of pI's in PIP was similar that observed by Pouvreau et al. (Pouvreau et al., 2001).

Structural changes in potato proteins

Changes in the secondary structure of patatin

The far-UV CD spectra of patatin and PAT-5 at pH 7 are shown in Figure 1A. PAT-5 was examined because we wanted to know if precipitation of patatin at its isoelectric pH affects its properties. The spectrum of untreated patatin is almost identical to the spectrum found for patatin (cv. Bintje) as published by Pots et al. (Pots et al., 1998a;Pots et al., 1998b) showing a zero-crossing at about 203 nm and negative extremes at about 208 and 220 nm. Precipitation of patatin at pH 5 proved not to induce significant irreversible changes in the far-UV CD spectrum.

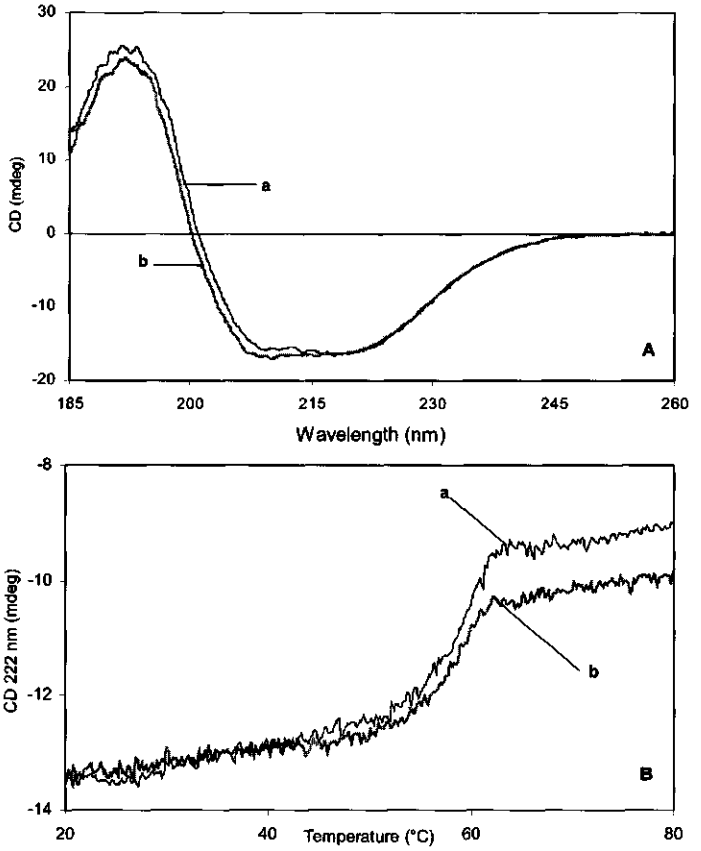

Figure 1: A: Far-UV CD spectra of patatin: **a:** patatin (pH 7) at 20°C **b:** PAT-5 (pH 7) at 20°C
B: Thermal unfolding of patatin as monitored by the CD-signal 222 nm **a:** patatin (pH 7)
b: PAT-5 (pH 7)

The thermal unfolding of patatin and PAT-5 were monitored by the ellipticity at 222 nm as a function of temperature and are shown in Figure 1B. The ellipticity at this wavelength originates from both α-helical and β-stranded structures, whereas contributions of non-structured parts are presumed to be virtually absent in this spectral region (Hennessy Jr. and Johnson Jr., 1981). Both curves show a small gradual decrease in absolute ellipticity with temperature up to about 50°C and above 63°C, with a sharp transition between these two temperatures. The midpoint of the transition for both curves was at 58°C as was determined from the second derivative of the curves (not shown). The difference between the ellipticities above 63°C of patatin and PAT-5 may have been caused by a difference in the aggregation state after unfolding.

Changes in the tertiary structure of patatin

Near-UV CD spectra give an indication of the interactions of aromatic side-chains with other side-chain groups and peptide bonds (Hennessy Jr. and Johnson Jr., 1981;Vuillemier et al., 1993).

In Figure 2A near-UV CD spectra of patatin at various temperatures are shown. The spectrum at 20°C shows two distinctive regions. A broad peak can be seen around 283 nm, which is mainly due to tyrosine and tryptophan contributions (Woody and Dunker, 1996;Pain, 1996).

A second peak can be observed around 258 nm with mainly phenylalanine contributions (Woody and Dunker, 1996;Pain, 1996). Interestingly, an appreciable loss of intensity at 283 nm was already observed when the temperature was increased from 20°C to 25°C, while at 258 nm no significant changes were observed (Fig. 2A). From 25°C to 50°C the intensity around 283 nm decreased gradually, whilst from 50°C up to 80°C no significant changes in the peak around 283 nm could be observed, whereas at 258 nm the largest decrease in intensity took place between 50°C and 60°C (Fig. 2A). The reversibility of the changes observed was limited, as cooling patatin to 20°C after heating at 80°C resulted only in small recovery of the intensities at 258 nm and 283 nm (results not shown).

Precipitation of patatin at pH 5 induced a 50 % decrease of the intensity of the peak around 258 nm (Fig. 2B), indicating changes in the surroundings of the phenylalanine residues. Increasing the temperature caused a further decrease of the intensity around 258 nm, especially between 50°C and 60°C. The behavior of the intensity around 283 nm with temperature had also changed, as compared to that of untreated patatin. Interestingly, it decreased with temperature only between 20°C and 25°C , while at higher temperature no further decrease was observed (Fig. 2B).

Figure 2C shows the ellipticity at 258 nm as a function of temperature for patatin and PAT-5. Both patatin and PAT-5 showed a transition between 45°C and 60°C with a midpoint at about 55°C, but the change in ellipticity was much smaller in the latter case.

It can be concluded that in contrast to the secondary structure, the tertiary structure of patatin is strongly affected by precipitation at pH 5.

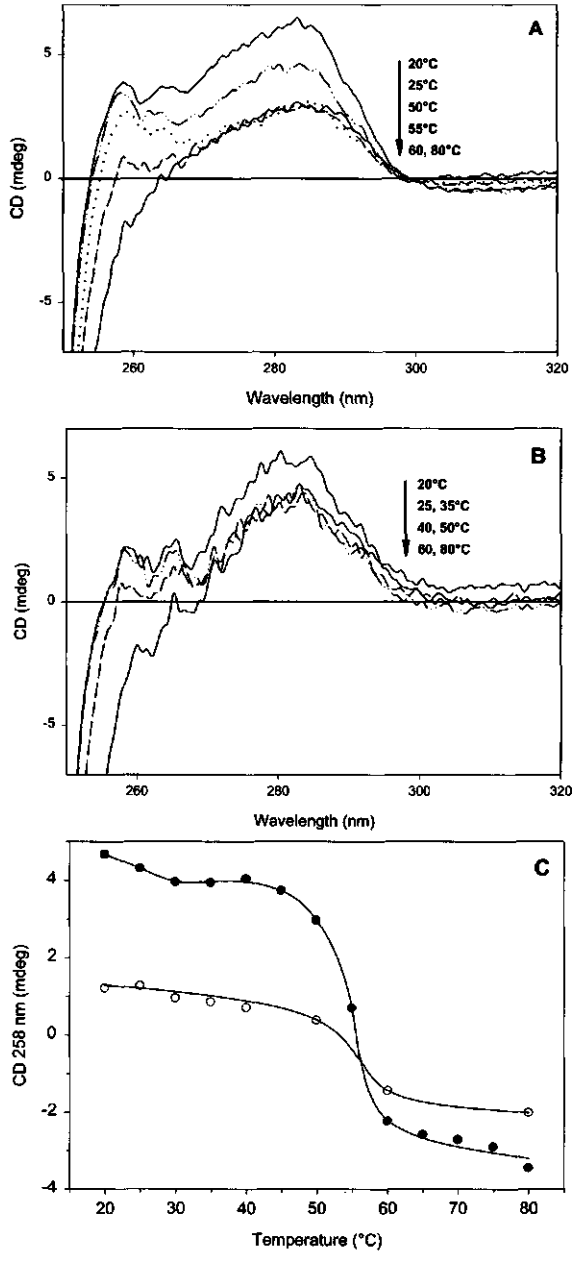

Figure 2: A: Near UV CD spectra of patatin (pH 7) at different temperatures B: Near UV CD spectra of PAT-5 (pH 7) at different temperatures C: CD signal at 258 nm as a function of temperature for patatin (●) and PAT-5 (○) at pH 7

Changes in the secondary structure of PIP

Far-UV CD spectra of PIP ($I \approx 15$ mM) at different temperatures are shown in Figure 3A. The spectrum at 20°C was similar to that described by Lindner et al. (Lindner et al., 1980) for a comparable protein mixture. The spectrum had a minimum at 195 nm and a maximum around 228 nm. As the temperature was increased, the absolute intensity at 195 nm decreased and the intensity at 228 nm also decreased and was inverted (Fig. 3A).

Figure 3B shows the ellipticity of PIP at 228 nm as a function of temperature at ionic strengths of 15 mM and 200 mM. Although the ellipticity at 228 nm contains no recognized structural information, it was monitored as a function of temperature because large changes could be observed at this wavelength (Fig. 3A). The influence of ionic strength on the thermal unfolding of PIP was studied because it could influence its denaturation temperature. An ionic strength of 200 mM is believed to be an approximation of the ionic strength in PFJ. At low

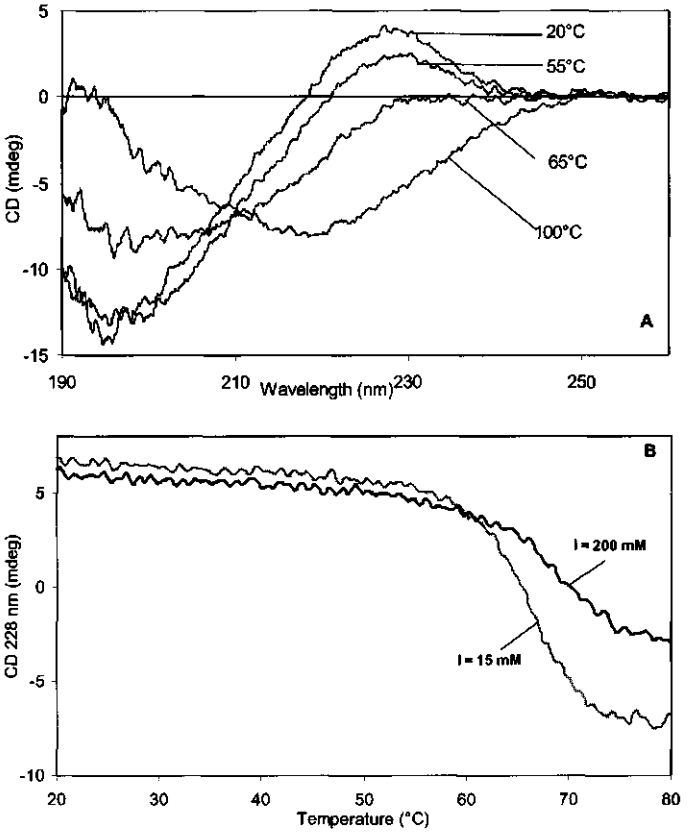

Figure 3: A: Far-UV CD spectra of PIP (pH 7, I =15 mM) at different temperatures B: CD signal at 228 nm as a function of temperature for PIP (pH 7) at I = 15 mM and I =200 mM

ionic strength, the ellipticity of PIP showed a transition between 58°C and 71°C with a midpoint at 66°C. At high ionic strength the curve of PIP showed a broader and weaker

transition between 58°C and 75°C. The transition midpoint of PIP had shifted from 66°C to 69°C.Adjusting the pH to pH 5 or pH 3 at ionic strengths of 15 mM and 200 mM did not affect the far-UV CD spectrum of PIP (no further results shown).

Differential scanning calorimetry

Examples of excess heat capacity profiles of various potato protein fractions are shown in Figure 4. The calorimetric enthalpies given in this section are the average of at least four experiments.

The DSC-profile of PFJ showed a large asymmetric peak and a small shoulder with transition temperatures (T_d's) of 66°C and 74°C, respectively. The total calorimetric enthalpy of unfolding of the proteins in PFJ was 20.5 ± 1.5 (SD) J/g protein.

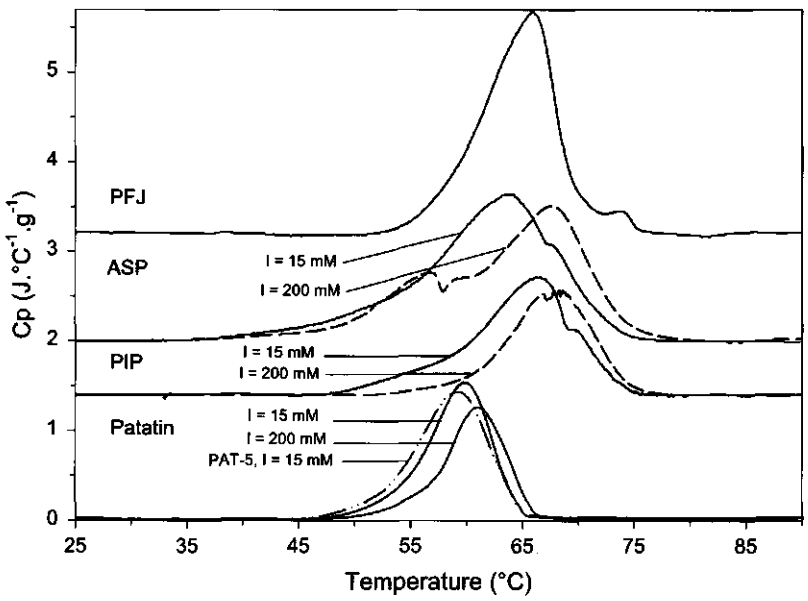

Figure 4: Samples of DSC thermograms of PFJ, ASP PIP and patatin at pH 7 and ionic strengths of 15 and 200 mM

ASP, which had a protein composition different from PFJ (see Table 1), showed different profiles. At low ionic strength ASP showed one peak at 64°C with a Δh_{cal} of 17.4 ± 1.0 (SD) J per gram protein. Increasing the ionic strength from 15 to 200 mM, which is more relevant to the ionic strength in PFJ, caused the main peak to shift from 64°C to 68°C and caused two shoulders to appear with transition temperatures of 56°C and 60°C, respectively (Fig. 4). The total Δh_{cal} of ASP at high ionic strength was 18.2 ± 1.4 (SD) J/g protein.

The same effect of ionic strength on T_d was observed for PIP. At low ionic strength PIP showed one major peak at 66°C with a Δh_{cal} of 25.2 ± 2 (SD) J/g protein. At high ionic strength the transition temperature had shifted to 68°C, with a total Δh_{cal} of 24.0 ± 2 (SD) J/g protein (Fig. 4). The major proteins in PIP thus showed a higher denaturation temperature at increased ionic strength, which is in agreement with the CD data (Fig. 3B).
The DSC profile of patatin at low ionic strength showed one asymmetric peak at 60°C with a Δh_{cal} of 12.6 ± 0.1 (SD) J/g (510 kJ/mole mononomer). At high ionic strength the denaturation temperature increased to 61°C (Fig. 4). The total Δh_{cal} was 11.4 ± 0.1 (SD) J/g. Figure 4 also shows that precipitation of patatin at pH 5 did not induce significant changes in the DSC-profile of patatin.

Solubility as a function of pH

The term solubility, which is used frequently throughout this paper, should be put between quotes because the authors do realize that the solubility measurements presented here are only an approximation of true solubility measurements, since these should be expressed as amount per unit volume. Instead the proportion of total protein that becomes "insoluble" is used. This property is not well defined because it does not give information about the changes in the solubilities of the proteins of which the saturation concentration is not reached (Chapter 2).

In Figure 5 solubility curves of different potato protein fractions are shown as a function of pH at high (Fig. 5A) and low ionic strength (Fig. 5B). Protein solubility at an ionic strength of 200 mM was studied because this ionic strength is similar to that in PFJ (estimated at about 0.2 M). The solubility of potato proteins in PFJ showed a gradual decrease with decreasing pH with a local minimum at pH 5 and the lowest solubility at pH 3 (Fig. 5A).
ASP, which was prepared as a representation of undenatured potato protein, showed a similar curve at high ionic strength (Fig. 5A). At low ionic strength the solubility curve of ASP showed a broad minimum around pH 5 with an increase in solubility at pH < 4.5 (Fig. 5B).
The same effect of ionic strength became apparent when the solubility curves of patatin at high and low ionic strength were compared. At low ionic strength patatin, being completely dissolved at pH 3 and below, showed a broad minimum in solubility around pH 4.5 (Fig. 5B). At high ionic strength patatin still showed a weak minimum in solubility at pH 4.5 but it was almost completely insoluble at pH 3.5. Below pH 3.5 a sharp rise of the solubility was observed (Fig. 5A). Ralet and Guéguen (Ralet and Guéguen, 2000), surprisingly, reported a minimum in solubility of a patatin-rich preparation at pH 4 when no salt or buffer was added. At high ionic strength they observed an increase in the solubility at pH 4, which they ascribed to salting-in of the protein. They did not observe a decrease in solubility at any pH when increasing the ionic strength.
PIP at low ionic strength also showed a broad minimum in solubility around pH 4.5. In this case increasing the ionic strength increased the protein solubility, especially at pH > 4. Ralet and Guéguen (Ralet and Guéguen, 2000), in contrast, did not find any change in solubility over the complete pH-range for a comparable preparation.

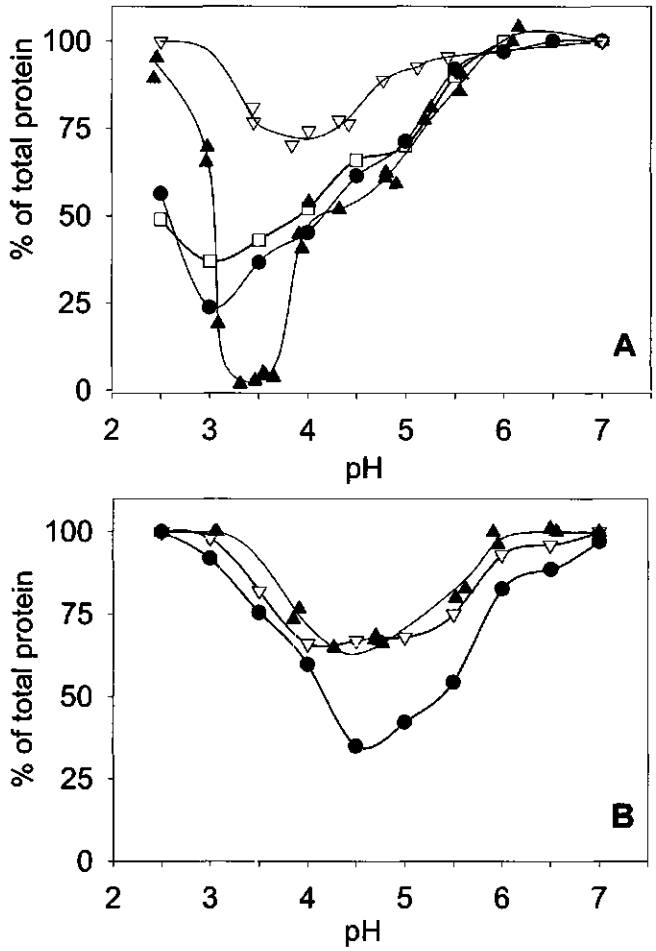

Figure 5: A Solubility of PFJ (□), ASP (●), PIP (▽) and patatin (▲) as a function of pH (I = 200 mM) B:Solubility of ASP (●), PIP (▽) and patatin (▲) as a function of pH (I = 15 mM)

Solubility as a function of heat treatment temperature

The proportion of protein remaining in solution as a function of heat treatment temperature is shown in Figure 6 at high (Fig. 6A) and low ionic strength (Fig. 6B). Precipitation in PFJ already occurred when heated above 40°C. After heating at 60°C, 50% of the protein originally present had precipitated, whilst precipitation was complete after heating at 70°C where > 90% of the protein had lost its solubility (Fig. 6A). In ASP, at high ionic strength, precipitation became apparent above 50°C (Fig. 6A). The decrease in solubility became steep above 60°C and precipitation was complete after heating ASP at 75°C, at which temperature 95 % of protein had precipitated. Decreasing the ionic strength had virtually no effect on the precipitation curve of ASP (Fig.6).

The solubility curve of PIP at high ionic strength showed a steep part between 50°C and 60°C, where almost all of the protein became insoluble (Fig. 6A). At low ionic strength the curve became much less steep and about 35 % of the protein remained in solution even after

heating at 100°C. Both curves are similar to the results reported by Ralet and Guéguen (Ralet and Guéguen, 2000) for a comparable protein preparation.

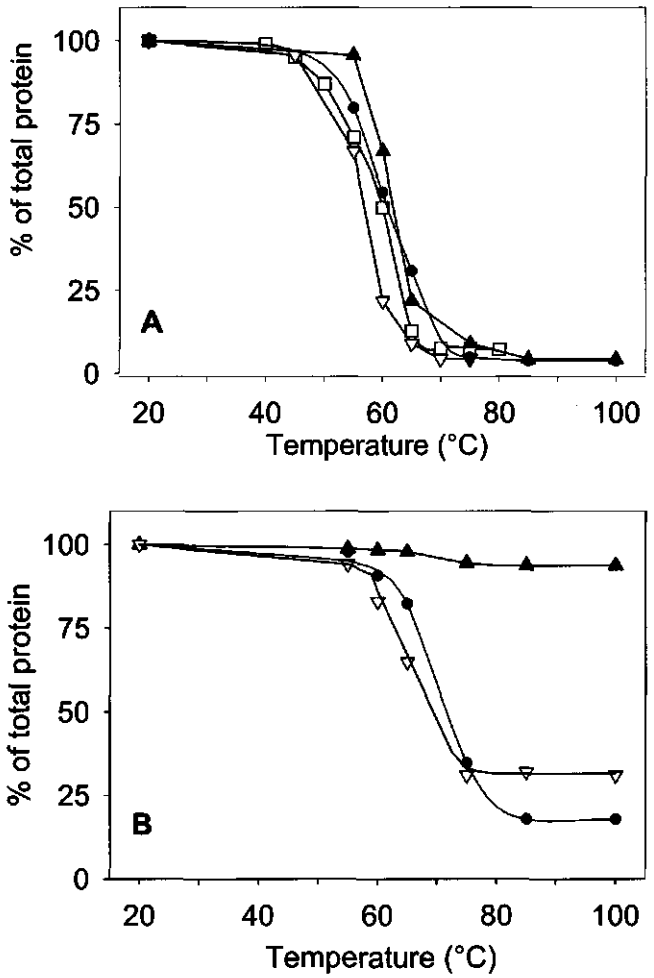

Figure 6: A: Solubility of PFJ (□), ASP (●), PIP (▽) and patatin (▲) as a function of heat treatment temperature (I = 200 mM) B:: Solubility of ASP (●), PIP (▽) and patatin (▲) as a function of heat treatment temperature (I = 15 mM)

The strongest effect of ionic strength on protein solubility after heating was seen with patatin. At high ionic strength the solubility curve of patatin (Fig. 6A) showed a steep decline between 60°C and 65°C and almost complete precipitation at 85°C, whereas at low ionic strength (Fig. 6B) only a small part of the protein became insoluble above 60°C. These results are similar to those reported by Ralet and Guéguen (Ralet and Guéguen, 2000) for a patatin-rich preparation.

Protease inhibitor activity as a function of heating temperature

Inactivation data of protease inhibitors are also important for food applications. Most of the potato protease inhibitors have molecular weights between 20 and 25 kDa (Pouvreau et al., 2001) and are present in PIP. The activity of these inhibitors in PIP was monitored as a function of heating temperature.

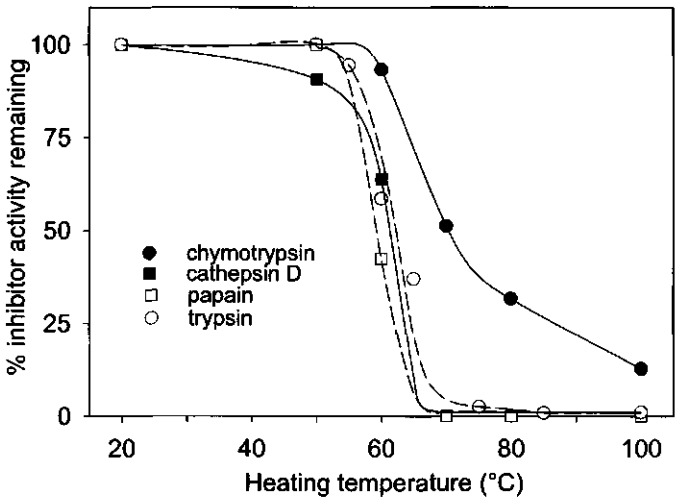

Figure 7: Protease inhibitor activities of PIP as a function of heating temperature (I = 15 mM)

As can be seen from Figure 7 most of the inhibitor activity was lost between 55°C and 70°C. The heating temperatures at which 50 % of the original activity was lost (T_{50}) were: 60°C for the papain inhibition, 63°C for the cathepsin D and trypsin inhibition and about 70°C for the chymotrypsin inhibition.

When the curves in Figure 7 are compared to the solubility curve of PIP in Figure 6A it can be seen that the activity of all inhibitors, except those inhibiting chymotrypsin, decreased at lower heating temperatures than protein solubility did. This would mean that most of the inhibitors became at least partly denatured before they precipitated.

The chymotrypsin inhibiting activity showed a different profile than the other inhibiting activities. The chymotrypsin inhibiting activity followed the solubility curve of PIP up to 75°C, where precipitation was complete. Hence, only at temperatures between 75°C and 100°C, at which point still 15 % of the original chymotrypsin inhibiting activity remained, the chymotrypsin inhibitor activity is solely decreased by heat denaturation and not by co-precipitation of active inhibitor.

DISCUSSION

Properties of patatin and PAT-5

The DSC-profiles of patatin showed one asymmetric peak. This asymmetry can be caused by several events: **1.** Dissociation of multimeric protein during or after denaturation **2.** Non

equilibrium denaturation 3. Multiple overlapping transitions. These possibilities are discussed in the following sections.

Patatin is known to be a dimeric protein at neutral pH and ambient temperature (Racusen and Weller, 1984). If dissociation of this dimer takes place during denaturation, the denaturation temperature (T_d) should rise with increasing protein concentration (Privalov and Potekhin, 1986;Sturtevant, 1987;Conejero-Lara and Mateo, 1996;Li et al., 1998). Variation of the patatin concentration with a factor of 10 did, however, only induce variations in T_d of 0.2°C (results not shown), which is within the accuracy limits of the instrument used. Moreover, using high performance size-exclusion chromatography at elevated temperatures (30 – 80°C) the presence of the monomeric form could not be detected (results not shown). The possible influence of kinetic effects on the shape and T_d of the peak was tested by applying different scan rates (10 to 90 K/hour). No differences were observed, hence the protein must be in equilibrium during the DSC experiments.

In order to obtain thermodynamic data from the thermal unfolding curve of patatin the latter was described using the model given by Van Mierlo et al (Van Mierlo et al., 1998), based on thermodynamic equations (Becktel and Schellman, 1987;Pace et al., 1989). The values of the van't Hoff enthalpy and T_d obtained from the CD-unfolding curve are shown in Table 2 together with the thermodynamic parameters obtained from the DSC data. Also, the ratios of the van't Hoff enthalpy (ΔH_{vH}) and the calorimetric enthalpy (ΔH_{cal}) are given. For a 2-state unfolding of a dimeric protein without dissociation the ratio of ΔH_{vH} and ΔH_{cal} should be approximately 0.5 when ΔH_{cal} is calculated on the basis of the concentration of monomer (Sturtevant, 1987;Makhatadze, 1998). As can be seen from Table 2 the ratio obtained for patatin is close to 1, which would indicate that patatin unfolds in the monomeric state or that the monomers unfold independently (Burova et al., 1999). The patatin monomers must therefore, either remain in some way associated during heating or immediately aggregate upon unfolding, since no change in apparent molecular weight was observed during HP-SEC up to 80°C.

Table 2: Thermodynamic data from fits of the CD thermal unfolding curve (222 nm) and DSC profile of patatin (pH 7)

	CD (222 nm)	**DSC**
T_m (°C) ± SD	58.8 ± 0.1	59.4 ± 0.2
ΔH_{cal} (kJ/mole) ± SD		510 ± 12
ΔH_{vH} (kJ/mole) ± SD	476 ± 6 [1]	529 ± 16 [2]
ΔH_{cal} / ΔH_{vH}	1.07	0.97

[1] Calculated using ΔH_{cal} from DSC data and ΔH_{vH} from CD data

[2] Calculated using ΔH_{cal} from DSC data and ΔH_{vH} from DSC data using: $\Delta H_{vH} = 4RT_m^2C_p/\Delta H_{cal}$

The asymmetry of the transition may be caused by sharpening of the peak at the high temperature side, since the low temperature side could be well fit a single 2-state transition with the parameters in Table 2 (results not shown). This exothermic sharpening is not uncommon in DSC-scans (Privalov and Potekhin, 1986) and is known to be associated with exothermic irreversible processes such as aggregation, de-amidation, proline isomerization, sulfhydryl oxidation, and so forth (Johnson et al., 1992).

The solubility of patatin at high ionic strength diminishes strongly near pH 3.5. This can be explained by realizing that patatin unfolds at pH<5 (Pots et al., 1998b). Increasing the ionic strength in the pH range where patatin is unfolded would decrease the thickness of the electric double layer around the proteins and thereby reduce the electrostatic repulsion between the positively charged proteins and favor association between the proteins via specific electrostatic interactions. At even lower pH these specific electrostatic interactions between the proteins are not possible any more because all the negative charges on the protein become protonated and charge repulsion prevails resulting in an increase in protein solubility (Fig. 5A). The same phenomenon is observed when thermally denatured whey proteins precipitate at their isoelectric pH, while in the native form they are soluble at that pH (Zhu and Damodaran, 1994).

PAT-5 was prepared to study the effects of precipitation at its isoelectric pH on patatin, an obvious step in industrial processes. Precipitation at pH 5 and resolubilization at pH 7 caused marked changes in the tertiary structure of patatin (Fig. 2). These changes were unexpected, since no changes in the far-UV CD spectrum were observed (Fig 1A). Also the lipid acyl hydrolase (LAH) activity (1.84 μmol/mg·min) was not significantly changed after precipitation at pH 5. A similar decrease in ellipticity around 260 nm at acidic pH was observed for α-chymotrypsinogen A (Kahn et al., 2000).

Properties of PIP

Most of the protease inhibitors in soluble potato protein can be inactivated by heat treatment. The protease inhibitors on average have a higher T_d and twice the calorimetric enthalpy of unfolding per unit mass than patatin. The CD and DSC results of PIP (Fig.3, Fig.4) showed that an increase of the ionic strength from 15 to 200 mM by addition of NaCl results in an increase in denaturation temperature. This increase in denaturation temperature is not uncommon (Radek and Castellino, 1989;Lim et al., 1994;Folawiyo and Owusu Apenten, 1996;Folawiyo and Owusu Apenten, 1997) and is usually ascribed to non-specific salt effects arising from the screening of protein destabilizing electrostatic repulsions in the structure of the native protein (Lim et al., 1994;Folawiyo and Owusu Apenten, 1996).

The T_{50} values of the different classes of protease inhibitors are in good agreement with the transition temperatures found for PIP by CD and DSC measurements (Figures 3, 4 and 7). Little is presently known about the thermal stability of potato proteinase inhibitors. Huang et al. (Huang et al., 1981) found that about 10 % of the chymotrypsin inhibiting still remained after cooking of potatoes, which agrees well with our results (Fig. 7). Our results indicate that for the application of potato proteins a heat treatment at 70°C would suffice to remove most of the protease inhibitor activity (Fig 4, Fig. 7). This treatment should be performed at the

lowest possible ionic strength in order to keep the solubility of the proteins as high as possible. An additional reason to use a low ionic strength is that a high ionic strength may stabilize the protease inhibitors in PIP, as higher denaturation temperatures were observed with CD and DSC of PIP when the ionic strength was increased (Fig.3, Fig.4).

Properties of ASP and PFJ

Potato proteins in PFJ were fractionated in order to study the properties of these fractions and to obtain better understanding of the behavior of these proteins in PFJ. ASP was assumed to be characteristic for the total undenatured potato protein. The relative patatin content of ASP is higher than that of PFJ (Table 1), which is also reflected in its lower total Δh_{cal}. The DSC results of ASP (Fig. 4) showed that some of the potato proteins show an increase in denaturation temperature when the ionic strength is increased from 15 to 200 mM by addition of NaCl.

As was shown in Figure 5 most potato proteins have a high solubility only at neutral or strongly acidic pH. The solubility of ASP at mildly acidic pH is governed by a large effect of ionic strength. An increase in ionic strength from 15 mM to 200 mM leads to a strong decrease in the protein solubility of ASP around pH 3.5. This can be explained by the presence of a relatively large amount of patatin. The same effects of ionic strength are observed when potato proteins are heat-denatured. Increasing the ionic strength leads to more extensive aggregation and precipitation. At low ionic strength precipitation takes place at temperatures above the denaturation temperature, where most of the proteins are already unfolded, whilst at high ionic strength only a small fraction of the proteins needs to be unfolded before precipitation takes place (Fig. 6).

In conclusion, this study showed that potato proteins unfold between 55°C and 75°C and that increasing the ionic strength generally causes an increase in denaturation temperature. It was also concluded that the dimeric protein patatin either unfolds in its monomeric state or that its monomers are loosely associated causing them to unfold independently. Thermal unfolding of the protease inhibitors is correlated with a decrease in protease inhibitor activities and results in an ionic strength dependent loss of protein solubility. Potato proteins were shown to be soluble at neutral and strongly acidic pH. The tertiary structure of patatin is severely perturbed by precipitation at pH 5. At mildly acidic pH the overall potato protein solubility is strongly dependent on ionic strength and the presence of unfolded patatin.

ACKNOWLEDGEMENT

Potatoes were kindly provided by AVEBE B.A. (Foxhol, The Netherlands). This research was supported by the Ministry of Economic Affairs through the programme IOP-Industrial Proteins and by AVEBE B.A.

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Chapter 4

The effects of ethanol on structure and solubility of potato proteins and the effects of its presence during the preparation of a protein isolate

ABSTRACT

In this study a protein isolate with a high solubility at neutral pH was prepared from industrial potato fruit juice by precipitation at pH 5 in the presence of ethanol. The effects of ethanol itself and the effects of its presence during precipitation on the properties of various potato protein fractions were examined. The presence of ethanol significantly reduced the denaturation temperature of potato proteins, indicating that the preparation of this potato protein isolate should be performed at low temperature in order to retain a high solubility. In the presence of ethanol the thermal unfolding of the tertiary and the secondary structure of patatin were shown to be almost completely decoupled. Even at 4°C precipitation of potato proteins in the presence of ethanol induced significant conformational changes. These changes did, however, only result in minor changes in the solubility of the potato protein fractions as a function of pH and heat-treatment temperature.

INTRODUCTION

Despite their high nutritional quality (Kapoor et al., 1975;Knorr, 1978) and possible functional properties, potato proteins are presently not used in food applications. This is mainly because their recovery by precipitation from industrial potato fruit juice (PFJ), a byproduct from industrial starch manufacture, results in poorly soluble protein products (Meister and Thompson, 1976;Knorr et al., 1977;Knorr, 1980;Knorr, 1982; Chapter 2), which hampers potential food applications (Kinsella, 1976). Recent research at our laboratory (Chapter 2) revealed that the use of organic solvents combined with a moderate lowering of pH resulted in potato protein precipitates with good solubility characteristics at neutral pH.

Although the use of organic solvents for protein precipitation is not uncommon (Cohn et al., 1946;Cohn et al., 1950) the structural properties of the proteins exposed to organic solvents may be severely affected (Lustig and Fink, 1992;Srinivasulu and Rao, 1995;Bakhuni, 1998;Grinberg et al., 1998). Unfolding of proteins due to ethanol treatment can also influence the solubility characteristics and the functional properties of proteins. These solubility characteristics are important for determining the conditions at which proteins can be applied in food systems. There is, however, no detailed information on the effects of ethanol on potato proteins.

Therefore, the effects of ethanol on the structure and structural stability of potato proteins were studied. Also the solubility characteristics of a potato protein isolate obtained using ethanol (PPI), were studied as a function of pH and temperature. We will attempt to link these effects to changes in structure and solubility of purified potato protein fractions.

MATERIALS AND METHODS

Preparation of protein fractions

1. Potato fruit juice (PFJ)

Potatoes (cv. *Elkana*) were washed thoroughly with water and cut into large pieces (max. 8 x 2.5 cm) which were immediately dipped in a 20 mg/ml solution of sodium bisulfite to prevent enzymatic browning. The potato pieces were ground in a domestic type juice extractor (AEG). The resulting turbid juice was allowed to settle for 15 min. Next, the liquid was decanted and centrifuged (15 min, 19000 × *g*, 10°C) and the supernatant filtered through a paper filter (Schleicher & Schuell, ref.no. 311653). The resulting clear yellowish filtrate, which has a pH of 5.7 - 6.0, is known to be similar to industrial PFJ (AVEBE B.A., Foxhol, The Netherlands) and is further denoted as PFJ. PFJ, on average, contained 3.59 ± 0.09 (SD) mg of nitrogen per ml. Of this nitrogen 60 ± 5.6 (SD) % could be precipitated with 12.5 % (w/v) TCA and was therefore assumed to be of protein origin, which leads to an average protein (N x 6.25) concentration of 13.4 ± 0.9 (SD) mg protein per ml of PFJ (Chapter 2).

2. Potato protein isolate (PPI)

Potato protein isolate (PPI) was prepared by slowly adding 95 % (v/v) ethanol (-20°C) to stirred PFJ (4°C) to a final concentration of 20 % (v/v) and adjusting the apparent pH of the clear mixture to 5.0 by addition of 0.5 M H_2SO_4. After 1 hour at 4°C the suspension was centrifuged (30 min, 19000 × *g*, 4°C) and the precipitate was washed twice with a 0.1 M

ammonium acetate buffer (pH 5) containing 20 % (v/v) ethanol. Subsequently, the precipitate was suspended in water and the suspension was adjusted to pH 7 using 0.1 M NaOH and then freeze-dried and stored at -20°C.

3. Patatin

Patatin was purified by applying PFJ, diluted ten times with water and adjusted to pH 8, on a Source 15 Q column (10 x 15 cm) (Amersham Pharmacia Biotech AB, Uppsala, Sweden) equilibrated with a 25 mM Tris-HCl buffer (pH 8) containing 0.5 g/l $NaHSO_3$. After washing out the unbound compounds the bound fraction was eluted with the same buffer containing 0.35 M NaCl. Further purification of the bound fraction was established by gel filtration on a Superdex 75 column (63 x 10 cm) (Amersham Pharmacia Biotech AB, Uppsala, Sweden) eluted with a 30 mM sodium phosphate buffer (pH 7) containing 0.5 g/l $NaHSO_3$ at a linear flow rate of 30 cm/h. The first peak, as observed from the absorbance at 280 nm, containing patatin of > 95 % purity (SDS-PAGE), was collected and concentrated 10 times using Xampler UFP-3-C cross-flow hollow fiber laboratory cartridges with a molecular weight cut-off of 10 kDa (A/G Technology Corp., Needham, USA) at 4°C. The patatin was subsequently diafiltered with 5 volumes of a 9 mM sodium phosphate buffer (pH 7) and stored at –20°C.
Ethanol precipitated patatin (PAT-5E) was prepared by slowly adding 95 % (v/v) ethanol (-20°C) to a stirred patatin solution (5 mg/ml, 4°C) to a final concentration of 20 % (v/v). The apparent pH of the clear mixture was adjusted to 5.0 by addition of small volumes of 0.5 M H_2SO_4. After 1 hour at 4°C the suspension was centrifuged (30 min 19000 × *g*, 4°C) and the precipitate was suspended in water, adjusted to pH 7 using 0.1 M NaOH, extensively dialyzed against 9 mM sodium phosphate buffer (pH 7) and then stored at -20°C.

4. Protease inhibitor pool (PIP)

Protease inhibitor pool (PIP) protein was prepared by gelfiltration of PFJ on a Superdex 75 column (63 x 10 cm) (Amersham Pharmacia Biotech AB, Uppsala, Sweden) eluted with a 30 mM sodium phosphate buffer (pH 7) containing 0.5 g/l $NaHSO_3$ at a linear flow rate of 30 cm/h. The fractions making up the second peak eluting, as observed from the absorbance at 280 nm, were collected, combined and concentrated 10 times using Xampler UFP-3-C cross-flow hollow fiber laboratory cartridges with a molecular weight cut-off of 5 kDa (A/G Technology Corp., Needham, USA) at 4°C. The concentrated PIP, which contained mainly proteins with a Mw 15-25 kDa (SDS-PAGE), was subsequently diafiltered with distilled water until no further decrease of the conductivity of the permeate could be observed. The concentrated PIP was subsequently freeze-dried and stored at -20°C.
PIP-5E was prepared by slowly adding 95 % (v/v) ethanol (-20°C) to a stirred PIP solution (6 mg/ml, 4°C) in 9 mM sodium phosphate buffer (pH 7) to a final concentration of 20 % (v/v). The apparent pH of the clear mixture to was adjusted to 5.0 using 0.5 M H_2SO_4. After 1 hour at 4°C the suspension was centrifuged (30 min 19000 × *g*, 4°C) and the precipitate was suspended in distilled water and adjusted to pH 7 using 0.1 M NaOH. Next, the solution was extensively dialyzed against 9 mM sodium phosphate buffer (pH 7) and then stored at -20°C.

Protein composition

Protein composition of PFJ, PPI, PIP and patatin was estimated by electophoresis and subsequent densitometric analysis of Phastgel IEF 3-9 gels, or IEF 2.5-6 for patatin, after separation on a Phast System (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and staining with Coomassie Brilliant Blue. The relative intensity of the separated bands was measured using a Molecular Dynamics 300 computing densitometer (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Circular dichroism(CD) spectroscopy

Sample preparation

CD experiments were performed to investigate the effect of various treatments on the conformation and the thermal stability of patatin and proteins in PIP. The conformation (20°C) and thermal stability of patatin and proteins in PIP were estimated at pH 7, using a 9 mM sodium phosphate buffer, in the presence and absence of 20 % (v/v) ethanol. The conformation and thermal stability of PAT-5E and PIP-5E were also estimated.

Far-UV CD

Far-UV CD spectroscopy was used to monitor changes in the secondary structure of patatin (0.1 mg/ml) and PIP (0.2 mg/ml) caused by different treatments. Far-UV CD spectra (190 – 260 nm) were recorded 10-fold and averaged on a Jasco J-715 spectropolarimeter (Jasco Corp., Japan) at ambient temperature in quartz cells with an optical path length of 1 mm. Far-UV CD spectra were also recorded at various temperatures after heating for 6 min at a specific temperature prior to recording the CD spectrum. The scan speed was 100 nm/min, the data interval 0.2 nm, the band width 1.0 nm, the sensitivity 20 mdeg and the response time 0.125 s. All recorded spectra were corrected by subtraction of the spectrum of a protein free sample.

Changes in the thermal stability of the secondary structure of patatin and PIP after various treatments, were also monitored, for patatin samples by measuring the ellipticity at 222 nm as a function of temperature at a heating rate of 20 K/h. PIP samples were heated at the same rate but in this case the ellipticity was monitored at 228 nm.

Near-UV CD

Near-UV CD was used to monitor changes in the structure of patatin at a tertiary level. Near-UV CD spectra (250-350 nm) were recorded 25 fold and averaged at temperatures in the range from 20°C to 80°C at a heating rate of 20 K/h and heated for 15 min at specific temperatures before spectra were recorded. Samples (4 mg/ml) were tested in a cuvette with an optical path length of 1.0 cm. The scan speed used was 50 nm/min, the data interval 0.5 nm, the bandwidth 1.0 nm, the sensitivity 10 mdeg and the response time was 0.25 sec. Recorded spectra were corrected by subtracting spectra of protein free samples.

The thermal unfolding of patatin in the presence or absence of 20 % (v/v) ethanol was estimated by recording the ellipticity at 258 nm as a function of temperature with a heating rate of 20 K/h.

Differential scanning calorimetry (DSC)

DSC experiments were performed on a VP-DSC MicroCalorimeter (MicroCal Inc., Northampton (MA), USA). Thermograms were recorded from 10°C to 90°C at a heating rate of 20 K/h. DSC experiments were conducted with untreated PFJ (13.5 mg protein/ml). ASP, PIP and patatin were used at concentrations of 10, 6 and 4 mg/ml, respectively. All fractions except PFJ were analyzed in 9 mM sodium phosphate buffer (pH 7) that contained no NaCl ($I \approx 15$ mM) or 185 mM of NaCl ($I = 200$ mM).

Gel filtration chromatography

The apparent molecular weight of patatin at various temperatures samples in the presence of 20 % (v/v) ethanol was estimated using gel filtration chromatography on a thermostatted Superdex 75 HR column (60 x 1 cm) (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Samples of patatin (1 mg/ml) in the presence of ethanol were preheated (10 min) at temperatures of 10°C, 25°C and 35°C. Samples of 1 ml were injected on the column that was equilibrated with a 10 mM sodium phosphate buffer (pH 7) containing 20 % (v/v) ethanol. The column was eluted with the same buffer at a flow-rate of 1 ml/min and the eluate was monitored at 280 nm. The effect of 20 % (v/v) in the eluens on the separation characteristics of the column was tested using ovalbumin (43 kDa; Sigma) as a standard. Samples of ovalbumin (1 mg/ml) showed the same retention time both in the presence and absence of 20 % (v/v) ethanol indicating that the separation characteristics of the column did not change in the presence of ethanol.

Protein solubility

Protein solubility experiments with PFJ were performed with undiluted PFJ. PPI and PIP were solubilized to final concentrations of 10 and 6 mg/ml, respectively, in a 9 mM sodium phosphate buffer (pH 7) that contained no NaCl ($I \approx 15$ mM) or 185 mM of NaCl ($I = 200$ mM). Solutions of patatin were adjusted to a final concentration of 4 mg/ml in the same buffers as mentioned above.

1. Effect of pH

Protein precipitation experiments as a function of pH were performed in duplicate by adjusting the pH of stirred samples to the desired values with 0.5 M H_2SO_4 in 15 ml Kimax tubes at room temperature. The acidified samples were left to settle for 1 hour at room temperature. The settled samples were centrifuged for at least 15 min ($3600 \times g$, 20°C) until clear supernatants were obtained. Supernatants were analyzed in duplicate for nitrogen content using the micro-Kjeldahl method (AOAC, 1980) when PFJ samples were used. When other protein preparations than PFJ were used, supernatants were analyzed for protein content using the method of Bradford (Bradford, 1976) with bovine serum albumin (Sigma A-7511)

(Lot 92H93131) as a standard. In the case of treated PFJ samples soluble protein nitrogen was calculated as protein nitrogen in PFJ minus precipitated nitrogen of the sample and expressed as proportion of 12.5 % (w/v) TCA-precipitable nitrogen present in PFJ. Precipitated nitrogen was assumed to be of protein origin. Soluble protein in solutions of PPI, PIP and patatin is given as the proportion of the protein originally present in solution at pH 7 and was corrected for the amount of liquid added during acidification.

2. Effect of heat treatment

Protein precipitation as a function of heat-treatment temperature was determined by heating 1.5 ml samples (pH 7; $I \approx 15$ mM or 200 mM) for 15 min in a closed Kimax tube in a thermostatted waterbath. After heating the samples were immediately cooled on ice. After a cooling time of 15 min the heat-treated samples were centrifuged for at least 15 min ($3600 \times g$, 20°C) until clear supernatants were obtained. Protein analysis of the supernatants was performed as described above.

Protease inhibitor activity

Trypsin from bovine pancreas (T-0134) (lot 100H0685), Type II α-chymotrypsin from bovine pancreas (C-4129) (lot 58H7001), papain from papaya latex (P-9886) (lot 66H7130), carboxypeptidase A from bovine pancreas (C-0261) (lot 116H8020) and cathepsin D from bovine spleen (C3138) (lot 103H8005) were obtained from Sigma Chemical Co. Trypsin inhibitor activity was estimated by the Kakade method as modified by Smith et al. (Smith et al., 1980) with 0.36 mM benzoyl-DL-Arg-*p*-nitroanilide (Merck) as a chromogenic substrate. Chymotrypsin inhibiting activity was estimated according to Geiger (Geiger, 1984) with 0.88 mM N-succinyl-L-Phe-*p*-nitroanilide (Sigma) as a substrate. Inhibition of papain was estimated by the method of Mole and Horton (Mole and Horton, 1973) with 2.53 mM of benzoyl-L-Arg-*p*-nitroanilide (Merck) as a substrate. Cathepsin D inhibiting activity was estimated according to Van Jaarsveld et al (Van Jaarsveld et al., 1997) with 0.67 % (w/v) of acid denatured hemoglobin as a substrate. Carboxypeptidase A inhibiting activity was estimated as described by Riordan and Holmquist (Riordan and Holmquist, 1984) with 0.1 mM of N-(furanacryloyl)-L-Phe-L-Phe (Bachem) as a substrate.

For all protease inhibition assays the degree of inhibition was measured as a function of protein concentration. Subsequent inhibition measurements were conducted in the concentration range where inhibition was linear with protein concentration. Residual inhibitor activity after heat treatment was measured in supernatants of heated PIP solutions and expressed as % activity remaining per volume of supernatant compared to the unheated solution.

RESULTS

In the present study we examined the effects of ethanol on the conformation and the conformational stability of potato proteins and we attempted to link these results to possible changes in the solubility characteristics of these proteins. The results from these experiments can provide more information about the effect of ethanol treatment on proteins and they also serve as a guide on how to modulate the preparation of potato protein isolate (PPI) in the presence of organic solvents.

Protein composition

PPI is a protein isolate that contains about 85 % of the total protein originally present in industrial potato fruit juice (PFJ) (Chapter 2). Table 1 shows the relative protein distributions of PFJ and PPI on the basis of isoelectric pH. The data in Table 1 are estimated from the optical density of protein bands on IEF gels within an arbitrary pI-range and expressed as proportion of the total density of the protein bands within each lane. It can be seen that PFJ and PPI showed small differences in relative protein composition. PFJ contained 11 protein bands with a pI < 5.2, representing different patatin isoforms and constituting about 38 % of the PFJ protein (Pouvreau et al., 2001). PPI contains, compared to PFJ, less patatin and a higher proportion of proteins with a high isoelectric pH (pI > 7). The presence of the latter suggests that the mechanism of precipitation in the presence of organic solvents is not, or only partly governed by the net charge on the proteins.

Table 1: Composition of various potato protein fractions on the basis of pI

Sample	pI < 5.2	5.2 < pI < 6	6 < pI < 7	7 < pI < 8	pI > 8
PFJ	38 %[a] (11)[b]	11 % (4)	15 % (5)	20 % (4)	17 % (2)
PPI	33 % (11)	12 % (3)	12 % (4)	22 % (6)	21 % (4)

[a]: expressed as proportion of the totalized density of the protein bands within one lane

[b]: the number between parentheses presents the number of protein bands detected

Structural changes in potato proteins

Changes in the secondary structure of patatin

Both the presence of 20 % (v/v) ethanol in the patatin solution as well as its presence during precipitation of patatin at pH 5 (PAT-5E) did not substantially affect the far-UV CD spectrum of patatin (Fig. 1A). Analysis of the spectra for their secondary structure content according to De Jongh et al. (De Jongh et al., 1994) revealed that treatment of patatin with ethanol would, however, induce a small increase in the α-helix content with a concomitant loss of β-stranded structures (Table 2). Repeated preparation of samples and subsequent CD-analysis confirmed this observation.

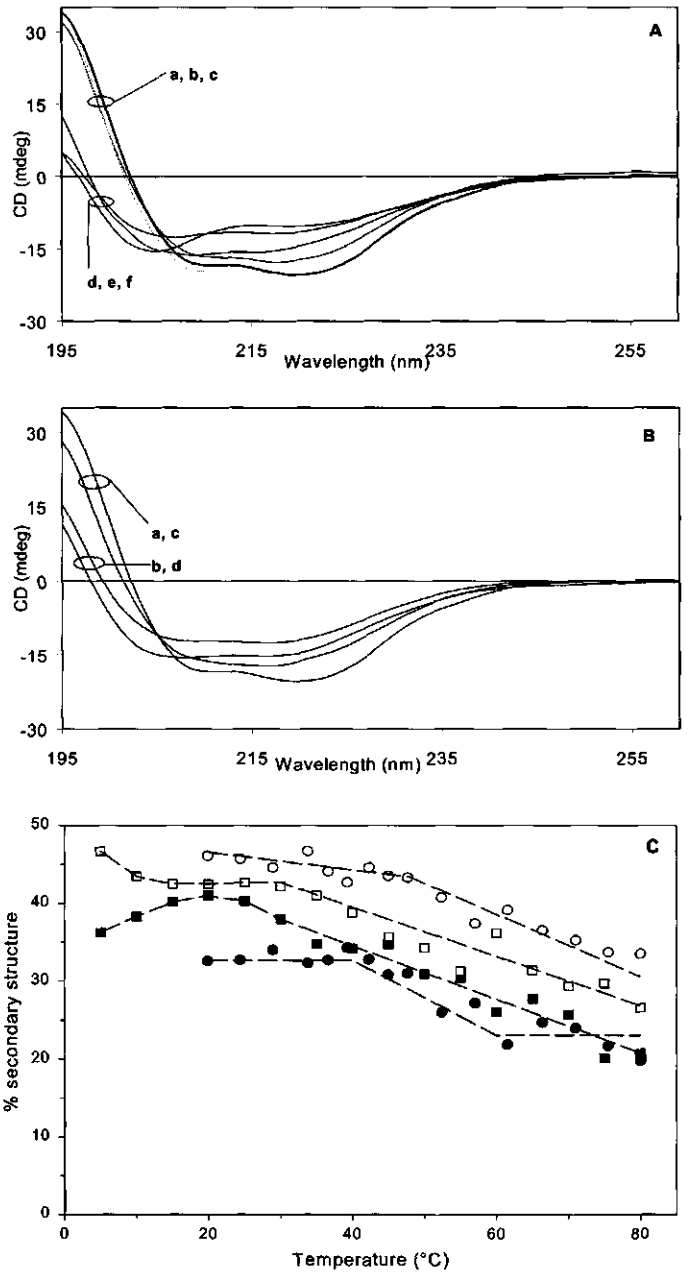

Figure 1: **A**: Far-UV CD spectra of patatin: **a:** patatin (pH 7) at 20°C **b:** patatin (20% (v/v) ethanol, pH 7) at 20°C **c:** PAT-5E (pH 7) at 20°C **d:** **a** at 80°C **e:** **b** at 80°C **f:** **c** at 80°C **B**: Far-UV CD spectra of patatin **a:** patatin (pH 7) at 20°C **b:** **a** heated at 80°C and cooled to 20°C **c:** patatin (pH 7) heated to 80°C in the presence of 20 % (v/v) ethanol and cooled to 20°C **d:** PAT-5E (pH 7) heated at 80°C and cooled to 20°C. **C**: Secondary structure content of patatin (pH 7): α-helix (●), β-strand (○) and patatin in 20 % ethanol (pH 7): α-helix (■), β-strand (□), as a function of temperature as obtained from analysis of far-UV CD spectra

This effect of ethanol of inducing helix formation in proteins has been observed by others (e.g. (Clark and Smith, 1997;Arunkumar et al., 1997;Karpenko et al., 1997) and is supposed to be driven by shielding of the unfavorable contacts of polar residues with the apolar solvent. These polar residues are shielded from the solvent by the formation of internal hydrogen bonds through formation of helices (Herskovits et al., 1970;Arakawa and Godette, 1985).
Heating patatin at 80°C resulted in a decrease in the secondary structure content (Table 2, Chapter 3). Similar results were obtained when PAT-5E was heated (Fig. 1A, Table 2). In the presence of ethanol, however, patatin had a different conformation at 80°C with a higher helical content and less β-strands (Fig. 1A, Table 2).

Table 2: Estimated secondary structure content of patatin samples at different temperatures

Sample	Temperature	α-helix	β-strand	aperiodic
patatin (pH 7)	20°C	34 %	48 %	14 %
	80°C	15 %	36 %	46 %
	20°C (after 80°C)	30 %	26 %	44 %
patatin (pH 7) 20 % (v/v) ethanol	20°C	41 %	42 %	13 %
	80°C	21 %	27 %	44 %
	20°C (after 80°C)	42 %	43 %	15 %
PAT-5E	20°C	40 %	42 %	18 %
	80°C	14 %	39 %	43 %
	20°C (after 80°C)	26 %	33 %	42 %

When samples of patatin and PAT-5E were heated to 80°C and subsequently cooled to 20°C, their temperature induced unfolding proved to be only partly reversible (Fig. 1B, Table 2). In the presence of 20 % (v/v) ethanol the thermal unfolding of patatin was, however, fully reversible upon cooling to 20°C.Figure 1C shows the secondary structure content of patatin in the absence and presence of 20 % (v/v) ethanol as a function of temperature. In the absence of ethanol the α-helix content in patatin showed a transition between 40°C and 60°C. The β-strand content showed a gradual decrease. These results are in agreement with those reported by Pots et al. (Pots et al., 1998). In the presence of ethanol the α-helix content of patatin showed an initial rise up to 15°C, followed by a gradual decrease up to 80°C. The β-strand content showed a course similar to the helical content (Fig. 1C). The presence of ethanol seemed to decrease the thermal stability of the secondary structure of patatin, as its secondary structure content started to decrease at about 25°C, instead of 40°C in the absence of ethanol.

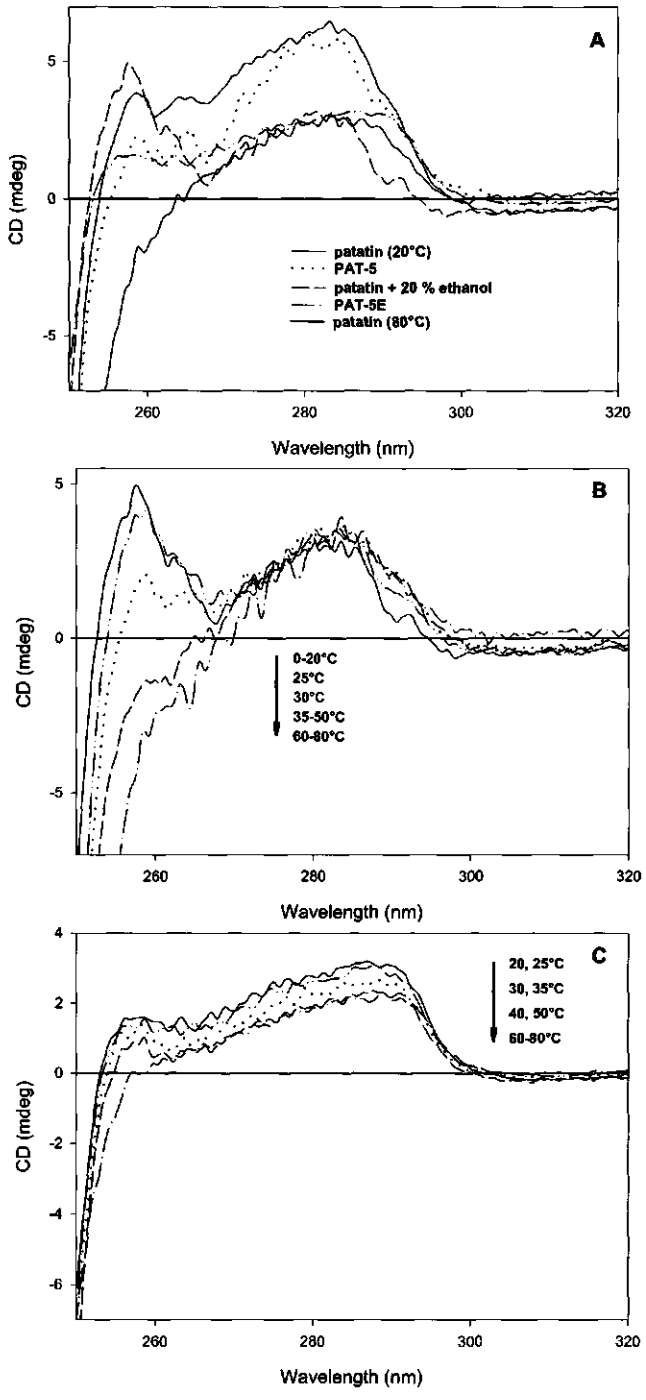

Figure 2: A: Near-UV CD spectra of patatin after different treatments B: Near UV CD spectra of patatin in the presence of 20 % (v/v) ethanol at various temperatures C: Near UV CD spectra of PAT-5E (pH 7) at various temperatures

Changes in the tertiary folding of patatin

Near-UV CD spectra provide an indication of protein tertiary structure and dynamics. It depends critically on the local environment and closeness of packing of aromatic amino acid residues (Pain, 1996).

Figure 2A shows the near-UV CD spectra of patatin after various treatments. The spectrum at 20°C shows a broad maximum around 283 nm, due to tyrosine and tryptophan contributions, and a sharp maximum around 258 nm presumably attributed to phenylalanine contributions (Woody and Dunker, 1996).

Addition of ethanol to patatin caused a small increase in the intensity around 258 nm and a shift of the maximum towards lower wavelengths (Fig. 2A). It also caused a large decrease in the intensity at 283 nm, indicating a substantial increase of the mobility of the tyrosine and tryptophan residues, and an almost complete loss of the ellipticity between 290 nm and 300 nm. The latter was also observed for myoglobin in the presence of methanol (Ravindra Babu and Douglas, 2000) and for lysozyme in the presence of trifluoroethanol (Bakhuni, 1998).

The spectrum of PAT-5E showed a combination of the effects of precipitation at pH 5 and contact with ethanol, which are characterized by a decrease in ellipticity around 258 nm (Chapter 3) and a decrease in ellipticty around 283 nm, respectively. The spectrum of patatin at 80°C was included to illustrate the maximal degree of thermal unfolding of the tertiary structure of patatin.

Figure 2B shows that in the presence of ethanol the major changes in the intensity near 258 nm occur between 25°C and 35°C. In the absence of ethanol, on the other hand, the major changes in the intensity at 258 nm took place between 50°C and 60°C (see Chapter 3). The near-UV CD spectra of PAT-5E at various temperatures in Figure 2C show that, although the intensities at 258 and 283 nm had already substantially decreased compared to untreated patatin, a further decrease with temperature was still observed. The major changes at 283 nm occurred between 25°C and 40°C, while at 258 nm the intensity seemed to decrease over the whole temperature range.

CD-temperature traces

Figure 3A shows the thermal unfolding of patatin (pH 7) in the absence and presence of ethanol and of PAT-5E as monitored by the ellipticity at 222 nm, which is known to be a combination of contributions of α-helical and β-stranded structures (Hennessy Jr. and Johnson Jr., 1981). The unfolding curve of PAT-5E showed a transition with a midpoint of 58°C and was almost identical to that of patatin (Fig 3A, Chapter 3). In the presence of ethanol the absolute ellipticity of patatin at 222 nm showed a gradual decrease that was most pronounced between 58°C and 100°C (Fig. 3A). No clear transition was observed and the slope of the unfolding curve above 58°C was substantially smaller than the one observed without ethanol (Fig. 3A), presumably indicating a non-cooperative unfolding.

The results described above, together with those in Figure 1C, indicate that the temperature induced unfolding of the secondary structure of patatin in the presence of ethanol is a non-cooperative process.

Figure 3B shows the unfolding curves of patatin at the conditions mentioned above, but in this case the ellipticity at 258 nm is plotted as a function of temperature. The unfolding curves of patatin and PAT-5E showed a clear transition with a midpoint at about 55°C, although the latter transition was much smaller, presumably due to irreversible changes in its tertiary structure at pH 5 (Chapter 3).

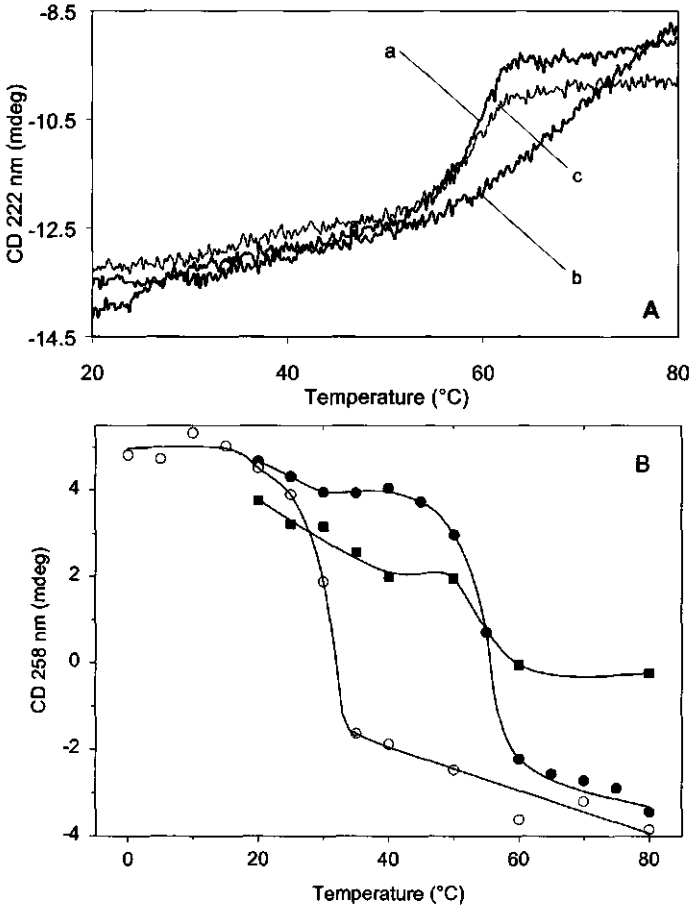

Figure 3: A: Thermal unfolding of patatin as monitored by the CD-signal 222 nm: **a:** patatin (pH 7) **b:** patatin (20% (v/v) ethanol, pH 7) **c:** PAT-5E (pH 7) B: Thermal unfolding of patatin as monitored by the CD-signal 258 nm of patatin (pH 7, ●), patatin (20% (v/v) ethanol, pH 7, ○) and PAT-5E (pH 7, ■)

In the presence of 20 % (v/v) ethanol the unfolding curve of patatin also showed a transition, but now between 15°C and 30°C. These results indicate that in the presence of ethanol the thermal unfolding of the tertiary structure of patatin, in contrast to that of the secondary structure, is still a cooperative process.

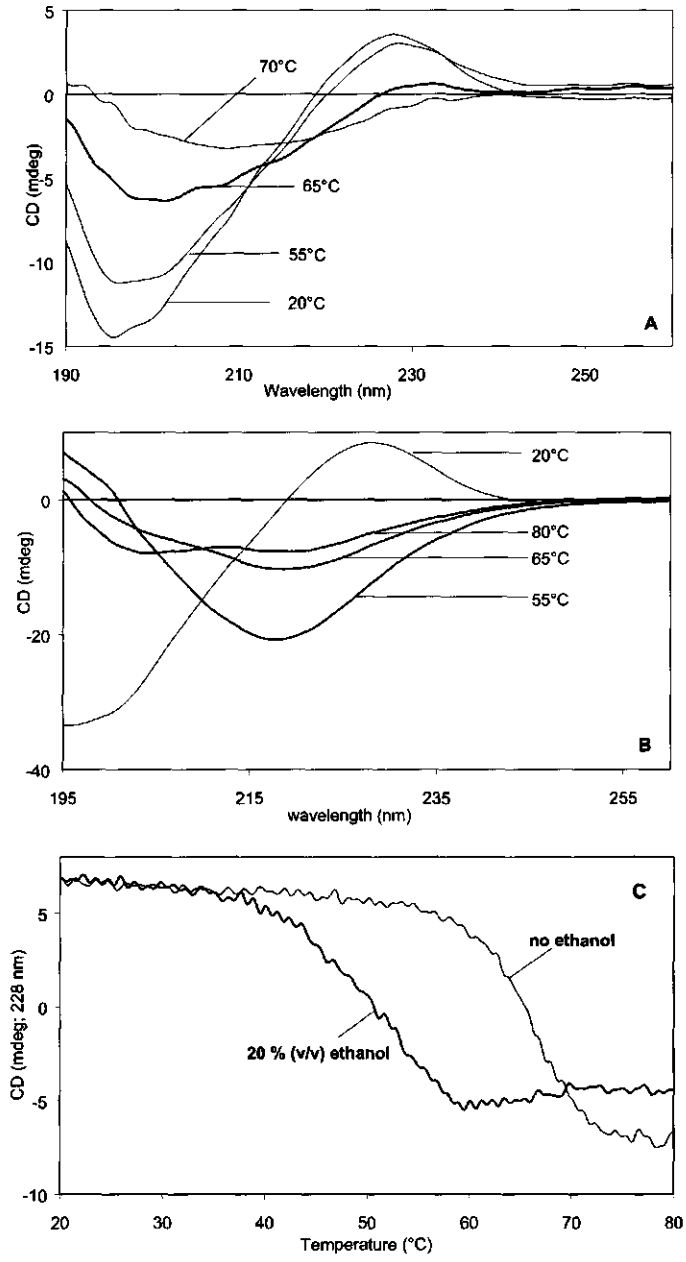

Figure 4: A: Far-UV CD spectra of PIP-5E (pH 7)) at various temperatures B: Far-UV CD spectra of PIP (20 % ethanol, pH 7, I =15 mM) at various temperatures C: Thermal unfolding of PIP as monitored by the CD-signal 228 nm in the presence and absence of 20 % ethanol

Changes in the conformation of PIP proteins

Since PIP is a mixture of proteins, far-UV CD spectroscopy was used only to monitor changes in the conformation of the proteins in PIP without attempting to interpret these as changes in

the content of specific secondary structure elements. The spectrum of PIP was similar to that described by Lindner et al. (Lindner et al., 1980) for a comparable protein mixture. The spectrum had a minimum at 195 nm and a maximum around 228 nm (see Chapter 3).

Figure 4A shows the far-UV CD spectra of PIP (pH 7) after precipitation at pH 5 in the presence of ethanol (PIP-5E) at various temperatures. Ethanol precipitation apparently did not change the spectrum of PIP at 20°C as it was similar to that of untreated PIP (Chapter 3). Also the changes in the spectrum with temperature (Fig. 4A) were similar to those observed for untreated PIP (Chapter 3).

Addition of ethanol did not induce significant changes in the far-UV CD spectrum of PIP at 20°C (Fig. 4B). It did, however, decrease the thermostability of the proteins in PIP, as the spectrum at 55°C was already completely different from that at 20°C, in contrast to that in the absence of ethanol (Fig 4A).

Figure 4C shows the thermal unfolding curves of PIP in the absence and presence of ethanol, measured as the ellipticity at 228 nm. The unfolding curve of PIP-5E is not shown, since it did not differ significantly from that of untreated PIP. The unfolding curve of PIP showed a transition with a midpoint of 66°C. In the presence of ethanol PIP showed a transition between 40°C and 60°C, with a midpoint of 51°C.

Thermal unfolding of potato protein fractions

The excess heat capacity profiles of different potato protein fractions are shown in Figure 5. The DSC-curve of PFJ showed a large and a small peak with transition temperatures of 66°C and 74°C, respectively (Figure 5) with a total calorimetric enthalpy (Δh_{cal}) of 20.5 ± 1.5 J/g.

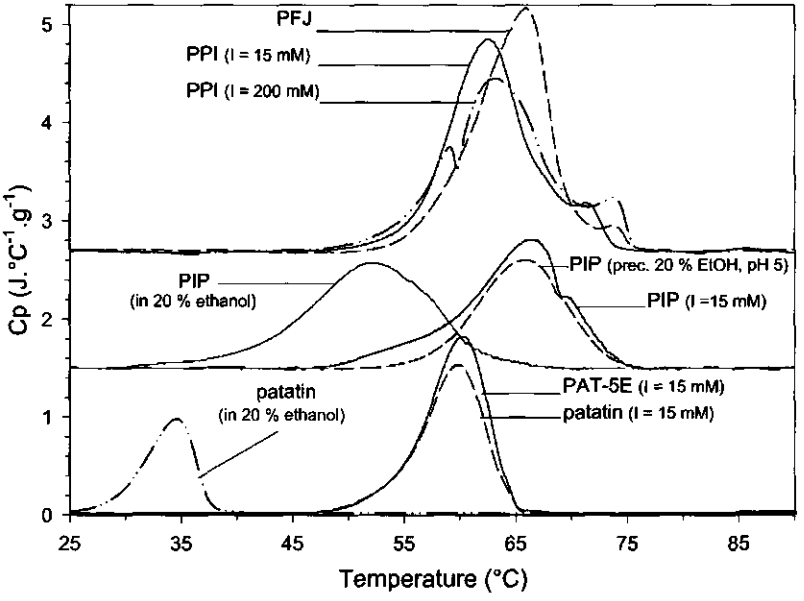

Figure 5: Examples of DSC thermograms of PFJ, PPI, PAT-5E and PIP-5E at pH 7

The DSC thermogram of PPI at low ionic strength showed a large endothermic peak at 62°C and a small peak at 71°C with a total Δh_{cal} of 19.6 ± 1.3 J/g. At high ionic strength the DSC thermogram of PPI showed a much broader transition zone, with peaks at 58°C, 64°C and 74°C (Fig. 5) and a total Δh_{cal} of 18.6 ± 0.5 J/g. The peak at 58°C is probably due to the presence of patatin.

The thermogram of PIP-5E was similar to that of untreated PIP. It showed one major transition at 66°C. The shoulder at 71°C present in untreated PIP had disappeared (Fig. 5) and also the total Δh_{cal} decreased from 25.2 ± 2 J/g to 21.2 ± 1.0 J/g. Both facts could be due to small differences in protein composition as well as to changes in the tertiary structure of the proteins in PIP. The thermogram of PIP in the presence of ethanol showed a broad transition around 52°C, which is in agreement with the far-UV CD data (Fig. 4C). The total Δh_{cal} was reduced in the presence of ethanol from 25.2 ± 2 J/g to 20.8 ± 2 J/g. Precipitation in the presence of ethanol at pH 5 did not induce significant changes in the thermogram of patatin, as can be seen in Figure 5. PAT-5E showed a single asymmetric transition at 59.5°C with Δh_{cal} of 10.8 ± 0.5 J/g (439 kJ per mole monomer), as compared to 12.6 ± 0.1 J/g (510 kJ/mole of monomer) for untreated patatin (Fig. 5). The presence of 20 % (v/v) ethanol caused a shift in the denaturation temperature of patatin from 59.4°C to 32.3°C (Fig. 5) and decreased Δh_{cal} by 50 % to 6.3 ± 0.2 J/g.

Solubility as a function of pH

The term solubility, which is used frequently throughout this paper, should be put between quotes because the authors do realize that the solubility measurements presented here are only an approximation of true solubility measurements, since these should be expressed as amount per unit volume. Instead the proportion of total protein that becomes "insoluble" is used. This property is not well defined because it does not give information about the changes in the solubilities of the proteins of which the saturation concentration is not reached (Chapter 2).

In Figures 6A and 6B the solubility curves of PFJ (200 mM only), PPI, PAT-5E and PIP-5E are shown as a function of pH at ionic strengths of 200 mM and 15 mM, respectively. The experiments with PAT-5E and PIP-5E were performed to examine the effect of the ethanol treatment used for obtaining PPI, on the solubility characteristics of patatin and PIP.

The solubility of potato proteins in PFJ showed a local minimum in their solubility at pH 5 and was lowest at pH 3 (Fig. 6A, Chapter 2). At high ionic strength the pH/solubility profile of PPI was similar to that of PFJ (Fig. 6A). At low ionic strength the solubility of PPI at low pH had strongly decreased; the proteins in PPI became almost insoluble at pH < 5.5 (Fig. 6B). This is surprising since more than 50 % of the mass in PPI is of proteins with an isoelectric pH above 6 (Table 1).

The solubility of PAT-5E at low ionic strength showed a broad minimum around pH 4.5 (Fig. 6B). At high ionic strength PAT-5E still showed a weak minimum around pH 5 but became almost insoluble at pH 3.5 and soluble again at pH < 3 (Fig. 6A). Both solubility profiles were very similar to those obtained for untreated patatin (Chapter 3) and therefore showed that the solubility of patatin was almost unaffected by precipitation in the presence of ethanol. The

solubility of PIP at low ionic strength showed minima around pH 4 and pH 5.5 showing that the solubility of PIP-5E around pH 5.5 had decreased as compared to untreated PIP (Fig.6B, Chapter 3). At high ionic strength the solubility of PIP-5E showed a broader solubility minimum as compared to untreated PIP (Fig. 6A, Chapter 3).

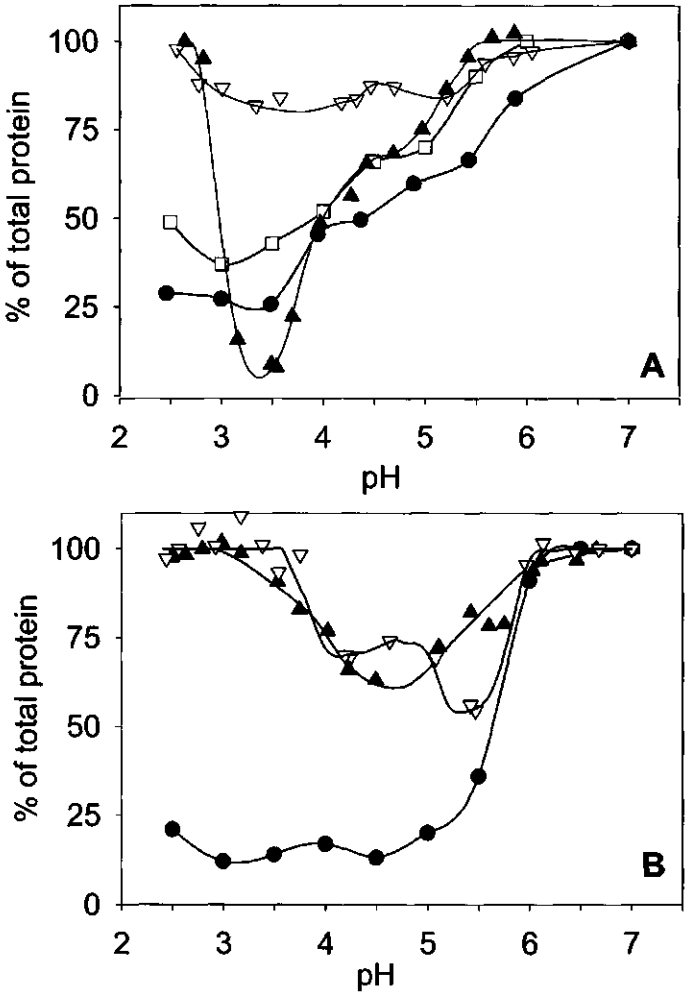

Figure 6: A: Solubility of PFJ (□), PPI (●), PIP-5E (▽) and PAT-5E (▲) as a function of pH (I =200 mM) B: Solubility of PPI (●), PIP-5E (▽) and PAT-5E (▲) as a function of pH (I =15 mM)

Solubility as a function of heat-treatment temperature

The proportions of protein that remained in solution as a function of the heat treatment temperature for PFJ and PPI are shown in Figure 7. Precipitation in PFJ already occurred when it was heated above 40°C and precipitation did not increase further above 70°C (Fig. 7). At high ionic strength (200 mM) the solubility of PPI as a function of heat treatment

temperature appeared very similar to that of PFJ, although precipitation started only after heating above 50°C. After heating at 60°C 50 % of the protein had precipitated, whilst at 70°C precipitation was complete and 10 % of the protein originally present remained in solution (Fig. 7).

At low ionic strength (15 mM) the solubility curve of PPI showed a steeper decline between 60°C and 70° than at high ionic strength. At heat treatment temperatures > 70°C about 20 % of the protein originally present remained soluble (Fig. 7).

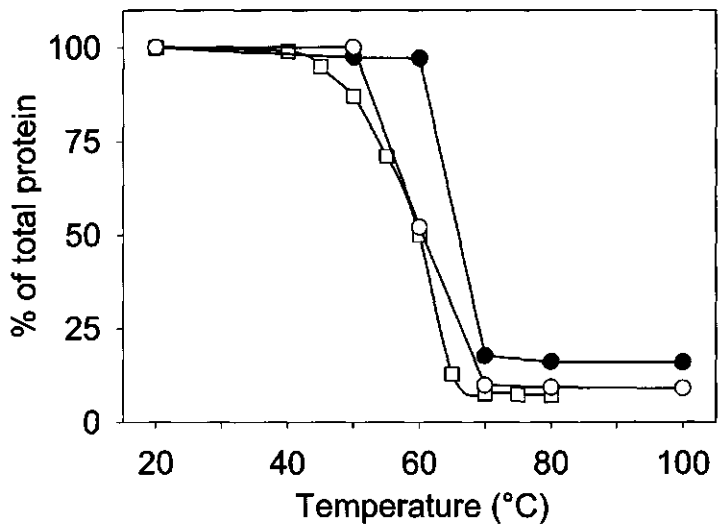

Figure 7: Solubility of PFJ (□) and PPI (I= 15 mM (●), I =200 mM (○)) as a function of heat treatment temperature

Protease inhibitor activity as a function of heat treatment temperature

The various protease inhibiting activities in PPI are shown in Figure 8 as a function of heat treatment temperature. The heating temperature at which 50 % of the original inhibiting activity was lost (T_{50}) was: 60°C for the trypsin and papain inhibitors, 65°C for the cathepsin D inhibitors and 72°C for the chymotrypsin inhibitors. For the carboxypeptidase A inhibitors the T_{50} was not reached at 100°C.

Most of the inhibiting activity was lost after heating at 70°C. Above 70°C about 15 % of original papain inhibiting activity, 15 % of the chymotrypsin inhibiting activity and 60 % of the carboxypeptidase A inhibiting activity remained (Fig. 8). The curves of chymotrypsin and carboxypeptidase A inhibiting activity (Fig. 8) show that these activities are at least partly lost in conjunction with or because of precipitation (Fig. 7), since at temperatures > 70°C, where precipitation increases no further, their activities decrease less steep with temperature.

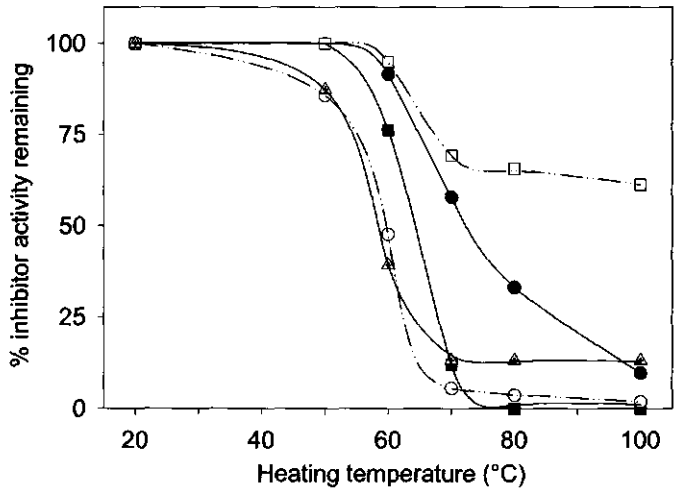

Figure 8: Protease inhibitor activities of PPI as a function of heating temperature (I = 15 mM): chymotrypsin ●, cathepsin D ■, carboxypeptidase A □, trypsin ○, papain △

DISCUSSION

Effect of ethanol on protein structure and stability

Effects of the presence of ethanol on the structure of potato proteins in various preparations were studied to determine within what temperature range ethanol precipitation could be used as a means of concentrating potato proteins from dilute solutions without having detrimental effects on their properties.

The presence of 20 % (v/v) ethanol was shown to decrease both the T_d and the Δh_{cal} of unfolding of potato proteins. The effect of ethanol on the T_d of the proteins in PIP is not uniform as can be seen from the increased width of the transition in the presence of ethanol as observed with CD (Fig. 4) and DSC (Fig. 5). This decrease of T_d is often observed in the presence of alcohols (e.g. Herskovits et al., 1970;Lustig and Fink, 1992;Grinberg et al., 1998) and is attributed to the effect of alcohols on the solvent structure thereby reducing the unfavorable free energy of solvating hydrophobic residues upon protein unfolding (Brandts and Hunt, 1967;Herskovits et al., 1970;Arakawa and Godette, 1985;Woolfson et al., 1993).

The presence of ethanol affects the structural changes that take place in patatin during heating. The tertiary structure cooperatively unfolds at low temperature while the secondary structure of the protein unfolds non-cooperatively at higher temperatures. Significant decoupling of the thermal unfolding of the tertiary and the secondary structure has been observed for ribonuclease A in the presence of methanol (Fink and Painter, 1987;Lustig and Fink, 1992). However, the decoupling in the case of patatin is more extensive than in the case of ribonuclease A and shows more similarity to the thermal unfolding of apo-α-lactalbumin via a molten-globule state (Griko et al., 1994).

Mechanistically, the effects of ethanol on the unfolding of patatin can be described as a destabilization of the tertiary structure of the protein, which is predominantly stabilized by

hydrophobic interactions (Fink and Painter, 1987), resulting in a marked decrease of T_d leading to the formation of an intermediate state. This intermediate state will have lost already a great deal of its tertiary structure, which will cause the remaining secondary structure to unfold non-cooperatively as the temperature is raised (Privalov and Gill, 1988;Mizuguchi et al., 2000;Griko, 2000). The non-cooperative unfolding of the secondary structure has been observed with DSC as a continuous change in heat capacity instead of an endothermic transition (Makhatadze and Privalov, 1995;Griko et al., 1994), which we also observed (not shown).

In the absence of ethanol the monomers of patatin were shown to unfold independently upon heating (Chapter 3). In the presence of ethanol, however, the denaturation temperature of patatin is lowered to 32°C. At such a low temperature the patatin monomers can be expected to be strongly associated and, therefore, to unfold as a dimeric protein. Gel filtration chromatography of patatin at various temperatures (15°C-35°C) (results not shown) confirmed that patatin remains dimeric upon unfolding in the presence of ethanol. The molar calorimetric enthalpy of unfolding (ΔH_{cal}) for patatin was, therefore, calculated on the basis of the concentration of dimeric patatin. This resulted in a ΔH_{cal} of 527 kJ per mole of patatin dimer.

In order to obtain thermodynamic data from the CD thermal unfolding curves of patatin in the absence and presence of ethanol, the curves in Figures 3A and B were described using a model reported by Van Mierlo et al (Van Mierlo et al., 1998). The data obtained from the CD unfolding curves, together with the DSC results, are summarized in Table 3. This table shows that the values of the van't Hoff enthalpy of unfolding (ΔH_{vH}), obtained from near-UV CD data, and of ΔH_{cal}, obtained from DSC profiles, do not change significantly in the presence of 20 % ethanol. The ΔH_{vH} obtained from the DSC profiles significantly increases in the presence of ethanol, causing the ratio of ΔH_{vH} over ΔH_{cal} to deviate significantly from unity (Table 3). The latter may indicate that the unfolding of the tertiary structure of patatin becomes more cooperative in the presence of ethanol (Sturtevant, 1987). Presently, we have no explanation for the discrepancy between the values obtained for ΔH_{vH} from the CD and DSC data.

Table 3: Thermodynamic parameters from fits of the CD thermal unfolding curves (222 nm, 258 nm) and DCS profiles of patatin (pH 7), in the absence and presence of ethanol (+ EtOH)

Sample	CD 222 nm T_m (°C)	CD 222 nm ΔH_{vH} ± SD (kJ/mole)	CD 258 nm T_m (°C)	CD 258 nm ΔH_{vH} ± SD (kJ/mole)	$\frac{\Delta H_{cal}}{\Delta H_{vH}}$[1]	DSC T_m (°C)	DSC ΔH_{cal} ± SD (kJ/mole)	DSC: ΔH_{vH} ± SD (kJ/mole)	$\frac{\Delta H_{cal}}{\Delta H_{vH}}$[2]
patatin	58.8	476 ± 6	58.2	498 ± 27	1.02	59.4	510 ± 12	529 ± 16	0.97
patatin + EtOH	not possible	not possible	31.1	502 ± 52	1.05	32.3	527 ± 6	630 ± 6	0.84

[1] Calculated using ΔH_{cal} from DSC data and ΔH_{vH} from the CD data (258 nm)

[2] Calculated using ΔH_{cal} and ΔH_{vH} from DSC data using: $\Delta H_{vH} = 4RT_m^2 C_p^{max}/\Delta H_{cal}$

These DSC results have practical implications for the preparation of PPI. As can be seen from Figures 5 the temperature during the precipitation of potato proteins in the presence of 20 % (v/v) ethanol should stay at 15°C or below to prevent protein unfolding. We observed that the preparation of PPI at ambient temperature resulted in a protein precipitate with a reduced solubility at neutral pH.

Effect of ethanol precipitation (pH 5) on protein structure and stability

PAT-5E and PIP-5E were used in this study to examine the effects of precipitation in the presence of ethanol at pH 5 on the structure and solubility of patatin and PIP. The changes observed in these individual protein fractions can be useful in explaining the properties of PPI in relation to those of untreated potato proteins.

This study showed that precipitation of PIP in the presence of ethanol (pH 5) did not significantly alter the structural properties of PIP. With DSC no change in T_d was observed, although the total Δh_{cal} of unfolding had decreased by 18 %. Since PIP is a mixture of proteins ethanol precipitation could have caused changes in the protein composition and hence the decrease of Δh_{cal} can not be ascribed to possible changes in the tertiary structure of the proteins.

Precipitation of patatin in the presence of ethanol (pH 5) did not influence the denaturation temperature. It did, however, result in an irreversible increase of the α-helix content (Table 2) and a perturbation of the tertiary structure, which caused a 14 % decrease of Δh_{cal} of unfolding. A perturbation of the tertiary structure due to exposure to ethanol was also observed for soybean lipoxygenase (Srinivasulu and Rao, 1995) and α-lactalbumin (Grinberg et al., 1998).

Properties of ethanol precipitated (pH 5) potato protein isolate (PPI)

Now that we have studied the properties of PAT-5E and PIP-5E, the question is how these relate to the properties of the crude preparation PPI that was prepared in the same way directly from PFJ.

The decrease of Δh_{cal} of unfolding of patatin and PIP after ethanol precipitation is only visible to a small extent in PPI. At high ionic strength the total calorimetric enthalpy of unfolding of PPI is about 90 % of that of PFJ, which may, however, also be due to differences in protein composition (Table 1).

The pH/solubility profile of PPI at high ionic strength is very similar to that of PFJ. At low ionic strength, however, PPI is almost insoluble at pH < 6 (Fig 6). On the other hand, both PAT-5E and PIP-5E, having undergone the same treatment as PPI, are quite soluble below pH 6 at low ionic strength. The low solubility could, therefore, result from the formation of specific electrostatic complexes between the different proteins in PPI, which fall apart when ionic strength is increased. PPI can, however, also be expected to contain more non-protein impurities than PIP and patatin, as it is only a crude protein preparation obtained by only one, rather non-selective, purification step from PFJ. The presence of these impurities could also have had a large effect on the solubility characteristics of PPI.

The solubility of PPI as a function of heat-treatment temperature is similar to that of PFJ and ASP (Chapter 3). At low ionic strength precipitation takes place at temperatures above the denaturation temperature, where most of the proteins are already unfolded, whilst at high ionic strength only a small fraction of the proteins needs to be unfolded before precipitation takes place (Fig. 2). The T_{50} values for PPI agree well with those found for PIP (Chapter 3), showing that these values were not significantly affected by precipitation in the presence of ethanol. The differences in protease inhibitor activity between PPI and PIP can be explained by compositional differences. PPI presumably contains the 85 kDa potato multicystatin (Walsh and Strickland, 1993;Waldron et al., 1993) and contains the heat-stable potato carboxypeptidase inhibitor (4.3 kDa) (Hass et al., 1975;Huang et al., 1981;Olivia et al., 1991), which, because of their Mw, are not present in PIP.
The results in this study can not explain the relatively high solubility of the protein isolate obtained in the presence of ethanol compared to potato protein isolates at low pH in the absence of ethanol (Chapter 2) (Meister and Thompson, 1976;Knorr et al., 1977;Knorr, 1978;Knorr, 1980;Knorr, 1982). The authors have strong indications that especially the reversibility of the precipitation of potato proteins from industrial PFJ is hindered by interactions of the proteins with endogenous phenolic compounds. We have also reported that the presence of ethanol or other water-miscible organic solvents significantly reduces the interaction between phenolic compounds and various plant proteins thereby significantly increasing the solubility of the resulting protein precipitate (Van Koningsveld et al., 2001).

From this study it can be concluded that the presence of ethanol considerably decreases the T_d of potato proteins. This imposes restrictions on the temperature at which potato proteins can be precipitated in the presence of ethanol, without causing significant irreversible structural damage. Precipitation of potato proteins in the presence of ethanol at pH 5 at 4°C did induce significant structural changes, but these changes only resulted in minor changes in the solubility of the proteins as a function of pH and heat-treatment temperature. The use of precipitation in the presence of ethanol does, however, also have great advantages because it is, to our knowledge, the only known large scale precipitation method for potato proteins that results in a protein isolate with a high solubility at neutral pH combined with a high yield.

ACKNOWLEDGEMENT

Potatoes were kindly provided by AVEBE B.A. (Foxhol, The Netherlands). This research was supported by the Ministry of Economic Affairs through the programme IOP-Industrial Proteins and by AVEBE B.A.

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Chapter 5

Formation and stability of foam made with various potato protein preparations

ABSTRACT

In the present study foam forming and stabilizing properties of potato proteins were studied using whipping and sparging tests. The soluble potato proteins are mainly composed of patatin and protease inhibitors The performed whipping tests showed that less foam could be formed from untreated patatin than from the protease inhibitors, but patatin foam was much more stable. The foam forming properties of patatin could be strongly improved by partial unfolding of the protein. Whipping tests, at both low (0.5 mg/ml) and high (10 mg/ml) protein concentration, also indicated that foams made with an ethanol precipitated protein isolate (PPI) were more stable than those made with β-casein and β-lactoglobulin. More generally it was concluded that when proteins are used as a foaming agent, a high concentration is required, because the protein available is inefficiently used. Also, the different methods used to make foam, result in changes in the mutual differences in foaming properties between the various protein preparations and may induce different instabilities to become apparent in foams made at the same conditions

INTRODUCTION

Potato fruit juice (PFJ) is a by-product from industrial starch manufacture and contains approximately 1.5 % (w/v) soluble protein. The soluble potato proteins can be tentatively classified into three classes: Patatin (41 kDa), protease inhibitors (5-25 kDa) and others (mostly high Mw) (Pots et al., 1999). Potato protein has a relatively high nutritional quality (Kapoor et al., 1975;Knorr, 1978), and it thereby has good potential for utilization in foods. However, recovering the protein by heat-coagulation from PFJ, results in a complete loss of most of its functional properties. Several efforts have, therefore, been made to recover potato proteins from PFJ that have retained their functional properties.

The ability to form and stabilize foams is considered an important functional property of food proteins, which is exploited in several food products. The foam properties of undenatured potato proteins have only been studied to a limited extent (Wojnowska et al., 1981;Jackman and Yada, 1988) and were observed to be inferior to the properties of whey proteins (Jackman and Yada, 1988). However, the foaming properties of potato protein obtained by ultrafiltration were shown to be very good in a number of food systems, being at least comparable to those of casein and egg albumin (Edens et al., 1997). The purpose of the present study was to examine foam forming and foam stabilizing properties of potato protein isolate prepared by precipitation in the presence of ethanol and to compare these properties to those of purified potato protein preparations as well as to the foam properties of β-casein and β-lactoglobulin, as a sort of reference proteins.

When studying foam properties a distinction should be made between formation and stability, although both processes can not be studied separately (Walstra and Smulders, 1997). For the making of a foam, air, liquid, energy and a surfactant, in this study protein, are needed. The surfactant serves two purposes, of which the most important is its ability to form interfacial tension gradients to stabilize the newly formed bubbles against immediate coalescence. Surfactants also lower the interfacial tension (γ) thus making the break-up of bubbles, opposed by the Laplace pressure ($p_L = 2\gamma/R$), easier (Walstra and Smulders, 1997). Proteins differ from small-molecule surfactants in that their conformation needs to change to effectively reduce γ. Proteins vary markedly in the rate at which these conformational changes take place.

In this study two different methods are used to make foam: 1. sparging and 2.beating or whipping. During sparging bubbles are released from a grit due to buoyancy forces. This method is more suited than whipping to produce foams from structurally rigid proteins, because the proteins have more time to adsorb and unfold at the interface. During whipping large bubbles are introduced in the solution, which are subsequently broken up into smaller ones. During whipping the making and breaking of bubbles occurs simultaneously and in this process whipping speed and whipping time are important parameters. During whipping the beaters cause velocity fluctuations, which, according to Bernoulli´s law, result in pressure fluctuations. These pressure fluctuations causes the bubbles in the solution to pulsate and their interfacial area (A) to change with time via $\Delta \ln A/dt = \rho \cdot v^3/3 \cdot L \cdot p$, in which L is the distance between the pins on the whisk, ρ the density of the liquid and v the velocity. The change in

area leads to a change in surface tension via $\Delta\gamma = \rho \cdot \eta_{SD}/3 \cdot L \cdot p$, in which η_{SD} is the surface dilational viscosity (= $d\gamma/(d \ln A/dt)$). This implies that above a certain whipping speed $\Delta\gamma$ will become too large and the film between two encountering bubbles will break and they will coalesce, according to Prins (Prins, 1988). When proteins are used, whipping time can also be expected to show an optimum. Increasing the whipping time may initially result in an increase in foam volume since more and smaller bubbles can be formed. When, however, whipping times become too long the continuous surface expansion and protein desorption, due to bubble breakage, may result in extensive protein unfolding and subsequent aggregation leading to a decrease in the effective molar protein concentration and a decrease in foam volume.

Once a foam is formed various instabilities may occur. Coalescence, i.e. the rupture of the film between two bubbles, can occur in foams when spreading particles (e.g. fat) are present or when hydrophobic particles with a diameter larger than the film thickness, are present (Prins, 1988). Drainage is the flow of liquid out of the foam due to gravity. The rate of drainage is, among other factors, affected by the bubble-size, the viscosity of the liquid and the amount of liquid in the foam. Compared to foam from small-molecule surfactants, drainage in protein foams is greatly retarded, as the adsorbed proteins can form fully stagnant surfaces (Prins, 1999). Ostwald ripening, the most important instability in most foams, is fast because of the high solubility of air in water. Due to a higher Laplace pressure the solubility of air in the liquid phase is higher around smaller than around larger bubbles, resulting in the growing of larger bubbles at the cost of small ones. This process can be retarded or stopped if the surfactant stays adsorbed at the interface of the shrinking bubble, as occurs with proteins, because then the surface tension will decrease when the surface area is reduced. The relation between the surface tension and change in surface area is given by $E_{SD} = d\gamma/d \ln A$, in which E_{SD} is the surface dilational modulus. It has been shown that Ostwald ripening in foam will completely stop if E_{SD} becomes equal to or larger than $\gamma/2$ (Lucassen, 1981). In this study the three described foam instabilities are monitored.

MATERIALS AND METHODS

Preparation of protein preparations

1. Potato fruit juice (PFJ)

Potatoes (cv. *Elkana*) were washed thoroughly with water and cut into large pieces (max. 8 × 2.5 cm) which were immediately dipped in a 20 mg/ml solution of sodium bisulfite to prevent enzymatic browning. The pieces were ground in a domestic type juice extractor (AEG). The resulting turbid juice was allowed to settle for 15 minutes. Next, the liquid was decanted and centrifuged (15 min, 19000 × *g*, 10°C) and the supernatant filtered through a paper filter (Schleicher & Schuell, ref.no. 311653). The resulting clear yellowish filtrate, which has a pH of 5.7 - 6.0, is known to be similar to industrial PFJ (AVEBE B.A., Foxhol, The Netherlands) and is further denoted as PFJ.

2. Potato protein isolate (PPI)

Potato protein isolate (PPI) was prepared by slowly adding 95 % (v/v) ethanol (-20°C) to stirred PFJ (4°C) to a final concentration of 15 or 20 % (v/v) and adjusting the apparent pH of the clear mixture to 5.0 by addition of 0.5 M H_2SO_4. After 1 hour at 4°C the suspension was centrifuged (30 min, 19000 × *g*, 4°C) and the precipitate was washed twice with a 0.1 M ammonium acetate buffer (pH 5) containing 15 or 20 % (v/v) ethanol. Subsequently, the precipitate was suspended in water and the suspension was adjusted to pH 7 using 0.1 M NaOH and then freeze-dried and stored at -20°C. The preparations obtained were denoted PPI (15 % EtOH) and PPI (20 % EtOH).

Potato protein isolate (PPI) contains most of the proteins present in PFJ, but these are known to be unfolded to some small extent due to their precipitation in the presence of ethanol (Chapter 4). PPI was prepared in two different variants: PPI (15 % EtOH) and PPI (20 % EtOH), which mainly differ in the lower protein solubility of the latter. The mere indication PPI in this chapter signifies PPI (15 % EtOH).

3. Ammonium sulfate precipitate (ASP)

Ammonium sulfate precipitate (ASP) was prepared from PFJ by adding $(NH_4)_2SO_4$ to 60 % saturation as proposed by (Seppälä et al., 1999) while keeping the pH at 5.7 by addition of small volumes of 0.5 M H_2SO_4. After 1 hour at 4°C the suspension was centrifuged (30 min, 19000 × *g*, 4°C) and the resulting precipitate was washed twice with half the starting volume of 50 mM sodium phosphate buffer (pH 7) that contained $(NH_4)_2SO_4$ up to 60 % saturation. Subsequently, the precipitate was suspended in distilled water and dialyzed (MWCO 3.5 kDa, Spectrum Medical Industries, Laguna Hills, CA., USA) against distilled water until the conductivity of the retentate remained constant. The retentate was subsequently freeze-dried and stored at -20°C.

Ammonium sulfate precipitate (ASP) was prepared as a simulant of total undenatured potato protein

4. Patatin

Patatin was purified by applying PFJ, diluted ten times with water and adjusted to pH 8, on a Source 15 Q column (10 × 15 cm) (Amersham Pharmacia Biotech AB, Uppsala, Sweden) equilibrated with a 25 mM Tris-HCl buffer (pH 8) containing 0.5 g/l $NaHSO_3$. After washing out the unbound compounds, the bound fraction was eluted with 0.35 M NaCl in the same buffer. Further purification of the bound fraction was established by gel filtration on a Superdex 75 column (63 × 10 cm) (Amersham Pharmacia Biotech AB, Uppsala, Sweden) eluted with a 30 mM sodium phosphate buffer (pH 7) containing 0.5 g/l $NaHSO_3$ at a linear flow rate of 30 cm/h. The first peak, as observed from the absorbance at 280 nm, containing patatin of > 95 % purity (SDS-PAGE), was collected and concentrated 10 times using Xampler UFP-3-C cross-flow hollow fiber laboratory cartridges with a molecular weight cut-off of 10 kDa (A/G Technology Corp., Needham, USA) at 4°C. The patatin was subsequently diafiltered with 5 volumes of a 9 mM sodium phosphate buffer (pH 7) and stored at –20°C.

Ethanol precipitated patatin (PAT-5E) was prepared by slowly adding 95 % (v/v) ethanol (-20°C) to a stirred patatin solution (5 mg/ml, 4°C) to a final concentration of 20 % (v/v) ethanol. The apparent pH of the clear mixture was adjusted to 5.0 by addition of small volumes of 0.5 M H_2SO_4. After 1 hour at 4°C the suspension was centrifuged (30 min, 19000 × *g*, 4°C) and the precipitate was suspended in water, adjusted to pH 7 using 0.1 M NaOH, extensively dialyzed against 9 mM sodium phosphate buffer (pH 7) and then stored at -20°C.
Patatin is the major protein in PFJ (Racusen and Foote, 1980), with a molecular weight of 41 kDa (Pots et al., 1999), and is estimated to make up 38 % of potato protein in PFJ. PAT-5E is patatin that has a partly unfolded tertiary structure due to precipitation at pH 5 in the presence of ethanol (Chapter 4).

5. Protease inhibitor pool (PIP)

Protease inhibitor pool (PIP) protein was prepared by gel filtration of PFJ on a Superdex 75 column (63 x 10 cm) (Amersham Pharmacia Biotech AB, Uppsala, Sweden) eluted with a 30 mM sodium phosphate buffer (pH 7) containing 0.5 g/l $NaHSO_3$, at a linear flow rate of 30 cm/h. The fractions making up the second peak, as observed from the absorbance at 280 nm, were collected, combined and concentrated 10 times using Xampler UFP-3-C cross-flow hollow fiber laboratory cartridges with a molecular weight cut-off of 5 kDa (A/G Technology Corp., Needham, USA) at 4°C. The concentrated PIP was subsequently diafiltered with water until no further decrease of the conductivity of the permeate could be observed. The concentrated PIP was subsequently freeze-dried and stored at -20°C.
Ethanol precipitated PIP (PIP-5E) was prepared by slowly adding 95 % (v/v) ethanol (-20°C) to a stirred PIP solution (6 mg/ml, 4°C) in 9 mM sodium phosphate buffer (pH 7) to a final concentration of 20 % (v/v). The apparent pH of the clear mixture was adjusted to 5.0 using 0.5 M H_2SO_4. After 1 hour at 4°C the suspension was centrifuged (30 min. 19000 × *g*, 4°C) and the precipitate was suspended in distilled water and adjusted to pH 7 using 0.1 M NaOH. Next, the solution was extensively dialyzed against 9 mM sodium phosphate buffer (pH 7) and then stored at -20°C.
Protease inhibitor pool (PIP) contains mainly protease inhibitors of molecular weights between 15-25 kDa (Chapter 3) and is estimated to contain 45 % of the protein present in PFJ (Pouvreau et al., 2001). PIP-5E has approximately the same protein composition as PIP but has been precipitated in the presence of ethanol, which is expected to have caused small irreversible changes in the conformation of the proteins (Chapter 4)

Other proteins

β-Lactoglobulin was obtained as described previously (Caessens et al., 1997b). β-Casein (90 % pure on dry weight) was purchased from Eurial (Nantes, France). β-Casein and β-lactoglobulin were used as reference proteins, because their foam properties have been extensively studied (Mulvihill and Fox, 1989) and have been repeatedly tested using the applied whipping test (Caessens et al., 1997a;Caessens et al., 1999).

Protein content

Protein content of protein solutions used was estimated using the method of Bradford (Bradford, 1976) with bovine serum albumin (Sigma A-7511; Lot 92H93131) as a standard.

Foam properties

Whipping method

Protein solutions of β-lactoglobulin, β-casein, PPI, ASP, PIP, PIP-5E, PAT-5E and patatin were dispersed to a protein concentration of 0.5 mg/ml in a 9 mM sodium phosphate buffer containing 35 mM NaCl (pH 7, I = 50 mM), unless stated otherwise. Formation and stability of foam was estimated by the whipping method described by Caessens and coworkers (Caessens et al., 1997a). Generally, a volume of 100 ml of protein solution in a graduated glass cylinder was stirred at a speed of 2500 rpm during 70 s. The foam volume was monitored during 60 min (the first measurement 2 min after stirring started), and the foam quality (bubble size, coalescence and Ostwald ripening) was evaluated visually. All measurements were performed at least in duplicate.

Variation of the stirring speed was tested on 0.5 mg/ml protein solutions using speeds from 2000-5000 rpm with a stirring time of 70 s. Variation of the stirring time (10 – 360s) was applied at 2500 rpm. Initial foam volumes were measured 50 s after the stirring was stopped. Foam measurements as a function of protein concentration were performed with solutions containing 0.1, 0.2, 0.35, 0.5 and 1.0 mg of protein per ml.

The effect of pH on the foam properties of PPI, ASP, PIP and patatin was investigated by dispersing these proteins to a concentration of 0.5 mg/ml in 9 mM sodium phosphate (pH 7), 24 mM sodium acetate (pH 5) or 17 mM sodium phosphate (pH 3) all adjusted to an ionic strength of 50 mM by addition of NaCl to a concentration of 35 mM. The effect of pH unfolding was tested for PPI, ASP and patatin by dissolving these proteins in 9 mM sodium phosphate buffer (pH 3). After 30 min the pH was adjusted to 7 by addition of 0.1 M NaOH and NaCl was added to a final concentration of 35 mM. Next, 9 mM sodium phosphate buffer (pH 7) containing 35 mM NaCl (pH 7, I = 50 mM) was added to make the final protein concentration 0.5 mg/ml. This treatment will be further indicated as pH 3 → pH 7.

The effect of heat treatment on the foam properties of PPI, ASP, PIP and patatin was investigated by dispersing these proteins to a final concentration of 2.5 mg/ml in 9 mM sodium phosphate buffer (pH 7; I = 15 mM) and subsequently heating these dispersions in a thermostatted waterbath at 80 ± 1°C during 10 min. After heating, the solutions were immediately cooled in ice. Next, NaCl was added to a final concentration of 35 mM and the solutions were filtered over a 0.2 µm filter (Schleicher & Schuell, Dassel, Germany). The protein concentration of the heated solutions was estimated and the final concentration was adjusted to 0.5 mg/ml using 9 mM sodium phosphate buffer containing 35 mM NaCl (pH 7, I = 50 mM).

Effects of ionic strength on foam properties were investigated by dispersing PPI, ASP, PIP and patatin to a final concentration of 0.5 mg/ml in 9 mM sodium phosphate buffer (pH 7) that contained no NaCl (I = 15 mM), 35 mM NaCl (I = 50 mM) or 185 mM NaCl (I = 200 mM).

Ledoux apparatus

Formation and stability of foam was also studied using a "Whipped-cream tester" (Ledoux Machine Factory, Dodewaard, the Netherlands), which was designed by the NIZO (Streuper and Van Hooydonk, 1986) and will be further denoted as Ledoux apparatus. The apparatus is schematically shown in Figure 1. In the beaker of the instrument 200 ml of protein solution was poured and the two whisks were stirred at a speed of 360 rpm (80%) during 70 or 360 sec. After whipping the whisks were kept in place. The foam height was monitored during 60 min (the first measurement was 50 s after stirring was stopped), and the foam quality (bubble sizes, coalescence and Ostwald ripening) was evaluated visually. All measurements were performed at least in duplicate. For foam prepared using the Ledoux apparatus 10 mg/ml solutions of β-lactoglobulin, β-casein, PPI and ASP in 9 mM sodium phosphate buffer containing 35 mM NaCl (pH 7, I = 50 mM) were used.

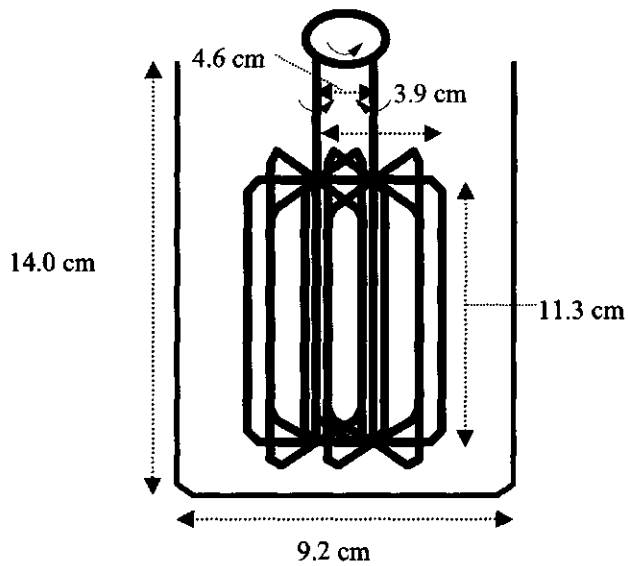

Figure 1: Schematic representation of the Ledoux apparatus

Sparging method

Foams were also made using a sparging method. Formation and stability of foam were estimated by introducing air at a constant flow rate during 40 s through a G-2 filter (pore-size: 40-90 μm) in a glass column (3.2 x 20 cm) with 40 ml of protein solution. The foam height was monitored during 60 min (the first measurement immediately after stopping the airflow), and the foam quality (bubble sizes, coalescence, etc.) was judged visually. All measurements were performed at least in duplicate. Protein dispersions (pH 7; I = 50 mM) with a protein concentration of 0.5 mg/ml prepared from β-lactoglobulin, β-casein, PPI, ASP, PIP and patatin were used.

RESULTS

Effects of whipping speed and whipping time using the whipping method

The effect of whipping speed on the foam volume formed from the various protein preparations is shown in Figure 2. For most preparations foam volume increased gradually with whipping speed between 2000 and 3000 rpm. Above 3000 rpm foam volume generally increased much stronger and the foam became much firmer and the bubble size became visibly smaller.

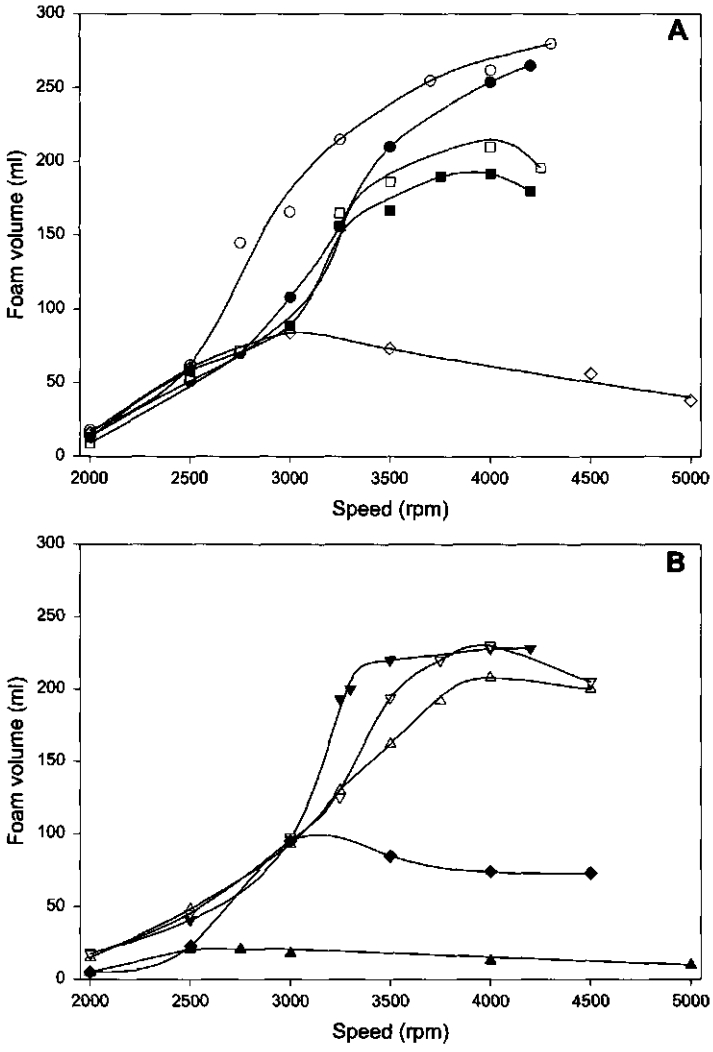

Figure 2: Effect of whipping speed on foam formation at pH 7 and $I = 50$ mM using a whipping time of 70 s, unless otherwise stated, with 0.5 mg/ml solutions of: (A) β-lactoglobulin●, β-caseinO, ASP□, PPI (20 % ethanol)◇, PPI (15 % ethanol)■ and (B) patatin (30 s)◆, patatin (70 s) ▲, PIP▼, PAT-5E△ and PIP-5E ▽

Speeds ≥ 3500 rpm resulted in protein aggregation, as the serum became turbid. Only in the case of β-casein the foam remained liquid-like at higher whipping speeds and in this case no aggregation was observed. For β-casein, β-lactoglobulin and PIP no optimal speed was found while for ASP, PPI (15 % EtOH), PAT-5E and PIP-5E foam formation seemed to be maximal at 4000 rpm. Foam formation of PPI (20 % EtOH) was maximal at 3000 rpm. Using the standard whipping time of 70 s for patatin almost no foam remained at any whipping speed. When the whipping time was reduced to 30 s substantially more foam remained, resulting in an optimum speed of about 3000 rpm.

The influence of whipping time on foam formation at 2500 rpm is shown in Figure 3. The curves in Figure 3 show two different shapes. The shape of the curves, obtained for both PPI variants and patatin, is characterized by a strong relative increase in foam formation with whipping time until a maximum is reached at 60-100 s. Longer whipping times resulted in a gradual decrease in foam formation. Another curve shape is characterized by an initial strong increase in foam formation with whipping time but with a longer optimum whipping time of 150-300 s; this concerned β-casein, β-lactoglobulin, ASP and PIP. During the whipping of these proteins distinct transitions at 80 and 130 s were also observed, in that whitening of the liquid and a strong increase in viscosity occurred; the resulting foams had visibly smaller bubbles and a greater firmness. In contrast to high whipping speeds, long whipping times usually did not result in visible protein aggregation. Only β-casein (at whipping times > 100 s) formed precipitates on the whisk.

Higher whipping speeds or longer whipping times had similar effects; both resulted in increased foam formation and foam firmness and decreasing bubble size, up to a certain level.

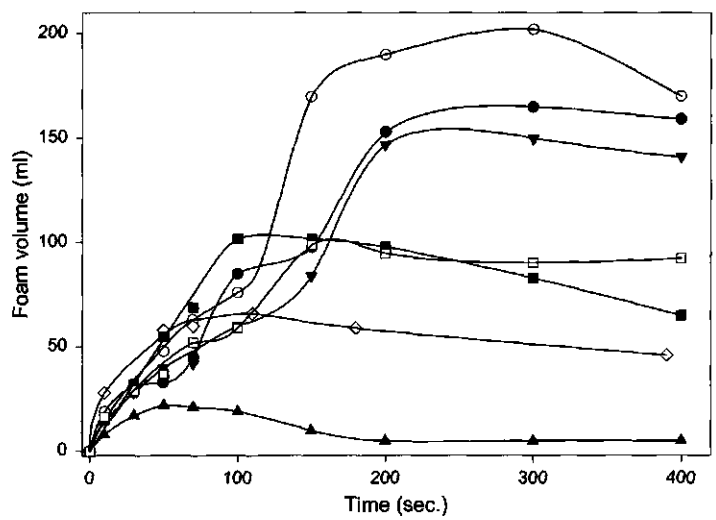

Figure 3: Effect of whipping time on foam formation at pH 7 and I = 50 mM using a whipping speed of 2500 rpm with 0.5 mg/ml solutions of: PPI (20 % ethanol)◇, PPI (15 % ethanol)■, ASP□, β-lactoglobulin ●, β-casein ○, patatin ▲ and PIP ▼

Effects of protein concentration

In Figure 4 the influence of protein concentration on foam formation can be seen. All of the protein preparations showed an increase in foam formation with increasing protein concentration. Two curve shapes could be distinguished. An S-shaped curve was found for PPI (20 % EtOH), patatin and β-lactoglobulin, with a remarkable increase in foam formation between 0.35 mg/ml and 0.5 mg/ml. The curve shape found for ASP, PPI (15 % EtOH), PIP and β-casein featured a strong increase in foam formation with concentration up to 0.35 mg/ml, followed by a more gradual increase above this concentration. For all protein preparations a visible decrease in bubble size was observed when the protein concentration was increased above 0.35 mg/ml. Therefore, if the surface area of the foam bubbles rather than the foam volumes were depicted, a much stronger increase would have been observed at higher concentrations. Indications that protein was not efficiently used during foam formation were obtained when the average bubble size was estimated by microscopy (results not shown). The average bubble size (d_{32}) of PPI at 0.5 mg/ml was estimated at 150 μm. If a surface load of 3 mg/m^2 is assumed then the amount adsorbed (in 60 ml of foam) is merely about 10 % of the protein available.

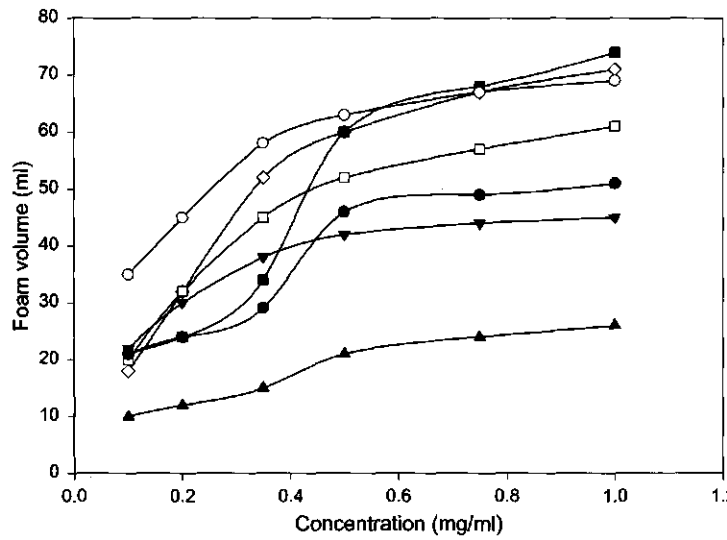

Figure 4: Effect of protein concentration on foam formation at pH 7 and I = 50 mM using a whipping speed of 2500 rpm and a whipping time of 70 s with solutions of: PPI (20 % ethanol) ◇, PPI (15 % ethanol) ■, ASP□, patatin▲, PIP ▼, β-lactoglobulin● and β-caseinO

Foam properties at various pH using the whipping method

In Figure 5A foam volume as a function of time is presented for foam made with various protein preparations (pH 7; I = 50 mM) using the whipping method. Curves of foam volumes as a function of time in Figure 5 are examples of the curves generally obtained. Further results obtained using the whipping method are summarized in Table 1.

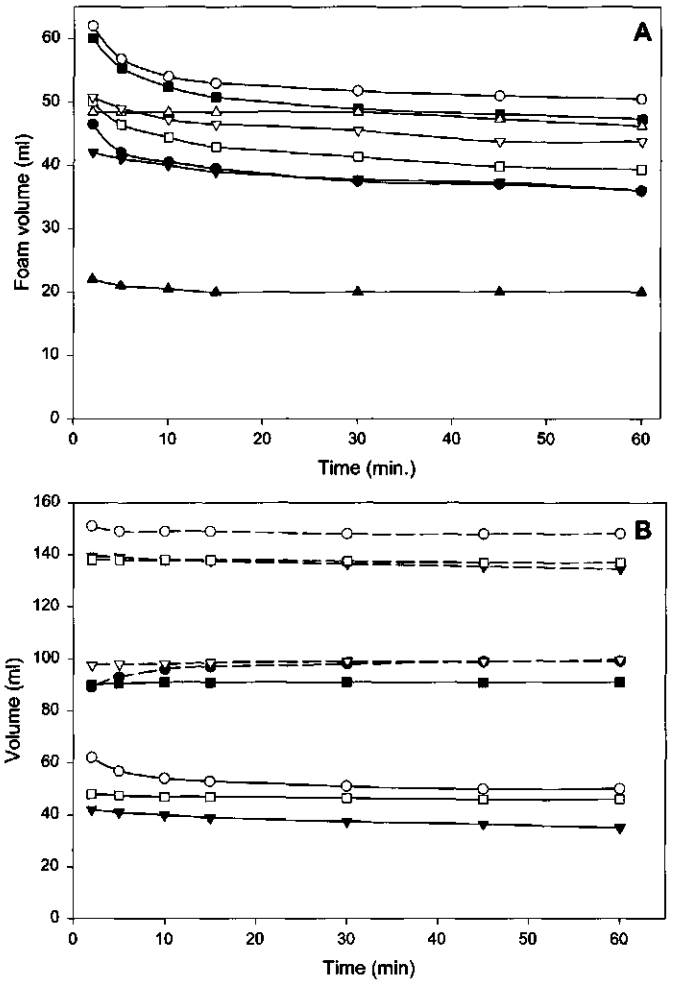

Figure 5: A: Foam volume as a function of time for foam formed using the whipping method (2500 rpm, 70s) at pH 7 and I = 50 mM with 0.5 mg/ml solutions of: β-lactoglobulin●, β-casein ○, ASP □, PPI ■, patatin ▲, PIP ▼, PAT-5E △ and PIP-5E ▽ B: Foam volume (solid line) and upper and lower foam boundaries (dashed lines) of foam formed using the whipping method (2500 rpm, 70s) at pH 7 and I = 50 mM with 0.5 mg/ml solutions of β-casein ○(volume), ○(upper), ●(lower); PIP ▼(volume), ▼(upper), ▽(lower) and PAT-5E □(volume), □(upper), ■(lower)

At pH 7 foam formation was highest for β-casein and PPI. Significantly less foam was formed with ASP, PIP-5E and PAT-5E. Foam formation was lowest for patatin. Remarkably, ethanol precipitation of patatin and, to a lesser extent, PIP resulted in a marked increase in foam formation compared to untreated patatin and PIP. Patatin, PAT-5E and PIP-5E formed the smallest bubbles, while in foam whipped from ASP, PPI and β-lactoglobulin the largest bubbles were observed.

In Figure 5B examples are shown of foam volume and the upper and lower foam boundaries as a function of time, for foam made with β-casein, PIP and PAT-5E. The change in the lower foam boundary gives an indication of the amount of liquid drained from the foam, while the upper foam boundary indicates foam volume decrease due to other instabilities. Data on drainage are also presented in Table 1 together with the initial volume fraction of air in the foam. From Figure 5B it can be seen that the decrease in foam volume with time in foam made with β-casein is mainly due to drainage, while in foam made with PIP other instabilities prevail. Foams whipped from patatin and PAT-5E showed the slowest drainage, while drainage of foams formed from the other preparations increased in the order PIP and PIP-5E < ASP < β-lactoglobulin < PPI and β-casein (Table 1). Foam formed from PAT-5E was also the most stable against Ostwald ripening, while foams prepared from PIP, PIP-5E and β-casein were the least stable against Ostwald ripening. Coalescence was only observed in the case of PIP and PIP-5E (Table 1).

Since the isoelectric pH of patatin is about 5 the effect of pH on the foam properties of various potato protein preparations was tested at pH 5, when protein solubility permitted, and at pH 3. Because patatin is known to irreversibly unfold at pH ≤ 4.5 (Pots et al., 1998) the influence of unfolding was also tested by adjusting the pH of patatin containing preparations to 3 and subsequently back to pH 7 (pH 3 → 7).

In Table 1 the influence of pH on formation and stability of foams formed by various protein preparations can be seen. PPI formed less foam at pH 3 than at pH 7, but the foam at pH 3 drained more slowly and was more stable against Ostwald ripening, resulting in a volume decrease of 12 % rather than 22 % in 60 min. When PPI was dispersed at pH 3 and subsequently brought to pH 7, foam formation was significantly affected. The foam drained faster than at pH 3 but it was more stable against Ostwald ripening and drained slower than that formed from PPI at pH 7, that had not previously been at pH 3. ASP formed similar volumes of foam at pH 5 and pH 7. The foam formed at pH 5 drained faster than foam prepared at pH 7 and showed a higher relative decrease in foam volume during 60 min; 27 % at pH 5 as compared to 22 % at pH 7. At pH 3 ASP formed a bit more foam than at pH 7 and pH 5 with significantly smaller bubbles, but it showed somewhat faster drainage. If the pH had been adjusted from 3 to 7, the foam formed showed similar properties to that formed at pH 3 but seemed to be more stable against Ostwald ripening (Table 1). PIP formed more foam at pH 5 than at pH 7 and pH 3, with smaller bubbles and a higher stability against Ostwald ripening. Also, the coalescence observed at pH 3 and pH 7 was not observed at pH 5. Foam properties of PIP at pH 3 were similar to those at pH 7, except for an increase in stability

Table 1: Summary of results obtained using the whipping method (2500 rpm; 70 s) with solutions containing 0.5 mg of protein per ml

				Foam volume (ml)					Occurence of:	
Fraction	pH	I (mM)	ΔT	2 min	60 min	φ (air)	Bubble size[1]	drainage (%)[2]	Coalesc.[3]	Ostwald ripening[4]
β-casein	7	50		62	50	0.67	+ +	49	-	+ +
β-lactog.	7	50		47	36	0.66	+ + +	38	-	+ + +
PPI	7	15		58	45	0.70	+ + + +	52	-	+ + + +
	7	50		60	47	0.68	+ + +	52	-	+ +
	7	200		64	46	0.66	+ + +	46	-	+ +
	7	50	yes	61	48	0.68	+ +	51	-	+
	3	50		41	36	0.67	+ +	30	-	+
	3→7	50		56	45	0.66	+ +	37	-	+
ASP	7	15		44	41	0.70	+ + + +	23	-	+ + +
	7	50		50	39	0.66	+ + +	29	-	+ +
	7	200		50	33	0.66	+ + +	41	-	+ +
	7	50	yes	37	33	0.71	+ + +	9	+	+
	5	50		51	37	0.63	+ + +	48	-	+ + +
	3	50		56	43	0.64	+ +	45	-	+ +
	3→7	50		58	44	0.66	+ +	46	-	+
PIP	7	15		33	30	0.79	+ + + +	14	+	+ + + +
	7	50		42	36	0.73	+ +	18	+	+ + +
	7	200		49	36	0.65	+ + +	41	-	+ + +
	7	50	yes	53	41	0.67	+ + +	40	+	+ + +
	5	50		60	49	0.65	+	19	-	+ +
	3	50		39	32	0.72	+ +	18	+	+ + +
PIP-5E	7	50		51	44	0.66	+	17	+	+ + + +
patatin	7	15		22	20	0.72	+ +	0	-	+ + +
	7	50		22	20	0.65	+	0	-	+ +
	7	200		31	29	0.61	+ +	0	-	+ +
	7	50	yes	32	30	0.80	+	0	-	-
	5	50		58	51	0.67	+ +	5	-	+ +
	3	50		55	42	0.68	+ +	23	-	+ + +
	3→7	50		60	46	0.67	+ +	45	-	+ +
PAT-5E	7	50		48	46	0.61	+	5	-	-

ΔT = heat treatment (80°C, 10 min.); φ = volume fraction; [1] more plus signs indicates larger bubbles; [2] % drained of liquid initially present in foam; [3] + coalescence observed and - coalescence not observed; [4] – slow Ostwald ripining·····++++ fast Ostwald ripening

against Ostwald ripening at pH 3. The relative decrease of foam volume after 60 min was 14 % at pH 7 while it was 18 % at pH 5 and pH 3 (Table 1).
The largest changes in foam formation with pH were observed with patatin. At pH 3 2.5 times more foam was whipped from patatin than at pH 7. The resulting foam, however, drained faster and was less stable against Ostwald ripening than at pH 7. When the pH of patatin was first adjusted to pH 3 and then to pH 7 (pH 3 → 7) it formed almost three times more foam than at pH 7, but in this case the foam was more stable against Ostwald ripening than at pH 3, although it drained faster. Foam whipped from patatin at its isoelectric pH (pH 5) was similar in foam volume and foam stability to patatin (pH 3 → 7). The relative decrease in foam volume after 60 min was only 9 % at pH 7 and 12 % at pH 5, while it was about 24 % for pH 3 and pH 3 → pH 7 (Table 1).

Effects of ionic strength

The effects of ionic strength on the formation and stability of foam whipped from PPI, ASP, PIP and patatin at pH 7 are also shown in Table 1. In the case of ASP and PIP a decrease in ionic strength resulted in a slight decrease in foam formation. Decreasing the ionic strength from 50 to 15 mM resulted in all potato protein foams in an increase in bubble size and a decreasing stability against Ostwald ripening (Table 1).
Increasing the ionic strength from 50 to 200 mM resulted in the case of PIP and patatin in an increase in foam formation but also in an increase in bubble size. Foam whipped from PIP at high ionic strength did not show coalescence. In the case of ASP and PIP, increasing the ionic strength resulted in the formation of foams that showed faster drainage (Table 1).

Effects of heat treatment

The effect of heating (80°C, 10 min) solutions of PPI, ASP, PIP and patatin at pH 7 on the foam properties are shown in Table 1. Heating of PPI did not result in increased foam formation. The resulting foam, however, contained smaller bubbles and was significantly more stable against Ostwald ripening. ASP formed less foam if it had been heated. The foam formed showed slower drainage but was less stable against coalescence. The foam volume had decreased by 22 % for unheated ASP and by 11 % for heated ASP after 60 min. PIP formed more foam after heat treatment. But the resulting foam drained faster, which may have resulted in the observed decrease in stability against coalescence. The relative volume decrease observed was by 14 % for unheated PIP and by 22 % for heated PIP. Heating of patatin resulted in the formation of more foam, which was very stable against Ostwald ripening (Table 1).

Foam properties using the sparging method

Sparging was used as a comparison to whipping because it is known that some proteins have a low foamability when whipped, while they do form foam when sparging is used. In Figure 6 foam height as a function of time is presented for foam formed from various protein preparations (pH 7; I = 50 mM) by sparging. It can be seen in Figure 6 that the amount of foam formed by sparging was nearly independent of the protein preparation, quite unlike the results obtained by whipping. Even patatin, which showed a low foamability when whipped (Figure 5), produced a foam height comparable to that of the other proteins. Only PIP formed somewhat less foam than the other preparations.

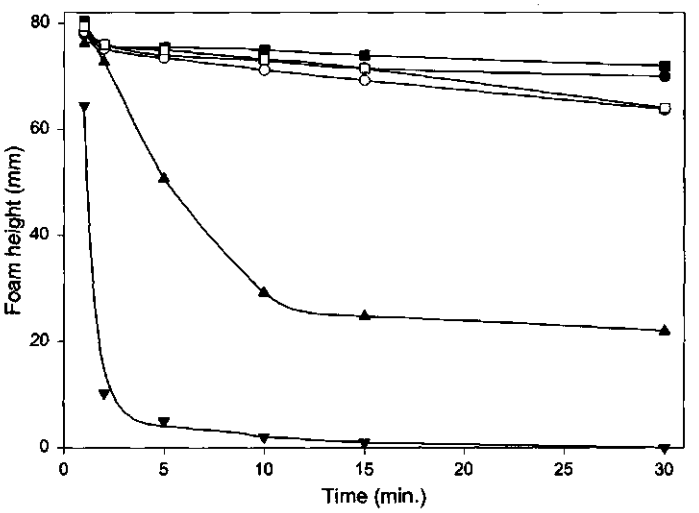

Figure 6: Foam height as a function of time for foam prepared using the sparging method at pH 7 and I = 50 mM with 0.5 mg/ml solutions of: β-lactoglobulin ●, β-casein ○, ASP □, PPI ■, patatin ▲ and PIP▼

Bubble sizes in sparged foams were much larger than in whipped foams and increased in the order β-casein and PPI < β-lactoglobulin < ASP << patatin << PIP. Foam formed by PPI and β-casein also drained least, while foam stabilized by patatin and PIP drained most and fastest. Possibly the fast and extensive drainage resulted in the poor stability against coalescence and Ostwald ripening observed in foam stabilized by patatin and PIP.

Foam properties using the Ledoux apparatus

With the whipping method, low protein concentrations were used to emphasize the differences in foam properties between the protein preparations. In order to test the foam properties of some potato protein preparations at conditions more similar to food manufacture, we used the Ledoux apparatus at a protein concentration of 10 mg/ml. Figure 7 shows the foam height as a function of time for foam whipped from solutions of ASP, PPI, β-casein and β-lactoglobulin. As can be seen in Figure 7 no significant differences in initial

foam formation were observed. Using a whipping time of 70 s foam prepared from ASP and PPI was visibly more stable against Ostwald ripening than foam prepared from β-casein and β-lactoglobulin. Also, foam made with ASP and PPI under these conditions showed less drainage than foam prepared from β-casein. Increasing the whipping time for ASP and PPI from 70 to 360 s caused a decrease in bubble size. At both whipping times a smaller bubble size was observed for foam made with PPI than with ASP.

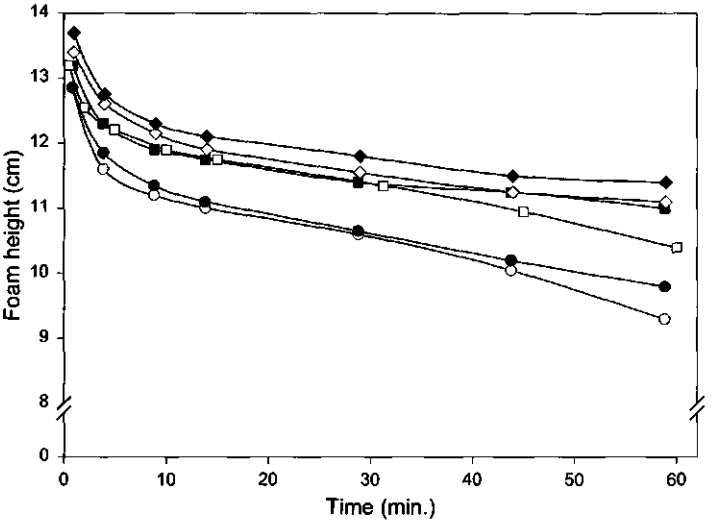

Figure 7: Foam height as a function of time for foam prepared with the Ledoux apparatus using whipping times of 70 and 360 s at pH 7 and I = 50 mM with 10 mg/ml solutions of: β-casein (70 s) ○, β-lactoglobulin (70 s) ●, PPI (70 s) ■, PPI (360 s) ◆, ASP (70 s) □ and ASP (360 s) ◇

DISCUSSION

Foam properties of patatin

The foam properties of patatin at pH 7 (Figure 5) resemble those of a structurally rigid protein like e.g. lysozyme (Townsend and Nakai, 1983;Bacon et al., 1988), being characterized by the formation of only a small volume of foam consisting of small bubbles. That patatin unfolds slowly at the interface would also agree with the results in Figure 2. At a whipping time of 30 s the optimum whipping speed was observed to be 3000 rpm, which is low compared to those for the other protein preparations. At higher whipping speeds patatin is presumably not able to unfold fast enough to produce a surface pressure sufficient to stabilize the newly formed bubbles against immediate coalescence by forming γ-gradients. Longer whipping times may result in increased foam formation due to the adsorption of surface denatured proteins, as is the case when whipping ovalbumin (Kinsella, 1981;Dickinson, 1992). The rate of unfolding of patatin at the interface must be very slow or its rate of refolding, when desorbed from the interface, must be fast, since longer whipping times do not result in increased foam formation. Foam formation from patatin by sparging, which is characterized by low surface expansion

rates as compared to whipping, is similar to that of other protein preparations (Fig. 6). But even then the unfolding of patatin is apparently too slow to stabilize the foam against extensive drainage, Ostwald ripening and coalescence (Fig.6).

When whipped at its isoelectric pH (pH 5), patatin forms much more foam, which has been observed for various proteins (Graham and Phillips, 1976;German and Phillips, 1991). However, in the case of patatin the tertiary structure is already affected at pH 5 (Chapter 3) which may have affected its foaming properties. As can be seen from Figures 2 and 5 and Table 1, PAT-5E, which differs from patatin in having a partially unfolded tertiary structure, forms much more foam than patatin when whipped at the same conditions. Apparently, as emphasized by Damodaran and coworkers (Song and Damodaran, 1987;Zhu and Damodaran, 1994), the flexibility of the tertiary structure is important in foam formation. The increase in foam formation is not accompanied by a decrease in foam stability, since drainage rate, presumably due to a smaller bubble size, and Ostwald ripening are lower than in foams produced from β-casein and β-lactoglobulin (Table 1). Patatin is unfolded more extensively and irreversibly at pH 3 (Pots et al., 1998), and foam formation then increases even more, though it also results in faster drainage (Table 1). Irreversible unfolding of patatin by heating it at 80°C resulted in a much smaller increase in foam formation, although the amount of residual structure in the protein after heat induced unfolding was shown to be similar to that after pH induced unfolding (Pots et al., 1998). A higher degree of aggregation after heating, resulting in a decrease of the effective molar protein concentration, may have caused this difference in foam formation.

The effect of various pre-treatments on the foaming properties of patatin indeed shows that these properties are very sensitive to the structural stability of the protein and to the degree of unfolding of the tertiary (Ibanoglu and Ibanoglu, 1999) and secondary structure (Wagner and Guéguen, 1999) of the protein. Unfolding may enhance both foam formation and foam stability but complete loss of protein structure may result in extensive aggregation and a decrease in solubility, and thus be detrimental to its foaming properties (Song and Damodaran, 1987;Zhu and Damodaran, 1994;Sorgentini et al., 1995).

Foam properties of the PIP

When whipped at pH 7 PIP forms more foam than patatin. The protease inhibitors in PIP generally have an even higher structural stability than patatin (Chapter 3). However, their pI's are known to cover a wide range (pH 5.1- > 9) (Pouvreau et al., 2001), which has been observed to increase foam formation when mixtures of proteins differing substantially in pI were compared to pure proteins (German and Phillips, 1991). The stability of foam formed from PIP at pH 7 did, however, show a low stability against Ostwald ripening and especially against coalescence (Table 1). The same instabilities became even more apparent when foam was produced from PIP by sparging. In contrast, at pH 5 no coalescence was observed. Since PIP was prepared by gel filtration chromatography the presence of coalescence inducing impurities in this preparation is very unlikely. The authors have, until now, no explanation for the coalescence in PIP stabilized foams.

Foam properties of PPI

PPI (15 % EtOH) formed much more foam than PPI (20 % EtOH) at whipping speeds > 3000 rpm (Fig. 2). This difference can be explained by the observation that dispersions of PPI (20 % EtOH) contain substantial amounts of large aggregates, as observed by gel filtration chromatography (results not shown), thereby lowering the effective molar protein concentration. At low whipping speeds (< 3000 rpm) this reduction of the effective protein concentration is not noticed, presumably because new surface is not formed extremely fast and the large aggregates may even contribute to the stabilization of the newly formed bubbles against coalescence. At high whipping speeds, however, the decrease in effective molar concentration presumably prevails, since the rate at which new interface is created is higher, and foam formation with PPI (20 % EtOH) decreases. In the remainder of this section only the properties of PPI (15 % EtOH) will be discussed.

PPI at pH 7 formed more foam than PAT-5E, PIP-5E and ASP. The stability of the foam formed from PPI at pH 7 is similar to that of PIP-5E and ASP (Table 1). When the foam properties of undenatured potato proteins in ASP are compared to those of ethanol precipitated proteins in PPI (Table 1), it can be seen that foam formation with PPI is higher. Foam prepared from ASP generally drains more slowly than that prepared from PPI, but the relative decrease in foam volume after 60 min is similar.

Foam properties of (partially) unfolded proteins

Various degrees of unfolding of the tertiary and secondary structure of potato proteins by ethanol, pH variations or heat treatment, generally resulted in a substantial increase in the stability against Ostwald ripening (Table 1). The driving force for Ostwald ripening is the Laplace pressure difference over the curved surface of a bubble: $\Delta p = 2\gamma/R$ (Prins et al., 1998). If the proteins do not desorb when the bubble shrinks then γ will continue to decrease and thereby reduce the driving force for Ostwald ripening (Prins et al., 1998). Restricted unfolding of proteins prior to foaming has been shown not only to increase foam formation, but also to increase intermolecular interactions between proteins in the interface in several cases (Kinsella, 1981;German and Phillips, 1991;Zhu and Damodaran, 1994;Wagner and Guéguen, 1999). The increase of these interactions will presumably reduce the probability that the proteins desorb when the interface is compressed, and thereby stabilize the bubbles against Ostwald ripening. Lowering of the ionic strength was shown to reduce the stability against Ostwald ripening (Table 1; (Yu and Damodaran, 1991)), which can possibly be explained by an increase in electrostatic repulsion distance in the interface thereby reducing the strength of attractive interactions between the proteins in the interface. Increasing the ionic strength, on the other hand, results in some cases in an increase in the amount of foam formed and more generally in an increased drainage rate (Table 1). When an increase in drainage rate was observed, with increasing ionic strength, the initial volume fraction of liquid in the foam was also found to have increased. In general, the drainage rate could not be related to the initial volume fraction of air in the foam. A strong correlation was, however, found between the total volume of liquid in the foam and the rate of drainage (results not shown), excluding the foams made with patatin.

In conclusion, it was shown that less foam could be formed from untreated patatin than from the protease inhibitors, but patatin foam was much more stable. The foam forming properties of patatin could be strongly improved by partial unfolding of the protein. Whipping tests, at a concentration of 0.05 % (w/v), also indicate that foams made with PAT-5E and the industrially more relevant PPI are more stable than those made with β-casein and β-lactoglobulin (Table 1), also at industrially more relevant protein concentrations (1 % (w/v)). More generally it can be concluded that when proteins are used as a foaming agent, a high concentration is required, because the protein available is inefficiently used. Also, the different methods used to make foam, result in changes in the mutual differences in foaming properties between the various protein preparations and may induce different instabilities to become apparent in foams made at the same conditions. Much more and more detailed research would be necessary in order to explain all the observations described in this study.

ACKNOWLEDGEMENT

Potatoes were kindly provided by AVEBE B.A. (Foxhol, The Netherlands). This research was supported by the Ministry of Economic Affairs through the programme IOP-Industrial Proteins and by AVEBE B.A.

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Chapter 6

Formation and stability of emulsions made with various potato protein preparations

ABSTRACT

Emulsions were made with various potato protein preparations and were characterized with respect to average droplet size, plateau surface excess and the occurrence of droplet aggregation. The average droplet size of the emulsions made with potato proteins was found to be presumably determined by the lipolytic release of surface active fatty acids and monoglycerides from the tricaprylin oil phase during the emulsification process. It was concluded that only trace amounts of patatin, the lipase activity of which has been strongly underestimated, sufficed to liberate significant amounts of these surfactants. The plateau surface excess of emulsions made with patatin was found to be 2.6 mg/m^2, while emulsion droplets made with protease inhibitors showed a significantly smaller surface excess. Of the various solvent conditions and treatments used only heat treatment resulted in a significant increase in surface excess. Droplet aggregation in emulsions made with potato protein preparations could be prevented at pH 3.

INTRODUCTION

Potato fruit juice (PFJ) is a by-product from industrial starch manufacture and contains approximately 1.5 % (w/v) soluble protein. The soluble potato proteins have been tentatively classified into three classes: Patatin (41 kDa), protease inhibitors (5-25 kDa) and others (mostly high Mw) (Pots et al., 1999). Potato protein has a relatively high nutritional quality (Kapoor et al., 1975;Knorr, 1978), and it thereby has good potential for utilization in foods. However, recovering the protein by heat-coagulation from PFJ, results in a complete loss of most of its functional properties for industrial application. Several efforts have, therefore, been made to recover potato proteins from PFJ that have retained their functional properties (Meister and Thompson, 1976;Knorr et al., 1977;Knorr, 1980;Wojnowska et al., 1981;Gonzalez et al., 1991).

In many food products proteins act as emulsifiers and emulsion stabilizers. The emulsifying properties of undenatured potato proteins have only been studied to a limited extent (Holm and Eriksen, 1980;Wojnowska et al., 1981;Jackman and Yada, 1988;Ralet and Guéguen, 2000). These properties were observed to be inferior to the emulsifying properties of whey proteins (Jackman and Yada, 1988) but superior to those of commercial soy isolate (Holm and Eriksen, 1980). The emulsifying properties of potato protein obtained by ultrafiltration were reported to be very good, being superior to those of whey protein, soy protein and casein (Edens et al., 1997). Most authors (Holm and Eriksen, 1980;Jackman and Yada, 1988;Ralet and Guéguen, 2000), however, have based their findings on parameters such as emulsifying capacity and on empirical emulsion stability tests, which have little practical value (Walstra and De Roos, 1993;Sherman, 1995). Also, the use of higher alkanes as dispersed phase for studying food emulsion properties (Ralet and Guéguen, 2000) may result in overlooking important aspects. The purpose of this study was to examine emulsion forming and stabilizing properties of potato protein isolate prepared by precipitation in the presence of ethanol and to compare these properties to those of purified potato protein preparations.

When studying emulsion properties a distinction should be made between formation and stability (Walstra and Smulders, 1997). For the making of an emulsion, two immiscible liquids, energy and a surfactant, in this study protein, are needed. It is quite easy to make an emulsion, but a stable emulsion requires the formation of very small droplets, which requires much more effort. During emulsion formation the presence of surfactant serves two purposes, of which the most important one is the ability to form interfacial tension gradients to stabilize the newly formed droplets against immediate coalescence. Surfactants also lower the interfacial tension (γ) thus making the break-up of droplets, which is opposed by the Laplace pressure ($p_L = 4\gamma/d$), into smaller ones easier (Walstra and Smulders, 1997). The droplet size of the emulsion formed is the result of a kind of steady state between droplet break-up and recoalescence and, therefore, depends on the time-scales of the different processes (Smulders, 2000). The extent to which a surfactant lowers the interfacial tension and stabilizes the newly formed droplets depends on the concentration and interfacial properties of the surfactant. Proteins differ from small-molecule surfactants, amongst other aspects, by the fact that their conformation generally needs to change to effectively reduce γ. Proteins vary markedly in the rate at which these conformational changes take place. Proteins often form a monolayer at

oil/water interfaces, resulting in a plateau value of the surface excess (Γ). In some cases multilayers are formed and no plateau is observed. Protein monolayer adsorption is generally almost irreversible and is, therefore, not an equilibrium process at the time-scales usually considered (Dalgleish, 1989).

Once an emulsion is formed various instabilities may occur. Creaming is the rise of droplets to the top of the emulsion due to the density difference between the dispersed phase and the continuous phase. The rate of creaming is, amongst other factors, affected by the droplet size and the width of its distribution, the viscosity of the continuous phase, the density difference between the two phases, and the volume fraction of dispersed phase (Walstra, 1996). Creaming is opposed by Brownian or heat motion of droplets and by convection. Generally, food emulsions are stable to creaming if the effective average droplet size is smaller than 1 μm.

Droplet aggregation may also occur in emulsions, i.e. droplets remain close for prolonged time without rupture of the film between them. Aggregation increases the effective average particle size and, therefore, usually results in a decreased stability of the emulsion against creaming. Droplet aggregation is governed by the balance of the attractive and repulsive interactions between the droplets, which is a function of the distance between the droplets and the properties of the interfacial layer. When proteins are used as surfactants the electrostatic interactions between the droplets, repulsive or attractive, may depend strongly on pH, ionic strength and the protein used. In emulsions specific mechanisms of aggregation may be observed that are called bridging and depletion flocculation. Bridging flocculation occurs at low concentrations of polymeric surfactants. In this situation one polymer molecule adsorbs at two interfaces thus forming a bridge between the droplets. Depletion flocculation may occur when high concentrations of non-adsorbing polymers are present, because then there is a zone near the particle interface that is, due to steric hindrance, depleted of this polymer. The concentration difference between this layer and the bulk, forces the droplets together in order to minimize the volume devoid of polymeric surfactant.

Coalescence occurs if the thin film between two droplets is ruptured and the droplets flow together in one droplet. At the end of this process an oil layer and a water layer will remain. For coalescence to occur the film between the droplets should be very thin for a prolonged time, and stability against coalescence will, therefore, be decreased by creaming and droplet aggregation. Coalescence is opposed by steric and electrostatic repulsion provided by the adsorbed layer.

In order to investigate the occurrence of most of the instabilities mentioned above, in the present study emulsions were made with various potato protein preparations, differing in protein composition and extent of unfolding, at various conditions such as pH and ionic strength. These emulsions were characterized with respect to their average droplet size, surface excess and stability against droplet aggregation.

MATERIALS AND METHODS

Preparation of protein preparations

1. Potato fruit juice (PFJ)

Potatoes (cv. *Elkana*) were washed thoroughly with water and cut into large pieces (max. 8 × 2.5 cm) which were immediately dipped in a 20 mg/ml solution of sodium bisulfite to prevent enzymatic browning. The pieces were ground in a domestic type juice extractor (AEG). The resulting turbid juice was allowed to settle for 15 minutes. Next, the liquid was decanted and centrifuged (15 min, 19000 × *g*, 10°C) and the supernatant filtered through a paper filter (Schleicher & Schuell, ref.no. 311653). The resulting clear yellowish filtrate, which has a pH of 5.7 - 6.0, is known to be similar to industrial PFJ (AVEBE B.A., Foxhol, The Netherlands) and is further denoted as PFJ.

2. Potato protein isolate (PPI)

Potato protein isolate (PPI) was prepared by slowly adding 95 % (v/v) ethanol (-20°C) to stirred PFJ (4°C) to a final concentration of 15 % (v/v) and adjusting the apparent pH of the clear mixture to 5.0 by addition of 0.5 M H_2SO_4. After 1 hour at 4°C the suspension was centrifuged (30 min, 19000 × *g*, 4°C) and the precipitate was washed twice with a 0.1 M ammonium acetate buffer (pH 5) containing 15 % (v/v) ethanol. Subsequently, the precipitate was suspended in water and the suspension was adjusted to pH 7 using 0.1 M NaOH, freeze-dried and stored at -20°C.

Potato protein isolate (PPI) contains most of the proteins present in PFJ, but these are known to be unfolded to some small extent due to their precipitation in the presence of ethanol (Chapter 4).

3. Ammonium sulfate precipitate (ASP)

Ammonium sulfate precipitate (ASP) was prepared as a simulant of total undenatured potato protein, and was prepared from PFJ by adding $(NH_4)_2SO_4$ to 60 % saturation as proposed by (Seppälä et al., 1999) while keeping the pH at 5.7 by addition of small volumes of 0.5 M H_2SO_4. After 1 hour at 4°C the suspension was centrifuged (30 min, 19000 × *g*, 4°C) and the resulting precipitate was washed twice with half the starting volume of 50 mM sodium phosphate buffer (pH 7) that contained $(NH_4)_2SO_4$ up to 60 % saturation. Subsequently, the precipitate was suspended in distilled water and dialyzed (MWCO 3.5 kDa, Spectrum Medical Industries, Laguna Hills, CA., USA) against distilled water until the conductivity of the retentate remained constant. The retentate was subsequently freeze-dried and stored at -20°C.

4. Patatin

Patatin was purified by applying PFJ, diluted ten times with water and adjusted to pH 8, on a Source 15 Q column (10 × 15 cm) (Amersham Pharmacia Biotech AB, Uppsala, Sweden) equilibrated with a 25 mM Tris-HCl buffer (pH 8) containing 0.5 g/l $NaHSO_3$. After washing out the unbound compounds, the bound fraction was eluted with 0.35 M NaCl in the same

buffer. Further purification of the bound fraction was established by gel filtration on a Superdex 75 column (63 × 10 cm) (Amersham Pharmacia Biotech AB, Uppsala, Sweden) eluted with a 30 mM sodium phosphate buffer (pH 7) containing 0.5 g/l $NaHSO_3$ at a linear flow rate of 30 cm/h. The first peak, as observed from the absorbance at 280 nm, containing patatin of > 95 % purity (SDS-PAGE), was collected and concentrated 10 times using Xampler UFP-3-C cross-flow hollow fiber laboratory cartridges with a molecular weight cut-off of 10 kDa (A/G Technology Corp., Needham, USA) at 4°C. The patatin was subsequently diafiltered with 5 volumes of a 9 mM sodium phosphate buffer (pH 7) and stored at –20°C.
Ethanol precipitated patatin (PAT-5E) was prepared by slowly adding 95 % (v/v) ethanol (-20°C) to a stirred patatin solution (5 mg/ml, 4°C) to a final concentration of 20 % (v/v) ethanol. The apparent pH of the clear mixture was adjusted to 5.0 by addition of small volumes of 0.5 M H_2SO_4. After 1 hour at 4°C the suspension was centrifuged (30 min, 19000 × *g*, 4°C) and the precipitate was suspended in water, adjusted to pH 7 using 0.1 M NaOH, extensively dialyzed against 9 mM sodium phosphate buffer (pH 7) and then stored at -20°C.
Patatin is the major protein in PFJ (Racusen and Foote, 1980), with a molecular weight of 41 kDa (Pots et al., 1999), and is estimated to make up 38 % of potato protein in PFJ. PAT-5E is patatin that has a partly unfolded tertiary structure due to precipitation at pH 5 in the presence of ethanol (Chapter 4).

5. Protease inhibitor pool (PIP)

Protease inhibitor pool (PIP) protein was prepared by gel filtration of PFJ on a Superdex 75 column (63 × 10 cm) (Amersham Pharmacia Biotech AB, Uppsala, Sweden) eluted with a 30 mM sodium phosphate buffer (pH 7) containing 0.5 g/l $NaHSO_3$, at a linear flow rate of 30 cm/h. The fractions making up the second peak, as observed from the absorbance at 280 nm, were collected, combined and concentrated 10 times using Xampler UFP-3-C cross-flow hollow fiber laboratory cartridges with a molecular weight cut-off of 5 kDa (A/G Technology Corp., Needham, USA) at 4°C. The concentrated PIP was subsequently diafiltered with water until no further decrease of the conductivity of the permeate could be observed. The concentrated PIP was subsequently freeze-dried and stored at -20°C.
Ethanol precipitated PIP (PIP-5E) was prepared by slowly adding 95 % (v/v) ethanol (-20°C) to a stirred PIP solution (6 mg/ml, 4°C) in 9 mM sodium phosphate buffer (pH 7) to a final concentration of 20 % (v/v). The apparent pH of the clear mixture was adjusted to 5.0 using 0.5 M H_2SO_4. After 1 hour at 4°C the suspension was centrifuged (30 min, 19000 × *g*, 4°C) and the precipitate was suspended in distilled water and adjusted to pH 7 using 0.1 M NaOH. Next, the solution was extensively dialyzed against 9 mM sodium phosphate buffer (pH 7) and then stored at -20°C.
Protease inhibitor pool (PIP) contains mainly protease inhibitors of molecular weights between 15-25 kDa (Chapter 3) and is estimated to contain 45 % of the protein present in PFJ of the cultivar used (Pouvreau et al., 2001). PIP-5E has approximately the same protein composition as PIP but has been precipitated in the presence of ethanol, which is expected to have caused small irreversible changes in the conformation of the proteins (Chapter 4).

Preparation of protein dispersions

Protein dispersions (7.5 mg/ml) at pH 7 were prepared from ASP, PPI, PIP, PIP-5E, patatin and PAT-5E by dispersing these proteins in 9 mM sodium phosphate buffer (pH 7) containing 0.2 g/l of sodium azide, when an ionic strength of ≈15 mM was tested. When an ionic strength of 50 mM was tested the same buffer was used, but 35 mM of NaCl was added.

Protein dispersions (7.5 mg/ml) at pH 5 were prepared from ASP, PPI, PIP and patatin by dispersing these proteins in 24 mM sodium acetate buffer (pH 5) containing 0.2 g/l of sodium azide and 185 mM of NaCl to reach an ionic strength of 200 mM.

Protein dispersions (7.5 mg/ml) at pH 3 were prepared from ASP, PPI, PIP and patatin by dispersing these proteins in 17 mM sodium acetate buffer (pH 3) containing 0.2 g/l of sodium azide, to inhibit microbial growth, and had and ionic strength of ≈ 15 mM. Parts of the protein dispersions of ASP, PPI and patatin at pH 3 were adjusted to pH 7 by addition of 1 M NaOH and their ionic strengths were adjusted to 50 mM by addition of appropriate amounts of NaCl; this is referred to as the pH 3→pH 7 treatment.

Protein samples for testing the effect of heat treatment were prepared by making dispersions of 10 mg/ml of ASP, PPI, PIP and patatin in 9 mM sodium phosphate buffer (pH 7) containing 0.2 g/l of sodium azide ($I \approx 15$ mM). The dispersions were filtered over a 0.2 μm filter (Schleicher & Schuell, Dassel, Germany), and subsequently heated in thermostatted waterbath at 80 ± 1°C for 10 min, after the temperature in the sample reached 80°C. Heated samples were immediately cooled in ice water and the ionic strength was adjusted to 50 mM by addition of NaCl and the dispersions were again filtered over a 0.2 μm filter (Schleicher & Schuell, Dassel, Germany).

All protein dispersions prepared were stirred overnight at 4°C and were subsequently equilibrated at room temperature and the pH was monitored and if necessary adjusted with small volumes of 1 M NaOH or 0.5 M H_2SO_4. Next, the protein dispersions were centrifuged (10000 × g, 20 min., 25°C) and subsequently filtered over a 0.2 μm filter (Schleicher & Schuell, Dassel, Germany). The protein concentration of the final dispersions was estimated using the method of Bradford (Bradford, 1976) with bovine serum albumin (Sigma A-7511; Lot 92H93131) as a standard.

Preparation of emulsions

Emulsions were prepared by mixing 18 ml of protein dispersion and 2 ml of tricaprylin oil (Sigma; $\rho = 0.95$ kg.m^{-3}; $n_D = 1.4466$). A coarse pre-emulsion was prepared using an Ultra Turrax type T-25B (Janke & Kunkel GmbH, Germany) at 11000 rpm during 1 min. The pre-emulsion was homogenized by passing it 30 times through a Delta Instruments HU 2.0 laboratory scale high-pressure homogenizer (Delta Instruments, Drachten, the Netherlands) operated at 6 MPa. Emulsions were prepared and tested at least in duplicate.

Droplet size estimation

The droplet size was calculated as the volume-surface average diameter (d_{32}) given by: $d_{32} = S_3/S_2 = \Sigma N_i.d_i^3/\Sigma N_i.d_i^2$, with N_i and d_i the number and diameter of droplets in size class i,

respectively. The relative width of the droplet size distribution is then given by: $c_s = (S_2.S_4/S_3^2 - 1)^{0.5}$ (Walstra, 1968), in which S_4 is $\Sigma N_i.d_i^4$.

The mentioned parameters were determined using the spectroturbidimetric method as decribed by Walstra (Walstra, 1965;Walstra, 1968). Emulsions were prepared in duplicate and the optical density of the emulsions was measured once for each emulsion at wavelengths from 380 nm to 1700 nm using a Zeiss spectrophotometer (type M 4 GII) with an attachment for turbidity measurements and an angle of acceptance of 1.5°. Before measurement the emulsions were diluted with 0.3 % (w/v) sodium dodecyl sulfate (SDS), to stabilize the droplets and disperse any aggregates present, until a final optical density between 0.2 and 0.8 was reached. The absence of aggregates was checked by light microscopy (magnification: × 400).

Estimation of the protein surface excess (Γ)

The protein surface excess of emulsions was estimated using an indirect depletion method, which is based on the estimation of the amount of unadsorbed protein and the interfacial area of the emulsion (Oortwijn and Walstra, 1979). For patatin (pH 7, I = 50 mM) surface excess (Γ) was determined as a function of the protein concentration over the interfacial area of the emulsion (c/A), in which c is the protein concentration (mg/m^3) and A is the specific area (m^2/m^3) of the emulsion. A can be calculated from: $A = 6.\varphi/d_{32}$ (Walstra, 1983), in which φ is the volume fraction of oil in the emulsion. For these experiments patatin concentrations ranging from 0.67 to 10 mg/ml were used. For the other emulsions Γ was determined at a single protein concentration. In order to determine the concentration of unadsorbed protein the emulsions were centrifuged (30 min, 14000 × g, 25°C) resulting in a cream layer and a serum layer. About one third of the bottom part of the serum layer was taken and centrifuged again. This procedure was repeated three times and the final serum was filtered over a 0.2 µm filter (Schleicher & Schuell, Dassel, Germany) and its protein content was estimated. The cream layers obtained after the first centrifugation step were redispersed in volumes of the buffer equal to those in the original emulsion. The washing liquid obtained after centrifuging (30 min, 14000 × g, 25°C) the redispersed emulsion, was centrifuged again when necessary and subsequently filtered over a 0.2 µm filter (Schleicher & Schuell, Dassel, Germany) and its protein content was determined if no coalescence was observed during the washing procedure. The washing procedure was repeated twice. Protein content of protein solutions used was estimated using the method of Bradford (Bradford, 1976) with bovine serum albumin (Sigma A-7511) (Lot 92H93131) as a standard. The surface excess was calculated as $\Gamma = \Delta c$ (mg/m^3)/A (m^2/m^3), in which $\Delta c = c_{emulsion} - c_{serum} - c_{washing\ 1} - c_{washing\ 2}$ and c is the protein concentration in mg per m^3.

Viscosity measurements

The viscosity of a number of emulsions was estimated as a function of shear rate (4 – 135 s^{-1}) using a Bohlin CVO Rheometer (Bohlin Instruments, Cirencester, U.K.) at 20°C using a finely grooved cylinder (C25) measuring body.

Extraction and separation of emulsion oil phase components

Samples of 1 ml of some typical emulsions were extracted by mixing them with 5 ml of 1:2 (v/v) methanol/chloroform in closed tubes. The tubes were centrifuged (3600 × *g*, 20°C, 30 min) and the lower organic layer was collected. The organic layer was evaporated at 40°C under a flow of nitrogen, and subsequently dissolved in 1 ml of a mixture of 2 % (v/v) water in methanol containing 0.1 % (v/v) of acetic acid and used for HPLC analysis.

HPLC separations were performed with a Spectra Physics P1000 solvent delivery system equipped with an AS3000 auto-sampler and UV3000 absorbance detector (Thermo Separations Products, Fremont CA, USA). Quantities of 20 µl of sample were injected on a Spherisorb 5 ODS-2 column (4.6 (ID) × 250 mm) (Chrompack, the Netherlands) equilibrated and eluted with a mixture of 2 %(v/v) water in methanol containing 0.05 % (v/v) acetic acid at a flow rate of 1.0 ml/min. Eluting compounds were detected by their absorbance at 210 nm and their retention times and peak areas were compared to those of the standard compounds. Standard compounds used were tricaprylin (Sigma) and caprylic acid (Fluka), which were dissolved in eluent at concentration of 95 and 91 mg/ml, respectively.

RESULTS

Aggregation characteristics of emulsions made at pH 7

At pH 7 (I = 50 mM) emulsions (10 % (v/v) oil) were made with protein dispersions (7.5 mg/ml) of PPI, ASP, PIP and patatin. Microscopy indicated that all protein preparations containing protease inhibitors, i.e. all preparations except patatin, resulted in emulsions that showed extensive droplet aggregation and, therefore, creamed fast.

The mechanism inducing this droplet aggregation was further investigated. The possible importance of bridging aggregation, resulting from a too low surface excess (Γ), was tested by reducing the volume fraction of oil (φ) in the emulsion from 0.10 to 0.025. Microscopy of the emulsion, however, showed that the extent of droplet aggregation was not significantly reduced. When depletion flocculation would be the mechanism inducing the observed droplet aggregation, then the interactions keeping the droplets together would be quite weak and easily broken. This mechanism was tested by measuring the viscosity of the aggregated emulsions as a function of shear rate, as is shown in Figure 1A. Figure 1A shows that at low shear rate the viscosity of the aggregated emulsions made with PPI and PIP was much higher than that of the non-aggregated patatin emulsion. Moreover, the viscosity decreased only slowly with increasing shear rate, which excludes the mechanism of depletion flocculation. Next, the presence of homogenization clusters in the aggregated emulsions was tested. If these clusters were present then the decrease in viscosity, as observed in Figure 1A, should be at least partly irreversible since the break-up of homogenization clusters should be irreversible. Figure 1B, however, shows that the viscosity of the emulsions after the first measurement was almost completely recovered. Moreover, microscopic inspection of emulsions that had been passed several times through the homogenizer at a low pressure, which is known to break up homogenization clusters, showed no significant reduction in aggregate size. Also, increasing the ionic strength and dilution of the emulsion in 10 % (w/v)

SDS did not break up the aggregates. Addition of dithiothreitol, which is a potent agent to reduce disulfide bridges, was able to break up the aggregates completely into separate droplets, and thus clearly showed the important involvement of disulfide reshuffling in droplet aggregation in emulsions containing cysteine-rich potato protease inhibitors. With this knowledge the formation of disulfide bridge dominated droplet aggregation could be completely prevented by carefully avoiding the inclusion of air during pre-homogenization (results not shown).

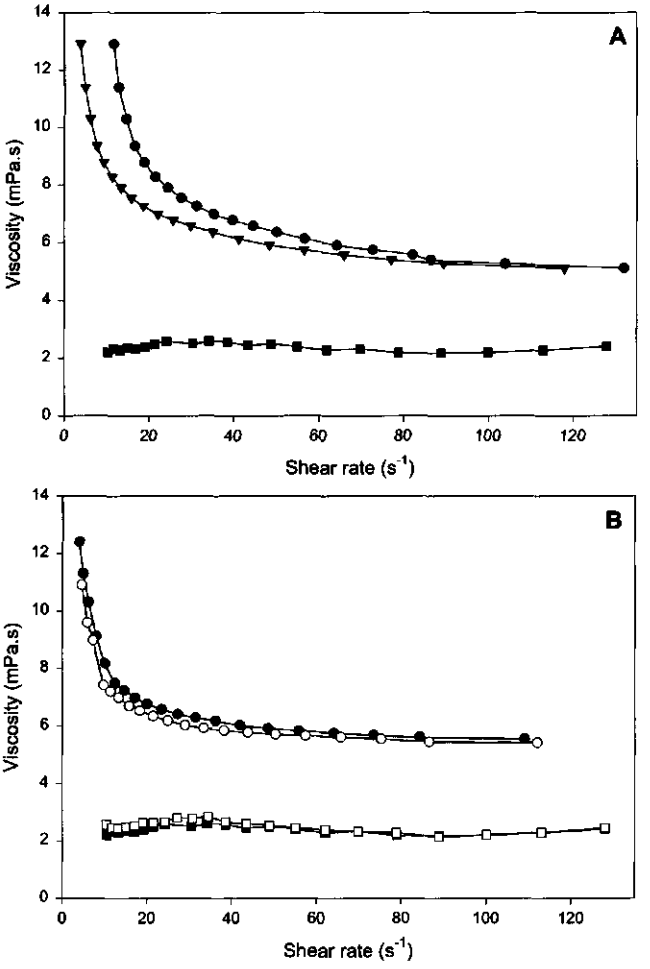

Figure 1: A: Viscosity as a function of shear rate for emulsions made with patatin ■, PPI ●, and PIP ▼, at pH 7 and I = 50 mM B: Viscosity as a function of shear rate for emulsions made with patatin (■ 1st measurement; □ 2nd measurement) and PPI (● 1st measurement; ○ 2nd measurement), at pH 7 and I = 50 mM

Emulsions made at pH 7

Even when the inclusion of air was prevented, emulsions made with PPI, ASP and PIP at pH 7 (I = 50 mM) showed droplet aggregation, as can be seen in Table 1. However, dilution (1:10) of these emulsions in 0.3 % (w/v) SDS before microscopic inspection, revealed that covalent interactions were no longer involved in droplet aggregation since only separate droplets were observed.

The accuracy of the Γ values mentioned in Table 1 was estimated as described in the appendix of the paper by Oortwijn and Walstra (Oortwijn and Walstra, 1979). The average standard deviation of the average droplet size, of all the emulsions mentioned in Table 1, was estimated as 0.12 μm. This resulted in the case of ASP (pH 3→pH 7) in a $\sigma(A)$ of 1.0 m^2, in which $\sigma(A)$ is the standard deviation of the surface area of 1 ml separated oil. The other parameters for this emulsion were estimated to be: Δc = 3.71 mg/ml; $\sigma(c)$ = 0.065 mg/ml; A = 10.3 m^2; φ = 0.1; $\sigma(\varphi)$ = 0.0005, in which Δc is the difference in protein concentration in the original protein solution and that in serum layer after centrifugation, A' is the surface area of 1 ml separated oil, and φ is the volume fraction of oil in the emulsion. $\sigma(c)$ and $\sigma(\varphi)$ are the standard deviations of Δc and φ, respectively. From these values the standard deviation of Γ ($\sigma(\Gamma)$) was calculated as being $0.29 \cdot \Gamma$. Therefore, when comparing the emulsions in Table 1 differences in surface excess of less than approximately 30 % and differences in average droplet size smaller than 0.12 μm were considered not significant. For details about the calculations the reader is referred to the original publication.

As can be seen from Table 1, emulsions made with potato proteins had very small volume-surface average droplet sizes (d_{32}), especially those preparations that contained substantial amounts of patatin (PPI, ASP and patatin). Emulsions made with PIP at pH 7 (I = 50 mM) contained significantly larger droplets than emulsions made with PPI, ASP and patatin. Surprisingly, emulsions made with PIP and patatin that had been precipitated in the presence of ethanol (PIP-5E, PAT-5E) had a much smaller average droplet size than those made with untreated proteins. From the emulsions made at pH 7 (I = 15 mM) the protease inhibitors in PIP showed a significantly lower surface excess (Γ) of 1.8 mg/m^2 (Table 1). The value found for emulsions made with PIP (pH 7; I I = 50 mM) was found to be unexplainably low and was, therefore, left out of the discussion.

Table 1: Characteristics of emulsions made with various potato protein preparations at various conditions

Fraction	pH	I (mM)	ΔT	e_0 (mg/ml)	d_{32} (μm)	d_{43} (μm)	c_S (-)	$\Gamma_{protein}$ (mg/m^2)[1]	Droplet aggregation[2]
PPI	7	15		7.0	0.35	0.83	1.16	2.6	+ +
	7	50		7.5	0.39	0.91	1.16	2.3	+ + +
	7	50	yes	11.3	0.49	0.90	0.92	7.8	+ + + +
	5	200		5.2	4.48	5.79	0.54	2.1	+ +
	3	15		8.1	0.51	0.94	0.92	2.7	-
	3→7	50		8.4	0.25	0.81	1.51	2.0	+ + + + +
ASP	7	15		7.5	0.62	1.03	0.81	2.5	+ + +
	7	50		7.4	0.59	1.08	0.92	2.9	+ + + +
	7	50	yes	11.4	0.52	0.96	0.92	7.7	+ +
	5	200		3.8	3.01	4.53	0.71	3.8	+
	3	15		7.4	0.43	0.73	0.83	2.6	-
	3→7	50		7.7	0.58	0.95	0.79	3.2	+ +
PIP	7	15		7.7	0.35	1.15	1.51	1.8	+ +
	7	50		7.5	0.71	2.73	1.68	0.8*	+ + +
	7	50	yes	11.2	0.42	0.99	1.16	6.1	+ + +
	5	200		7.2	0.73	2.19	1.41	1.7	+ + +
	3	50		7.8	0.72	1.23	0.84	2.1	-
PIP-5E	7	50		8.9	0.24	0.77	1.51	1.5	+ +
patatin	7	15		7.5	0.31	0.85	1.31	2.5	-
	7	50		7.8	0.32	0.63	0.99	2.7	-
	7	50	yes	9.8	0.46	1.08	1.16	5.9	±
	5	200		4.4	0.45	0.73	0.79	2.7	+
	3	15		4.8	0.59	0.84	0.64	3.1	-
	3→7	50		3.7	0.48	1.84	1.68	2.4	+ + +
PAT-5E	7	50		11.5	0.20	0.55	1.31	2.6	-

ΔT = heat treatment (80°C, 10 min); e_0 = protein concentration before emulsification; [1] plateau values of surface excess (Γ); [2] ±···+++++ increasing size of droplet aggregates, - droplet aggregation not observed;* unexplainable value; c_S relative width of the droplet size distribution; d_{32}: volume-surface average droplet size (μm)

Droplet size and surface excess of patatin emulsions

Figure 2 shows the volume-surface average droplet size of emulsions made with patatin as a function of the patatin concentration. Figure 2 shows that at low patatin concentrations (< 2 mg/ml) the average size of the droplets formed decreased steeply with increasing patatin concentration. At concentrations higher than about 3 mg/ml an excess of protein was present in solution and the obtained droplet size became about constant.

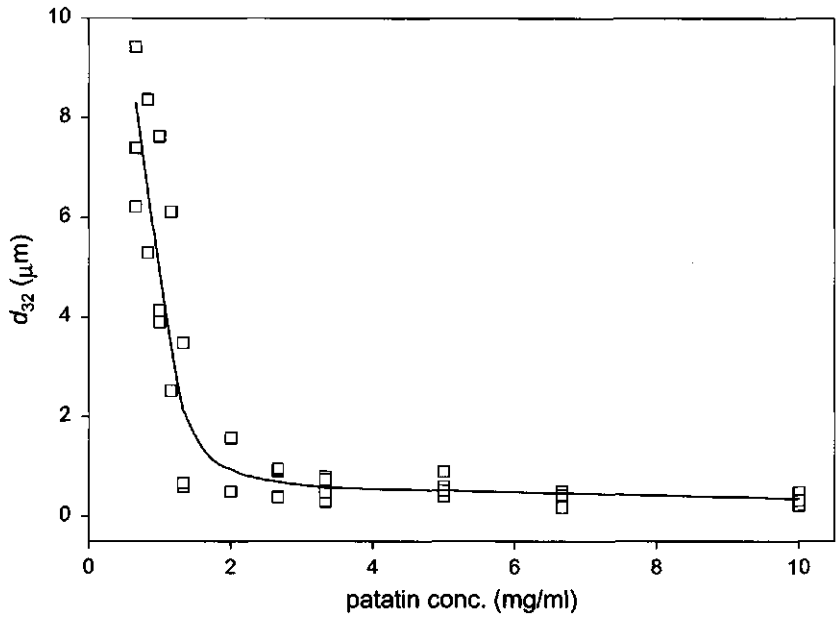

Figure 2: Average droplet diameter (d_{32}) of emulsion made with patatin (pH 7; I = 50 mM) as a function of protein concentration (mg/ml)

In Figure 3 the surface excess of emulsion droplets made with patatin at pH 7 (I = 50 mM) is shown. The surface excess is given as a function of the protein concentration (c) over the specific interfacial area (A) in order to enable comparison with emulsions made with other proteins and different interfacial areas. At low concentrations the surface excess of emulsion droplets made with patatin increased fast with increasing concentration and most of the available patatin was adsorbed at the droplet interface. In Figure 3 also the maximum possible surface excess at any value of c/A is shown as a dashed line. It can be seen that at low concentration the experimental curve for patatin was close to this line and remained almost parallel to this line, even at higher concentrations. At higher concentrations the droplets became saturated with protein and a plateau value for patatin was reached at 2.6 mg/m^2.

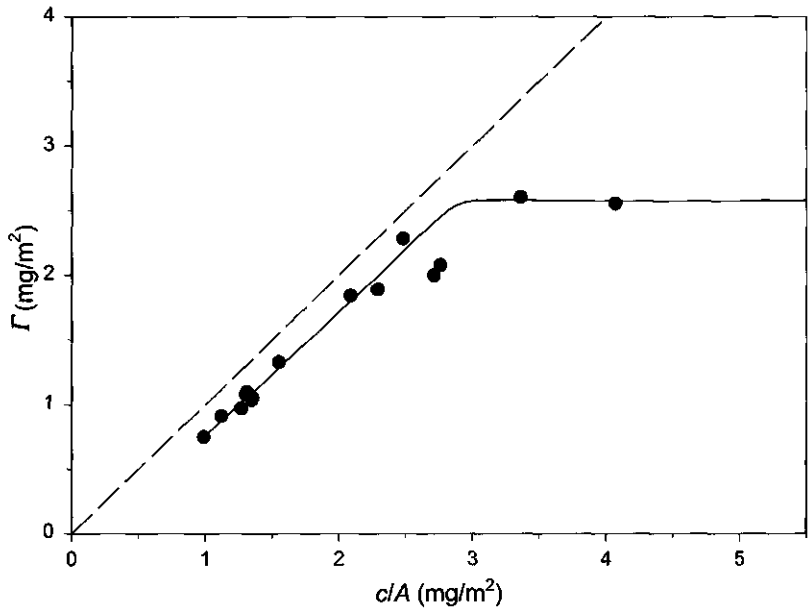

Figure 3: Surface excess (Γ; mg/m^2) of emulsions made with patatin (pH 7; I = 50 mM) as a function of protein concentration over specific surface area (c/A; mg/m^2)

Emulsions made at various pH

The effect of pH on the emulsifying properties of PPI, ASP, PIP and patatin was also studied. To this end emulsions were prepared at pH 5 and pH 3. For the patatin containing protein preparations (PPI, ASP and patatin) also a pH 3→pH 7 treatment was conducted in order to study at pH 7 the effect of the presence of patatin that had been unfolded at low pH (Pots et al., 1998b).

Emulsions at pH 5 were made at ionic strength of 200 mM in order to keep protein solubility in the preparations containing patatin, at its pI, as high as possible. Table 1 shows that the average droplet sizes of emulsions made with PPI and ASP were much larger than at pH 7. In emulsions made with PIP and patatin the droplet sizes at pH 5 were only somewhat larger than at pH 7. This difference can, however, not be completely attributed to the change in pH, since also the ionic strength was increased from 50 to 200 mM. When prepared at pH 5 the emulsion droplets made with PPI, ASP and PIP were still aggregated. Droplet aggregation was also observed in emulsions made with patatin at pH 5, which may also have been due to the increase in ionic strength. Droplet aggregates in emulsions made with ASP and PPI at pH 5 were smaller than at pH 7. No significant differences in surface excess were found between emulsions made at pH 5 and at pH 7 (Table 1).

Emulsions at pH 3 were made at an ionic strength of about 15 mM to prevent extensive aggregation and insolubility of pH-unfolded patatin (Chapter 3). In emulsions prepared at pH 3 with PPI, ASP and PIP droplet aggregation was no longer observed (Table 1). The average droplet size in emulsions made at pH 3 became significantly larger for PIP and patatin, and

significantly smaller for ASP, compared to the droplets in emulsions prepared at pH 7 (I = 15 mM). The surface excess did not change significantly on emulsion droplets prepared at pH 3, compared to the surface excess obtained at pH 7 (Table 1).

The pH 3→pH 7 treatment resulted for all protein preparations in droplet aggregation. For patatin and PPI the droplet aggregates became larger, while for ASP and PIP they became smaller than at pH 7 without prior pH adjustment. After the pH 3→pH 7 treatment the average droplet size in emulsions made with patatin was significantly larger than at pH 7 without pH 3→pH 7 treatment. Surprisingly, significantly smaller droplets were obtained with PPI after pH 3→pH 7 treatment than at pH 7 without pH adjustments. The pH 3→pH 7 treatment did not result in significant changes in the surface excess on the emulsion droplets (Table 1).

Effect of ionic strength and heat treatment at pH 7

Generally, no large effects on emulsion properties were observed when the ionic strength was reduced from 50 mM to ≈15 mM at pH 7, as can be seen from Table 1. For emulsions made with PIP lowering of the ionic strength resulted in a significantly smaller average droplet size. Generally, the size of the droplet aggregates became somewhat smaller when the ionic strength was decreased. The surface excess on emulsion droplets did not significantly change upon lowering the ionic strength (Table 1).

Heating (10 min, 80°C) of the protein preparations before emulsification always resulted in emulsions with aggregated droplets (Table 1). Generally, heat treatment had only a small effect on average droplet size, except in the case of PIP where the droplets became significantly smaller after heat treatment. For all protein preparations heat treatment resulted in emulsion droplets with a much higher surface excess, presumably due to adsorption of protein aggregates (Saito and Taira, 1987;Rientjes and Walstra, 1993;Smulders, 2000).

Changes in the oil composition

The average droplet sizes obtained for several of the emulsions made with potato proteins (Table 1) were observed to be unusually small when compared to those obtained for emulsions made with various other proteins using the same homogenizer and triglyceride oil (Smulders, 2000). Since patatin possesses lipid acyl hydrolase (LAH) activity (Galliard, 1971;Galliard and Dennis, 1974), it was hypothesized that patatin might have hydrolyzed tricaprylin and, thereby, have released surface active components such as free caprylic acid and monocaprylin. In order to verify the validity of this hypothesis the oil phase of several emulsions was extracted and analyzed using reversed phase HPLC. The chromatograms obtained for the extracts of these emulsions are shown in Figure 4. The peak identification was performed on the basis of the retention times of caprylic acid and tricapryline, while the elution order of the mono- and diglyceride was deduced from reported chromatograms of similar mixtures (Marcato and Cecchin, 1996;Hampson and Foglia, 1998;Xu et al., 2000).

The estimated concentrations of the different components, based on peak areas, are given in Table 2. The data in Figure 4 and Table 2, of extracts obtained within 1 hour after

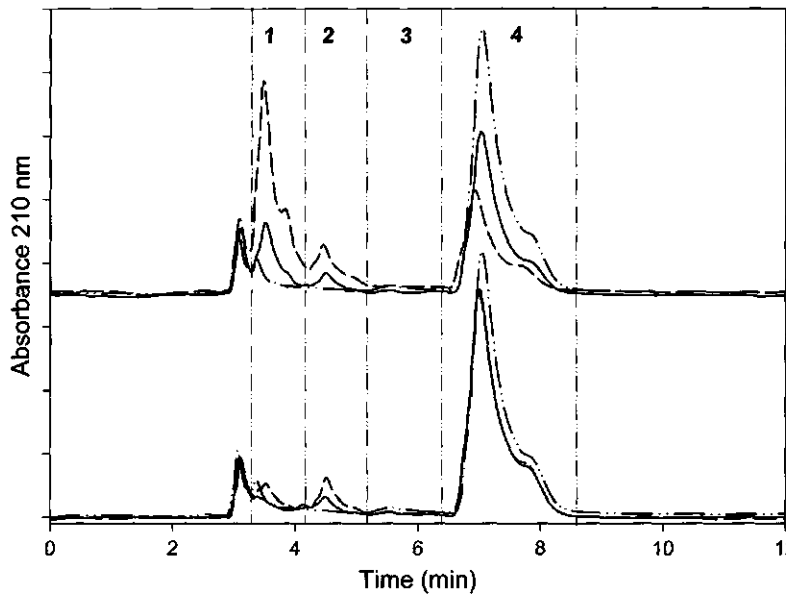

Figure 4: HPLC chromatograms of the lipid fractions of typical emulsions: **1.** caprylic acid; **2.** presumably monocaprylin; **3.** presumably dicaprylin; **4.** tricaprylin
Top: untreated tricaprylin (— ·· —), emulsified with PPI at pH 7 (solid) and PAT-5E (dashed)
Bottom: untreated tricaprylin (— ·· —), emulsified with heated ASP (solid) and PIP-5E (dashed)

emulsification, show that indeed significant amounts of both caprylic acid and monocaprylin were formed in the various emulsions. Table 2 shows that even in emulsions made with PIP-5E significant amounts of caprylic acid and especially monocaprylin were formed. PIP-5E was not expected to contain any patatin as judged from SDS-PAGE. The lowest rate of hydrolysis, as judged from Table 2, is obtained for heated ASP, in which the patatin present is almost completely irreversibly heat denatured. The highest rate of hydrolysis was obtained for PAT-5E, which contains > 95 % patatin as judged from SDS-PAGE. An extract was also made from an emulsion made with patatin at pH 7 directly after emulsification. The composition of this extract showed that during emulsification already 20 % of the tricaprylin present had been hydrolyzed and that the ratio monocaprylin/caprylic acid in the emulsion directly after preparation was higher than after storage for some time (Table 2). The activity of lipases on emulsion droplets can be very high, turnover numbers of up to 3000 s^{-1} have been reported (Eskin, 1990) and its lipolysis rate is proportional to the amount of interfacial area formed, when the amount of enzyme is not limiting (Desnuelle, 1961).

Table 2: Compositions of the lipid fractions of typical emulsions as obtained from the peak areas from HPLC chromatograms

	Caprylic acid (mg/ml)	Monocaprylin (mg/ml)	Dicaprylin (mg/ml)	Tricaprylin (mg/ml)
Before homogenization	1 (4) [1]	0 (0)	0 (0)	94 (200)
ASP (heated)	5 (25)	5 (22)	1 (2)	81 (173)
PPI (pH 7)	15 (95)	4 (19)	1 (3)	58 (123)
PIP-5E (pH 7)	8 (48)	7 (33)	1 (4)	81 (172)
PAT-5E (pH 7)	42 (287)	9 (40)	0 (0)	34 (72)

[1] Between parenthesis the concentration in mM is given

DISCUSSION

Correlation between expected LAH-activity and droplet size

It has been reported that patatin has only a very low activity on di and triacylglycerols (Andrews et al., 1988;Macrae et al., 1998). From the results shown it has become very clear that the activity of patatin on triglycerides has until now been greatly underestimated. The presence of monoglycerides, and perhaps also fatty acids, in addition to proteins may decrease the average droplet size of emulsions significantly as do other oil soluble small molecule emulsifiers (Courthaudon et al., 1991;Dickinson and Tanai, 1992;Dickinson et al., 1993;Gelin et al., 1996;Cornec et al., 1998). They cause a stronger reduction of the interfacial tension during emulsification than with protein alone (Dickinson, 1994), resulting in the ability to form larger γ-gradients at the droplet interface.

Smulders (Smulders, 2000) found a lowest average droplet size of about 0.8 μm for emulsions made with various proteins using the same homogenizer and triglyceride oil as was used in this study. If we correct this value for the higher homogenization pressure and the lower volume fraction of oil used in this study, we can estimate that the lowest average droplet size (d_{32}) attainable with only protein as a surfactant would be about 0.6 μm. The question now is, whether we can correlate the droplet sizes (< 0.6 μm) in Table 1 to the expected LAH-activity in that protein preparation. For the emulsions made with patatin this seems possible, since the droplets are larger after heating and at pH 3, which conditions are known to decrease the activity of patatin (Pots et al., 1998a;Pots et al., 1998b). The activity of patatin in PAT-5E was shown to be significantly higher than that of untreated patatin (Chapter 4), which may indirectly have resulted in the lower average droplet size obtained (Table 1). For PPI this relation is less clear because it does not explain the low average droplet size obtained after the pH 3→pH 7 treatment, which would reduce the LAH activity of patatin by 35 % (Pots et al., 1998b). For ASP the average droplet size obtained seems not related to the expected LAH-activity, since the average droplet size obtained seems to be almost independent of the conditions used, except at pH 5. The smallest droplets with ASP were obtained at pH 3, where the activity of patatin should be much lower than at pH 7. In PIP the presence of patatin could

not even be made visible using SDS-PAGE and emulsions made with PIP should therefore have average droplet sizes larger than 0.6 μm. Figure 3 and Table 2, however, indicate that traces of patatin, or a similar enzyme activity, must be present, because significant tricaprylin hydrolysis is observed and also the average droplet size becomes smaller than 0.6 μm in some cases (Table 1). From these results it seems therefore likely that only a trace amount of active patatin is enough to liberate enough surface active compounds from the oil to significantly reduce the surface tension during emulsification resulting in a decrease in droplet size. Whether this reduction in droplet size is in practice obtained, will depend also on the proteins used and the conditions applied as these will also determine the γ-gradients occurring at the droplet surface and hence determine the rate of recoalescence during emulsification and the resulting droplet size. In the present study, no care was taken to limit the time needed to make the final emulsions, since the effects of the formation of fatty acids and monoglycerides was established afterwards. In industrial practice much less time is consumed between mixing oil and protein solution and the liberation of small-molecule surfactants during emulsification may then be less significant. During storage of the emulsion lipolysis will proceed further. The production of free fatty acids will also in industrially produced emulsions, especially food emulsions, generally be considered undesired.

It is also clear that the small molecule surfactants liberated during emulsification do not strongly displace the proteins from the droplet surface, since the values obtained for the surface excess of emulsions made with non-heated potato proteins (1.4 - 3.8 mg/m2) are comparable to the values normally found for protein stabilized emulsions (Smulders, 2000). The absence of protein displacement, or even an increase in the protein surface excess (Dickinson et al., 1993), by not too high concentrations of oil-soluble small molecule surfactants, however, seems to be quite general (Walstra and De Roos, 1993;Cornec et al., 1998).

Effects of pH, ionic strength and heat treatment.

Compared to emulsions made at pH 7, emulsions made at pH 5 with the patatin containing preparations (PPI, ASP and patatin) showed droplet aggregation and in most cases an increase in droplet size, which is often observed for emulsions near the isoelectric pH of the protein used (Halling, 1981;Smulders, 2000). Droplet aggregation in emulsions made with PPI, ASP and PIP could only be prevented at pH 3. All of these preparations contain, in contrast to patatin, large amounts of protease inhibitors with pI's covering the range pH 5.1 – 9 (Chapter 5; Pouvreau et al., 2001). From this pI-range it can be argued that maximum electrostatic repulsion between emulsion droplets covered with these proteins would be obtained at acidic pH and could, thereby, explain that droplet aggregation in emulsions made with these preparations is absent only at pH 3. In contrast to our observations, Ralet and Guéguen (Ralet and Guéguen, 2000) did not report any droplet aggregation in the crude emulsions they prepared from whole potato protein or a potato protease inhibitor preparation. Moreover, they found that the stability against creaming in emulsions from these protein preparations was lowest at pH 4. They also concluded, using hexadecane as dispersed phase, that the emulsifying properties of a patatin rich preparation were inferior to those of the protease

inhibitor preparation (Ralet and Guéguen, 2000), which is very different from our results. Since in these emulsions hexadecane was used as the dispersed phase, obviously no lipolytic activity will have occurred. Also, in emulsions made with potato protein and soy oil (Holm and Eriksen, 1980) or corn oil (Jackman and Yada, 1988) no lipolysis was reported.
Heating of potato proteins resulted in emulsions with a much higher surface excess, which is often obtained for heated proteins and can be explained by the adsorption of heat induced protein aggregates (Saito and Taira, 1987;Rientjes and Walstra, 1993;Smulders, 2000). Lowering of the ionic strength, from 50 to ≈ 15 mM, at pH 7 resulted in emulsions with a decreased aggregate size, which can be possibly explained by an increase in the electrostatic repulsion distance between the protein layers on the droplet interface. The reduction of the droplet size of emulsions made with PIP upon lowering the ionic strength can, however, not yet be explained.

It can be concluded that if potato proteins are to be used in fat containing foods, which is the majority of foods, the activity of patatin should be reduced to a minimum, e.g. by extensive heat treatment, because otherwise the liberated fatty acids may almost immediately cause a "soapy" off-flavor. This study shows that only trace amounts of patatin, the lipase activity of which has been strongly underestimated, suffice to cause extensive lipolysis.
The application of potato protein in emulsions would, as can be concluded from this study, preferably be done at acidic pH. Heat denaturation of the patatin present, which would also diminish the protease inhibitor activities (Chapters 3 & 4), could be done at low pH as suggested by Ralet and Guéguen (Ralet and Guéguen, 2000) in order to prevent excessive protein aggregation.

ACKNOWLEDGMENT

Potatoes were kindly provided by AVEBE B.A. (Foxhol, The Netherlands). This research was supported by the Ministry of Economic Affairs through the programme IOP-Industrial Proteins and by AVEBE B.A.

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Chapter 7

General Discussion

INTRODUCTION

From the numerous publications on potato protein recovery (e.g. Meister and Thompson, 1976;Knorr et al., 1977;Knorr, 1980;Lindner et al., 1981;Wojnowska et al., 1981;Strætkvern et al., 1999) it has become clear that the recovery of a high quality protein product from industrial potato fruit juice is difficult and not straightforward. The main reason for this is that, in fact, only little is known about the protein and non-protein composition of PFJ and the behavior of these components under various conditions. Many, mostly globular proteins have been identified in potato tuber and potato fruit juice (see Chapter 1). However, when considering the SDS-PAGE analysis in Figure1, showing PFJ and two PFJ derived protein preparations described in this thesis, it can be seen that the majority of the proteins present in PFJ have molecular masses of 41 and 20 – 22 kDa, respectively, which correspond to patatin and protease inhibitors, respectively.

Recently, it was shown that the proteins in PFJ from cultivar *Elkana* consist for 90 % of patatin and protease inhibitors (Pouvreau et al., 2001).

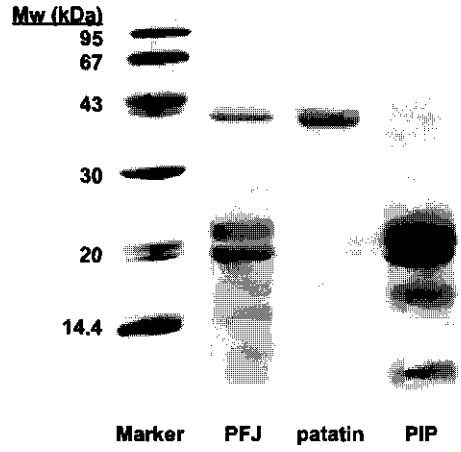

Figure 1: SDS-PAGE pattern (Coomassie staining) of PFJ, patatin and protease inhibitors (PIP)

The most important question which lead to the research described in this thesis was: Can potato proteins be recovered from PFJ at large scale, cost-effective, and in such a way that they retain their functional properties, most importantly their solubility? In addressing this question the effect of conditions during protein recovery on the foam and emulsion properties of the resulting protein product will be discussed first. Next, the effects of various conditions during protein recovery will be discussed in relation to the mechanisms and interactions involved. Because of the predominance of patatin and protease inhibitors, the research in this

thesis was not only aimed at protein preparations representing all proteins (ASP, PPI) but was also focussed on the properties of these two classes of proteins separately (patatin, PIP).

Behavior of potato proteins in foams and emulsions in relation to extent of unfolding

In Chapters 5 and 6 it was shown that the effects of the conditions applied during or after potato protein recovery have a large effect on the behavior of the proteins in emulsions and, especially, in foams.

By Pots and coworkers (Pots et al., 1998a;Pots et al., 1998b) and in Chapters 3 and 4, it was shown that patatin unfolds to various extents when heated, when subjected to low pH (3 – 5), and in the presence of ethanol, respectively. All these conditions were shown to enhance, to different extents, both foamability and, to a lesser extent, foam stability against Ostwald ripening, as compared to the foaming properties of untreated patatin at pH 7 (Chapter 5). The same conclusions possibly hold also for the emulsifying properties of patatin, but the experimental results on these properties are probably dominated by the hydrolytic release of small molecule surfactants (fatty acids and monoglycerides) from the oil phase during emulsification due to the unexpectedly high lipolytic activity of patatin (Chapter 6).

The structure of potato protease inhibitors (PIP) seems not to be affected at low pH and by precipitation in the presence of ethanol (Chapters 3 and 4). Ethanol precipitation does, however, result in a strong increase in the foamability of PIP and in a reduction of the average droplet size in emulsions, as compared to untreated PIP (Chapters 5 and 6). Heating does induce structural changes in the protease inhibitors, resulting in the loss of the largest part of their inhibiting activity (Chapter 3), and induces an increase in foamability and a decrease in emulsion droplet size (Chapters 5 and 6). However, the positive effects on foam formation are counteracted by a decrease in foam stability against drainage, which may be caused by a loss of protein solubility due to aggregation (Chapter 5).

The use of PPI, which can be regarded as ethanol precipitated ASP, also resulted in a higher foamability and in smaller emulsion droplets being formed than obtained by using ASP (Chapters 5 and 6). Further unfolding of the proteins in PPI, at low pH or by heating, had only a minor effect on its foaming and emulsifying properties (Chapters 5 and 6).

The general conclusion that can be drawn from this is, that partial unfolding of potato proteins, thereby increasing their structural flexibility, improves their performance at the surface of foam bubbles and possibly emulsion droplets, provided that this unfolding does not result in extensive aggregation and loss of solubility. Restricted unfolding of proteins, resulting in a greater structural flexibility, prior to their use as surfactant has been previously reported to improve their functionality in several cases (German and Phillips, 1991;Zhu and Damodaran, 1994;Nir et al., 1994;Wagner and Guéguen, 1999a;Wagner and Guéguen, 1999b). This effect is generally more important in foams than in emulsions.

POTATO PROTEIN RECOVERY AND PROTEIN SOLUBILITY

Protein solubility in relation to solvent quality and protein structure

Definition of solubility

Solubility of potato proteins, much like their performance in foams and emulsions, is affected

by protein structure and solvent conditions. As stated in Chapter 2, it should be realized that the solubility data presented in this thesis are not really solubility data, since these should be expressed as amount per unit volume. The solubility of a component is a well-defined property and is the amount of that component remaining in solution when it is in equilibrium with its crystals (or a liquid or gas phase), under well-defined conditions. Most proteins have well defined solubilities, which depend on factors like solvent quality, temperature, pH and ionic strength (see Chapter 1). Moreover, proteins can change their conformation (e.g. denaturation) in such a way that their solubility greatly alters (mostly decreases). In the case of a single protein, solubility data, e.g. at various pH, can be readily obtained if the protein is available in crystalline form. For protein mixtures this requires more effort, because the solution has to be saturated with every single protein present in the mixture and this would thus require large amounts of protein. The potato protein mixture in PFJ can not be processed in such a way that the solution becomes saturated with every single protein present. Instead the proportion of total protein that becomes "insoluble" was used, as has been done by several workers (Meister and Thompson, 1976;Knorr et al., 1977;Knorr, 1980;Knorr, 1982), even though the property determined is not well defined and does not give information about the changes in the solubilities of the proteins of which the saturation concentration has not been reached. When precipitates are resolubilized, the situation is even more complex because the resolubilization volume was kept constant, regardless of the amount of precipitate, and the resolubility was again expressed as proportion of total protein originally present. This means that, although a precipitate was always present after resolubilization the solution need not be saturated with all proteins, since some may have a high solubility and others may be present in small amounts. This means that both precipitation and resolubility data are not real solubility data; they indicate how average protein solubility differs from that at the starting conditions.

Heat induced changes in potato protein structure and solubility

In Chapters 3 and 4 it was shown that most potato proteins unfold at temperatures between 55 and 75°C. However, protein precipitation in PFJ already occurred at temperatures > 40°C. Patatin and protease inhibitors showed a somewhat different behavior upon heating. The rate of aggregation of the unfolded proteins, and their subsequent precipitation, will depend on the electrostatic repulsion between the proteins. This electrostatic repulsion depends on the net charge density on the protein surface, and thus on pH, and on the distance over which this repulsion acts, and thus on ionic strength. Pots and coworkers (Pots et al., 1999) showed that the rate of aggregation of patatin at pH 7 strongly increased with ionic strength at ionic strengths > 50 mM. This ionic strength dependence was also observed for the precipitation of patatin in Chapter 3; the fraction precipitating at pH 7 increased from about 5 % at $I \approx 15$ mM to about 95 % at $I = 200$ mM, although precipitation occurred only at temperatures > 60°C (Chapter 3). Analysis of heat induced aggregates of patatin, using SDS-PAGE, by Pots and coworkers (Pots et al., 1999) revealed the formation of a stable trimer. They presumed that covalent interactions kept the trimer together. In Chapter 3 results were obtained strongly indicating that patatin, which in non-dissociating media is a dimer (Racusen and Weller,

1984), thermally unfolds in its monomeric form. The formation of patatin monomers during heating would then induce the formation of trimeric patatin. It can even be hypothesized that the reactive particle in the reaction scheme proposed by Pots and coworkers (Pots et al., 1999), is the monomeric patatin.

Heat induced precipitation of the protease inhibitors was shown to be less dependent on ionic strength. Although in general they unfold at a higher temperature than does patatin, they start to precipitate already at 50°C. At low ionic strength heat induced unfolding of potato proteins, a mixture of patatin and protease inhibitors, does not result in extensive aggregation and precipitation (Chapter 3). At high ionic strength, which resembles the situation in PFJ, potato proteins were shown to be more thermostable. Their heat-induced unfolding at high ionic strength, however, immediately results in extensive aggregation and precipitation. Only a small fraction of the proteins needs to be unfolded before precipitation takes place and will cause precipitation to start at a lower temperature (Chapter 3).

Although the behavior of potato proteins in PFJ upon heating can be largely explained from the behavior of more purified protein preparations, it is likely that also interactions between proteins and non-protein PFJ components (Table 1 of Chapter 1) play a role (Haslam, 1998).

Changes in potato protein structure and solubility induced by pH in purified preparations

As can be seen in Figure 2, solubility of potato proteins in PFJ as a function pH, at various ionic strengths, showed an unusual trend. When increasing the ionic strength the apparent isoelectric pH shifted to lower pH-values, and solubility at that pH decreased as the ionic strength was increased.

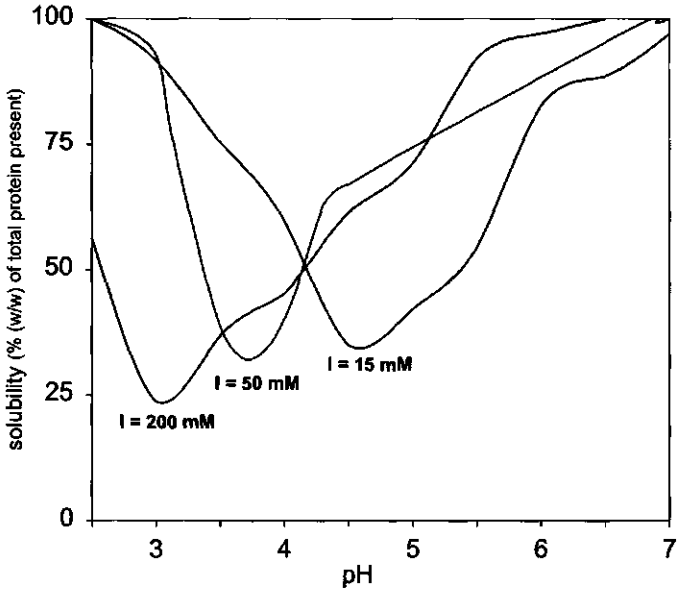

Figure 2: Solubility (pH 7 = 100 %) of potato proteins in PFJ as a function of pH at various ionic strengths

The aggregation of patatin unfolded at low pH was observed to depend on ionic strength much like patatin unfolded at high temperature, i.e. a much higher aggregation rate at high ionic strength. It was shown that at low pH and low ionic strength, presumably due the thickness of the electric double layer, the net charge on patatin provides enough electrostatic repulsion to prevent extensive aggregation and precipitation. At high ionic strength attractive interactions prevail and result in extensive aggregation and precipitation (Chapter 3). Solubility of the protease inhibitors as a function of pH was shown to be much less sensitive to ionic strength. Their solubility, however, decreased somewhat upon increasing the ionic strength (Chapter 3).

Protein solubility in PFJ at low pH

The same processes and interactions seem to take place in PFJ, as is shown in Figure 2, but these do not explain the shift in apparent pI that is observed with increasing ionic strength of previously dialyzed PFJ. It must also be noted that besides a difference in ionic strength, a difference in the presence of non-protein components is expected between the PFJ preparations depicted in Figure 2. The curves at ionic strengths of 15 and 50 mM were obtained for dialyzed PFJ, while the curve for ionic strength of 200 mM was obtained for untreated PFJ. Another difference between protein precipitation in purified protein fractions and that in PFJ is that protein precipitation in PFJ is always largely irreversible, i.e. the protein cannot be redissolved at neutral pH (Chapter 2), whereas in the more purified protein fractions protein precipitation was always reversible. The extent of resolubility, or more precisely redispersibility, was shown to be only weakly dependent on the pH at which the precipitate was formed (Chapter 2). If, however, the mechanism of protein precipitation would be, at least partly, changed to one that is less dependent on pH, resolubility could be increased. As was shown in Chapter 2 this can be done by adding metal salts, such as $FeCl_3$ and $ZnCl_2$, and even more efficiently by addition of organic solvents. Metal salts may, via complexing with amino acid residues, non-covalently and reversibly cross-link proteins and cause them to precipitate. By complexing with amino acid residues they may add to the stability of the proteins. Organic solvents were shown to irreversibly affect protein structure (Chapter 4); at high solvent concentrations, depending on the polarity of the solvent, this caused irreversible protein precipitation (Chapter 2). At moderate concentrations, and low temperature, these solvents are effective precipitants (Chapter 1), yielding protein precipitates with a high solubility at neutral pH (Chapter 2). In the next section it will be made plausible that both metal salts and organic solvents may also have affected potato protein precipitation and especially its reversibility in a completely different manner.

Interactions between proteins and phenolic compounds in relation to protein solubility

As mentioned in Chapter 1 (Table 1), PFJ contains several non-protein components, of which the phenolic compounds, mainly chlorogenic acid and caffeic acid, are the most interesting in relation to protein solubility, because of their recognized ability to bind to proteins (Haslam, 1998).

Among plant polyphenols, or more correctly plant phenolic compounds, polymeric and

monomeric compounds can be distinguished. The polymeric or perhaps more properly oligomeric phenolic compounds, also called tannins, include the widely spread proanthocyanidins (condensed tannins) and the less widely spread gallotannins (hydrolyzable tannins). Proanthocyanidins are oligomers of catechin or epicatechin monomers linked together via so-called interflavan bond (see Figure 4A). The gallotannins consist of glucose, which is esterified with a number of gallic acid residues, and which may also be polymerized via further esterification of these gallic acid residues.

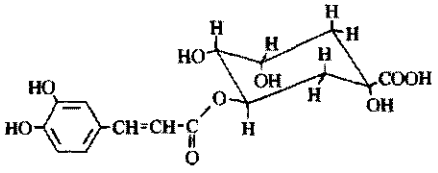

Figure 3: Structure of chlorogenic acid (1,3,4,5-tetrahydroxycyclohexanecarboxylic acid 3-(3,4-dihydroxycinnamate; pK_a 2.66)

The monomeric phenolic compounds include, among others, monomeric flavonoids (e.g. catechins), cinnamic acid derivatives and hydroxy benzoic acid derivatives. In potato the most important monomeric phenolic compounds, quantitatively, are chlorogenic acid (an ester of caffeic aicd and quinic acid, see Figure 3) and caffeic acid (Friedman, 1997;Lewis et al., 1998). The interactions between phenolic compounds and proteins can be subdivided into non-covalent and covalent interactions.

Non-covalent interactions

The non-covalent interactions between oligomeric phenolic compounds are believed to be dominated by hydrophobic bonding and also hydrogen bonds (Artz et al., 1987;Baxter et al., 1997). The initial interaction will presumably be hydrophobic and when water is removed from the hydrophobic patches on the protein hydrogen bonding may become important. The same interactions are reported to be involved in the binding of monomeric phenols to proteins (Muralidhara and Prakash, 1995;Suryaprakash et al., 2000). Oligomeric, and probably also monomeric, phenolic compounds were shown to have a higher affinity for less structured and unfolded proteins than for highly structured native proteins (Artz et al., 1987) Binding of phenolic compounds in itself may also cause protein unfolding (Muralidhara and Prakash, 1995;Kawamoto et al., 1997).

In contrast to monomeric phenolic compounds, oligomeric phenolic compounds are multidentate and can thus non-covalently cross-link proteins. The size of the aggregates formed by this cross-linking is dependent on the phenol-protein ratio. If a much lower amount of oligomeric phenolics than proteins is present then the aggregate size will be smaller than when about equimolar amounts of proteins and phenolic compounds are present. When an excess of oligomeric or monomeric phenols is present each of the available binding sites on the proteins will be bound with one phenolic compound only, and cross-linking will be virtually absent causing protein precipitation to occur due to a decrease of the polarity of the protein (McManus et al., 1985;Naczk et al., 1996;Siebert, 1999). Since the latter situation

may also apply to monomeric phenolics, it can be argued that the protein precipitating capacity of chlorogenic acid will be highest at acidic pH as its carboxyl group (pK_a = 2.66) will be protonated and electrostatic repulsion will be absent.
Generally, the non-covalent binding of monomeric as well as oligomeric phenolic compounds to proteins is observed to decrease protein solubility at pH values, 0.1 to 3.1 pH-units (Naczk et al., 1996), below their pI (Hagerman and Butler, 1981;Kawamoto and Nakatsubo, 1997).

Covalent interactions
At low pH proanthocyanidins may depolymerize due to a specific acid-catalyzed mechanism that is shown in Figure 4A (Beart et al., 1985). The carbocation that results from this reaction is extremely reactive and may react with nucleophilic groups on the protein surface, such as lysine, methionine, cysteine and tryptophan side-chains, and thus cause covalent protein cross-linking.

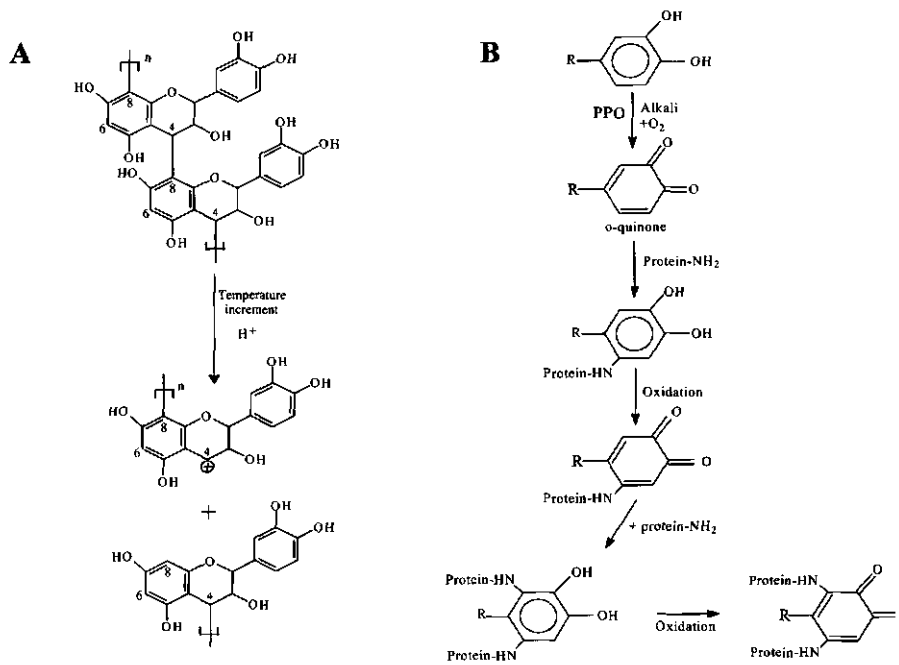

Figure 4: A: Depolymerization of proanthocyanidins at low pH or high temperature
B: Mechanism of covalent protein cross-linking by oxidized monomeric phenolic compounds (Adapted from (Kroll et al., 2000))

Also monomeric phenolic compounds may cause covalent protein cross-linking. *o*-diphenols, such as chlorogenic and caffeic acid, may be easily oxidized in the presence of polyphenoloxidase (PPO) or at alkaline pH to their corresponding quinones (Figure 4B). These quinones are highly reactive and normally react further to high molecular weight brown pigments. They may, however, also react with protein groups, as indicated in Figure 4B, such as the side-chains of lysine, methionine, cysteine and tryptophan (Kroll et al., 2000).

Rawel and coworkers (Kroll et al., 2000;Rawel et al., 2000;Rawel et al., 2001) have shown for various proteins that, at alkaline pH, these reactions do occur and result in protein modification and protein cross-linking with an average of three phenolic residues bound per protein molecule. The resulting proteins were shown to become insoluble at pH-values lower than their pI.

Possible effects of protein-phenolic compound interactions in PFJ

It can be concluded that, in general, phenolic compounds may cause proteins to become insoluble at pH-values below their isoelectric pH and may thus provide an alternative explanation for the behavior seen in Figure 2 and explain the irreversibility of potato protein precipitation at low pH. The presence of polymeric phenolics has not been reported in potato, although monomeric catechin has been found (Lewis et al., 1998) and proanthocyanidins may therefore be present in potato. Large amounts of especially chlorogenic acid, however, are present in PFJ (Table 1 of Chapter 1) and may thus, via a covalent or non-covalent mechanism, cause potato protein precipitation to become irreversible. The molar ratio of chlorogenic acid over protein may vary in PFJ from 0.6 to 4 (see Table 1 of Chapter 1). The reaction as indicated in Figure 4B should be inhibited by the presence of sulfite, which is added during the production of PFJ, but seems in practice not capable of completely inhibiting oxidation since browning in PFJ was always observed to some extent. Moreover, covalently bound phenolics were shown to be present in protein precipitates obtained from PFJ at low pH, since they co-eluted, as observed from their absorbance at $\lambda > 300$ nm, with the protein during gel filtration chromatography in 8 M urea (results not shown). The involvement of phenolics in potato protein precipitation also sheds a different light on the effects of metal salts and organic solvents on the reversibility of potato protein precipitation. Metal ions are known to form stable soluble or insoluble complexes with plant phenolic compounds (Haslam, 1989;Mila and Scalbert, 1996;McDonald et al., 1996;Ferrali et al., 1997). Addition of metal ions may, therefore, also prevent the formation of complexes between polyphenols and proteins. Also, organic solvents may reduce the interaction between phenolics and proteins (Hagerman et al., 1998;Siebert, 1999;Van Koningsveld et al., 2001). Moreover, at the low temperature at which organic solvents are applied (Chapters 2 and 4), hydrophobic association can be expected to be negligible. Both additives may thus prevent potato protein precipitation from becoming irreversible.

CONCLUDING REMARKS

In Table 1 a summary is given of the properties of patatin and protease inhibitors separately, and when both are together in PFJ. It is clear that both patatin and the protease inhibitors behave differently in PFJ and when separated, especially at low pH. More detailed information would, however, be needed on the protein and non-protein composition of PFJ, and the participation of phenolic compounds in protein precipitation, to completely understand the mechanisms involved in protein precipitation in PFJ.

Table 1: Properties of various potato protein preparations

	patatin	protease inhibitors	PFJ
Molar mass	41 kDa	16 – 22 kDa	4.3 - ?·kDa
T_D (°C)	60	60 - 70	55 - 75
Δh_{cal} (J/g)	13	25	21
Effect of pH on protein structure	yes	no	yes
pH ranges of reduced solubility	low *I*: 3.5 – 5.5 (**m**) high *I*: 3.0 – 4.0 (**l**) 4.0 – 5.5 (**m**)	low *I*: 3.2 – 5.7 (**m**) high *I*: 3.2 – 5.0 (**h**)	low *I*: 3.0 – 6.0 (**m**) high *I*: 2.5 – 4.0 (**l**) 4.0 – 5.5 (**m**)

T_D = denaturation temperature; Δh_{cal} = calorimetrically determined enthalpy of unfolding per unit mass **l** = relatively low solubility; **m** relatively moderate solubility; **h** relatively high solubility

In chapter 6 it was shown that the emulsifying properties of various potato protein preparations were mainly dominated by the lipolytic release of surface active fatty acids and monoglycerides from the oil phase by patatin, the lipase activity of which has been strongly underestimated. Therefore, if potato proteins are to be used in food emulsions, the lipolytic activity of patatin should be reduced to a minimum, e.g. by extensive heat treatment, because otherwise the liberated fatty acids may cause the product to taste "soapy" almost immediately. It was shown that only trace amounts of patatin are enough to cause significant lipolysis. Much more research would be necessary to completely characterize the emulsion forming and stabilizing properties both in the complete absence and in the controlled presence of fatty acids and monoglycerides.

In addition to the differences summarized chapter 5 it was shown that less foam could be formed from untreated patatin than from the protease inhibitors, but patatin foam was much more stable. The foam forming properties of patatin could be strongly improved by partial unfolding of the protein. Foams formed from ethanol precipitated potato proteins (PPI) were observed to be more stable than those made with β-casein and β-lactoglobulin under the same conditions.

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In potato starch manufacture an aqueous byproduct remains that is called potato fruit juice (PFJ). On a dry matter basis PFJ contains about 20-25 % protein and amino acids, 15 % sugars, 20 % minerals, 14 % organic acids and other components, such as phenolic compounds. Potato protein has a relatively high nutritional quality, comparable to that of whole egg, and it therefore has high potential for utilization in food applications. Protein recovery from industrial PFJ is presently achieved through heat coagulation by steam injection after pH adjustment. This method is very efficient in removing protein from solution. However, it leads to protein precipitates that exhibit a poor solubility, which hampers potential food applications.

An economic method to efficiently recover soluble potato protein would considerably increase its possibilities for use in food and add to its commercial value. Therefore, the important question resulting in this study was: can potato proteins be recovered from PFJ in such a way that they retain their functional properties, most importantly their solubility? This recovery method should be applicable at a large scale and result in a high yield. Potato protein recovery was expected to be complicated by the presence of and the interactions with non-protein components in PFJ. The objective in this study was to examine how extrinsic factors like pH, ionic strength and temperature would influence the structure of potato proteins, this in relation to the functionality of the proteins in making and stabilizing foams and emulsions.

Three groups of potato proteins can be distinguished in PFJ. Patatin, the major potato tuber protein, comprises 38 % of the protein in PFJ from cultivar *Elkana*. The protease inhibitors make up about 50 % and other proteins up to 12 % of total protein in PFJ from cultivar *Elkana*.

In Chapter 2 the effects of pH and various additives on the precipitation and (re)solubility at pH 7 of potato proteins from industrial PFJ are studied. Addition of various strong and weak acids caused the same extent of protein precipitation, which comprised at the most 60 % of total protein at pH 3. The use of weak acids, however, resulted in an increase in the resolubility of the precipitates at pH 7, as compared to strong acids. At pH 5 addition of $FeCl_3$ or $ZnCl_2$ increased both precipitation and resolubility. The largest increase in precipitation and resolubility was achieved by using organic solvents, resulting in a maximum precipitation (pH 5) of 91 % of total protein and a maximum resolubility of 91 % of precipitated protein. The results described in Chapter 2 lead to the hypothesis that precipitation and resolubilization of potato proteins from PFJ is not so much determined by their isoelectric pH but by their interactions with low molecular weight components.

In Chapter 3 it was shown, using DSC and both far-UV and near-UV CD spectroscopy, that potato proteins unfold between 55°C and 75°C. Increasing the ionic strength from 15 to 200 mM generally caused an increase in denaturation temperature. It was concluded that the dimeric protein patatin unfolds either in its monomeric state or that its monomers are loosely associated and unfold independently. Thermal unfolding of the protease inhibitors was correlated with a decrease in protease inhibitor activities and resulted in an ionic strength dependent loss of protein solubility. Potato proteins were best soluble at neutral and strongly

acidic pH. At mildly acidic pH the overall potato protein solubility was dependent on ionic strength and the presence of unfolded patatin.

In Chapter 4 a protein isolate with a high solubility at neutral pH prepared from industrial PFJ by precipitation at pH 5 in the presence of ethanol is described. The effects of ethanol itself and the effects of its presence during precipitation on the properties of various potato protein fractions were examined. The presence of ethanol significantly reduced the denaturation temperature of potato proteins, indicating that preparation of this potato protein isolate should be done at low temperature to retain a high solubility. In the presence of ethanol the thermal unfolding of the tertiary and the secondary structure of patatin were shown to be almost completely decoupled. Even at 4°C precipitation of potato proteins in the presence of ethanol induced significant conformational changes. These changes did, however, only result in minor changes in the solubility of the potato protein preparations.

In Chapter 5 foam forming and stabilizing properties of potato proteins are described; whipping or sparging was used to make foam. The performed whipping tests showed that less foam could be formed from untreated patatin than from the protease inhibitors, but also that patatin foam was much more stable against coalescence, Ostwald ripening and drainage. The foam forming properties of patatin could be strongly improved by partial unfolding of the protein. Whipping tests, at both low (0.5 mg/ml) and high (10 mg/ml) protein concentrations, also indicated that foams made with an ethanol precipitated protein isolate (PPI) were more stable against Ostwald ripening and drainage than those made with β-casein and β-lactoglobulin. More generally it was concluded that when proteins are used as a foaming agent, a high concentration is required, because the available protein is inefficiently used. Also, the different methods used to make foam, result in changes in the mutual differences in foaming properties between the various protein preparations and may induce different instabilities to become apparent in foams made at the same conditions.

In Chapter 6 emulsions made with various potato protein preparations were characterized with respect to average droplet size, plateau surface excess and the occurrence of droplet aggregation. The average droplet size of the emulsions made with potato proteins appeared to be determined by the lipolytic release of surface active fatty acids and monoglycerides from the tricaprylin oil phase during the emulsification process. It was concluded that only trace amounts of patatin, the lipase activity of which has been strongly underestimated in literature, sufficed to liberate significant amounts of these surfactants. The plateau surface excess of emulsions made with patatin was found to be 2.6 mg/m^2, while emulsion droplets made with protease inhibitors showed a significantly smaller surface excess. Of the various solvent conditions and treatments applied only heat treatment resulted in a significant increase in surface excess. Droplet aggregation in emulsions made with potato protein preparations other than patatin, could in contrast to at pH 5 and at pH 7 be prevented at pH 3.

In Chapter 7 the relations between potato protein structure, solubility and foam and emulsion forming and stabilizing properties are discussed. Also, the different mechanisms by which phenolic compounds may affect protein solubility are discussed in relation to the solubility and resolubility behavior of potato proteins in PFJ and when separated. A summary of the most important differences in the properties of patatin and protease inhibitors is also given.

Fysisch-chemische en functionele eigenschappen van aardappeleiwitten

In de aardappelzetmeelindustrie blijft na de zetmeelisolatie een vloeibaar bijproduct over dat aardappelvruchtwater (PFJ) wordt genoemd. De droge stof die dit PFJ bevat bestaat voor ca. 20 % uit eiwitten, voor ca. 15 % uit suikers, voor ca. 14 % uit organische zuren en uit ander componenten zoals fenolische verbindingen. Aardappeleiwit heeft een relatief hoge voedingswaarde, zeker voor een planteneiwit, die vergelijkbaar is met die van eiwit uit kippeneieren. Aardappeleiwit zou daarom goed in levensmiddelen toegepast kunnen worden. Het winnen van aardappeleiwit uit PFJ wordt tegenwoordig uitgevoerd door middel van hitteprecipitatie met behulp van stoominjectie. Deze methode, die erg efficiënt is, leidt tot eiwitproducten die slecht oplosbaar zijn, wat hun toepassing als levensmiddeleningrediënt verhindert. Het zou daarom voor de toepassing van aardappeleiwit in levensmiddelen van groot belang zijn als er een methode zou zijn waarmee op een economisch haalbare manier goed oplosbaar eiwit uit het PFJ zou kunnen worden gewonnen. De belangrijkste onderzoeksvraag, die leidde tot deze studie was dan ook: kunnen aardappeleiwitten zo worden gewonnen, dat hun oplosbaarheid en andere mogelijk functionele eigenschappen niet verloren gaan? Deze winningmethode zou op een grote schaal toepasbaar moeten zijn en een hoge eiwitopbrengst moeten opleveren. Ook werd op voorhand rekening gehouden met de mogelijkheid dat de aanwezigheid van niet-eiwitverbindingen deze eiwitwinning aanzienlijk zou kunnen bemoeilijken. Het doel van deze studie was te onderzoeken hoe factoren, zoals pH, ionsterkte en temperatuur de structuur van de aardappeleiwitten zouden beïnvloeden en wat de invloed van eventuele structuurveranderingen zouden zijn op de oplosbaarheid en het gedrag van de aardappeleiwitten in schuim en emulsies.

De eiwitten in PFJ kunnen worden verdeeld in drie groepen. Patatine, het meest voorkomende oplosbare aardappeleiwit, beslaat ongeveer 38 % van het eiwit in PFJ gemaakt van het ras *Elkana*;. 50 % bestaat uit protease remmers en 12 % uit andere eiwitten.

In hoofdstuk 2 worden de effecten van pH en diverse toevoegingen op de precipitatie en heroplosbaarheid, bij pH 7, van aardappeleiwitten uit PFJ beschreven. Het gebruik van verschillende sterke en zwakke zuren leidde niet tot wezenlijke verschillen in de mate van eiwitprecipitatie, die hoogstens 60 % van het aanwezige eiwit bedroeg. Het gebruik van zwakke zuren tijdens precipitatie leidde echter wel tot een toename van de heroplosbaarheid van het eiwitprecipitaat bij pH 7. Het toevoegen van $ZnCl_2$ of $FeCl_3$ verhoogde zowel de precipitatie bij pH 5, als de heroplosbaarheid van het precipitaat bij pH 7. De hoogste waarden voor precipitatie en heroplosbaarheid werden verkregen door de toevoeging van organische oplosmiddelen bij pH 5, waar bij 91 % van het aanwezige eiwit kon worden geprecipiteerd en 91 % daarvan weer kon worden opgelost bij pH 7. De resultaten in hoofdstuk 2 leidden ook tot de hypothese dat de precipitatie en de heroplosbaarheid van aardappeleiwitten uit PFJ niet alleen worden bepaald door hun isoelektrische pH maar ook door interacties met laag moleculaire verbindingen.

In hoofdstuk 3 wordt aangetoond, uit resultaten verkregen met DSC en zowel ver-UV als nabij-UV circulair dichroïsme spectroscopie, dat aardappeleiwitten ontvouwen bij een temperatuur tussen 55°C en 75°C. Verhoging van de ionsterkte van 15 naar 200 mM leidde in het algemeen tot een toename van de thermostabiliteit van aardappeleiwitten. Er kon ook worden geconcludeerd dat de thermische ontvouwing van het dimere eiwit patatine plaatsvindt òf in de monomere toestand van het eiwit, òf dat de beide monomeren slechts een zwakke onderlinge interactie vertonen en onafhankelijk van elkaar ontvouwen. De thermische ontvouwing van de proteaseremmers ging gepaard met een verlies van hun remmende activiteit en oplosbaarheid. Er werd waargenomen dat aardappeleiwitten het best oplosbaar zijn bij neutrale en sterk zure pH. Bij zwak zure pH bleek de oplosbaarheid van aardappeleiwitten vooral te worden bepaald door de ionsterkte en de aanwezigheid van ontvouwen patatine.

In hoofdstuk 4 word een methode beschreven om aardappeleiwitisolaat (PPI) te maken met een hoge oplosbaarheid bij neutrale pH. Dit isolaat werd vervaardigd met behulp van precipitatie van het in PFJ aanwezige eiwit in aanwezigheid van 15-20 % (v/v) ethanol bij pH 5. De effecten van de aanwezigheid van ethanol, al dan niet tijdens precipitatie, op de eigenschappen van verschillende aardappeleiwitpreparaten werd onderzocht. De aanwezigheid van ethanol verlaagde de ontvouwingstemperatuur van verschillende aardappeleiwitten aanzienlijk, wat er op wees dat de productie van aardappeleiwitisolaat bij lage temperatuur zou moeten plaatsvinden om een goede oplosbaarheid te behouden. In de aanwezigheid van ethanol bleken de secundaire en tertiaire structuur van patatine bij temperatuurverhoging volledig onafhankelijk van elkaar te ontvouwen. Experimenten toonden aan dat, zelfs bij 4°C, de precipitatie van aardappeleiwitten in aanwezigheid van ethanol leidde tot significante veranderingen in de eiwitstructuur. Deze veranderingen bleken echter slechts tot kleine veranderingen te leiden in het oplosbaarheidsgedrag van aardappeleiwitpreparaten, als functie van zowel pH als verhittingstemperatuur.

In hoofdstuk 5 worden de schuimvormende en –stabiliserende eigenschappen van diverse aardappeleiwitpreparaten beschreven met behulp van opklop- en "sparging"testen. De gebruikte opkloptesten wezen uit dat van patatine minder schuim kon worden gevormd dan van proteaseremmers, maar dat de stabiliteit van het patatine schuim tegen coalescentie, Ostwald vergroving en drainage aanzienlijk hoger was. De schuimvorming door patatine kon sterk worden verhoogd door het eiwit gedeeltelijk te laten ontvouwen. Opkloptesten, bij zowel lage (0.5 mg/ml) als hoge eiwitconcentratie (10 mg/ml), toonden aan dat schuim gemaakt met ethanol geprecipiteerd eiwitisolaat stabieler was tegen drainage en Ostwald vergroving dan schuim gemaakt met de melkeiwitten β-caseïne en β-lactoglobuline. In het algemeen kon worden geconcludeerd, dat als eiwitten worden gebruikt als schuimvormers ze in hoge concentratie aanwezig moeten zijn, omdat het aanwezige eiwit inefficiënt wordt gebruikt voor de schuimvorming. Ook kunnen verschillende methoden om schuim te maken, leiden tot geheel andere onderlinge verschillen in de schuimeigenschappen van de verschillende eiwitpreparaten en zelfs tot het ontstaan van andere instabiliteiten in schuim gemaakt van hetzelfde eiwit onder dezelfde fysisch-chemische condities.

In hoofdstuk 6 worden emulsies gemaakt met verschillende aardappeleiwitpreparaten beschreven en gekarakteriseerd wat betreft gemiddelde druppelgrootte, eiwitbelading en het voorkomen van druppelaggregatie. De gemiddelde druppelgrootte van emulsies gemaakt met aardappeleiwitten bleek naar alle waarschijnlijkheid volledig te worden bepaald door het vrijkomen van oppervlakteactieve vetzuren en monoglyceriden, die het gevolg zijn van door patatine gekatalyseerde lipolyse, gedurende het emulgeren. Er kon worden geconcludeerd dat slechts een kleine hoeveelheid actieve patatine, waarvan de lipase-activiteit tot nut toe in de literatuur sterk is onderschat, voldoende was om significante hoeveelheden van deze oppervlakteactieve stoffen te produceren. Voor de eiwitbelading van emulsies gemaakt met patatine werd een plateauwaarde van 2.6 mg/m^2 gevonden. Voor emulsies gemaakt met protease remmers werd een significant lagere waarde gevonden. Van de verschillende condities en behandelingen die gebruikt werden, leidde alleen verhitten van het eiwit vóór het emulgeren tot een hogere eiwitbelading. In emulsies van aardappeleiwitpreparaten kon bij pH 3, in tegenstelling tot bij pH 5 en pH 7, het aggregeren van de emulsiedruppels worden voorkomen.

In hoofdstuk 7 wordt de structuur van aardappeleiwitten onder verschillende condities bediscussieerd, in relatie tot hun oplosbaarheid en schuim- en emulsievormende en -stabiliserende eigenschappen. Ook worden verschillende mechanismen bediscussieerd waardoor fenolische verbindingen de eiwitoplosbaarheid kunnen beïnvloeden, dit in relatie tot de oplosbaarheid en de heroplosbaarheid van aardappeleiwitten uit PFJ en in geïsoleerde vorm. Een vergelijking wordt ook gemaakt tussen de belangrijkste eigenschappen van patatine en proteaseremmers.

Zo, na het afronden van dit proefschrift kan ik, na vijf jaar afwezigheid, terugkeren naar het "echte" leven met zijn vrije tijd, hobby's en dergelijke. Het is zowel op niet-wetenschappelijk als wetenschappelijk gebied een leerzame periode geweest, die zelfs ik niet zonder de hulp van anderen zou zijn doorgekomen.

Allereerst wil ik mijn beide promotoren, Pieter Walstra en Fons Voragen, bedanken. Pieter, je brede kennis op velerlei gebieden is van onschatbare waarde voor dit proefschrift geweest. Ondanks dat je jouw mening nooit onder stoelen of banken stak, accepteerde en waardeerde je ook dat ik soms een andere mening had. Fons, doordat je wat verder van mijn onderzoek af stond dan de rest van mijn begeleiders, wist je vaak de zwakke en onbegrijpelijke passages in de manuscripten moeiteloos aan te geven.

Ook mijn beide co-promotoren, Tiny van Boekel en Harry Gruppen, wil ik hierbij bedanken voor hun begeleiding. Tiny, je nauwkeurige correctie van de manuscripten en je belangstelling voor het onderzoek heb ik erg gewaardeerd. Harry, jij was altijd degene die overal de grote lijn in wist aan te brengen. Vooral je inzet in de laatste fase van het afronden van dit proefschrift was enorm en heeft er voor gezorgd dat het "op tijd" af was. Bovendien lever je als kamergenoot een grote dosis relativerend vermogen. Daarnaast is ook de rol van Harmen de Jongh zeker niet te verwaarlozen geweest. Zijn capaciteiten in het organiseren en focussen van onderzoek zijn samen met zijn uitgebreide theoretische en praktische kennis betreffende de structuur van eiwitten van doorslaggevende betekenis geweest voor de hoofdstukken 2, 3 en 4. Ook wil ik Martin Hessing en Gerrit Wijngaards en hun collegae bedanken voor hun bijdragen aan dit proefschrift vanuit TNO-Voeding in Zeist. Daarnaast wil ik ook de leden van de IOP-begeleidingscommissie bedanken voor de prettige sfeer en de nuttige commentaren tijdens de vergaderingen, en in het bijzonder Rick Nijssen voor zijn belangstelling en nuttige bijdragen aan het project vanuit AVEBE.

Ook een aantal andere mensen zou ik willen bedanken voor een bijdrage waaraan en in welke vorm dan ook. Allereerst, aardappelvriend van het eerste uur en paranimf, André Pots. Jouw proefschrift is voor mijn onderzoek van essentieel belang geweest als mede ook de discussies tijdens en na jouw verblijf hier op de vakgroep. The research on potato proteins is now successfully continued by my other paranimf and friend Laurice Pouvreau. Ook de bijdragen van drie studenten zijn van belang geweest voor het tot stand brengen van dit proefschrift en toekomstig onderzoek op het gebied van aardappeleiwit. Julia, Susan en Eef, bedankt. De laatste fase van mijn onderzoek zou ik niet succesvol hebben kunnen afronden zonder de hulp van Franklin Zoet en Katja Grolle. Hierbij wil ik dan ook meteen alle rokers binnen het Biotechnion bedanken voor de ontspannen pauzes en Bruno en Pieter voor hun gastvrijheid in de proceshal, na de invoering van het algehele rookverbod.

I also want to thank all colleagues from the Centre for Protein Technology, Food Chemistry and the WCFS for the pleasant working atmosphere and for listening to my mostly useless comments.

Verder wil ik alle mensen in mijn naaste omgeving bedanken voor hun steun en geduld; blijkbaar is het niet voor niets geweest. Nicoliene, het wordt tijd dat we meer vrije tijd gaan krijgen.

Curriculum vitae

Gerrit van Koningsveld werd op 6 november 1968 geboren te Leeuwarden. In 1988 behaalde hij het VWO-diploma aan het Andreas College te Drachten. In datzelfde jaar startte hij met de opleiding HLO-chemie, met als specialisatie levensmiddelen, aan de Noordelijke Hogeschool te Leeuwarden. Deze opleiding rondde hij in 1992 af met het behalen van het diploma. Vervolgens volgde hij een driejarig doorstroomprogramma scheikunde, met als specialisatie biochemie, aan de Rijksuniversiteit Groningen, alwaar hij in 1995 afstudeerde.
Vanaf december 1995 tot oktober 2000 werkte hij als AIO aan de Landbouwuniversiteit Wageningen bij de leerstoelgroep Levensmiddelenchemie. Het onderzoek tijdens de periode staat beschreven in dit proefschrift.
Sinds oktober 2000 werkt hij als post-doc bij de leerstoelgroep levensmiddelenchemie.

Printing: Ponsen & Looyen bv, Wageningen

The research described in this thesis was financially supported by the Dutch Ministry of Economic Affairs through the program IOP Indutrial Proteins and AVEBE ba, the Netherlands