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Effects of Ethanol on Structure and Solubility of Potato Proteins and the Effects of Its Presence during the Preparation of a Protein Isolate

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In this study, a protein isolate with a high solubility at neutral pH was prepared from industrial potato 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 was shown to be almost completely independent. 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.

KEYWORDS: Potato; *Solanum tuberosum*; proteins; solubility; ethanol; patatin; protease inhibitors; structural stability; circular dichroism; differential scanning calorimetry

INTRODUCTION

Despite their high nutritional quality (*1*, *2*) 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 manufacturing, results in poorly soluble protein products (*3*−*7*), which hampers potential food applications (*8*). Recent research at our laboratory (*7*) 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 (*9*, *10*), the structural properties of the proteins exposed to organic solvents may be severely affected (*11*−*14*). 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 (PPI) obtained using ethanol, were studied as a function of pH and temperature at two ionic strengths (15 and 200 mM), which represent salt concentrations that can be expected in foods. 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. PFJ.* Potatoes (cv. Elkana) were washed thoroughly with water and cut into large pieces (maximum 8 cm × 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

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was decanted and centrifuged (15 min, 19 000*g*, 10 °C), and the supernatant was filtered through a paper filter (Schleicher & Schuell, reference 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 (standard deviation (SD)) mg of nitrogen per milliliter. Of this nitrogen, 60% ± 5.6 (SD) could be precipitated with 12.5% (w/v) trichloroacetic acid (TCA) and was therefore assumed to be of protein origin, which leads to an average protein ($N \times 6.25$) concentration of 13.4 ± 0.9 (SD) mg of protein per milliliter of PFJ (*7*).

2. PPI. 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. The ethanol solution was cooled to −20 °C to prevent the temperature of the PFJ raising too much due to the heat of mixing. After 1 h at 4 °C, the suspension was centrifuged (30 min, 19 000*g*, 4 °C) and the precipitate was washed twice with 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. Experiments showed that freeze-drying at pH 5 resulted in a reduced solubility of the dry product.

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 cm × 15 cm) (Amersham Pharmacia Biotech AB, Uppsala, Sweden) equilibrated with a 25 mM Tris-HCl buffer (pH 8) containing 0.5 g/L $NaHSO_3$ at a flow rate of 19 cm/h. After the unbound compounds were washed out, the bound fraction was eluted with the same buffer containing 0.35 M NaCl at a flow rate of 46 cm/h. Further purification of the bound fraction was established by gel filtration on a Superdex 75 column (63 cm × 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 flow rate of 30 cm/h. The first peak, as observed from the absorbance at 280 nm, containing patatin of >95% purity (sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE)), was collected and concentrated 10 times using Xampler UFP-3-C cross-flow hollow fiber laboratory cartridges with a molecular mass cutoff of 10 kDa (A/G Technology Corp., Needham, U.S.A.) 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 h at 4 °C, the suspension was centrifuged (30 min, 19 000*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). PIP protein was prepared by gel filtration of PFJ on a Superdex 75 column (63 cm × 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 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 mass cutoff of 5 kDa (A/G Technology Corp.) at 4 °C. The concentrated PIP, which contained mainly proteins with a molecular mass of 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 was adjusted to 5.0 using 0.5 M H_2SO_4. After 1 h at 4 °C, the suspension was centrifuged (30 min, 19 000*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 electrophoresis and subsequent densitometric analysis of Phastgel isoelectric focusing (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). All samples were analyzed at least in duplicate.

Circular Dichroism (CD) Spectroscopy. *Sample Preparation.* CD experiments were performed to investigate the effect of various treatments on the conformation and 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. All samples were analyzed in at least in duplicate.

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 was 0.2 nm, the bandwidth was 1.0 nm, the sensitivity was 20 mdeg, and the response time was 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 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 was 0.5 nm, the bandwidth was 1.0 nm, the sensitivity was 10 mdeg, and the response time was 0.25 s. 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). Thermograms were recorded from 10 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 ($I \approx 200$ mM). All samples were analyzed at least in triplicate.

Gel Filtration Chromatography. The apparent molecular weight of patatin at various temperature samples in the presence of 20% (v/v) ethanol was estimated using gel filtration chromatography on a thermostated Superdex 75 HR column (60 cm × 1 cm) (Amersham Pharmacia Biotech AB). Samples of patatin (1 mg/mL) in the presence of ethanol were incubated (10 min) at temperatures of 10, 25, 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 eluents 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 in the absence of 20% (v/v) ethanol indicating that the separation characteristics of the column did not change in the presence of ethanol. All samples were analyzed in duplicate.

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 ($I \approx 200$ 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 h at room temperature. The settled samples were 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 (*15*) when PFJ samples were used. When other protein preparations than PFJ were used, supernatants were analyzed for protein content using the method of Bradford (*16*) 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 a proportion of 12.5% (w/v) TCA precipitable nitrogen present in PFJ. Precipitated nitrogen was assumed to be of protein origin. Samples for all conditions were prepared in duplicate, and the duplicates were analyzed in triplicate for their protein or nitrogen content. 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. The average absolute SD in the estimation of protein solubility was 3%.

2. Effect of Heat Treatment. Protein precipitation as a function of temperature of heat treatment was determined by heating 1.5 mL samples (pH 7; $I \approx 15$ or 200 mM) for 15 min in a closed Kimax tube in a thermostated waterbath. After they were heated, 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*g*, 20 °C) until clear supernatants were obtained. Protein analysis of the supernatants was performed as described above. Samples for all conditions were prepared in duplicate, and the duplicates were analyzed in triplicate for their protein or nitrogen content. Samples for all conditions were prepared in duplicate, and the duplicates were analyzed in triplicate for their protein or nitrogen content.

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. (*17*) with 0.36 mM benzoyl-DL-Arg-*p*-nitroanilide (Merck) as a chromogenic substrate. Chymotrypsin inhibiting activity was estimated according to Geiger (*18*) 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 (*19*) 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. (*20*) with 0.67% (w/v) of acid-denatured hemoglobin as a substrate. Carboxypeptidase A inhibiting activity was estimated as described by Riordan and Holmquist (*21*) 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 percent activity remaining per volume of supernatant as compared to the unheated solution. All samples were analyzed at least in duplicate. Average absolute SDs were 2% for trypsin, 5% for chymotrypsin, 7% for carboxypeptidase A, 2% for papain, and 11% for cathepsin D.

RESULTS

In this 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 PPI in the presence of organic solvents.

Table 1. Protein Distribution in Various Potato Protein Fractions on the Basis of PI as Estimated from the Density of the Various Bands on Coomassie-Stained IEF Gels

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)
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.

Protein Composition. PPI is a protein isolate that contains about 85% of the total protein originally present in industrial PFJ (*7*). **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 p*I* 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 p*I* < 5.2, which is known to represent different patatin isoforms (*22*) and constitute about 38% of the PFJ protein (*23*). PPI contains, as compared to PFJ, less patatin and a higher proportion of proteins with a high isoelectric pH (p*I* > 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. In aqueous ethanol, local electrostatic interactions may play a more prominent role than in water alone due to a change of the dielectric constant. Moreover, the mechanism of protein precipitation in the presence of water miscible organic solvents seems to be similar to that of "salting-out", which is not dominated by net charge (*24*).

Structural Changes in Potato Proteins. *Changes in the Secondary Structure of Patatin.* Both the presence of 20% (v/v) ethanol in the patatin solution and its presence during precipitation of patatin at pH 5 (PAT-5E) did not substantially affect the far UV CD spectrum of patatin (**Figure 1A**). Analysis of the spectra for their secondary structure content was performed according to De Jongh et al. (*25*). Analysis of replicate samples revealed that the average absolute SD in the estimation of the secondary structure content was 2%. Analysis 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. This effect of ethanol of inducing helix formation in proteins has been observed by others (e.g., see refs *26*−*28*) 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 (*29*, *30*).

Heating patatin at 80 °C resulted in a decrease in the secondary structure content (**Table 2**) (*31*). Similar results were obtained when PAT-5E was heated (**Figure 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 (**Figure 1A**, **Table 2**).

When samples of patatin and PAT-5E were heated to 80 °C and subsequently cooled to 20 °C, their temperature-induced

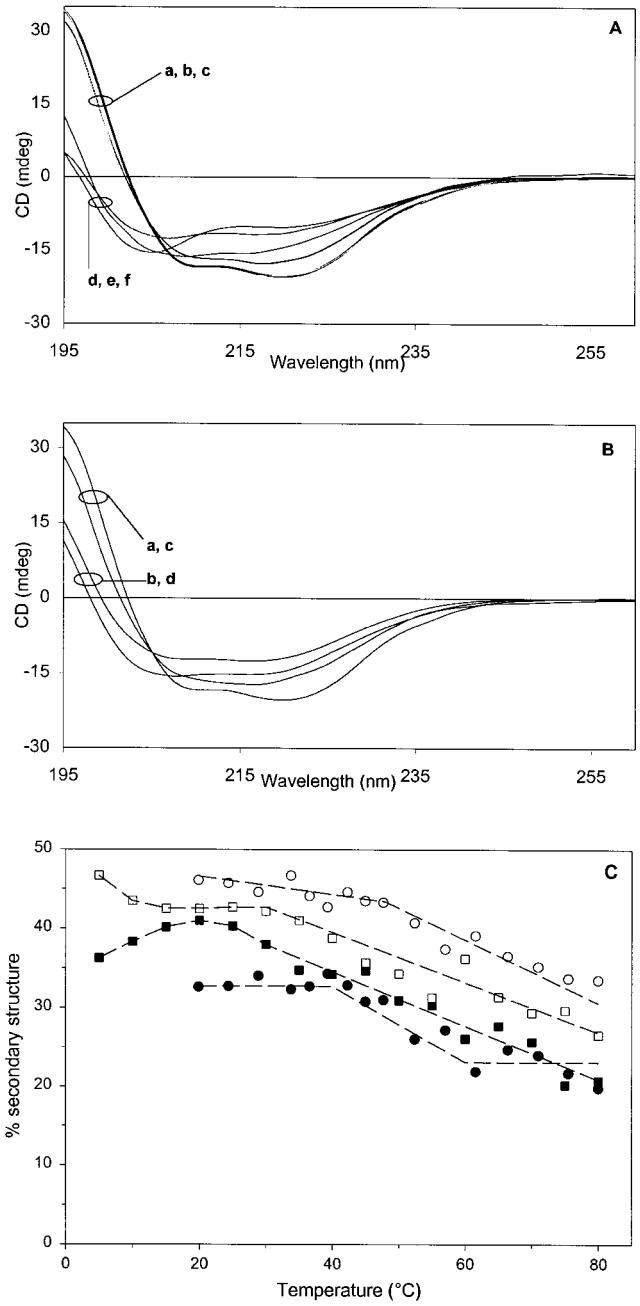

Figure 1. (**A**) Far UV CD spectra of patatin (0.1 mg/mL) at pH 7 and $I = 15$ mM. (**a**) Patatin (pH 7) at 20 °C; (**b**) patatin (20% (v/v) ethanol, pH 7) at 20 °C; (**c**) PAT-5E (pH 7) at 20 °C; (**d**) **a** at 80 °C; (**e**) **b** at 80 °C; (**f**) **c** at 80 °C. (**B**) Far UV CD spectra of patatin (0.1 mg/mL) at pH 7 and $I = 15$ mM. (**a**) Patatin at 20 °C; (**b**) **a** heated at 80 °C and cooled to 20 °C; (**c**) patatin heated to 80 °C in the presence of 20% (v/v) ethanol and cooled to 20 °C; (**d**) PAT-5E heated at 80 °C and cooled to 20 °C. (**C**) α-Helix and β-strand content of patatin (0.1 mg/mL) at pH 7 and $I = 15$ mM; α-helix (●), β-strand (○), and patatin (0.1 mg/mL) at pH 7 and $I = 15$ mM in 20% ethanol (pH 7); α-helix (■) and β-strand (□) as a function of temperature as obtained from analysis of far UV CD spectra.

unfolding proved to be only partly reversible (**Figure 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

Table 2. Content of Various Elements of Secondary Structure of Patatin Samples (0.1 mg/mL; pH 7; $I = 15$ mM) at Various Temperatures (Average Absolute SD, 2%)

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

in patatin showed a transition between 40 and 60 °C. The β-strand content showed a gradual decrease. These results are in agreement with those reported by Pots et al. (*32*). 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 (**Figure 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.

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 (*33*).

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, attributed to phenylalanine contributions (*34*).

Addition of ethanol to patatin caused a small increase in the intensity around 258 nm and a shift of the maximum toward lower wavelengths (**Figure 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 and 300 nm. The latter was also observed for myoglobin in the presence of methanol (*35*) and for lysozyme in the presence of trifluoroethanol (*13*), indicating that this is a more general effect of organic solvents on proteins. The spectrum of PAT-5E showed a combination of the effects of precipitation at pH 5 and contact with ethanol, which is characterized by a decrease in ellipticity around 258 nm (*31*) and a decrease in ellipticity 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 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 and 60 °C (*31*). 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 as compared to untreated patatin, a further decrease with temperature was still observed. The major changes at 283 nm occurred between 25 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

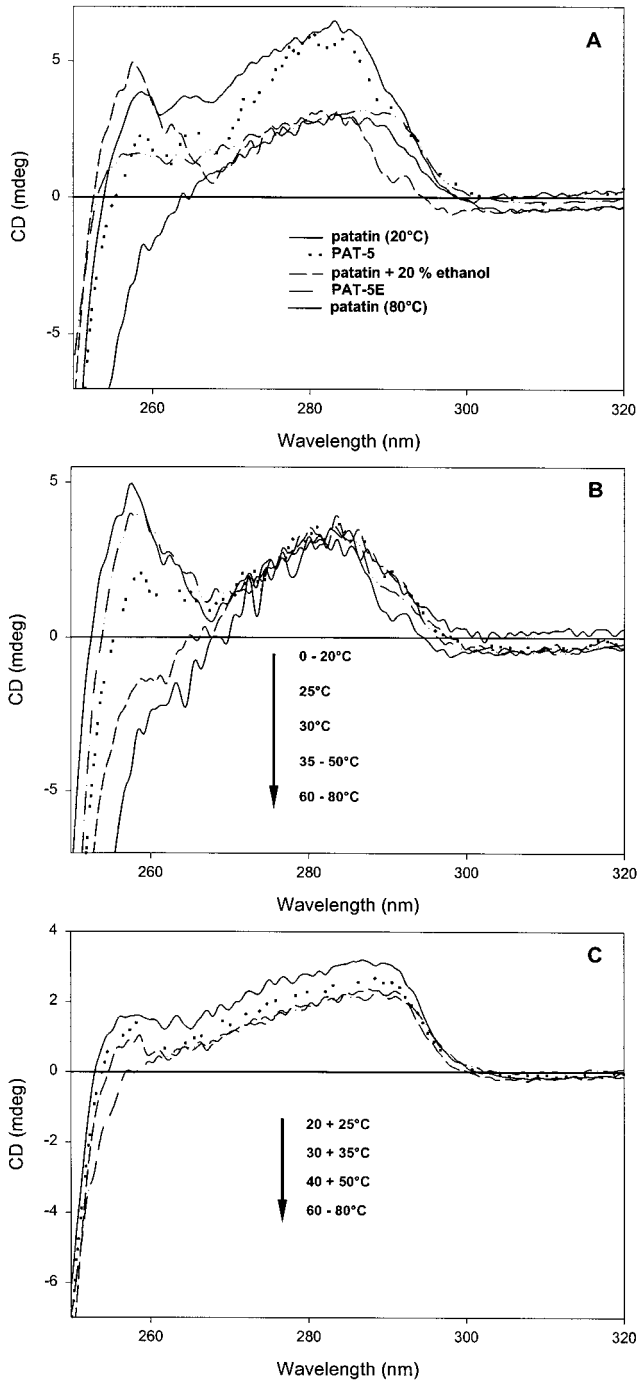

Figure 2. (**A**) Near UV CD spectra of patatin (4 mg/mL) at pH 7 and I = 15 mM after different treatments. (**B**) Near UV CD spectra of patatin (4 mg/mL) at pH 7 and I = 15 mM in the presence of 20% (v/v) ethanol at various temperatures. (**C**) Near UV CD spectra of PAT-5E (4 mg/mL) at pH 7 and I = 15 mM at various temperatures.

α-helical and β-stranded structures (*36*). The unfolding curve of PAT-5E showed a transition with a midpoint of 58 °C and was almost identical to that of patatin (**Figure 3A**) (*31*). In the presence of ethanol, the absolute ellipticity of patatin at 222 nm showed a gradual decrease that was most pronounced between 58 and 100 °C (**Figure 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 (**Figure 3A**), presumably indicating a noncooperative unfolding. The results described above, together with those in **Figure 1C**,

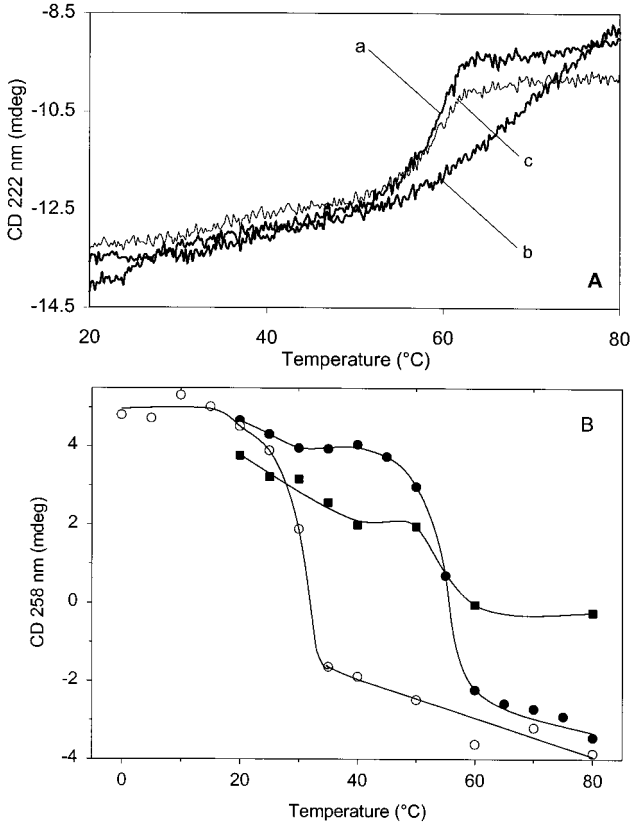

Figure 3. (**A**) Thermal unfolding of patatin (0.1 mg/mL) at pH 7 and I = 15 mM as monitored by the CD-signal 222 nm. (**a**) Patatin; (**b**) patatin in 20% (v/v) ethanol; (**c**) PAT-5E. (**B**) Thermal unfolding of patatin (4 mg/mL) at pH 7 and I = 15 mM as monitored by the CD signal 258 nm; patatin (●), patatin (20% (v/v) ethanol ○), and PAT-5E (■).

indicate that the temperature-induced unfolding of the secondary structure of patatin in the presence of ethanol is a noncooperative 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. This wavelength was chosen because it gives an indication of the mobility of the Phe residues, which are believed to be located more on the inside of proteins than Tyr and Trp and thus should be more indicative for global state of folding of the protein than at 283 nm. 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 (*31*). In the presence of 20% (v/v) ethanol, the unfolding curve of patatin also showed a transition but now between 15 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.

Changes in the Conformation of PIP Proteins. Because 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. (*37*) for a comparable protein mixture. The spectrum had a minimum at 195 nm and a maximum around 228 nm (*31*).

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

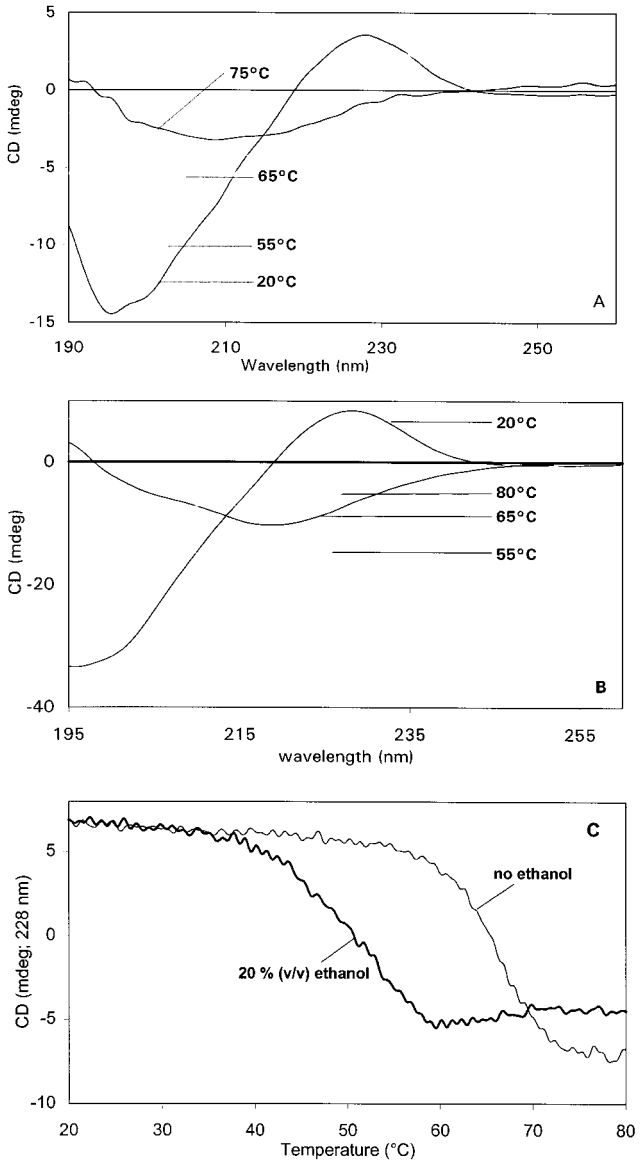

Figure 4. (**A**) Far UV CD spectra of PIP-5E (0.2 mg/mL) at pH 7 and I = 15 mM at various temperatures. (**B**) Far UV CD spectra of PIP (0.2 mg/mL) at pH 7 and I = 15 mM in 20% (v/v) ethanol at various temperatures. (**C**) Thermal unfolding of PIP (0.2 mg/mL) at pH 7 and I = 15 mM in the presence and absence of 20% (v/v) ethanol as monitored by the CD signal at 228 nm.

change the spectrum of PIP at 20 °C as it was similar to that of PIP (*31*). Also, the changes in the spectrum with temperature (**Figure 4A**) were similar to those observed for untreated PIP (*31*).

Addition of ethanol did not induce significant changes in the far UV CD spectrum of PIP at 20 °C (**Figure 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 (**Figure 4A**).

Figure 4C shows the thermal unfolding curves of PIP in the absence and presence of ethanol, measured as the ellipticity at 228 nm. 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 4A**,**B**). The unfolding curve of PIP-5E is not shown, since it did not differ substantially from that of untreated PIP. The unfolding curve of PIP showed a transition

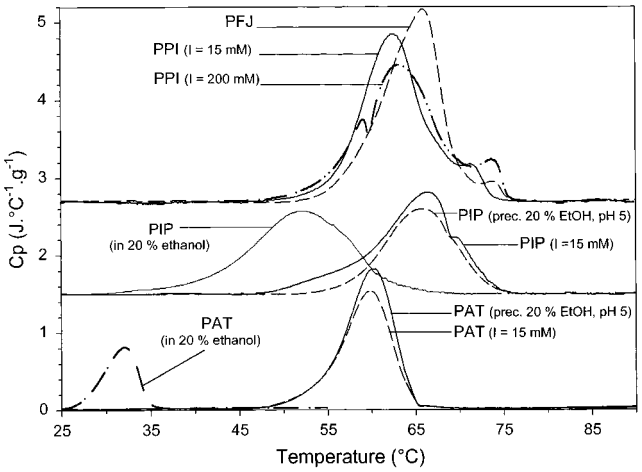

Figure 5. Examples of DSC thermograms of PFJ (13.5 mg/mL), PPI (10 mg/mL), PAT-5E (4 mg/mL), and PIP-5E (6 mg/mL) at pH 7 and I = 15 mM.

with a midpoint of 66 °C. In the presence of ethanol, PIP showed a transition between 40 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 of 74 °C, respectively (**Figure 5**), with a total calorimetric enthalpy (Δh_{cal}) of 20.5 ± 1.5 J/g.

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, 64, and 74 °C (**Figure 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 (**Figure 5**), and also, the total Δh_{cal} decreased from 25.2 ± 2 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 (**Figure 4C**). The total Δh_{cal} was reduced in the presence of ethanol from 25.2 ± 2 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/mol monomer), as compared to 12.6 ± 0.1 J/g (510 kJ/mol of monomer) for untreated patatin (**Figure 5**). The presence of 20% (v/v) ethanol caused a shift in the denaturation temperature of patatin from 59.4 to 32.3 °C (**Figure 5**) and decreased Δh_{cal} by 50% to 6.3 ± 0.2 J/g.

Solubility as a Function of pH. In **Figure 6A**,**B**, 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 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

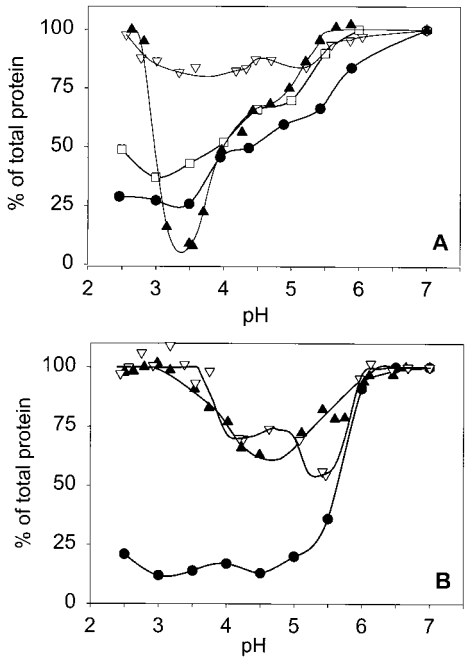

Figure 6. (**A**) Solubility at 25 °C of PFJ (13.5 mg/mL; □), PPI (10 mg/mL; ●), PIP-5E (6 mg/mL; ▽), and PAT-5E (4 mg/mL; ▲) as a function of pH (I = 200 mM). (**B**) Solubility at 20 °C of PPI (13.5 mg/mL; ●), PIP-5E (6 mg/mL; ▽), and PAT- 5E (4 mg/mL; ▲) as a function of pH (I = 15 mM).

(**Figure 6A**) (*31*). At high ionic strength, the pH/solubility profile of PPI was similar to that of PFJ (**Figure 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 (**Figure 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 (**Figure 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 (**Figure 6A**). Both solubility profiles were very similar to those obtained for untreated patatin (*31*) 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 indicating that the solubility of PIP-5E around pH 5.5 had decreased as compared to untreated PIP (**Figure 6B**) (*31*). At high ionic strength, the solubility of PIP-5E showed a broader solubility minimum as compared to untreated PIP (**Figure 6A**) (*31*).

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 (**Figure 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, while at 70 °C precipitation was complete and 10% of the protein originally present remained in solution (**Figure 7**). At low ionic strength (15 mM), the solubility curve of PPI showed a steeper decline between 60 and 70 °C than at high ionic strength. At heat treatment

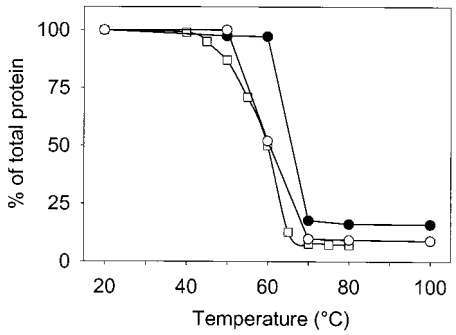

Figure 7. Solubility at pH 7 of PFJ (13.5 mg/mL; □) and PPI (10 mg/mL; I = 15 mM (●); I =200 mM (○)) as a function of heating temperature.

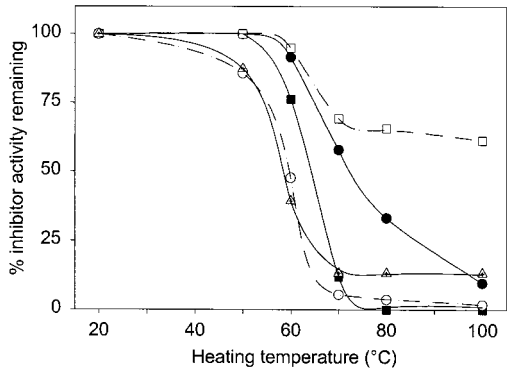

Figure 8. Protease inhibitor activities of PPI determined at 20 °C as a function of heating temperature (pH 7; I = 15 mM): chymotrypsin ●, cathepsin D ■, carboxypeptidase A □, trypsin ○, and papain △.

temperatures >70 °C, about 20% of the protein originally present remained soluble (**Figure 7**).

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 (**Figure 8**).

The curves of chymotrypsin and carboxypeptidase A inhibiting activity (**Figure 8**) show that these activities are at least partly lost in conjunction with or because of precipitation (**Figure 7**), since at temperatures >70 °C, where precipitation increases no further, their activities decrease less steeply with temperature.

DISCUSSION

Effect of Ethanol on Protein Structure and Stability. Effects of the presence of ethanol on the structure of various potato protein fractions 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

Table 3. Thermodynamic Parameters from Fits of the CD Thermal Unfolding Curves (222 and 258 nm) and DCS Profiles of Patatin (pH 7; $I = 15$ mM) in the Absence and Presence of Ethanol

sample	CD 222 nm T_m (°C)	CD 222 nm $\Delta H_{vH} \pm$ SD (kJ/mol)	CD 258 nm T_m (°C)	CD 258 nm $\Delta H_{vH} \pm$ SD (kJ/mol)	$\Delta H_{cal}{}^a/\Delta H_{vH}$	DSC T_m (°C)	DSC $\Delta H_{cal} \pm$ SD (kJ/mol)	DSC: $\Delta H_{vH} \pm$ SD (kJ/mol)	$\Delta H_{cal}{}^b/\Delta H_{vH}$
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

[a] Calculated using ΔH_{cal} from DSC data and ΔH_{vH} from the CD data (258 nm). [b] Calculated using ΔH_{cal} and ΔH_{vH} from DSC data using $\Delta H_{vH} = 4RT_m{}^2C_p{}^{max}/\Delta H_{cal}$.

as can be seen from the increased width of the transition in the presence of ethanol as observed with CD (**Figure 4**) and DSC (**Figure 5**). This decrease of T_d is often observed in the presence of alcohols (e.g., see refs *11*, *14*, and *29*) 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 (*29*, *30*, *38*, *39*).

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 noncooperatively 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 (*11*, *40*), indicating that alcohols may generally cause such a decoupling in proteins. However, the decoupling in the case of patatin is more extensive than in the case of ribonuclease A. Mechanistically, the effects of ethanol on the thermal unfolding of patatin can be described as a destabilization of the tertiary structure of the protein, which is predominantly stabilized by hydrophobic interactions (*40*), resulting in a marked decrease of T_d leading to the formation of an intermediate state upon heating, that will have lost already a great deal of its tertiary structure. Because of this, the remaining secondary structure will unfold noncooperatively as the temperature is raised (*41*−*43*). The noncooperative unfolding of the secondary structure has been observed with DSC as a continuous change in heat capacity instead of an endothermic transition (*44*, *45*), which we also observed (not shown).

In the absence of ethanol, the monomers of patatin were shown to unfold independently upon heating (*31*). 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 to remain dimeric. Gel filtration chromatography of patatin at various temperatures (15−35 °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/mol. Apart from the effects of ethanol itself, also the absence of the dissociation of the patatin monomers before unfolding may have affected the properties of patatin during heating, especially the unfolding of its tertiary structure.

To obtain thermodynamic data from the CD thermal unfolding curves of patatin in the absence and presence of ethanol, the curves in **Figure 3A**,**B** were described using a model reported by Van Mierlo et al. (*46*). 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} value obtained from the DSC profiles significantly increases in the presence of ethanol, causing the ratio of ΔH_{vH} to ΔH_{cal} to deviate significantly from unity (**Table 3**). Presently, we have no explanation for the discrepancy between the values obtained for ΔH_{vH} from the CD and DSC data, although it cannot be excluded that the CD/temperature traces at 258 nm represent a more local unfolding of the protein than do DSC measurements.

These DSC results have practical implications for the preparation of PPI, since they show that this should be done at 15 °C or below to prevent protein unfolding (**Figure 5**). 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 (pH 5) on the structure and solubility of patatin and PIP. The changes observed in these individual protein preparations 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%. Because PIP is a mixture of proteins, ethanol precipitation could have caused changes in the protein composition; hence, the decrease of Δh_{cal} cannot 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 (*12*) and α-lactalbumin (*14*), thereby suggesting that this may be a general effect of alcohols on protein structure.

Properties of Ethanol Precipitated (pH 5) 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 fraction 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 the Δh_{cal} PPI, since it is about 90% of that of PFJ. This difference 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 (**Figure 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 nonprotein impurities than PIP and patatin, as it is only a crude protein fraction obtained by only one, rather nonselective, purification step from PFJ. The presence of these impurities could also have 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 (*31*). At low ionic strength, precipitation takes place at temperatures above the denaturation temperature, where most of the proteins are already unfolded, while at high ionic strength only a small fraction of the proteins needs to be unfolded before precipitation takes place (**Figure 2**). The differences in protease inhibitor activity between PPI and PIP can be explained by compositional differences. PPI contains the 85 kDa potato multicystatin (*47*, *48*) and the heat stable potato carboxypeptidase inhibitor (4.3 kDa) (*49*, *50*), which because of their molecular masses are not present in PIP.

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. However, precipitation in the presence of ethanol is, to our knowledge, the only known large scale precipitation method for potato proteins that results in protein isolate with a high solubility at neutral pH combined with a high yield.

ABBREVIATIONS

CD, circular dichroism; DSC, differential scanning calorimetry; I, ionic strength; LAH, lipid acyl hydrolase; 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; PIP-5E, PIP after precipitation at pH 5 in the presence of 20% (v/v) ethanol; PPI, potato protein isolate prepared by precipitation at pH 5 in the presence of 20% (v/v) ethanol; T_d, denaturation temperature; Δh_{cal}, calorimetric enthalpy of unfolding per unit mass; ΔH_{cal}, molar calorimetric enthalpy of unfolding; ΔH_{vH}, Van't Hoff enthalpy $= 4RT_m^2C_p^{max}/\Delta H_{cal}$.

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