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Effect of Heat-Induced Aggregation on the IgE Binding of Patatin (*Sol t 1*) Is Dominated by Other Potato Proteins

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The interaction of the major potato allergen patatin, *Sol t 1*, with IgE was investigated on a quantitative level as a function of heat treatment at different temperatures. On the basis of a number of publications, potato is considered to be a heat-labile allergen, but the molecular explanation for this behavior was not given. In this work, heat treatment of patatin in the absence and presence of other potato proteins mimicking the proteinaceous environment of the potato was studied. Using far-UV circular dichrosim spectroscopy, tryptophan fluorescence spectroscopy, and differential scanning calorimetry, the molecular transitions during heating of patatin were investigated. It was found that as long as patatin is not aggregated, denaturation of patatin on a secondary or tertiairy folding level is reversible with only a minor effect on the IgE affinity. Aggregation of patatin results in a nonreversible unfolding and a concomitant important decrease in affinity for IgE (25-fold). Aggregation of patatin in the presence of other potato proteins results in a less condensed aggregate compared to the situation of isolated patatin, resulting in a more pronounced decrease of affinity for IgE (110-fold). It is concluded that the heat lability of patatin−IgE interaction is explained by aggregation of patatin with other potato proteins rather than by denaturation of patatin itself.

KEYWORDS: Patatin; IgE binding; aggregation; potato proteins

INTRODUCTION

Patatin is the major protein in potato tubers as it accounts for 40% of the protein content. Although its physiological role is not entirely understood, its lipid acylesterase activity may indicate a role in the plant defense system (*1*). Due to the accumulation in the tuber, patatin is generally considered to be a storage protein (*2*, *3*). Because potatoes are widely consumed, patatin has an important nutritional contribution to the Western diet. Patatin is a glycosylated protein (*4*) consisting of 366 amino acids (*5*) appearing in isolated form as a dimer (*6*). The protein is highly structured at both secondary and tertiary levels (*7*). Heat denaturation of the isolated protein occurs at ∼55 °C but does not result in a complete unfolding of the protein (*7*). The protein retains a considerable degree of secondary structure and shows a strong aggregation tendency upon denaturation. The conformational properties and the thermostability of other potato proteins are not reported. About 75% of the total potato protein content is extractable in aqueous media (*28*).

Sensitization to potato is documented, and IgE binding is demonstrated for several potato proteins using Western blotting (*8*). Sensitization to potato often occurs in combination with birch pollen allergy (*9*−*11*). Because pollen allergens possess structural similarities with, for example, patatin [recently reviewed by Breiteneder and co-workers (*12*) and Aalberse (*13*)], native patatin is considered to be the most important potato allergen. This was established by Seppala (*14*), who identified patatin from raw potatoes as an in vivo reactive allergen [assigned as *Sol t 1* (*14*)] among a group of 27 atopic individuals from which 9 had birch pollen sensitization, too. A number of cases of allergic reactions are described in which raw potatoes were the offending allergen source. Skin contact with potato flesh (*14*−*16*) or inhalation of aerosols during potato scraping or peeling (*17*, *18*) are examples of contact with raw potatoes. Some studies show that raw potatoes give rise to allergic reactions, whereas cooking results in a partial or total loss of

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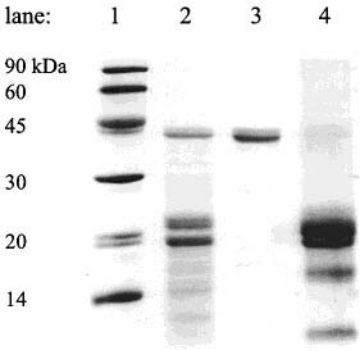

Figure 1. SDS-PAGE of potato fruit juice (PFJ), patatin, and potato protease inhibitor pool (PIP): (lane 1) MW markers, indicated in left margin; (lane 2) PFJ; (lane 3) peak 1 from PFJ fractionation; (lane 4) peak 2 from PFJ fractionation.

allergenicity (*10*, *18*). Fairly uncommon are allergic reactions to boiled potatoes present in foods. Nevertheless, allergy for boiled potatoes is documented (*17*, *19*) and considered to be of clinical importance for a certain group of patients. These patients typically have atopy, asthma, and, in some cases, birch pollen allergy. IgE recognition sites on patatin are not yet identified, and it is not known whether linear or structural IgE epitopes are important in evoking symptoms.

In view of the limited conformational changes and the reported aggregation behavior of patatin upon heat denaturation (*7*), it can be hypothesized that this latter phenomenon is responsible for the diminished allergenic character of boiled potatoes. It is the aim of this work to study the IgE binding properties of heat-treated patatin in relation to its aggregation behavior. Because aggregation processes can be strongly affected by the presence of other protein components, the role of other potato proteins in these processes is investigated as well. In particular, a group of proteins assigned as protease inhibitors [protease inhibitor pool (PIP)] that, together with patatin, comprises >80% of the soluble potato tuber protein was studied.

EXERIMENTAL PROCEDURES

Preparation of Potato Fruit Juice (PFJ). Potatoes (cv. Bintje) were washed thoroughly with water, peeled, and cut into large pieces (maximum = 8 × 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 juice extractor (AEG). The remaining 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 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, was used in the fractionation experiments and is further denoted PFJ. PFJ was stored at −20 °C.

Fractionation of PFJ. Fractionation of PFJ was obtained by gel filtration on a Superdex 75 column (63 × 10 cm, Pharmacia) eluted with a 10 mM sodium phosphate buffer (pH 6.8) at a flow rate of 30 cm/h. The first peak eluting from the column, as monitored by the absorbance at 280 nm, contained a 43 kDa protein band [~95%, previously assigned as patatin (*6*)] as analyzed by SDS-PAGE (**Figure 1**, lane 3). Fractions of this peak were collected and concentrated 10 times using Xampler UFP-3-C cross-flow hollow fiber laboratory cartridges with a molecular weight cutoff of 10 kDa (A/G Technology Corp., Needham, MA) at 4 °C. The concentrated patatin solution was subsequently diafiltrated with 5 volumes of 10 mM sodium phosphate buffer (pH 6.8) and stored at −20 °C. This preparation, denoted patatin, was used for further experiments.

The second peak eluting in the above-mentioned fractionation contains mainly proteins with a molecular weight between 20 and 25 kDa [previously assigned as protease inhibitors (*20*)], as analyzed by SDS-PAGE (**Figure 1**, lane 4). Fractions of the second peak were collected and combined in the PIP. The pooled fraction was concentrated 10 times using Xampler UFP-3-C cross-flow hollow fiber laboratory cartridges with a molecular weight cutoff of 5 kDa (A/G Technology Corp.) at 4 °C. The concentrated solution was subsequently diafiltrated with 5 volumes of 10 mM sodium phosphate buffer (pH 6.8) and stored at −20 °C. This preparation, denoted PIP, was used for further experiments.

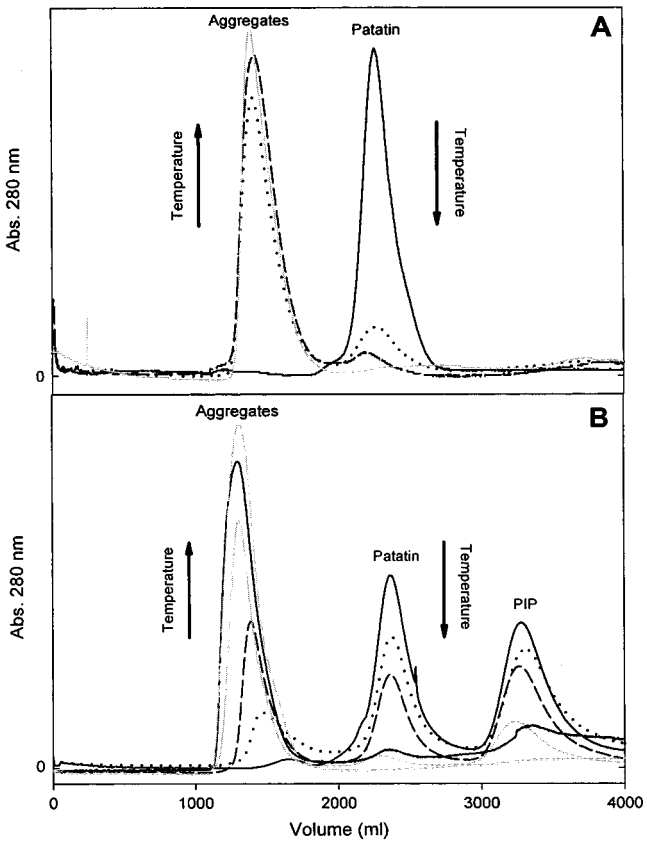

Figure 2. GPC of patatin samples after different treatments: (A) fractionation of patatin and aggregates after heating of patatin in the absence of PIP; (B) fractionation of patatin, PIP, and aggregates after heating of patatin in the presence of PIP.

Heat Treatments. Samples of patatin (40 mL) were mixed with either 60 mL of PIP or 60 mL of 10 mM sodium phosphate buffer (pH 6.8). This ratio was chosen to mimic the protein composition in potato. The mixtures were heated during 20 min to reach the desired temperature in a water bath set at temperatures of 45, 50, 55, 60, 65, and 80 °C. After heating, the mixtures were immediately cooled on ice and subsequently filtered through a 0.2 μm filter. Next, the filtered samples were applied on a Superdex 200 column (63 × 10 cm) (Pharmacia) and eluted with a 10 mM sodium phosphate buffer (pH 6.8) with a flow rate of 30 cm/h. Fractions of the first peak were collected and combined as the aggregated protein fraction (see **Figure 2**). The second peak, which contained nonaggregated patatin, was also collected (see **Figure 2**). The third peak consisted of nonaggregated PIP proteins and was discarded. The aggregated ptotein and nonaggregated patatin fractions were stored at −80 °C until used in further experiments.

Patient Sera. Serum from adult patients sensitized for potato proteins was collected. RAST (Pharmacia & Upjohn Diagnostics) values for potato ranged from 0.6 to 53 kU/L (mean = 28 kU/L), and skin prick test with fresh potato ranged from 2+ to 4+ and was in one patient negative. Anamnesis revealed that the most frequently experienced symptoms were urticaria and itching eyes during potato peeling. The presence of patatin-specific IgE in the patients sera was demonstrated using direct ELISA and Western blotting. Seven sera were selected on the basis of their potato-specific IgE levels and were used for further studies. A nonallergic individual and an allergic, but not potato-allergic,

individual were used as controls. Venous blood was withdrawn from the individuals and allowed to clot. Serum was collected by centrifugation and stored in aliquots at −20 °C until use. All studies were approved by the Medical and Ethical Committee of the University Medical Center Utrecht (Utrecht, The Netherlands), and informed consent was obtained form each patient.

IgE-Binding Experiments. Affinities of patatin for IgE were measured using IgE-binding experiments similar to an earlier reported assay for peanut allergen *Ara h1* (*21*). In short, dilutions of differently treated patatin samples [final patatin concentrations of 0.5 ng/mL to 1 mg/mL, calculated on the basis of the OD_{280} (OD_{280} 1 mg/mL = 0.72)] were incubated with a 1 to 30 dilution of patient serum in phosphate-buffered saline (PBS) containing 1% BSA and 0.1% Tween 20. In this fluid phase, patatin was allowed to bind to IgE for 1 h at room temperature under gentle shaking conditions. To determine the non-bound IgE fraction, the incubation mixtures were transferred to the 96-well plates pretreated as follows. Ninety-six-well plates were coated with 10 μg/mL patatin from non-heat-treated potatoes in PBS and subsequently blocked with BSA (1% in PBS containing 0.1% Tween 20) to diminish nonspecific binding. IgE bound to the patatin-coated wells was detected using an anti-human IgE antibody conjugated to horseradish peroxidase (MELIE 1/2, DPC, Los Angeles, CA). Between each step, the plates were washed five times with PBS containing 0.1% Tween 20. The inhibition of IgE binding as a function of the amount of patatin present in the preincubation sample reflects the affinity of patatin for IgE. The concentrations needed for half-maximal inhibition were calculated using a semilogarithmic equation and were used to compare the affinities of the different forms of patatin for IgE. Triplicate experiments resulted in averages with standard deviations <15% of the shown values. In control experiments, a possible role for PIP (in the absence of patatin) in the inhibition of patatin−IgE interaction was analyzed and appeared to be negligible. The specificity of the patatin−IgE ELISA was demonstrated using serum from a peanut-allergic individual that did not give rise to any substantial ELISA signal (OD < 0.090).

Circular Dichroism (CD) Measurements. Far-UV CD spectra of 0.15 mg/mL samples of the patatin fraction in 10 mM bis-Tris buffer (pH 7.0) were recorded as averages of 16 spectra on a Jasco J-715 spectropolarimeter (Jasco Corp.) at 20 °C. Quartz cells with an optical path of 0.1 cm were used. The scan range was 190−260 nm, the scan speed 100 nm/min, the spectral resolution 0.5 nm, the bandwidth 1 nm, and the response time 0.125 s. Spectra were corrected for a protein-free spectrum obtained under identical conditions, and subsequent noise reduction was applied using the Jasco software. The spectra were analyzed from 240 to 190 nm with a 1-nm resolution to estimate the secondary structure content of the proteins. Spectra were fitted using a nonlinear least-squares regression procedure with reference spectra of polylysine in the α-helix, β-strand, and nonstructured conformation (*22*), and the spectrum of β-turn structures was extracted from 24 proteins with known X-ray structure (*23*). Such a fitting procedure gives the relative contributions of the reference spectra that make up the best fit of the measured spectrum and from which the secondary structure can be calculated.

Fluorescence Measurements. Fluorescence spectra of 30 μg/mL patatin in 10 mM bis-Tris buffer (pH 7.0) were recorded at 20 °C as averages of four spectra on a Perkin-Elmer LS 50 B luminescence spectrometer equipped with a pulsed xenon light source. Excitation was at, respectively, 274 and 295 nm to monitor the tyrosine and tryptophan fluorescence emission. Spectra were recorded in the 305−420 nm spectral region with a resolution of 0.5 nm with a scan speed of 100 nm/min. The slits were set at 5 nm. All spectra were corrected for the emission spectrum of a protein-free sample.

To determine the ability of iodide to quench the fluorescence as a measure for solvent accessibility of the chromophores, the fluorescence intensity was quantified by determining the area of the spectrum in the region recorded as a function of the iodide concentration. This area is presented as the relative fluorescence obtained by dividing the peak area by that of the spectrum in the absence of iodide.

Differential Scanning Calorimetry (DSC). DSC was performed on a Perkin-Elmer DSC-7 equipped with 1 mL vessels and a detection limit of 2.3 μJ g^{-1} $°C^{-1}$. Samples containing 0.15 mg/mL patatin fraction in 10 mM bis-Tris buffer (pH 7.0) were scanned from 15 to 85 °C with a heating rate of 0.5 °C/min. Determination of ΔH_{cal} was achieved by taking the integral of the recorded peak using an automated baseline correction. All samples were recorded twice. Because previous results demonstrated the irreversibility of the thermodynamic transitions (*7*), no cooling or reheating scans were recorded.

Table 1. Relative Content of Patatin in Aggregates, Obtained by Heat Treatment at Various Temperatures of Mixed Samples of Patatin and PIP, Determined by Analysis of the GPC Elution Profile (**Figure 2**)

temp (°C)	% patatin	temp (°C)	% patatin
45	no aggregates formed	60	65 ± 1
50	86 ± 3	65	59 ± 1
55	71 ± 1	80	52 ± 1

RESULTS

Patatin Isolation and Heat-Induced Aggregation. Patatin and PIP are isolated from PFJ using gel permeation chromatography (GPC). Patatin migrates as a single band on SDS-PAGE (**Figure 1**, lane 3), and PIP is composed of a number of distinct protein bands with molecular weights ranging from 5 to 25 kDa (**Figure 1**, lane 4) previously assigned as protease inhibitors (*20*). It can be seen from **Figure 1** (lane 2) that PFJ is almost completely composed of patatin and PIP. Mixtures of patatin and PIP were prepared to mimic the proteinaceous content of the potato. To study the effect of heating on structural and IgE-binding characteristics of patatin, patatin itself and mixtures of patatin with PIP are heat-treated at various temperatures. A treatment of 20 min was chosen because it was previously shown that equilibrium in denaturation (*7*) and completion of aggregation (*24*) were achieved and because it reflects a typical time span for the preparation of foods. Heat treatment results in the formation of aggregates in a temperature-dependent way as demonstrated by GPC (**Figure 2**). The area of the aggregate peak increases with increasing temperatures, whereas both the patatin and PIP peaks decreased with increasing temperatures. The overall intensity remains constant. It can be seen that heating in the presence of PIP results also in aggregation of the PIP proteins because the intensity of the PIP peak decreases with heating temperature. The aggregates elute in the void volume of the Superdex 200 column and have estimated molecular masses between 200 and 600 kDa. No indications were observed that aggregate size distribution differs for aggregates formed in the absence or presence of PIP upon analysis of the samples on a Superose 6 column (not shown). The relative content of patatin in the aggregates ranges from 86% at 50 °C to 52% at 80 °C (**Table 1**), indicating that patatin is the major constituent. SDS-PAGE analysis of aggregates demonstrates a similar trend (not shown). All further results presented are corrected for the actual patatin content of the aggregated samples.

Interaction of Patatin with IgE. The different patatin preparations were subjected to IgE-binding analysis using a competition ELISA in which the patatin samples compete with immobilized patatin. **Figure 3A** shows a typical inhibition curve for native patatin. A dose-dependent inhibition is observed in the nanograms to micrograms per milliliter range. The concentration required for half-maximal inhibition (IC_{50}) was used for comparing the IgE affinities for the various patatin samples. For native patatin, the IC_{50} is 0.041 μg/mL. Inhibition curves were made for all tested samples, and **Figure 3B** shows the IC_{50} values as a function of the heating temperature. Heating patatin to 45 °C leads to a 4-fold decrease in affinity for IgE.

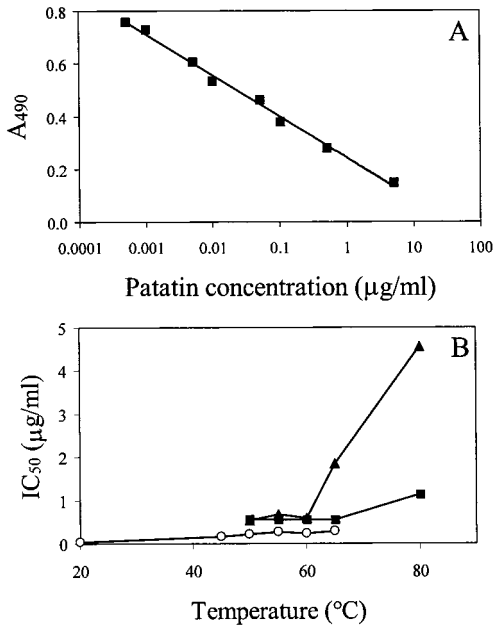

Figure 3. Patatin–IgE interaction: (A) inhibition curve of native patatin; (B) IC_{50} values as a function of heating temperature; (○) non-aggregated patatin; (■) patatin aggregates (heated in the absence of PIP); (▲) aggregates of patatin with PIP (heated in the presence of PIP).

This affinity is not changed for patatin heated to 65 °C (**Figure 3B**). However, part of the patatin becomes aggregated at temperatures exceeding 45 °C. This aggregated fraction was studied for IgE-binding properties separately (**Figure 3B**). Compared to nonaggregated patatin, the patatin aggregates exhibit an additional 3-fold decrease in IgE affinity between 45 and 65 °C. At 80 °C, this affinity is further decreased to 1.1 μg/mL, that is, a 25-fold decrease as compared to non-heat-treated patatin. In the presence of PIP, the IgE affinities of the aggregates are affected in a more pronounced way. Up to 60 °C, the IC_{50} values are similar to those of patatin aggregates, whereas at 65 and 80 °C the IgE affinity decreases significantly to 4.8 μg/mL. This is a 110-fold decrease compared to non-heat-treated patatin.

Structural Properties. The above results suggest that reduction of IgE-binding affinity is predominantly caused by patatin aggregation rather than changes in structural epitope recognition on a molecular level. To test this, studies are performed to investigate the structural properties of the proteins at secondary and tertiary folding levels and at a quaternary organization.

Secondary Level. **Figure 4A** displays far-UV CD spectra of patatin in the nonaggregated (dimer) and aggregated forms after a heat treatment at 55 °C in the absence of the PIP fraction. The nonaggregated protein exhibits two strong negative extremes at 222 and 207 nm and a zero-crossing around 201 nm, a spectrum comparable to that reported previously for native patatin (*7*). The aggregated material shows distinct spectral features with, for example, a zero-crossing at much lower wavelength. Such spectral differences point to a more unfolded character of the protein in the aggregated form (*23*). Spectral analysis, based on a nonlinear least-squares fitting procedure using reference spectra to describe the recorded spectrum (*22, 25*), can provide estimates of the secondary structure content, and the results of such analysis are presented in **Table 2**. Indeed, it can be seen that the nonaggregated heat-treated patatin possesses a secondary structure content comparable to that of the reference non-heat-treated protein. In fact, CD analysis of all nonaggregated patatin fractions heated from 45 to 65 °C

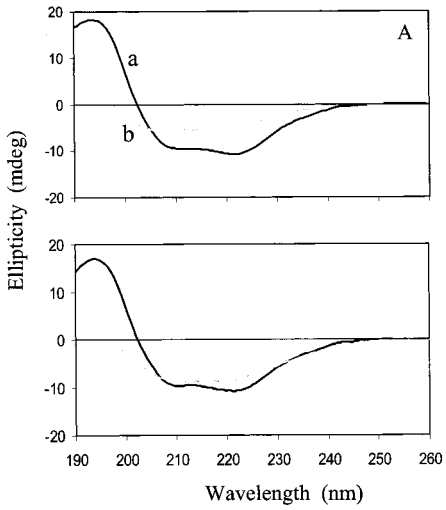

Figure 4. Far-UV CD spectra of 0.15 mg/mL patatin fractions after heating at 55 °C in the absence (A) and presence (B) of the PPI fraction. Spectra a represent the native fraction and spectra b the aggregated form.

Table 2. Secondary Structure Estimates Obtained from CD Spectra of Patatin Heated at 55 °C in the Absence or Presence of PPI Fraction in the Native and Aggregated Forms

patatin sample	α-helix	β-strand	β-turn	nonstructured
non-heat-treated	38	53	1	8
nonaggregated, –PIP	38	50	2	10
aggregated, –PIP	24	31	10	35
nonaggregated, +PIP	35	54	1	10
aggregated, +PIP	14	47	2	37

displayed a similar secondary structure content (results not shown). It is interesting to note that after heating well above the denaturation temperature of patatin (*7*) for a sufficiently long period to reach equilibrium, the protein regains its nativelike conformation upon cooling to 20 °C. Apparently, the reversibility of the heat-induced conformational changes is prevented by aggregation. Aggregated patatin contains ~25% more nonstructured parts (**Table 2**) compared to nonaggregated material, and again the secondary structure content was independent of the heating temperature (results not shown).

When the patatin fraction is heated in the presence of PIP (**Figure 4B**), the CD spectrum of the nonaggregated patatin is similar to that of heated, nonaggregated, patatin in the absence of PIP, whereas the aggregated protein displays a spectral difference compared to the spectrum in **Figure 4A**. Spectral analysis shows that indeed PIP has no effect on the secondary structure of nonaggregated heated patatin (**Table 2**), irrespective of the heating temperature (results not shown). Compared to aggregates of patatin heated in the absence of PIP, aggregates of patatin heated in the presence of PIP have 10% lower helical content and 15% higher β-strand content (**Table 2**). Again, no strong effect of the heating temperature is observed (results not shown). As shown in **Table 1** these latter aggregates do contain from 14% (50 °C) to 48% (80 °C) PIP proteins. The CD spectrum of PIP, however, has a molar ellipticity ~6 times smaller than observed for patatin (unpublished results), explaining why the CD spectra as shown, for example, in **Figure 4B**, and the spectral analysis (**Table 2**) are not severely influenced by the presence of PIP.

Because at a secondary level no temperature dependence is observed for aggregated proteins, it can be concluded that the decrease of affinity with increasing temperature (**Figure 3**) is not related to differences at the secondary folding level. It is,

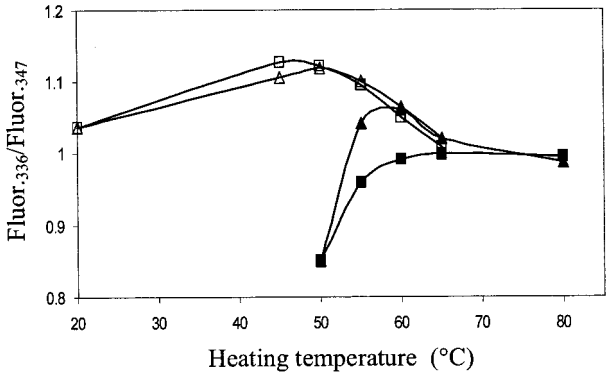

Figure 5. Ratios of fluorescence intensities at 336 and 347 nm, as a measure for the solvent exposure of tryptophan in patatin samples, plotted as a function of the heating temperature for both the aggregated (solid symbols) and nonaggregated (open symbols) forms. The samples were heated either in the absence (squares) or in the presence of PIP (triangles).

however, possible that the slightly (3-fold) lower IgE affinity observed for aggregated material compared to nonaggregated patatin is related to its lower secondary structure content.

Tertiary Level. To get insight at a tertiary folding level, fluorescence spectroscopy is used to monitor the local environment of the tryptophan residues. The fluorescence spectra of patatin exhibit two local maxima at 336 and 347 nm, representing a population of buried and more solvent-exposed tryptophan residues, respectively. The ratio of the fluorescence intensities at these two wavelengths represents a measure for the relative solvent exposure of the tryptophan, reflecting the tertiary packing of the protein. The lower this ratio, the more solvent-exposed the tryptophans are and, thus, the more tertiary-destabilized the protein is. These ratios are presented in **Figure 5** for the aggregated and nonaggregated proteins heated in the absence and presence of PIP. The values of the aggregated proteins heated in the presence of PIP are corrected for the calculated PIP contribution based on the fraction denoted in **Table 1** and the corresponding fluorescence spectrum of PIP (not shown). Remarkable is the significant increase of the ratio for the nonaggregated patatin upon heat treatment at 45 and 50 °C in the absence and presence of PIP compared to nontreated material (20 °C). Although at a secondary folding level no temperature effect was observed, at a tertiary level the protein appears to be more closely packed at intermediate temperatures, whereas heating at temperatures >50 °C causes the proteins to become more flexible. The observation that PIP seems to shift this maximum to slightly higher temperatures could indicate that PIP interacts with patatin in solution and thereby affects the tertiary interactions of patatin. The aggregated material has a looser packing of the tertiary structure than the nonaggregated material. This correlates to the more unfolded character of the material at a secondary level. Fluorescence analysis (**Figure 5**) also demonstrates that in the absence of PIP with increasing temperature the aggregated protein becomes more compact until a temperature of 65 °C is reached. Above this temperature the solvent exposure of the tryptophans does not change any more. Whereas at 50 °C the aggregated protein is strongly tertiary-destabilized, in the presence of PIP the solvent exposure of the tryptophans first approaches the curve of the nonaggregated patatin with increasing temperature. This suggests that in the aggregates formed in the presence of PIP nondenatured or reversibly denatured patatin is enclosed. Excitation at 274 nm instead of 295 nm gave comparable results (not shown). The band shape was identical, and the total intensity was ~2.4 times higher due to the energy transfer from tyrosine to tryptophan residues. Attempts to confirm these observations using near-UV CD failed, because for many of the heat-treated samples the protein concentration was too low to obtain reliable spectra (results not shown). The decision was made not to concentrate as could be done, for example, by ultrafiltration or lyophilization, because it was known that patatin is prone to aggregation if subjected to such treatment (unpublished observations).

Table 3. DSC Analysis of Aggregated and Nonaggregated Protein Fractions of Patatin in the Absence and Presence of PIP

patatin sample	heating temp (°C)	*H* (kJ/mol)
non-heat-treated		145 ± 11
nonaggregated, –PIP	45	143 ± 18
nonaggregated –PIP	65	106 ± 8
aggregated, –PIP	65	0 [a]
nonaggregated, +PIP	65	110 ±16
aggregated, +PIP	65	42 ± 11

[a] No endothermic transition observed.

Table 4. Concentrations of Iodide Required for Obtaining a 50% Decrease of Tryptophan Fluorescence for Various Patatin Samples (Results Are Averages of Two Separate Experiments)

patatin sample	heating temp (°C)	I^- concn (M)
non-heat-treated		0.42 ± 0.01
nonaggregated, –PIP	45	0.48 ± 0.02
nonaggregated, –PIP	65	0.32 ± 0.01
aggregated, –PIP	65	0.64 ± 0.03
nonaggregated, +PIP	65	0.38 ± 0.01
aggregated, +PIP	65	0.36 ± 0.02

An alternative way to get insight in the tertiary packing of the protein is by using DSC (**Table 3**). Generally, a single broad endothermic transition was observed with a maximum between 55 and 57 °C (thermograms not shown). For the non-heat-treated patatin an ΔH_{cal} of 145 kJ/mol was found. Heat treatment at 45 °C does not have an effect on the nativity of the material, whereas heating at 65 °C results in a decrease of 27% of the enthalpy change for the nonaggregated material compared to the native protein. The presence of PIP during the heat treatment does not affect the nativity of the nonaggregated patatin, whereas it strongly affects the nativity of the aggregated material. Whereas in the absence of PIP the aggregated patatin is fully irreversibly denatured, the aggregates obtained in the presence of PIP enclose non-denatured protein, which is in agreement with the fluorescence data. On the basis of the shape of the thermogram at least 70% of this non-denatured protein is patatin.

From the above data no clear correlation between the tertiary packing of the individual proteins and their IgE-binding affinity (**Figure 3**) can be deduced. On the other hand, these results do indicate that the types of aggregate might be different in the absence and presence of PIP.

Quaternary Organization. Quenching of the tryptophan fluorescence by iodide can be used to investigate the nature of protein aggregate formed. The efficiency of quenching depends on the distance between the protein chromophores and the quencher. The more compact an aggregate structure is, the less efficient a quenching process will be. **Table 4** summarizes the concentration iodide required to obtain a 50% quenching for the aggregates obtained in the absence and presence of PIP with respect to the nontreated patatin. In the absence of PIP the aggregated patatin requires much more iodide to quench the fluorescence, suggesting that some of the proteins are not solvent-exposed. Significantly less iodide is required to achieve quenching for the aggregates formed in the presence of PIP

compared to the pure patatin aggregates. Apparently the aggregates containing PIP are looser in structure because iodide is able to penetrate into the aggregate, resulting in an even more efficient quenching compared to the dimeric form of the nontreated patatin. It is also interesting to note that the nonaggregated patatin exhibits a more condensed structure when heated at 45 °C, because the protein is less sensitive to quenching, whereas the protein heated at 65 °C has a more open structure compared to that of the nontreated protein. This is in full agreement with the tryptophan data shown in **Figure 5**. **Table 4** also illustrates that the presence of PIP does affect the tertiary stability of nonaggregated patatin heated at 65 °C, as reflected in the somewhat reduced sensitivity for iodide quenching.

DISCUSSION

Sensitization for potato is characterized by the presence of potato-specific IgE in serum from patients found in a subpopulation of pollen-allergic patients. Potato-specific IgE can be demonstrated in vitro and in vivo, but in the majority of cases, clinical symptoms are not present. If symptoms are present, they are almost exclusively evoked by raw potatoes, such as during peeling or scraping potatoes, resulting in local reactions such as contact urticaria or rhinoconjuctivitis. The vast majority of patients can eat cooked potatoes without any problem. This implies that patatin is a heat labile allergen. The heat-induced denaturation and aggregation of purified patatin in relation to its molecular structure were published by our group earlier (*7*). In the present work we provide molecular insight in the mechanism for the reduced allergenicity of boiled potatoes.

Patatin in its isolated form denatures on secondary and tertiary folding levels upon heating (*7*). The present work surprisingly shows that none of these denaturation processes affect the affinity for IgE substantially, whereas the aggregation of patatin leads to some decrease of IgE affinity. However, this cannot fully explain the heat lability of the allergenicity of patatin. To investigate what determines the reduction of allergenicity of boiled potatoes compared to their raw counterparts, we developed a model system that mimics the proteinaceous environment of the potato by the addition of potato protease inhibitors (PIP). In this model system, patatin was heat-treated at different temperatures and the effect on IgE binding was studied. With increasing temperatures, patatin and PIP formed increasing amounts of aggregates (**Figure 2**). As a consequence the fraction of free patatin and PIP was decreased at higher temperatures. Determination of the IgE affinity for patatin in a series of samples heat-treated at different temperatures below and above the reported denaturation temperature (50−55 °C), and in the absence or presence of other potato proteins, provided three main observations. These observations are discussed separately below.

Heating of patatin at 45 °C or higher resulted in a 4-fold decrease of the IgE-binding affinity (**Figure 3B**). At a secondary folding level (**Figure 4** and **Table 2**) no correlation between the heating temperature and IgE affinity could be found. At a tertiary level, however, significant changes in tertiary packing are observed for the nonaggregated protein. Whereas heating at 45−50 °C (below the reported denaturation temperature) resulted in a more compact structure (**Figure 5** and **Table 4**), heating at higher temperatures provided a more loosely folded protein (**Figure 5**) with a concomitant loss of nativity as determined by DSC (**Table 3**). This temperature dependence of the tertiary packing of the protein is not reflected in a temperature dependence of the IgE affinity. Changes in the tertiary packing already occurring after heat treatment at <50 °C might be responsible for the 4-fold decrease of IgE affinity, irrespective of conformational changes taking place at higher temperatures.

From **Figure 3B** it can also be seen that aggregation of patatin upon denaturation causes an additional 3-fold decrease of IgE-binding affinity. At a secondary folding level two interesting observations were made (**Figure 4** and **Table 2**). First, it is demonstrated that as long as patatin does not aggregate, heat-induced conformational changes on the secondary folding level are fully reversible. Second, upon aggregation, the protein appears to be more unfolded (∼25% of the total protein), which could be responsible for the 3-fold decrease of IgE affinity. At a tertiary level, no correlations are found between patatin structure and IgE affinity. It is also shown that in the absence of PIP the aggregate is compact, making it difficult for a soluble fluorescence quencher to approach the chromophores (**Table 4**). From GPC the aggregate size was estimated to range from 200 to 600 kDa, corresponding to 5−15 patatin molecules per aggregate. Most likely, part of the patatin molecule is buried inside these aggregates and not available for interaction with IgE, explaining the 3-fold difference in affinity.

Finally, up to 60 °C no effect of the presence of other potato proteins on the IgE affinity is observed, whereas at higher temperatures a strong additional decrease of the IgE-binding affinity due to the presence of PIP is found (**Figure 3B**). Clearly, the protein aggregate in the presence of PIP differs from that formed by heat treatment in the absence of other potato proteins. The proteins in these first aggregates (i) have a distinct shift from α-helical to β-stranded structures (**Figure 4**; **Table 2**), (ii) enclose patatin that is not irreversibly denatured (**Figure 5**; **Table 3**), and (iii) have a much more open character (**Table 4**). The observation that the presence of PIP is of importance only above 60 °C can be explained by the fact that PIP proteins start to denature between 60 and 70 °C. Assuming that in these systems the PIP proteins are the driving force in the aggregation process, the enclosure of non-irreversibly denatured patatin in the aggregates can be explained. A possible interference of potato carbohydrates in the aggregation and concomitant sensitization of patients for patatin cannot be excluded. However, the majority of the carbohydrates present in potato are nonreducing, and the time intervals that are chosen in this study are known not to result in Maillard products. Furthermore, there is no literature that indicates a complex coacervation of starch components with proteins during heat treatment. Therefore, we decided not to include the carbohydrate fraction in our model system.

In summarizingy, by studying both the thermal effects and the structural effects on the IgE-binding properties, this work demonstrates that the heat-labile character of patatin allergenicity is not a result of molecular conformation changes only but that the consequence of the denaturation, namely, an aggregation phenomenon, is more important. Because such an aggregation process can depend strongly on the presence of other compounds, as illustrated in this work with potato protease inhibitors, it is questionable to study isolated allergens in relation to (food) processing conditions. None of the previously published papers on IgE−patatin interaction took the aggregation phenomena of patatin into account. Another point that emphasizes the importance of studying potato allergens in their natural occurrence is provided by sensitization routes. It was postulated over 50 years ago that pollen-sensitized patients often had clinical sensitivity to foods such as hazelnut, carrot, apple, and potato (*26*). It is now generally believed that sensitization takes place via inhalation of pollen and that the clinical reactions to several

foods are due to IgE cross-reactivity with patatin (*10*, *27*). Because sensitization takes place via inhalation of pollen, rather that via (heat-treated) foods, the allergens that cause sensitization are in their native conformational state. In our study, we have described the IgE-binding characteristics of patatin in relation to heat treatment as it occurs during boiling of potatoes. We have provided an explanation for the heat-induced decrease of IgE-binding properties. The fact that not the denaturation itself, but the aggregation of patatin with other potato proteins affects the allergenicity of patatin may provide new insights in the assessment of allergenicity of processed foods.

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