# Effects of pH and Heat Treatments on the Structure and Solubility of Potato Proteins in Different Preparations

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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 and 75 °C. Increasing the ionic strength from 15 to 200 mM generally caused an increase in denaturation temperature. It was concluded that either the dimeric protein patatin unfolds 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 values. 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.

**Keywords:** *Potato; Solanum tuberosum; patatin; protease inhibitor; solubility; structural stability; differential scanning calorimetry*

## INTRODUCTION

Potato protein has a relatively high nutritional quality, comparable to that of whole egg (*1*, *2*), and it, therefore, has high potential for utilization in food applications. The soluble proteins can be tentatively classified into three classes: patatin (43 kDa), protease inhibitors (5−25 kDa), and others (mostly high $M_w$) (*3*). 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.

A more profitable utilization of potato proteins would be of economical interest for potato starch manufacturers, so several efforts have been made to recover potato proteins from PFJ that have retained their native properties, of which solubility is most important. Because most of the proteins in PFJ have isoelectric points at acidic pH (*4*), most research has been devoted to precipitation of potato protein at low pH (*5*−*8*). Precipitation at low pH may, however, severely affect the molecular structure of potato proteins (*9*). A recent publication by Strætkvern et al. (*10*) uses expanded bed adsorption ion exchange chromatography as a large-scale technique for the isolation of patatin, but this method will in practice be too expensive for the recovery of food proteins.

The effects of heat treatments on potential food proteins need also to 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 (*11*).

The purpose of this study was to examine the effects of pH and heat treatment on the conformation and conformational stability of various potato protein fractions. Because protein solubility is a prerequisite for functional application of proteins in foods (*12*), the effects of structural changes on the solubility characteristics of potato proteins were also studied.

## MATERIALS AND METHODS

**Preparation of Protein Fractions.** *1. PFJ.* Potatoes (cv. Elkana) were washed thoroughly with water and cut into pieces (maximum = 8 × 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, Dassel, Germany). The resulting clear yellowish filtrate has a pH of 5.7−6.0. The procedure used was provided by a potato starch manufacturer (AVEBE B.A., Foxhol, The

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Netherlands) and is known to result in a PFJ that is comparable in composition to industrial PFJ and is further denoted as PFJ. PFJ, on average, contained 3.59 ± 0.09 (SD) mg of nitrogen/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 × 6.25) concentration of 13.4 ± 0.9 mg of protein/mL of PFJ.

*2. Ammonium Sulfate Precipitate (ASP).* ASP was prepared from PFJ by adding $(NH_4)_2SO_4$ to 60% saturation as proposed in ref *13* while the pH was maintained at 5.7 by the addition of small volumes of 0.5 M $H_2SO_4$. After 1 h at 4 °C, the suspension was centrifuged (30 min, 19000*g*, 4 °C) and the resulting precipitate 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) 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 10 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 the unbound compounds had been washed out using 5 column volumes of the above-mentioned buffer, 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) 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 an MWCO of 10 kDa (A/G Technology Corp., Needham, MA) 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).* PIP proteins were prepared by gel filtration of PFJ on a Superdex 75 column (63 × 10 cm) (Amersham Pharmacia Biotech AB) eluted with a 30 mM sodium phosphate buffer (pH 7) containing 0.5 g/L $NaHSO_3$ with a 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 an MWCO of 5 kDa (A/G Technology Corp.) 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.** The protein compositions of PFJ, ASP, PIP, and patatin were 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) 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).

**Circular Dichroism (CD) Spectroscopy.** *Sample Preparation.* CD experiments were performed to investigate the effect of various treatments on the conformations and thermal stabilities of patatin and PIP. Conformational characteristics and thermal stabilities 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 the addition of NaCl.

The conformation and 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 h, the turbid solutions were centrifuged (15000*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 PAT-5.

The effect of pH on the conformation of proteins in PIP was studied at pH 7, 5, and 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 ($I \approx 200$ 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.) 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 6 min of heating 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 stabilities of the secondary structures 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 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 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 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). Thermograms were recorded from 20 to 90 °C with a heating rate of 20 K/h. DSC experiments were conducted with untreated PFJ (13.5 mg of 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 NaCl ($I \approx 200$ mM).

**High-Performance Size Exclusion Chromatography (HP-SEC).** HP-SEC experiments at various temperatures (30−80 °C) were performed with a Spectra Physics P1000 solvent delivery system equipped with an AS3000 autosampler (Thermo Separations Products, Fremont, CA) and an SpH 99 column oven (Spark Holland, Emmen, The Netherlands). Patatin (0.85 mg/mL) samples were preheated during 10 min in a thermostated water bath. Quantities of 100 μL of preheated sample were injected on a TSK gel G2500PWXL column [7.8 (i.d.) × 300 mm] (TosoHaas, Montgomeryville, PA) equilibrated and eluted with a thermostated 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 Separations Products) 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 ($I \approx 200$ 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 h 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 (*14*) when PFJ samples were used. When protein fractions other than PFJ were used, supernatants were analyzed for protein content using the method of Bradford (*15*) 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 a 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 thermostated water bath (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.** The LAH activity of samples containing patatin was measured using *p*-nitrophenyl (PNP) butyrate as a substrate. Further conditions were as described by Pots et al. (*9*). The substrate concentration used was 0.41 mM. The specific LAH activity was determined from the initial rate of PNP release and expressed as micromoles per minute per milligram of 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. (Zwijndrecht, The Netherlands). Trypsin inhibitor activity was estimated by using the Kakade method as modified by Smith et al. (*16*) with 0.36 mM benzoyl-DL-Arg-*p*-nitroanilide (Merck, Darmstadt, Germany) as a chromogenic substrate. Chymotrypsin inhibiting activity was estimated according to the method of Geiger (*17*) with 0.88 mM *N*-succinyl-L-Phe-*p*-nitroanilide (Sigma) as a substrate.

Inhibition of papain was estimated according to the method of Mole and Horton (*18*) with 2.53 mM benzoyl-L-Arg-*p*-nitroanilide (Merck) as a substrate. Cathepsin D inhibiting activity was estimated according to the method of Van Jaarsveld et al. (*19*) with 0.67% (w/v) of acid-denatured hemoglobin as a substrate. Carboxypeptidase A inhibiting activity was measured as described by Riordan and Holmquist (*20*) with 0.1 mM *N*-(furanacryloyl)-L-Phe-L-Phe (Bachem, Bubendorf, Switzerland) 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 percent activity remaining per volume of supernatant relative to the activity present in an unheated solution.

**Table 1. Relative Composition of Various Potato Protein Fractions on the Basis of p*I***

| sample | p*I* < 5.2 | 5.2 < p*I* < 6 | 6 < p*I* < 7 | 7 < p*I* < 8 | p*I* > 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 total density of the protein bands within one lane. [b] The number in parentheses is the number of protein bands detected.

## RESULTS

**Protein Composition.** The IEF gels (not shown) of the various potato protein fractions all showed several protein bands in the p*I* range from pH 4.5 to >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 p*I* range and expressed as a proportion of the total density of the protein bands within one sample. The data for PIP and patatin in Table 1 are presented proportionally to their relative presence in PFJ of 45 and 38%, respectively (*21*). The number of bands indicated in 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 shows that the potato juice (PFJ) contained ~38% patatin, consisting of 11 protein bands with p*I* values between 4.5 and 5.2 representing different isoforms of patatin (*22*). About half of the total amount of protein in PFJ had an isoelectric pH below pH 6 (Table 1). The ASP, which represents ~75% of total potato protein, contained relatively more patatin and proteins with a high isoelectric pH (p*I* > 8) as compared to PFJ. The 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 evenly distributed over a wide range of isoelectric pH values. The distribution of p*I* values in PIP was similar that observed by Pouvreau et al. (*21*).

**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. (*9*, *11*), 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.

The thermal unfoldings 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 nonstructured parts are presumed to be virtually absent in this spectral region (*23*). Both curves show a small gradual decrease in absolute ellipticity with temperature up to about 50 and >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

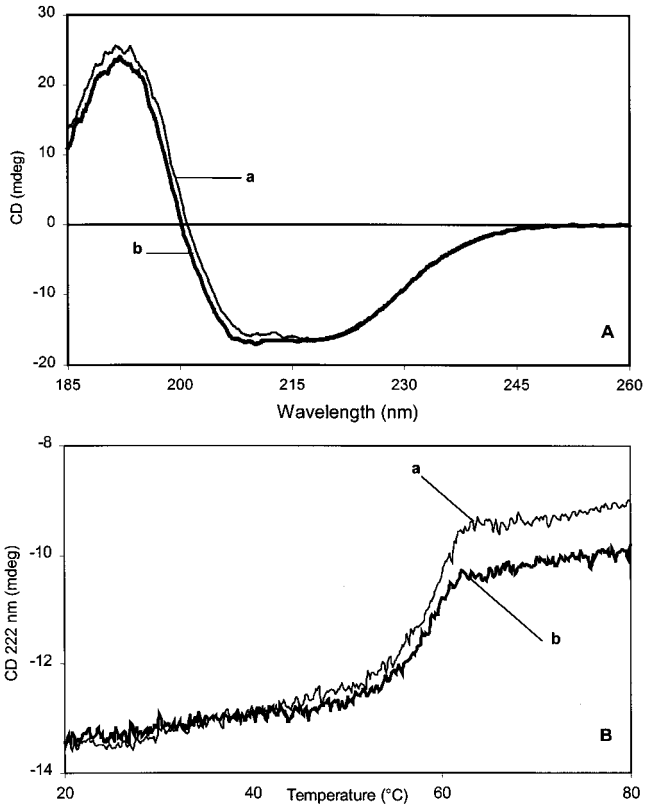


**Figure 1.** (A) Far-UV CD spectra of patatin: (a) patatin (pH 7) at 20 °C; (b) PAT-5 (pH 7) at 20 °C. (B) Thermal unfolding of patatin as monitored by the CD signal at 222 nm: (a) patatin (pH 7); (b) PAT-5 (pH 7).

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 (*23*, *24*).

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 (*25*, *26*). A second peak can be observed around 258 nm with mainly phenylalanine contributions (*25*, *26*). Interestingly, an appreciable loss of intensity at 283 nm was already observed when the temperature was increased from 20 to 25 °C, whereas at 258 nm no significant changes were observed (Figure 2A). From 25 to 50 °C the intensity around 283 nm decreased gradually, whereas from 50 to 80 °C no significant changes in the peak around 283 nm could be observed; at 258 nm the largest decrease in intensity took place between 50 and 60 °C (Figure 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 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 (Figure 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 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 and 25 °C, whereas at higher temperature no further decrease was observed (Figure 2B).

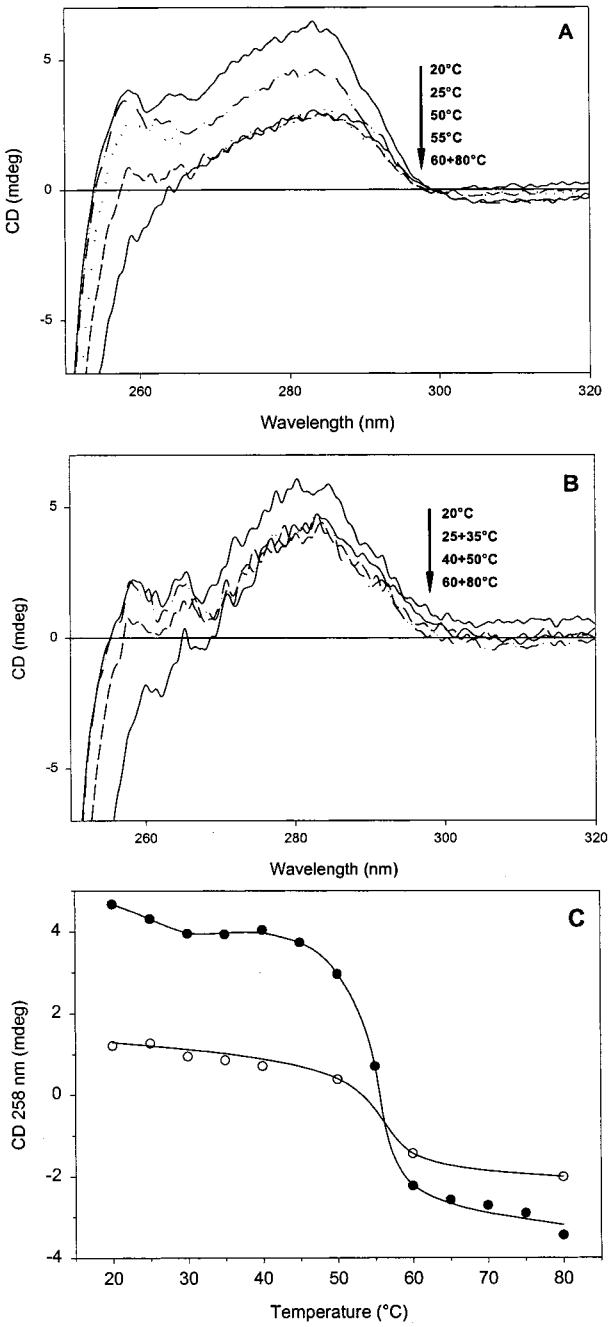


**Figure 2.** (A) Near-UV CD spectra of patatin (pH 7) at different temperatures; (B) near-UV CD spectra of PAT-5 (pH 7) at different temperatures; and (C) CD signal at 258 nm as a function of temperature for patatin (●) and PAT-5 (○) at pH 7.

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 and 60 °C with a midpoint at ~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.

*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. (*27*) for a

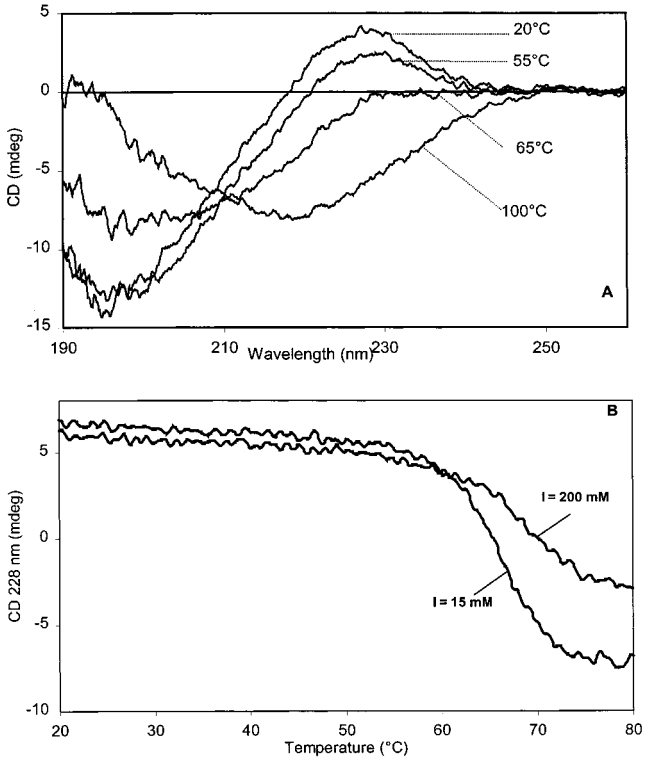


**Figure 3.** (A) Far-UV CD spectra of PIP (pH 7, $I$ =15 mM) at different temperatures and (B) CD signal at 228 nm as a function of temperature for PIP (pH 7) at $I$ = 15 and 200 mM.

comparable protein mixture. The spectrum had a minimum at 195 nm and a maximum at ~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 (Figure 3A).

Figure 3B shows the ellipticity of PIP at 228 nm as a function of temperature at ionic strengths of 15 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 (Figure 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 industrial PFJ. At low ionic strength, the ellipticity of PIP showed a transition between 58 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 and 75 °C. The transition midpoint of PIP had shifted from 66 to 69 °C.

Adjusting the pH to 5 or 3 at ionic strengths of 15 and 200 mM did not affect the far-UV CD spectrum of PIP (no further results shown).

**DSC.** 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 ($T_d$) of 66 of 74 °C, respectively. The total calorimetric enthalpy of unfolding of the proteins in PFJ was 20.5 ± 1.5 J/g of protein.

ASP, which had a protein composition different from that of PFJ (see Table 1), showed different profiles. At low ionic strength ASP showed one peak at 64 °C with

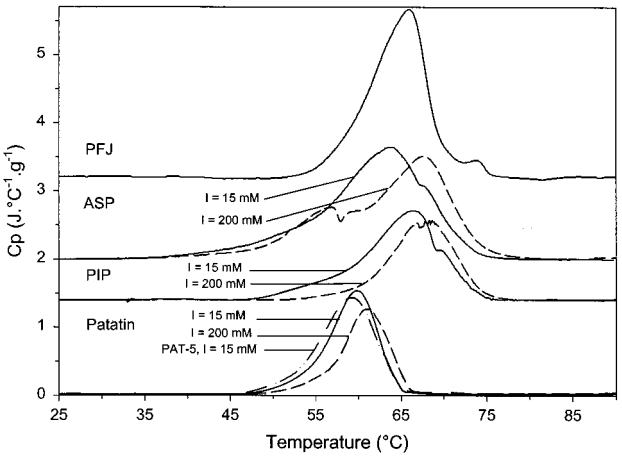


**Figure 4.** Samples of DSC thermograms of PFJ, ASP, PIP, and patatin at pH 7 and ionic strengths of 15 and 200 mM.

a $\Delta h_{cal}$ of 17.4 ± 1.0 J/g of 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 to 68 °C and caused two shoulders to appear with transition temperatures of 56 and 60 °C, respectively (Figure 4). The total $\Delta h_{cal}$ of ASP at high ionic strength was 18.2 ± 1.4 J/g of 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 $\Delta h_{cal}$ of 25.2 ± 2 J/g of protein. At high ionic strength the transition temperature had shifted to 68 °C, with a total $\Delta h_{cal}$ of 24.0 ± 2 J/g of protein (Figure 4). The major proteins in PIP thus showed a higher denaturation temperature at increased ionic strength, which is in agreement with the CD data (Figure 3B).

The DSC profile of patatin at low ionic strength showed one asymmetric peak at 60 °C with a $\Delta h_{cal}$ of 12.6 ± 0.1 J/g (510 kJ/mol of mononomer). At high ionic strength the denaturation temperature increased to 61 °C (Figure 4). The total $\Delta h_{cal}$ was 11.4 ± 0.1 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 not be taken literally; we do realize that the solubility measurements presented here are only an approximation of true solubility measurements, and 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.

In Figure 5 solubility curves of different potato protein fractions are shown as a function of pH at high (Figure 5A) and low ionic strength (Figure 5B). Protein solubility at an ionic strength of 200 mM was studied because this ionic strength is similar to that in PFJ (estimated at ~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 (Figure 5A).

ASP, which was prepared as a representation of undenatured potato protein, showed a similar curve at high ionic strength (Figure 5A). At low ionic strength the solubility curve of ASP showed a broad minimum

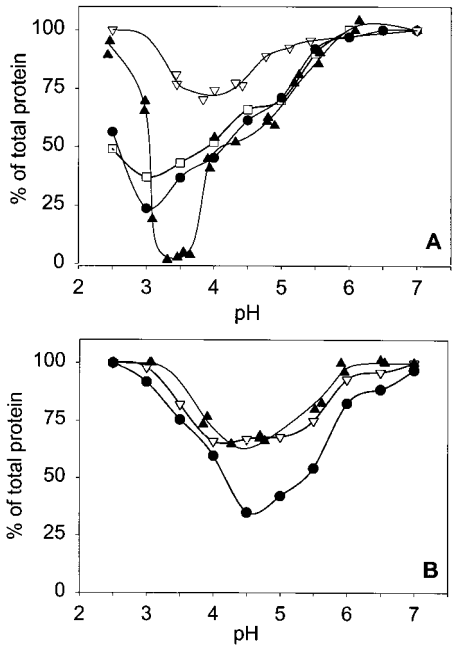


**Figure 5.** (A) Solubility of PFJ (□), ASP (●), PIP (▽), and patatin (▲) as a function of pH ($I = 200$ mM) and (B) solubility of ASP (●), PIP (▽), and patatin (▲) as a function of pH ($I =$ 15 mM).

around pH 5 with an increase in solubility at pH <4.5 (Figure 5B).

The same effect of ionic strength became apparent when the solubility curves of patatin at high and low ionic strengths 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 (Figure 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 (Figure 5A). Ralet and Guéguen (*28*), surprisingly, reported a minimum in solubility of a patatin-rich fraction 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 (*28*), in contrast, did not find any change in solubility over the complete pH range for a comparable fraction.

**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 (Figure 6A) and low ionic strengths (Figure 6B). Precipitation in PFJ already occurred when heated above 40 °C. After heating at 60 °C, 50% of the protein originally present had precipitated, whereas precipitation was complete after heating at 70 °C, at which >90% of the protein had lost its solubility (Figure 6A).

In ASP, at high ionic strength, precipitation became apparent above 50 °C (Figure 6A). The decrease in solubility became steep above 60 °C, and precipitation was complete after heating of ASP at 75 °C, at which temperature 95% of the protein had precipitated. Decreasing the ionic strength had virtually no effect on the precipitation curve of ASP (Figure 6).

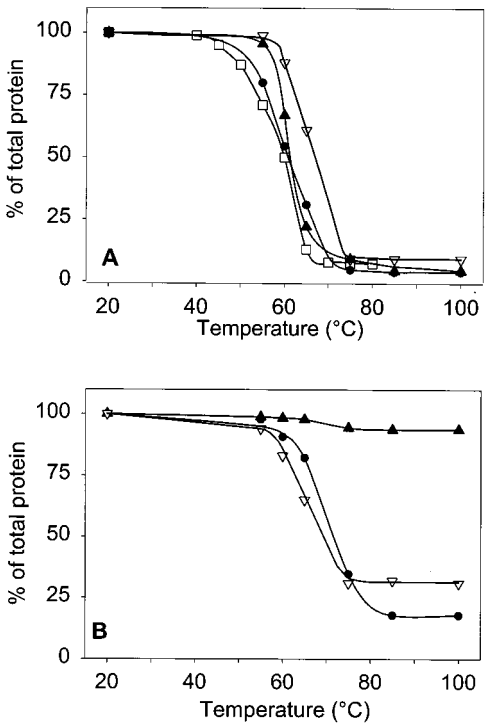


**Figure 6.** (A) Solubility of PFJ (□), ASP (●), PIP (▽), and patatin (▲) as a function of heat treatment temperature ($I =$ 200 mM) and (B) solubility of ASP (●), PIP (▽), and patatin (▲) as a function of heat treatment temperature ($I = 15$ mM).

The solubility curve of PIP at high ionic strength showed a steep part between 50 and 60 °C, where almost all of the protein became insoluble (Figure 6A). At low ionic strength the curve became much less steep, and ~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 (*28*) for a comparable protein fraction.

The strongest effect of ionic strength on protein solubility after heating was seen with patatin. At high ionic strength the solubility curve of patatin (Figure 6A) showed a steep decline between 60 and 65 °C and almost complete precipitation at 85 °C, whereas at low ionic strength (Figure 6B) only a small part of the protein became insoluble above 60 °C. These results are similar to those reported by Ralet and Guéguen (*28*) for a patatin-rich fraction.

**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 (*21*) and are present in PIP. The activity of these inhibitors in PIP was monitored as a function of heating temperature.

As can be seen from Figure 7 most of the inhibitor activity was lost between 55 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 ~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

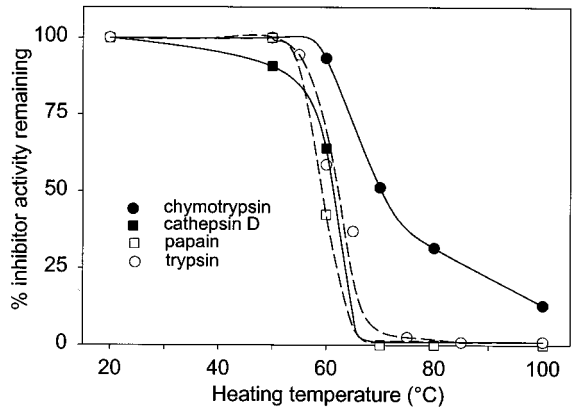


**Figure 7.** Protease inhibitor activities of PIP as a function of heating temperature ($I$ = 15 mM).

of the inhibitors became at least partly denatured before they precipitated.

The chymotrypsin inhibiting activity showed a different profile from the other inhibiting activities. The chymotrypsin inhibiting activity followed the solubility curve of PIP up to 75 °C, at which precipitation was complete. Hence, only at temperatures between 75 and 100 °C, at which point 15% of the original chymotrypsin inhibiting activity remained, is the chymotrypsin inhibitor activity solely decreased by heat denaturation and not by coprecipitation 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) nonequilibrium denaturation; or (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 (*29*). If dissociation of this dimer takes place during denaturation, the denaturation temperature ($T_d$) should rise with increasing protein concentration (*30*−*33*). Variation of the patatin concentration with a factor of 10 did, however, induce variations in $T_d$ of only 0.2 °C (results not shown), which is within the accuracy limits of the instrument used. Moreover, using HP-SEC 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 (10−90 K/h). No differences were observed; hence, the protein must be in equilibrium during the DSC experiments.

To obtain thermodynamic data from the thermal unfolding curve of patatin, the latter was described using the model given by Van Mierlo et al. (*34*), based on thermodynamic equations (*35*, *36*). 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 ($\Delta H_{vH}$) and the calorimetric enthalpy ($\Delta H_{cal}$) are given. For a two-state unfolding of a dimeric protein without dissociation, the ratio of $\Delta H_{vH}$ and $\Delta H_{cal}$ should be ~0.5 when $\Delta H_{cal}$ is calculated on the basis of the concentration of monomer (*31*, *37*). 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 (*38*). The patatin monomers must, therefore, either remain in some way associated during heating or immediately aggregate upon unfolding because 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 |
| $\Delta H_{cal}$ (kJ/mol) ± SD | | 510 ± 12 |
| $\Delta H_{vH}$ (kJ/mol) ± SD | 476 ± 6[a] | 529 ± 16[b] |
| $\Delta H_{cal}/\Delta H_{vH}$ | 1.07 | 0.97 |

[a] Calculated using $\Delta H_{cal}$ from DSC data and $\Delta H_{vH}$ from CD data. [b] Calculated using $\Delta H_{cal}$ from DSC data and $\Delta H_{vH}$ 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, because the low-temperature side could be fitted well to a single two-state transition with the parameters in Table 2 (results not shown). This exothermic sharpening is not uncommon in DSC scans (*30*) and is known to be associated with exothermic irreversible processes such as aggregation, deamidation, proline isomerization, and sulfhydryl oxidation (*39*).

The solubility of patatin at high ionic strength diminishes greatly near pH 3.5. This can be explained by realizing that patatin unfolds at pH <5 (*9*). Increasing the ionic strength in the pH range over which 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 of the negative charges on the protein become protonated and charge repulsion prevails, resulting in an increase in protein solubility (Figure 5A). The same phenomenon is observed when thermally denatured whey proteins precipitate at their isoelectric pH, whereas in the native form they are soluble at that pH (*40*).

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 (Figure 2). These changes were unexpected because no changes in the far-UV CD spectrum were observed (Figure 1A). Also, the LAH activity (1.84 $\mu$mol/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 (*41*).

**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 (Figures 3 and 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 (*42*−*45*) and is usually ascribed to nonspecific salt effects arising from the screening of protein destabiliz-

ing electrostatic repulsions in the structure of the native protein (*43*, *44*).

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. (*46*) found that ~10% of the chymotrypsin inhibiting activity remained after cooking of potatoes, which agrees well with our results (Figure 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 (Figures 4 and 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 (Figures 3 and 4).

**Properties of ASP and PFJ.** Potato proteins in PFJ were fractionated 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 $\Delta h_{cal}$. The DSC results of ASP (Figure 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 the addition of NaCl.

As was shown in Figure 5 most potato proteins have a high solubility only at neutral or strongly acidic pH values. The solubility of ASP at mildly acidic pH is governed by a large effect of ionic strength. An increase in ionic strength from 15 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, at which most of the proteins are already unfolded, whereas at high ionic strength only a small fraction of the proteins needs to be unfolded before precipitation takes place (Figure 6).

In conclusion, this study showed that potato proteins unfold between 55 and 75 °C and that increasing the ionic strength generally causes an increase in denaturation temperature. It was also concluded that either the dimeric protein patatin unfolds in its monomeric state or 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.

Despite the promising nutritional value and functional properties of potato proteins, no economic method is as yet available to recover resoluble and functional potato proteins from PFJ. This study also does not provide such a method, but it does show what the effects of various combinations of ionic strength, pH, and temperature are on protein structure, activity, and solubility and therefore provides useful information about the effects of various conditions that may be applied during industrial recovery and processing of potato proteins.

## ABBREVIATIONS USED

ASP, ammonium sulfate precipitate; CD, circular dichroism; DSC, differential scanning calorimetry; HP-SEC, high-performance size exclusion chromatography; $I$, ionic strength; LAH, lipid acyl hydrolase; PAT-5, patatin resolubilized at pH 7 after precipitation at pH 5; PFJ, potato fruit juice; PIP, protease inhibitor pool; $T_d$, denaturation temperature; $\Delta h_{cal}$, calorimetric enthalpy of unfolding per unit mass; $\Delta H_{cal}$, molar calorimetric enthalpy of unfolding; $\Delta H_{vH}$, van't Hoff enthalpy $= 4RT_m^2C_p^{max}/\Delta H_{cal}$.

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