The pH Dependence of the Structural Stability of Patatin

André M. Pots,[†] Harmen H. J. de Jongh,[†,‡] Harry Gruppen,[§] Martin Hessing,[||] and Alphons G. J. Voragen*[,§]

Centre for Protein Technology, TNO-WAU, Wageningen, The Netherlands, Department of Food Technology and Nutritional Sciences, Food Science Group, P.O. Box 8129, Wageningen Agricultural University, 6700 EV Wageningen, The Netherlands, and Department of Protein Technology, Division of Biochemistry and Gene Technology, TNO Nutrition and Food Research Institute, Zeist, The Netherlands

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

Keywords: *Structural stability; pH dependence; patatin; Solanum tuberosum*

INTRODUCTION

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

The amino acid sequence of patatin (366 amino acids) shows neither extended hydrophilic nor hydrophobic clusters (Stiekema et al., 1988). The positive and negative charges of the side chains are randomly distributed over the sequence. The protein contains 17 tyrosines and 2 tryptophans, the latter being positioned closely together in the primary sequence (residues 256 and 260). It is glycosylated at the 60 and 90 asparagine residues for about 4% (w/w) according to Sonnewald and co-workers (1989). Patatin has an estimated MW of 43 000 on SDS−PAGE, whereas in media without SDS or urea it forms a dimer with an apparent MW of about 80 000 (Racusen and Weller, 1984).

In a previous study (Pots et al., 1998), it was shown that patatin at pH 8 at room temperature is a highly structured molecule both at the secondary and the tertiary structure level. It is estimated from far-UV CD data that about 33% of the residues adopt an α-helical and 46% a β-stranded structure. Upon heating at pH 8, parts of the α-helices unfold cooperatively between 45 and 55 °C, whereas the β-stranded parts unfold more gradually at temperatures from 50 to 90 °C. The observed unfolding of the protein coincides with the inactivation of its enzymatic activity and with the precipitation that occurs in the so-called potato fruit juice upon heating (Knorr et al., 1977). At elevated temperatures (80−90 °C), patatin still contains some helical and β-stranded structures. Upon cooling the protein refolds partly, where mainly the α-helical structures were able to refold (Pots et al., 1998).

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

MATERIALS AND METHODS

Preparation of Patatin Solutions. Patatin from *Solanum tuberosum* cv. Bintje was purified and characterized as described elsewhere (Racusen and Foote, 1980; Pots et al., 1998).

* Corresponding author [telephone +31 (0)317 482888; fax +31 (0)317 484893; e-mail Fons.Voragen@chem.FdSci.wau.nl].
[†] TNO-WAU.
[‡] Present address: Wageningen Centre for Food Sciences, Wageningen, The Netherlands.
[§] Wageningen Agricultural University.
[||] TNO Nutrition and Food Research Institute.

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After purification, the protein solutions were dialyzed (Visking V20, Carl Roth GmbH + Co., Karlsruhe, Germany) at 4 °C against sodium phosphate buffers at different pH (2.0, 3.0, 4.0, 6.0, 7.0, and 8.0) with a calculated ionic strength of 0.035 M, which was kept constant by using phosphate concentrations of 82, 39, 35, 30, 22, and 13 mM, respectively. These buffers will be denoted as buffer pH 2, pH 3, etc. In addition, patatin solutions dialyzed as described above against similar sodium phosphate buffers pH 3, 6, and 8 containing 0.165 M NaCl (I = 0.2 M) were also used. After dialysis, samples were frozen to − 20 °C in small aliquots till use. Between pH 4.3 and pH 5.7, no representative sample of patatin could be obtained since iso-electric focusing and solubility experiments showed that the iso-electric point of patatin is about pH 4.7 (unpublished results).

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

Far-Ultraviolet Circular Dichroism. Far-UV CD wavelength scan spectra of 0.1 mg of patatin/mL in buffers pH 2, 3, 4, 6, 7, and 8 were recorded as averages of eight spectra on a Jasco J-715 spectropolarimeter equipped with a thermostated cell holder (Jasco Corp., Japan) at 20 °C. Furthermore, 1.0 mL of pH 3 and pH 8 (denoted as pH 8 heated sample) samples were heated for 15 min at 80 °C in closed glass tubes (13 × 100 mm Kimax culture tube, Kimble Glass Inc., USA) and cooled to 0 °C by placing the tube on ice. Next, the pH 3 sample was brought by dialysis as described above to pH 8 (denoted as pH 3 heated sample). Quartz cells with an optical path length of 0.1 cm were used. The scan range was 260−190 nm, the scan speed 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. Spectra were corrected for a protein-free spectrum obtained under identical conditions, and noise reduction was applied subsequently using the Jasco software. Analysis of far-UV wavelength scan spectra to determine the secondary structure content of the protein was performed using a nonlinear regression procedure, as described previously (Pots et al., 1998).

Far-UV CD temperature scans were monitored as the ellipticity at 222 nm (for buffers pH 3, 6, 7, and 8) or 208 nm (for buffers pH 2, 3, and 4) from 20 to 80 °C at a heating rate of 20 °C/h and a step resolution of 0.2 °C. The response time was 16 s, the bandwidth was 1.0 nm, and the sensitivity was 20 mdeg.

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

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

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

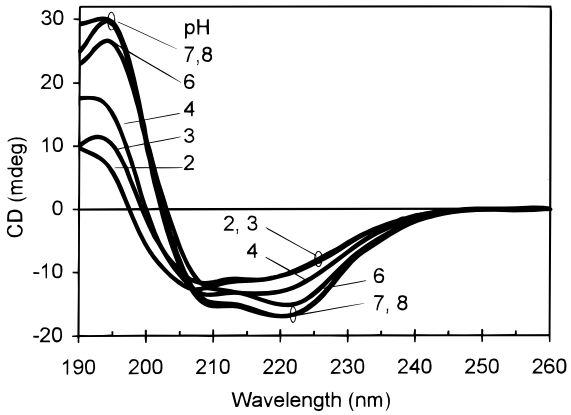

Figure 1. Far-UV CD of 0.1 mg of patatin at 20 °C/mL of phosphate buffer at pH 2−8 (I = 0.035 M).

Lipid Acyl Hydrolase (LAH) Activity. Patatin solutions were diluted to a final concentration of 0.65 μM in a 30 mM Tris-HCl buffer at pH 8.2. In a microtiter plate well, 200 μL of these solutions was equilibrated at 30 °C before 50 μL of a 5.26 μM *p*-nitrophenyl laurate in the same buffer was added (Racusen, 1985). After incubation at 30 °C for 5 min the absorbance at 410 nm was measured. The experiment was performed four times, and the LAH activity was expressed as the specific activity.

RESULTS

In this study, the structural and physicochemical properties of patatin at 20 °C in phosphate buffers with pH ranging from 2 to 8 were determined. Next, the pH dependency of the thermal stability of patatin is presented. Since the structure and structural stability of proteins can be affected by both the type and the ionic strength of the buffer (Brouilette et al., 1987; Griko et al., 1994; Tan et al., 1995; Boye et al., 1996), all sodium phosphate buffers were set to have a constant ionic strength (I = 0.035 or in some cases 0.2 M).

Structural Properties of Patatin. In Figure 1 it can be seen that the far-UV CD spectra of patatin obtained at pH 6, 7, and 8 show a zero-crossing about 203 nm and a negative extrema at 208 and, more intense, at 220 nm, which point at a high level of structured conformations (Johnson, 1988; Hennessey and Johnson, 1981). On the basis of spectral similarity, the spectra indicate a virtual identical secondary folding of the protein in this pH range (I = 0.035 M). Results of curve fitting analysis (de Jongh et al., 1994; Pots et al., 1998), as presented in Table 1, show indeed a highly structured protein, containing about 30% and 45−50% helical and stranded structures, respectively.

Spectra recorded at pH 3 and pH 4 show a smaller negative extreme around 220 nm as compared to those at pH 6 or pH 8; furthermore, the zero-crossing of the curves has shifted to 200 nm. This suggests a lower level of secondary structured domains of patatin at pH 3 and pH 4 as compared to the pH range of 6−8. Indeed, spectral analysis illustrated a decreased level

Table 1. Structural Stability of Patatin as a Function of pH and Temperature

	20 °C			80 °C
secondary struct[a]	pH 2	pH 3−4	pH 6−8	pH 8
α-helix	20	20−25	30	20−25
β-strand	30	35−40	45−50	35−40
random	45	35	15−20	35

	midpoint of unfolding (°C)					
	pH 2	pH 3	pH 4	pH 6	pH 7	pH 8
secondary[b]	−[c]	−	−	58	55	49
tertiary	−	−	−	precip[d]	nd[e]	48

[a] Percentage of secondary structured elements. [b] Structure level of the protein. [c] − indicates that only minor transitions could be observed. [d] precip, precipitation occurred. [e] nd, not determined.

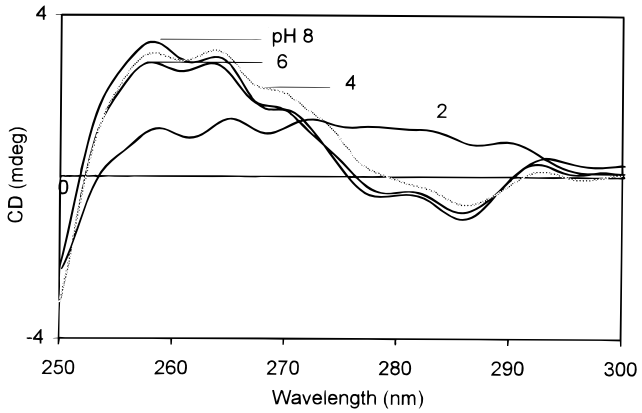

Figure 2. Near-UV CD spectra of 0.4 mg of patatin/mL of buffer at pH 2, 4, 6, and 8 (I = 0.035M) at 20 °C.

of the α-helix and β-strand content at pH 3 and pH 4 as compared to near-neutral pH (Table 1).

At pH 2, a small decrease in the ellipticity can be observed as compared to the spectrum obtained at pH 3, especially in the 190−200 nm region. Furthermore, the zero-crossing of the spectrum at pH 2 is located around 197 nm, both indicating a further unfolded protein as compared to pH 3 and pH 4. Curve fitting analysis as presented in Table 1 supports this conclusion. The presence of 0.165 M NaCl, resulting in a total ionic strength of 0.2 M, had no influence on the CD spectra at the various pH and, hence, on the secondary structure of patatin (results not shown).

Near-UV CD spectra provide a measure for interactions of side chain aromatic rings with other groups such as side chain amide and carboxylate groups and main chain peptide bonds and, therefore, are applicable as a measure for the tertiary structure of a protein (Vuilleumier et al., 1993). The near-UV CD spectra obtained at pH 6 and pH 8, as presented in Figure 2, show similar positive extremes at 292, 270, 263, and 258 nm and negative ellipticities at 277 and 286 nm, suggesting a comparable tertiary fold.

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

Patatin at pH 2 exhibits a completely different spectrum, showing a positive signal from 255 to 290 nm instead of the negative extreme in the 280−290 nm region observed at pH 4, 6, and 8. A protein with a

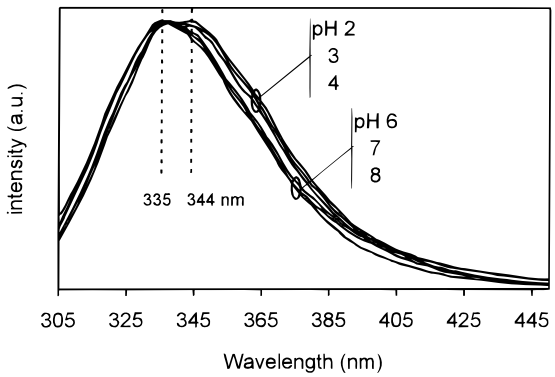

Figure 3. Fluorescence spectra of 0.1 mg of patatin/mL of buffer pH 2, 3, 4, 6, 7, and 8 at 20 °C.

negligible tertiary structure can still exhibit some ellipticity in the 250−350 nm region (Das et al., 1995; Sarkar and DasGupta, 1996); therefore, it is suggested that patatin at pH 2 is significantly more unfolded than at pH 4, despite the observed ellipticity.

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

Fluorescent probes such as ANS are widely used as a measure for changes in the exposure of hydrophobic sites of proteins due to heat treatment or at acidic or alkaline pH values (Andley and Chakrabarti, 1981; Goto and Fink, 1989; Vanderheeren and Hanssens, 1994). Interpreted carefully, they can provide useful additional information on the conformational state of a protein (Shi et al., 1994). In Figure 4, it can be seen that no differences in the normalized fluorescence intensity of the ANS−protein complex could be observed in the pH range 2−8. Apparently, no significant changes in the accessibility of solvent-exposed hydrophobic sites occur in this pH range. The absolute amount of ANS fluorescence increased with decreasing pH at identical ANS/protein ratios, whereas an ANS solution in a 50% (v/v) methanol/buffer mixture did not show pH-dependent differences in its fluorescence emission intensity (no results shown). This could be caused by a different interaction of the hydrophobic sites of the protein surface with the fluorescent probe due to a decreased level of electrostatic repulsion.

Thermal Unfolding. To investigate thermal unfolding of the secondary structure of patatin at various pH, the ellipticity of the protein at 222 nm was measured during heating from 20 to 80 °C (Figure 5). The protein

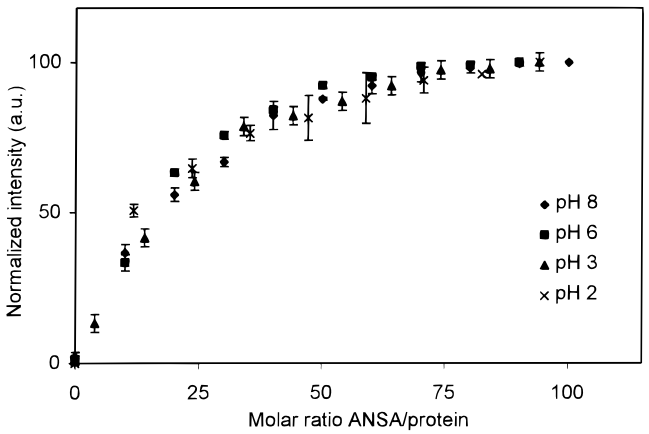

Figure 4. pH dependence of the exposed hydrophobic sites of patatin determined as ANS fluorescence. The fluorescence intensity is plotted as function of the ANS-protein ratio at pH 8 (◆), pH 6 (■), pH 3 (▲), and pH 2 (×).

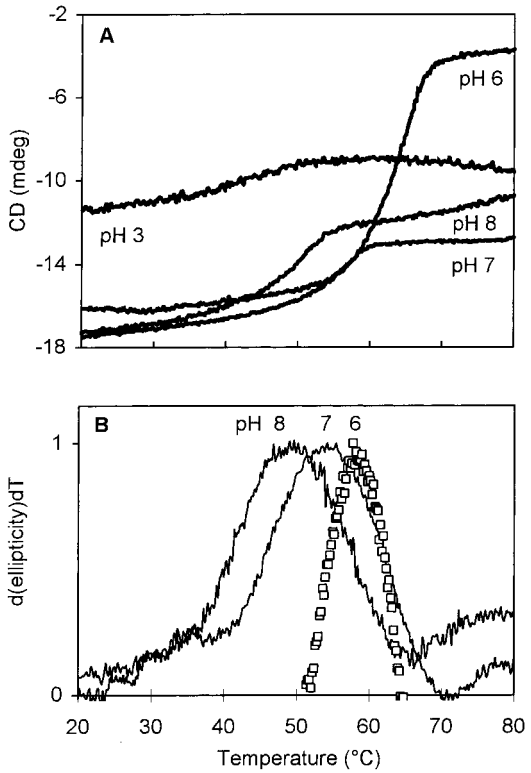

Figure 5. (A) Thermal unfolding of patatin in phosphate buffer (I = 0.035 M) at pH 6, 7, and 8 measured as CD at 222 nm and at pH 3 measured at 208 nm. (B) The first derivative of the pH 7 and 8 unfolding curves and the calculated (see description below) first derivative of unfolding at pH 6 (□).

structure was monitored at that wavelength since at 222 nm the combination of α-helical and β-stranded structures is measured, whereas the influence of random coil conformations is negligible (Hennessey and Johnson, 1981). The CD curve of patatin at pH 8 shows a small linear decrease in the absolute ellipticity up to 35 and above 55 °C (Figure 5A). Between 35 and 55 °C, a decrease of about 4 mdeg is observed, suggesting a transition toward a less structured conformation, which is in agreement with previous data (Pots et al., 1998). The first derivative of the unfolding curve, as obtained using the Jasco software, shows a symmetric band with a maximum at 49 °C (Figure 5B). The symmetry suggests that the unfolding possibly follows a two-state mechanism, since the integral of a symmetric peak

Table 2. Parameters Related to the Denaturation of Patatin Determined with a Thermodynamic Fit of the Fat-UV Unfolding Curves As Described in the Text

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

forms a sigmoidal curve which can be indicative for a two-state unfolding (Pace et al., 1988). Patatin at pH 7 shows a comparable unfolding behavior as at pH 8; however, the midpoint of unfolding is shifted about 6 °C to higher temperatures (55.5 °C; Figure 5B).

To obtain further evidence that the unfolding could be a two-state process as well as an estimate of the enthalpy of unfolding (ΔH_u), the temperature curves were fitted with a procedure based on thermodynamic equations (Elwell and Schellman, 1977; Becktel and Schellman, 1987; Pace et al., 1988; van Stokkum et al., 1995). Identical unfolding curves at both half and twice the above-mentioned heating rate indicated that the unfolding was in equilibrium, being a prerequisite to apply such a fitting procedure (results not shown). In Table 2, it can be seen that the midpoint of unfolding at pH 8 and pH 7 is at 48.8 °C and 55.4 °C, respectively. This thermodynamic fit of the thermal unfolding curves of patatin at pH 7 and pH 8 has a small error (χ^2) implying that the conformational change indeed follows a two-step mechanism. The calculated ΔH_u is about 89 and 74 kJ/mol for pH 7 and pH 8, respectively (Table 2). Interestingly, DSC results revealed an ΔH_{cal} of only 20 kJ/mol for the cooperative transition between 50 and 55 °C (Pots et al., 1998). This indicates that a restricted part of the protein unfolds in a small temperature range. The wavelength scan spectra at 80 °C of patatin at pH 7 and pH 8 indicate a similarly structured protein (results not shown). In both cases the protein is soluble up to at least 80 °C. At pH 8, a continued decrease in the ellipticity is observed above the transition temperature range, which is not exhibited by the protein at pH 7.

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

Heating from 20 to 80 °C has a negligible effect on the CD signal at 222 nm when the protein is in buffer pH 3 (no results presented), whereas around 208 nm a small transition can be seen between 30 and 50 °C (Figure 5A). The zero-crossing of the wavelength scan at 80 °C shifts approximately 2 nm to higher wavelengths, and in the 190−200 nm region no significant differences are observed when compared to the spectrum

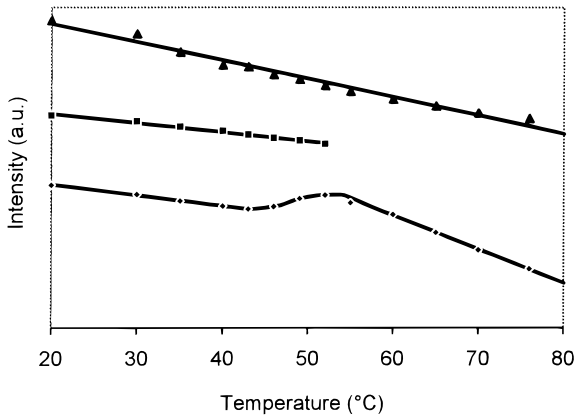

Figure 6. Thermal unfolding of patatin in phosphate buffer (I = 0.035 M) at pH 3 (▲), 6 (■), and 8 (◆) measured as fluorescence at 343 nm.

obtained at 20 °C (no further data shown). These changes as well as the observation that the transition was the most pronounced at 208 nm could be indicative for a helix-to-strand transition (Johnson, 1988; Hennessey and Johnson, 1981). Curve-fitting analysis indeed indicated that the protein at 80 °C still contains about 10% of helical and 50% of stranded structures (Table 1). The thermal unfolding behavior as was observed for pH 3 was also seen for patatin at pH 2 and pH 4 (no further data shown). No effect of 0.165 M NaCl on the thermal stability of patatin in buffer pH 3, 6, and 8 could be observed (data not shown).

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

The curve obtained of patatin at pH 8 shows a transition between 45 and 55 °C as indicated by an increase in its fluorescence emission due to a lower level of quenching of the two tryptophan residues upon unfolding (Pots et al., 1998). As was already observed with far-UV CD, patatin at pH 6 precipitates between 52 and 55 °C. Patatin at pH 3 shows no transition upon heating but only the linear decrease of the fluorescence intensity. This linear decrease is expected because the tryptophan environment of patatin at acidic pH appears to be already maximally solvent exposed (Figure 3) and because a heat treatment did not result in a significant change in tertiary structure (Figure 5). The different slopes of the fluorescence decay with increasing temperature may be due to different packing of the tryptophan residues in the protein at various pH.

To study the conformational changes of patatin at the tertiary level, the CD signal at 263 nm was monitored as a function of temperature for samples at various pH (Figure 7). This wavelength was chosen arbitrarily to present the typical trend indicative for the potential tertiary unfolding of the protein. Patatin at pH 8 shows a distinctive change of the CD signal upon heating from 42 to 60 °C, in agreement with previous results (Pots et al., 1998). In this temperature range, the

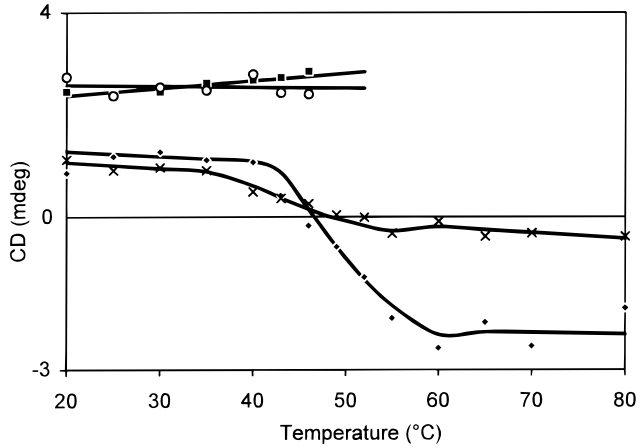

Figure 7. Near-UV CD at 263 nm of patatin showing thermal tertiary destabilization in phosphate buffers (I = 0.035 M) at pH 2 (×), 4 (○), 6 (■), and 8 (◆) measured as near-UV CD.

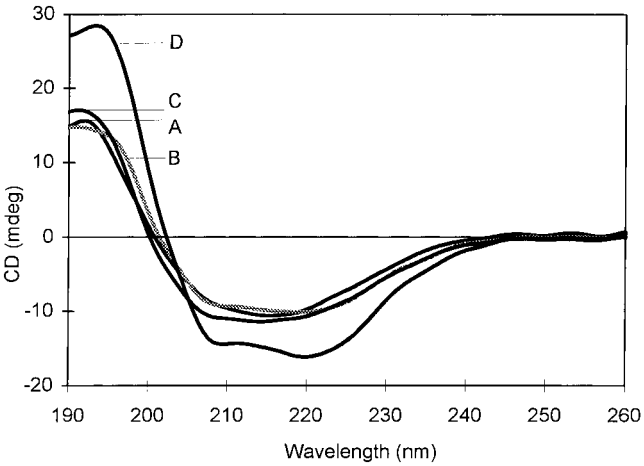

Figure 8. Reversibility of the unfolding of patatin at acidic pH at a secondary level of folding measured using far-UV CD at 20 °C. All samples contained 0.1 mg of patatin/mL of buffer. Samples at pH 3 were dialyzed to pH 8 prior to the measurement. Samples were (A) heated at pH 3 (15 min, 80 °C), (B)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

tertiary structure of the protein appears to change significantly.

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

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

UV CD spectra are shown of these pH 3 and pH 8 samples.

When patatin has been dialyzed to pH 3 and subsequently to pH 8, its secondary structure has been irreversibly unfolded as compared to the untreated protein structure at pH 8. The spectrum obtained from the pH 8 heated sample indicates a highly similar secondary structure as compared to both samples that have been at pH 3, irrespective of the heat treatment at pH 3. Interestingly, LAH measurements of patatin that have been at pH 3—but not heated—showed a residual specific activity of 1.16 ± 0.04 μmol min^{-1} (mg of protein)$^{-1}$ (64%) as compared to 1.82 ± 0.1 μmol min^{-1} mg^{-1} (100%) for the unheated pH 8 sample that has not been dialyzed to pH 3. Furthermore, the unheated pH 3 sample showed, after dialysis to pH 8, upon heating from 20 to 80 °C a small transition in the ellipticity at 222 nm. This transition had a midpoint of unfolding of 52.1 ± 0.2 °C ($\Delta H_u = 123 \pm 10.8$ kJ/mol; $\chi^2 = 1.74$; unfolding curve not shown) as determined with the fitting procedure as described above. After heat treatment of samples at any pH, no residual LAH activity could be detected.

DISCUSSION

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

Structural Stability of Patatin at Near-Neutral pH. Patatin at near-neutral pH at ambient temperatures is a highly structured protein at both a secondary and a tertiary level of folding (Figures 1−3; Table 1). Restricted parts of patatin exhibit a relatively high tendency to unfold, whereas at elevated temperatures the protein still contains a significant amount of helical and β-stranded structures (Figure 5A). Apparently, patatin has one or more relatively thermostable domains. In a previous study, it was shown that at pH 8 mainly the α-helical parts of patatin appear to unfold in the 45−55 °C region, whereas the β-stranded parts of the molecule unfold more gradually at temperatures from 50 to 90 °C (Pots et al., 1998). This could imply that particularly the β-stranded areas of the protein are part of these stable domains. The linear decrease of the ellipticity above the transition temperature at pH 8, which could not be observed at pH 7 (Figure 5A), could be due to differing structural stabilities of the stable domains (Creighton, 1996). At near-neutral pH the protein shows a thermal unfolding (Figure 5) that can be described using a fit based on thermodynamic parameters. The midpoint of unfolding can be deduced precisely with such a method; particularly the fact that the observed unfolding can be fitted accurately with this procedure (Table 2) suggests that the unfolding could follow a two-state transition (Pace et al., 1988). Formally the application of the thermodynamic fit is only allowed for a two-state fully-reversible transition. These conditions are not entirely fulfilled here because the thermal unfolding is not completely reversible. Nevertheless, the unfolding was in equilibrium, and the obtained fit was of good quality (Table 2). The midpoints of unfolding at pH 6, 7, or 8 (58, 55, and 49 °C, respectively; Table 2; Figure 5) indicate a decreased stability of the protein with increasing pH (Figure 5B). This is expected since proteins tend to have higher stability at pH values closer to their iso-electric point (Creighton, 1996). pH-induced changes in electrostatic interactions would be the major forces influencing the protein conformation and stability in this pH range. Minor changes in the protein structure are expected, since only the six histidine residues of patatin can be protonated in this pH range (pK_a of histidine, 6.5−7; Creighton, 1996). A protein like patatin, having its iso-electric point around 4.7, contains at pH 8 more negative net charge as compared to pH 6 or pH 7, possibly influencing the structural stability of the protein. Interestingly, at pH 6 where patatin has the thermally most stable conformation (Figure 5), it precipitates between 52 and 55 °C. This difference in behavior as compared to pH 7 or pH 8 was shown to be not an effect of pH-dependent differences in this pH range since in Figures 1−3 it can be seen that there are no significant differences in the structure of the protein. Upon heating at near-neutral pH the protein unfolds (Figure 5B), generally resulting in an increased exposure of hydrophobic sites. At a pH further away from its iso-electric point, the solubility of a protein is generally higher (Damodaran, 1997). The effect of the increased exposure of hydrophobic sites is smaller at higher pH, which can explain the precipitation of patatin at pH 6 whereas at pH 7 or pH 8 the protein remains soluble up to at least 80 °C.

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

compared to the far-UV CD measurements (Figure 7; Boye et al., 1997; Jiménez, et al., 1995).

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

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

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

ABBREVIATIONS USED

ANS, 8-anilino-1-naphthalenesulfonic acid; CD, circular dichroism; DSC, differential scanning calorimetry; LAH, lipid acyl hydrolase; MW, molecular weight; SDS−PAGE, sodium dodecyl sulfate−poly(acrylamide) electrophoresis; UV, ultraviolet.

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