

Allergenicity of Potato Proteins and of Their Conjugates with Galactose, Galactooligosaccharides, and Galactan in Native, Heated, and Digested Forms

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S Supporting Information

ABSTRACT: The effect of glycation of potato proteins on their immunoreactivity was studied by using a pool of human sera with specific IgE to potato proteins. Patatin conjugates were more immunoreactive than protease inhibitors ones. To better understand this behavior, the changes in patatin structure upon glycation and heat treatment were investigated. Patatin demonstrated an increase in total immunoreactivity when glycated with galactose and galactooligosaccharides. However, galactan conjugation to patatin resulted in a decrease in immunoreactivity by restricting IgE's access to the epitopes. Although the heat treatment resulted in a decrease in patatin's immunoreactivity through aggregation, it was less effective when patatin conjugates were used due to the decrease in aggregation and the secondary structural changes. Upon digestion, native patatin exhibited the largest decrease in immunoreactivity resulting from the disruption of both conformational and sequential epitopes. Patatin conjugates were less digested and had higher IgE-immunoreactivity as compared to the digested patatin.

KEYWORDS: Maillard reaction, potato proteins, patatin, galactan, galactose, galactooligosaccharides, immunoreactivity, protein structure

INTRODUCTION

Food allergy occurs in 2–6% of the population.¹ The mechanism underlying an allergen response involves an exposure of the allergenic patient to the causative allergen leading to increased terminally differentiated B lymphocyte (plasma cell) immunoglobulin E (IgE) antibody production. The IgE binds to IgE receptors of mast cells. When the antigen interacts with the bound IgE, mast cells release various substances, such as histamine, to the surrounding environment, which are responsible for the clinical symptoms typical of immediate hypersensitivity.² Although food allergy to potato is rare, a number of IgE binding proteins have been identified in potato tubers.³ It has been demonstrated that the sensitization to potato proteins occurs in conjunction with birch pollen allergy due to the structural similarities between the proteins.⁴ Most documented allergic reactions involve contact with raw potatoes^{5,6} and, to a lesser extent, boiled potatoes.⁷

Compared to proteins from other sources, potato proteins are considered higher in nutritional quality as they contain a high proportion of lysine similar to egg proteins.⁸ Potato proteins, being abundant in the potato byproducts, are emerging as interesting replacements for many existing food proteins.⁹ There are three major fractions of potato proteins: (1) patatin (PTT, ~40%); (2) protease inhibitors (PIs, ~50%); and (3) other high molecular weight (MW) proteins (~10%).¹⁰ PTT, which is the major allergen found in potato,⁶ is a dimer glycosylated protein with up to two carbohydrate chains, resulting in isoforms with MWs ranging from 40 to 45 kDa.¹¹ In addition to its anticarcinogenic¹² and antimicrobial effects,¹³ PTT has demonstrated emulsifying and foaming properties, which make it

suitable for diverse applications;¹⁴ however, its low solubility and low denaturation temperature have limited its applications.¹⁵ PIs have also been involved in allergic reactions to potato tuber¹⁶ and are composed of diverse proteins with a MW ranging from 5 to 25 kDa. PIs can promote satiety by increasing the cholecystokinin circulation.¹⁷

The glycation of potato proteins through the Maillard reaction has been demonstrated as a promising method to improve their functional properties.¹⁸ However, overall, the conjugation of food protein allergens with carbohydrates may decrease¹⁹ or enhance²⁰ their allergenicity.²¹ Indeed, the decrease in the allergenicity of proteins upon conjugation with carbohydrates was attributed to the steric inhibition of some epitope bindings, as a result of protein conformational change and/or glycation of the amino acid residues located on the epitopes.²² However, the formation of new epitopes and/or the exposure of the buried epitopes upon glycation have contributed to the increase in the allergenicity of glycoproteins.^{23,24} To the best of the authors' knowledge, the effect of glycation on the IgE-immunoreactivity of potato proteins has not yet been investigated.

The presented study was aimed at the investigation of the effect of glycation on the IgE-immunoreactivity of glycated potato proteins, PTT and PIs, with galactose, galactooligosaccharides (GOS), and galactan, through the Maillard reaction. PTT being the major potato allergen, the IgE-binding properties

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of their conjugates were investigated in relation to their protein structures. Because heat treatment and gastric digestion can affect the allergenic character, the immunoreactivity of PTT conjugates was assessed after these two treatments.

MATERIALS AND METHODS

Materials. Potato protein Solanin 206P (PTT $\approx$ 75%, PIs $\approx$ 25%) was kindly provided by Solanin (Foxhol, Ne). Potato galactan and endo-1-4- β -D-galactanase from *Aspergillus niger* were purchased from Megazyme (Wicklow, Ir). Furosine standard was purchased from Neosystem Laboratories (Strasbourg, Fr). Biogel P2 extra fine, Bradford reagent, and broad range SDS-PAGE standards were purchased from Bio-Rad (Philadelphia, PA). Four potato-sensitive human sera (Table SI-1, Supporting Information) were acquired from PlasmaLab (Everett, WA) (8.75–22.40 kU_A/1 potato specific IgE, ImmunoCAP; Pharmacia, Uppsala). Sera from nonallergic (no specific IgE) and dust-mite allergic (>100.00 kU_A/1 dust-mite specific IgE) individuals as controls were also obtained from PlasmaLab. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of Conjugates. PTT and PIs present in Solanin 206P were separated by ultrafiltration using a stirred ultrafiltration unit (Amicon system, Millipore, Billerica, MA) fitted with a 30 kDa molecular mass cut-off membrane. The SDS-PAGE gels (Figure SI-2, Supporting Information) of the purified PTT and PIs were analyzed using AlphaView Software (Cell Biosciences, Santa Clara, CA). The retentate recovered upon ultrafiltration was composed of 90% PTT, while the filtrate consisted of 95% protease inhibitors. The retentate and the filtrate containing PTT and PI-enriched extracts were freeze-dried separately. GOS (MW of 1,990.9 g/mol) were prepared from potato galactan using endo-(1-4)- β -D-galactanase from *A. niger* as described previously.²⁵ PTT or PIs and each of the galactose or GOS or galactan at the molar ratio of 1:9 were dissolved in 0.1% (w/v) of 0.05 M sodium phosphate buffer (pH 7.0). Protein-carbohydrate mixtures were lyophilized and incubated in sealed glass desiccators at 48 °C for 1 to 3 days under a controlled a_w value of 0.65 equilibrated with a saturated solution of potassium iodide. Control experiments (control PTT and PIs) were performed with selected proteins incubated under the same conditions in the absence of carbohydrates.

Determination of Glycation Extent of Potato Proteins with Selected Carbohydrates. To determine the extent of glycation of PTT and PIs, the percent blocked lysine was estimated from the total free amino content and the acid-generated furosine content, according to a modified method of Seo et al.²⁵ The free amino groups of native PTT and PIs were determined using a trinitrobenzene sulfonic acid (TNBS) assay. Then, 0.25 mL of 0.01% (v/v) TNBS was added to 0.5 mL of native protein solutions (10 mg/mL) in 0.1 M sodium bicarbonate buffer (pH 8.5). After 2 h of incubation at 37 °C, 0.25 mL of 10% (w/v) sodium dodecyl sulfate (SDS) and 0.125 mL of 1 M HCl were added to the mixtures to solubilize the protein and to avoid their precipitation. The assays were run in triplicate, and the standard curve was constructed using L-leucine. To estimate the ϵ -N-2-(furoylmethyl)-L-lysine (furosine), 500 μ L of the 20 mg/mL of protein sample in water was hydrolyzed with 200 μ L of 8 M HCl for 23 h at 110 °C capped under nitrogen. The hydrolyzed sample was cooled to room temperature and centrifuged at 13,000g for 15 min. The supernatant (500 μ L) was applied to a previously activated Sep-Pak C18 cartridge (Waters Corp, Milford, MA). Furosine was eluted with 3 M HCl and evaporated until dryness under nitrogen. The sample was resolubilized in 200 μ L of a water/acetonitrile/formic acid mixture (95:4.5:0.5, v/v/v) and analyzed through high-pressure liquid chromatography (HPLC). A Waters Bioseparations module and a photodiode array detector equipped with Empower 2 software for data collection were used. A sample volume of 20 μ L was separated using a Waters Symmetry Shield RP8 3.5 μ m (4.6 $\times$ 50 mm) column using an isocratic elution of 5 mM octanesulfonic acid in water/acetonitrile/formic acid mixture (79.8:20:0.2, v/v/v) for 20 min at a flow rate of 0.3 mL/min. The detection of furosine was performed at 280 nm. The calibration curve was constructed using a furosine standard. All assays were run in triplicate and injected with and without the internal standard. No

furosine was detected in the control samples, nonincubated mixtures of protein and carbohydrate. The final percentage of blocked lysine was indirectly estimated from the total free amino content and furosine content using the following equation, considering that the Amadori compound, tagatosyllysine, formed during the Maillard reaction, generates about 42% furosine upon 8 M HCl hydrolysis.²⁶

$$\text{Percent blocked lysine} = (1/0.42 \times \text{furosine}) \times 100/\text{total lysine} \quad (1)$$

Purification of Patatin Conjugates. Native PTT, incubated control PTT, as well as PTT/galactose, PTT/GOS, and PTT/galactan conjugates were subjected to ultrafiltration using a stirred ultrafiltration unit (Amicon system, Millipore) fitted with a 30 kDa or 300 kDa molecular mass cutoff membrane to remove the smaller Maillard reaction species and the unreacted saccharides present in the mixture. The retentates were collected for further analyses.

Simulated Gastric Digestion. Native PTT, incubated control PTT, and PTT conjugates were subjected to simulated gastric digestion according to the modified method of Moreno et al.²⁷ Proteins and glycoproteins (1 mg/mL) were solubilized in simulated gastric fluid (0.75 M NaCl, pH 2.5). To initiate the enzymatic digestion, porcine pepsin (7 mg/mL, $\geq$ 2,500 units/mg protein) was then added at a ratio of 1:50 (v/v). After the digestion was performed at 37 °C for 2 h, the pH was raised to 7.5 with 0.125 M ammonium carbonate. To simulate the duodenal environment, the following solutions were added: (1) 10 μ L of the following bile salt mixture (0.025 M sodium taurocholate, 0.025 M sodium glycodeoxycholic acid, 0.4 M CaCl₂, and 0.5 M Bis-Tris) and (2) 6 μ L of porcine trypsin (3 mg/mL, $\geq$ 6,000 units/mg protein) and 10 μ L of bovine α -chymotrypsin (4.77 mg/mL, $\geq$ 39 units/mg protein). The samples were then incubated at 37 °C for 1 h. The reaction was stopped by heating the samples in boiling water for 5 min. The control without the incubation steps was prepared as the unhydrolyzed control, and the native PTT was used as the hydrolyzed control. All assays were done in triplicate.

The quantification of the digested species was performed with a Beckman HPLC (Beckman Coulter, Fullerton, CA) system equipped with a programmable solvent module (model 126), a photodiode array detector and 32 Karat software for data collection. The separation was performed on a Zorbax SB-C8 (4.6 $\times$ 150 mm, 5 μ m, Agilent Technologies, Mississauga, ON, Canada). The digested sample (20 μ L) was injected in triplicate. The absorbance was measured at 214 nm. A linear gradient at a flow of 0.5 mL/min was used using water/acetonitrile/formic acid (90:10:0.1, mL/mL/mL) as solvent A and water/acetonitrile/formic acid (10:90:0.07, mL/mL/mL) as solvent B. The elution gradient was as follows: 1–45 min, 50% B; 45–50 min, 100% B; 50–55 min, 58% B; 55–60 min, 0% B; and 60–80 min, 0% B. The unhydrolyzed control was used as a standard to quantify the unhydrolyzed PTT in the samples.

$$\text{Percent protein digestion} = 100$$

$$- \left(\frac{\text{the peak area of unhydrolyzed PTT}}{\text{the peak area of the PTT control}} \right) \times 100 \quad (2)$$

The samples were kept at –20 °C before the assessment of their immunoreactivity.

Determination of IgE-Immunoreactivity. Native PTT or PIs were diluted with PBS buffer containing 0.1% (v/v) Tween 20 (PBS-T) to reach a final concentration of 5.4 μ g/mL, coated onto flat-bottomed high binding polystyrene Costar 3590 microtiter plates with 96 wells (50 μ L per well, Corning, Tewksbury, MA), and incubated overnight at 4 °C. The sera pool or control serum (diluted 1:3 with 1% w/v BSA in PBS-T) was mixed with conjugates as inhibitor (0.0012000 μ g/mL in 1% w/v BSA in PBS-T) at a ratio of 1:1 (v/v) and incubated overnight at 4 °C. As a positive control, a BSA solution (0.001–2000 μ g/mL in 1% w/v BSA in PBS-T) was incubated with the antipotato sera pool. The pool was composed of sera from 4 subjects with high levels of specific IgE antibody to potato proteins (PlasmaLab). Human serum with specific IgE antibody to dust mites but none to potato proteins was used as the control. The microtiter plate coated with native proteins was

washed 3 times with 300 μL of PBS-T using a Biotek ELx405 microplate washer (Winooski, VT) and was blocked using 5% (w/v) BSA in PBS-T for 2 h at 25 $^{\circ}\text{C}$. After washing 3 times with PBS-T, 50 μL of PTT conjugates and serum pool mixtures were added onto the PTT-coated microtiter plate. The plate was incubated at 25 $^{\circ}\text{C}$ for 2 h and washed with PBS-T three times. After incubation with 50 μL of anti-human IgE conjugated with horseradish peroxidase (1:2000 dilution with 1% w/v BSA in PBS-T) for 1 h at 25 $^{\circ}\text{C}$, the plate was washed 3 times and developed with 50 μL of substrate solution (3,3',5,5'-tetramethylbenzidine, TMB, Bioshop, Burlington, ON, Canada) at 25 $^{\circ}\text{C}$ for 15 min. The reaction was stopped by adding 50 μL of 0.5 M sulfuric acid solution. The absorbance was measured at 450 nm. The loss of the specific IgE binding ability of the patients' sera resulting from the conjugation of PTT with galactose/GOS/galactan was expressed as follows:

$$\text{Percent inhibition} = 100 - \left(\frac{\text{absorbance of the glycosylated samples}}{\text{preincubated with sera pool/absorbance of positive control}} \right) \times 100 \quad (3)$$

The experimental values were fitted to a four-parameter logistic equation using the SigmaPlot software from SPSS Inc. (Chicago, IL) and were subjected to normality (Shapiro-Wilk) and to constant variance tests to validate assumptions of one-way analysis of variance (ANOVA). IC_{50} was defined as the concentration of inhibitor at the inflection of the fitted curve. All assays were done in triplicate. p values ≤ 0.05 were considered to be statistically significant. To assess the effect of heat treatment, PTT and its conjugates (20 mg/mL) were incubated at 80 $^{\circ}\text{C}$ for 15 min before assessing their immunoreactivity.

Protein Primary Sequence Analysis. The primary sequence of PTT and its conjugates were analyzed through protein mass mapping, which consists of the tryptic hydrolysis of proteins followed by mass spectrometric analysis of the released peptides.

Tryptic Digestion. The digestion of PTT and its conjugates were performed on a MassPrep liquid handling robot (Micromass, Manchester, U.K.) according to the protocol of Shevchenko et al.²⁸ with modifications of Havlis et al.²⁹ The native PTT, the incubated control PTT, and the PTT conjugates (10 μL each; 1 $\mu\text{g}/\text{mL}$) were resolved by electrophoresis (Mini-Protean Tetra system, Bio-Rad) on Mini-Protean TGX precast gels (Bio-Rad). Proteins bands taken from the SDS-PAGE gel were reduced with 10 mM dithiothreitol and alkylated with 55 mM iodoacetamide. Trypsin digestion was performed using 105 mM of modified porcine trypsin (sequencing grade, Promega, Madison, WI) at 58 $^{\circ}\text{C}$ for 1 h. Digestion products were extracted using a formic acid/acetonitrile/water mixture (1:2:97, v/v/v) followed by a formic acid/acetonitrile/water mixture (1:50:49, v/v/v). Recovered extracts were pooled, dried by vacuum centrifugation, resuspended into 10 μL of 0.1% (v/v) formic acid, and analyzed by mass spectrometry.

Mass Spectrometry. Peptide samples were separated by online reversed-phase (RP) nanoscale capillary liquid chromatography (nanoLC) and analyzed by electrospray-mass spectrometry (ES-MS/MS). Analyses were performed with a Thermo Surveyor MS pump connected to an LCQ Deca XP mass spectrometer (Thermo Electron, San Jose, CA) supplied with a nanoelectrospray ion source (Thermo Electron, San Jose, CA). Peptides were bound on a cap trap (Michrom Bioresources, Auburn, CA) at 10 $\mu\text{L}/\text{min}$ after which chromatographic separation took place using a PicoFrit column BioBasic C18 (10 cm $\times$ 0.075 mm) (New Objective, Woburn, MA) set with a linear gradient from 2 to 50% of solvent B (acetonitrile and 0.1% v/v formic acid) for 30 min, at 200 nL/min (obtained by flow-splitting). Mass spectra were acquired using a data-dependent acquisition mode using Xcalibur software, version 1.2. Each full scan mass spectrum (400–2000 m/z) was followed by collision-induced dissociation of the three most intense ions. The dynamic exclusion (30 s exclusion duration) function was enabled, and the relative collisional fragmentation energy was set to 35%.

Database Searching. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.4.1) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)). X! Tandem was set up to search the UR12_5_SolanumTub_20120718

database (2615 entries) also assuming trypsin. Mascot was set up to search the UR12_5_SolanumTub_20120718 database assuming the digestion enzyme trypsin. Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.100 Da and a parent ion tolerance of 0.100 Da. Carbamidomethyl of cysteine was specified in Mascot and X! Tandem as a fixed modification. Dehydrated of the N-terminus, Glu $\rightarrow$ pyro-Glu of the N-terminus, ammonia-loss of the N-terminus, Gln $\rightarrow$ pyro-Glu of the N-terminus, deamidated of asparagine and glutamine, oxidation of methionine, and hex of lysine and arginine were specified in X! Tandem as variable modifications. Glu $\rightarrow$ pyro-Glu of the N-terminus, Gln $\rightarrow$ pyro-Glu of the N-terminus, deamidated of asparagine and glutamine, oxidation of methionine, hex of lysine and arginine, galactosyl of lysine, and galactosyl (R) of arginine were specified in Mascot as variable modifications.

Criteria for Protein Identification. Scaffold (version 3.6.2, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm.³⁰ Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.³¹ Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Secondary Structure Analysis. The secondary structure analysis of PTT and its conjugates was analyzed by Fourier transform infrared spectroscopy (FTIR). The samples were dissolved in $^2\text{H}_2\text{O}$ to achieve a final concentration of 100 mg protein/mL. Infrared spectra were recorded using a 8210 Nicolet FTIR spectrometer (Thermo Electron Corporation, Madison, WI) equipped with a deuterated triglycine sulfate (DTGS) detector. The spectrometer was continuously purged with dry air from Balston dryer (Balston, Haverhill, MA). The samples (9 μL) were placed between two CaF_2 windows separated by a 25 μm Teflon film spacer (Harrick Scientific, Pleasantville, NY). Each spectrum was obtained by coadding 256 scans to minimize the signal-to-noise ratio. Fourier self-deconvolution (FSD) in the amide I' region was performed using Omnic 6.0 software (Thermo Electron Corporation, Madison, WI) applying a bandwidth of 40 cm^{-1} and a resolution enhancement parameter of 3. The curve fitting was performed by using Origin 8 (OriginLab, Northampton, MA). The Gaussian function was chosen to fit the overlapped peaks. The amide I' region of PTT solution in $^2\text{H}_2\text{O}$ revealed five components upon deconvolution and curve-fitting. The band at 1628 cm^{-1} had been assigned to strongly bonded β -strands indicative of an aggregated strands type structure.³² The remaining structures were assigned as follows: bands at 1637 $\pm$ 3.0 and 1675 $\pm$ 5.0 cm^{-1} as β -sheets, band at 1645 $\pm$ 4.0 cm^{-1} as random coils, bands at 165 $\pm$ 4.0 cm^{-1} as α -helices, and bands at 1671 $\pm$ 3.0 and 1689 $\pm$ 2.0 cm^{-1} as β -turns.³³

Three-Dimensional Mapping. The FASTA format of amino acid sequence of PTT (accession number Q2MY50, UniProtKB) was submitted to Swissmodel.³⁴ This server used the template of SeMet Patatin (Protein Data Bank ID: 1OXW) for the prediction. The predicted model had a Q-MEAN Z-score, a useful measure for the description of the absolute quality of theoretical models, of -1.12 , which indicates acceptable quality. The predicted model was viewed by Jmol.³⁵

RESULTS AND DISCUSSION

Glycation Extent of Potato Proteins. Table 1 shows the percent blocked lysine of conjugates of PTT and PIs. As compared to GOS and galactan, higher levels of glycation of PTT and PIs with galactose were obtained with percent blocked lysine of 33.3 and 25.2%, respectively. These results are in agreement with other studies³⁶ in which the higher reactivity of monosaccharides was attributed to their higher proportion in the open-ring form and/or to their high affinity to the glycation sites of PTT.³⁷ The difference in the number of glycosylated residues between PTT and PIs can be explained by the different accessibilities of Lys ϵ -amino groups.³⁸ Indeed, there are 24

Table 1. Extent of Glycation of PTT and PIs with Selected Carbohydrates

conjugates	incubation time (days)	blocked lysine (%) ^a
PTT/galactose	1	33.3 (±0.5)
PTT/galactose	3	77.0 (±1.7)
PTT/GOS	3	4.0 (±0.0)
PTT/galactan	3	9.1 (±0.0)
PIs/galactose	1	25.2 (±0.0)
PIs/GOS	3	10.3 (±0.0)

^aExperimental results are averages of triplicates, and the standard deviations are given.

and 10–14 reactive amino groups on PTT and on major PIs, respectively (accession numbers Q2MY50, P58514, Q41448, and P16348, UniProtKB). The higher percent blocked lysine of PTT is attributed to the high number of its more exposed Lys residues as compared to the PIs. However, the lower level of conjugation of potato proteins with GOS can be attributed to the high rate of the glycoxidation reaction of GOS competing with the glycation rate through the progress of the Maillard reaction.^{39,40} The results also show that the percent level of blocked lysine of PTT (4%) upon glycation with GOS was lower as compared to that of PIs (10.3%). These results reveal the higher susceptibility of PTT to form protein aggregates upon the generation of protein cross-linking dicarbonyls through the glycoxidation/autoxidation of GOS,³⁹ therefore leading to the lower accessibility of the glycation sites as compared to PIs.

Effect of Glycation on the Immunoreactivity of PTT and PI Conjugates.

To determine the allergenic potential of native and conjugated potato proteins, IgE binding activity of the native protein was investigated by an inhibition ELISA assay using a serum pool of four subjects with specific IgE to potato proteins. The sera from different donors were first tested individually against native PTT and protease inhibitors to confirm the extent of their immunoreactivity (data not shown). The sera were then pooled to obtain a more general immunoreactivity profile. The IgE's in the serum pool showed immunoreactivity to the patatin band (Sol t 1 allergen, Figure SI-3, Supporting Information) but also to some protease inhibitors (Sol t 2–4 allergen, Figure SI-3, Supporting Information). An anti-dust mite serum sample was used as a negative control. Figure 1A demonstrates a typical inhibition curve for the native PTT and the PTT conjugates. Control experiments performed with incubated PTT without carbohydrates demonstrated a decrease in the concentration required for half-maximal inhibition of IgE binding (IC_{50} of 0.82 $\mu\text{g/mL}$, $p < 0.01$) as compared to the native PTT (IC_{50} of 1.19 $\mu\text{g/mL}$, $p < 0.01$). This increase in IgE binding affinity of PTT upon incubation is attributed to a change in PTT structure, leading to a higher exposure of its epitopes. The IC_{50} of PTT/galactose and PTT/GOS conjugates were estimated to be 0.70–0.90 $\mu\text{g/mL}$ (1–3 days incubation; $p < 0.01$) and 0.38 $\mu\text{g/mL}$ ($p < 0.01$), respectively. These experimental findings reveal an increase in the level of IgE binding extent upon the glycation of PTT with galactose and GOS as compared to the native PTT. These results are attributed to an increased exposure of the epitopes and/or the formation of novel IgE-binding sites upon covalent modifications of PTT during glycation with small carbohydrates.^{41,42} As compared to PTT/galactose conjugates formed upon 1 day of incubation, the slightly lower immunoreactivity obtained with PTT/galactose conjugates obtained upon 3 days of incubation indicates further structural changes due to extensive glycation leading to lower exposure of

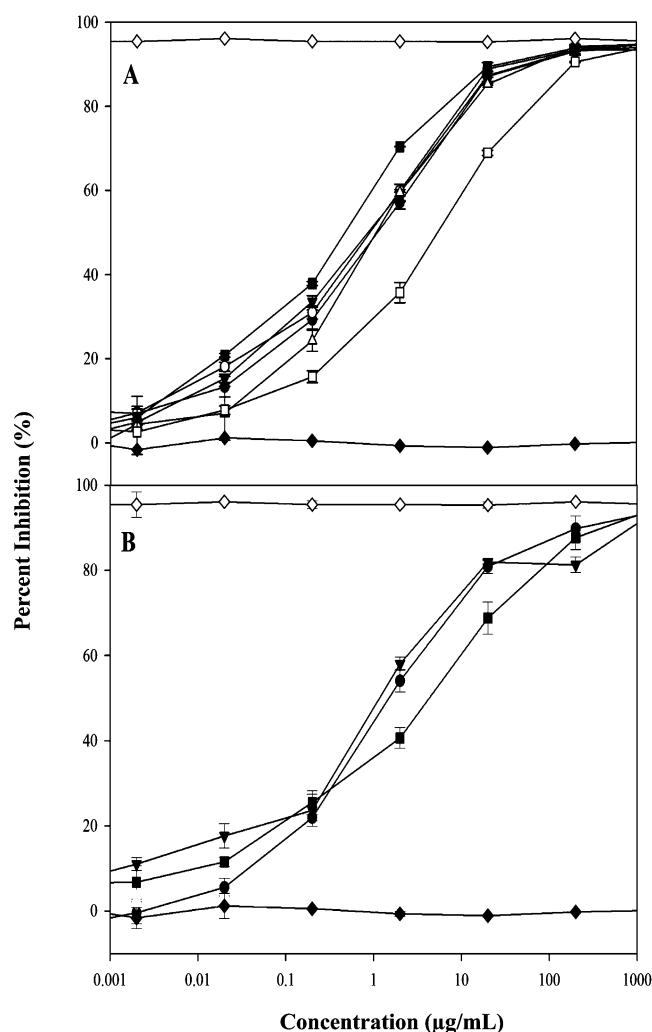


Figure 1. Effect of glycation on PTT and PI immunoreactivity obtained through inhibition ELISA: (A) PTT (●), incubated PTT (○), PTT/galactose conjugates (1-day) (▼), PTT/galactose conjugates (3-day) (Δ), PTT/GOS conjugates (■), PTT/galactan conjugates (□), positive control (◆), and negative control (◇). (B) PIs (●), PIs/galactose conjugates (▼), PIs/GOS conjugates (■), positive control (◆), and negative control (◇).

the epitopes. In contrast, lower immunoreactivity of ovalbumin after glycation with glucose was reported by Ma et al.⁴³ However, the results show that the glycation of PTT with galactan (IC_{50} of 5.49 $\mu\text{g/mL}$; $p < 0.01$) resulted in a significant decrease in the affinity for the specific IgE as compared to the native PTT (IC_{50} of 1.19 $\mu\text{g/mL}$, $p < 0.01$). Similarly, Aoki et al.⁴¹ have reported a decrease in the IgE binding activity of a major allergen of Japanese cedar, Cry j 1, conjugated with galactomannan and attributed this decrease in immunoreactivity to the effectiveness of polysaccharide chains at masking the molecular surface epitopes of an allergen.

Control experiments performed with control incubated PIs without carbohydrates did not show any difference in IgE binding level compared to that of the native proteins (data not shown). Contrary to the results obtained with PTT (Figure 1A), the conjugation of PIs to carbohydrates resulted in lower immunoreactivity as compared to the native compound (IC_{50} of 1.11 $\mu\text{g/mL}$, $p < 0.01$), where the conjugation with GOS (IC_{50} of 5.17 $\mu\text{g/mL}$, $p < 0.01$) resulted in a higher decrease in immunoreactivity than that with galactose (IC_{50} of 1.21 $\mu\text{g/mL}$,

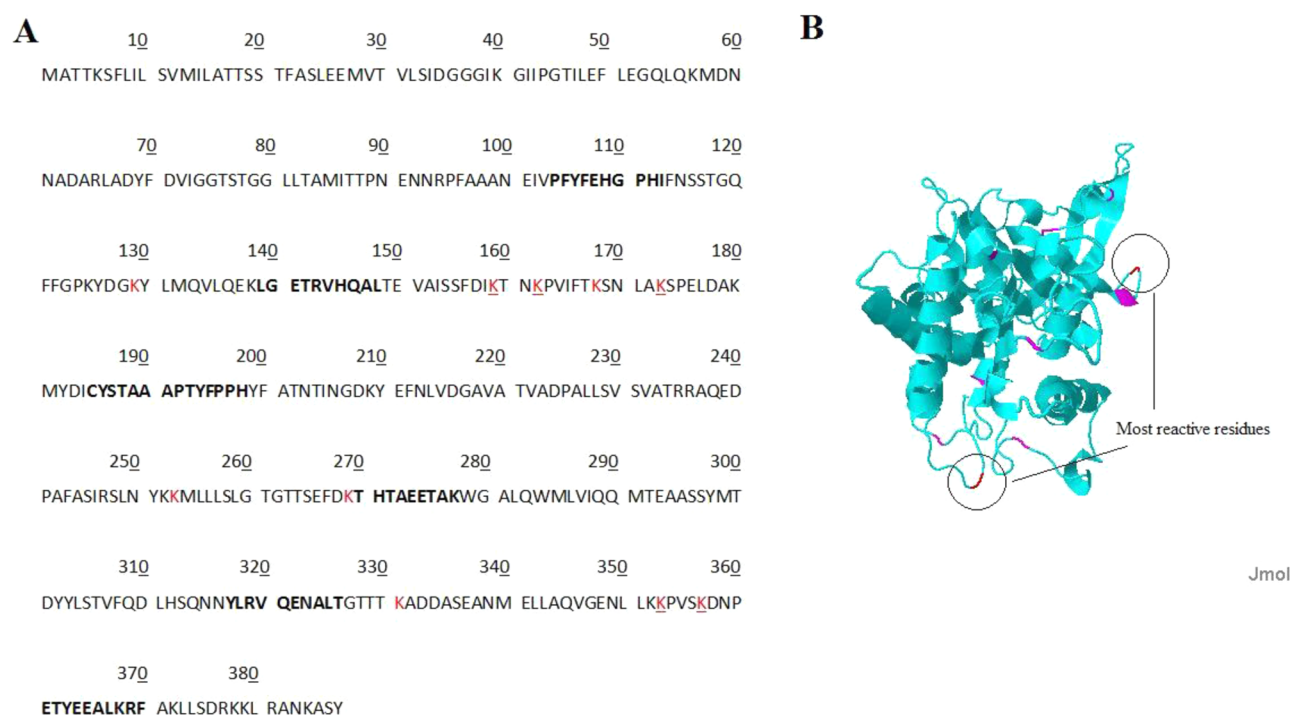


Figure 2. (A) Amino acid sequence of PTT glycated with galactose (red), glycated with GOS (red, underlined), and documented epitopes (bold). (B) Three-dimensional mapping of PTT with glycated residues (magenta) and the most reactive glycated residues (red).

mL, $p < 0.01$) (Figure 1B). It is likely that the conjugation of PIs to galactose or GOS resulted in epitope modification and/or to epitope shielding, thereby affecting the antigen–antibody recognition.⁴³ A decrease in the immunoreactivity of lysozyme was also obtained, in our previous study, upon its conjugation with galactose and GOS.⁴⁴

The overall results emphasize that the immunoreactivity effect is allergen and carbohydrate dependent.¹⁹ For further studies, PTT, being a heat-labile allergen, was selected to study the effects of the primary and secondary molecular modifications upon glycation and thermal treatment on immunoreactivity.

Location of Conjugated PTT Residues. To determine the conjugated residues on PTT, the skipped trypsin cleavage sites due to glycation of lysine residues and the increase in molecular weight of the digested peptides were identified by peptide mapping of conjugated PTT (Figure 2A). Table SI-4 in the Supporting Information summarizes the peptide masses identified through MS analysis. As expected, the highest number of glycated residues was found in PTT glycated with galactose, after one day of incubation, (Lys 129, 159, 162, 168, 173, 253, 269, 331, 353, and 357, in red) as compared to PTT glycated with GOS, after three days of incubation (Lys 159, 162, 173, 353, and 357, underlined in red) confirming the higher reactivity of the smaller carbohydrate compared to that of the larger one. The most reactive residues were Lys 159 and 357, which were found in the highest proportion in their conjugated state. The glycated residues did not overlap with the amino acid sequence of the epitopes previously identified in PTT (in bold),⁴⁵ which demonstrates that the direct blocking of these epitopes did not occur. This supports our inhibition ELISA findings where no decrease in the immunoreactivity of PTT was observed upon its conjugation with galactose (Figure 1A). On the contrary, the minor increase observed in the immunoreactivity might be explained by the conjugation of the carbohydrate to the residues that are found in close proximity to the documented epitopes,

such as Lys 269 and 357, which could have resulted in an increased exposure of these epitopes. The three-dimensional mapping of the PTT (Figure 2B) demonstrated that the glycation sites (in magenta) were mostly found toward the termini of individual PTT secondary structures, such as the α -helix and β -sheet, which indicates a minimal change in the secondary structure due to glycation. The most reactive residues (in red) are very easily accessible according to the three-dimensional mapping of the native PTT.

Investigation of the Secondary Structure of PTT Conjugates. Table 2 shows the percent change in the secondary

Table 2. Effect of Glycation on the Secondary Structures of PTT and Its Conjugates Obtained through FTIR^a

PTT sample	α -helix (%)	β -sheets (%)	β -turns (%)	aggregated sheets (%)
PTT	33.75	43.27	9.16	13.82
incubated PTT	34.19	42.36	9.07	14.38
PTT/galactose	39.02	43.14	8.62	9.22
PTT/GOS	34.54	43.29	9.43	12.74
PTT/galactan	31.10	37.99	6.94	23.96

^aEach spectrum was obtained by coadding 256 scans.

structure of the conjugates compared to that of the native PTT estimated through FTIR. The FTIR spectrum of native PTT shows the presence of 13.8% aggregated strands, 43.3% β -sheet, 9.2% β -turns, and 33.7% α -helix. The FTIR spectrum of the control incubated PTT showed negligible change (<1%) in the protein's secondary structure upon incubation at mild temperature of 48 °C. The results also show that the glycation with galactose had minimal effects on the secondary structure of PTT with 9.2, 43.1, 8.6, and 39.1% of aggregated strands, β -sheet, β -turn, and α -helix, respectively. These results confirm the high affinity of galactose to exposed glycation sites located at the

termini of PTT secondary structures (Figure 2B), leading to minimal secondary structural changes of PTT upon glycation. Similar low structural changes were exhibited by PTT/GOS conjugates with 12.7, 43.3, 9.4, and 34.6% of aggregated strands, β -sheet, β -turn, and α -helix, respectively. The most significant changes in the secondary structure of PTT were observed upon glycation with galactan with 24.0, 38.0, 6.9, and 31.1% of aggregated strands, β -sheet, β -turn, and α -helix, respectively; these results are due to the disruption of intermolecular noncovalent interactions that stabilize the secondary structure of PTT.⁴⁶ The significant structural changes observed in the PTT/galactan conjugates resulted in the burying of IgE specific epitopes between the protein molecules and to the subsequent decrease in IgE binding affinity.⁴⁷ Although relatively small, the changes in PTT secondary structure upon conjugation with the smaller carbohydrates induced an exposure of inner epitopes, leading to an increase in the immunoreactivity as observed in Figure 1A. Ma et al.⁴³ have reported that the increase in the immunoreactivity of the ovalbumin conjugated with glucose was due to the exposure of hydrophobic epitopes upon glycation.

Effect of Heat Treatment on the Immunoreactivity and the Secondary Structures of PTT Conjugates. The effect of heat treatment (80 °C for 15 min) on the immunoreactivity of native PTT and PTT conjugates was assessed (Table 3). The

Table 3. Effect of Heat Treatment on PTT Conjugates' Immunoreactivity Obtained through Competition ELISA^a

conjugates	incubation time (days)	IC ₅₀ (μ g/mL) ^a	
		nonheated	heated
PTT		1.19 ($\pm$ 0.11)	2.77 ($\pm$ 0.66)
incubated PTT	3	0.82 ($\pm$ 0.16)	2.64 ($\pm$ 0.28)
PTT/galactose	1	0.70 ($\pm$ 0.10)	0.98 ($\pm$ 0.65)
PTT/GOS	3	0.38 ($\pm$ 0.05)	0.03 ($\pm$ 0.01)
PTT/galactan	3	5.49 ($\pm$ 0.79)	0.81 ($\pm$ 0.05)

^aIC₅₀ is the concentration of PTT conjugates that produced 50% inhibition of antibody binding to the native PTT. All assays were done in triplicate. *p* values $\leq$ 0.05 were considered to be statistically significant.

heat treated native PTT demonstrated a significant decrease in the IgE binding affinity with an IC₅₀ value of 2.77 μ g/mL (*p* < 0.01) as compared to the nonheat treated PTT (IC₅₀ value of 1.19 μ g/mL (*p* < 0.01). Koppelman et al.⁴⁷ have obtained similar results to native PTT after heat treatment and attributed the decrease in the immunoreactivity to the hiding of the epitopes through their aggregation, caused by the exposure of hydrophobic regions of PTT during heat induced denaturation. However, the heat treatment did not affect the IgE binding affinity of PTT/galactose conjugates (IC₅₀ of 0.98 μ g/mL, *p* < 0.01). Contrary to PTT/galactose conjugates, an increase in the IgE binding affinity of PTT/GOS conjugates (IC₅₀ of 0.03 μ g/mL, *p* < 0.01) and of PTT/galactan conjugates (IC₅₀ of 0.81 μ g/mL, *p* < 0.01) were obtained after heat treatment. These results can be explained by an increase in the exposure of the epitopes after a partial protein unfolding and/or by the efficiency of longer carbohydrate chains (GOS, galactan) in preventing thermal protein aggregation upon glycation, leading to a better accessibility of the epitopes by IgE.⁴⁷ The increase in the immunoreactivity observed with the PTT conjugates are in direct contrast to the results obtained by Vissers et al.⁴⁸ where carbohydrate conjugated 2S albumins had lower IgE binding capacity after heat treatment.

To better understand the effect of the conformational changes on the immunoreactivity of PTT conjugates, the changes in secondary structures after heat treatment (Figure 3) were

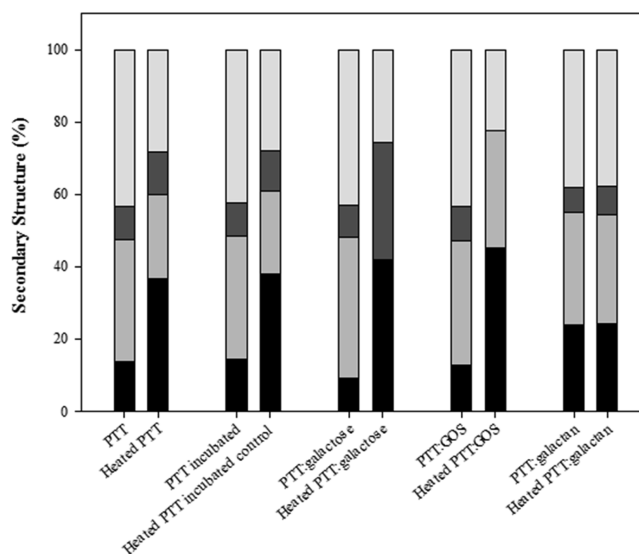


Figure 3. Effect of heat treatment on PTT and its conjugates' secondary structure obtained through FTIR: intermolecular β -sheet (black rectangle), α -helix (medium gray rectangle), β -turn (dark gray rectangle), and intramolecular β -sheet (light gray rectangle).

assessed. The results demonstrate that the partial unfolding of PTT and its conjugates upon heat treatment supports previous findings.⁴⁹ Indeed, an increase in the intermolecular β -sheets (>23.03%) after heat treatment was observed for PTT and its conjugates, except for PTT/galactan conjugates. These results confirm the strong tendency of native PTT and its conjugates with galactose and GOS to aggregate upon partial denaturation. Although the largest increase in the aggregated sheets was observed with PTT/galactose (32.67%) and PTT/GOS (32.45%) conjugates upon heat treatment, these changes did not lead to a decrease in the immunoreactivity of the conjugates, as observed with the native and the control PTT (Table 3). The major secondary structural changes observed with PTT/galactose and PTT/GOS conjugates were the complete loss of α -helices and β -turns, respectively; such structural modifications resulted in an increased exposure of the PTT epitopes, leading to an increase in the immunoreactivity of these conjugates even after aggregation (Table 3). On the contrary, galactan stabilizes PTT against heat denaturation with only 2.71% total change in the secondary structure. However, the relatively small change in the secondary structure resulted in an exposure of the hydrophobic epitopes. In addition, the lack of subsequent protein aggregation after exposure of the hydrophobic groups due to galactan conjugation resulted in a significant increase in immunoreactivity (Table 3).

Effect of Digestion on the Immunoreactivity of PTT Conjugates. The effect of in vitro simulated gastric digestion on the allergenic potential of native PTT and its conjugates was assessed by subjecting the samples to simulated gastric digestion before measuring their immunoreactivity by inhibition ELISA (Figure 4). The highest percent digestion was obtained with the native PTT (59.6%) as compared to PTT conjugates. The low percent digestion of PTT conjugates is explained by the fact that the cleavage site of one of the digestive enzymes, trypsin, is specific toward the Lys residues, which are involved in the

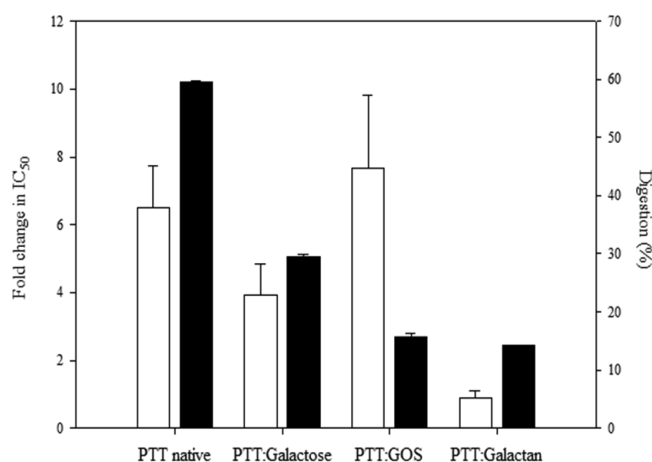


Figure 4. Effect of simulated gastric digestion on PTT conjugates' immunoreactivity: fold change in IC₅₀ (white bar) and percent digestion (black bar).

conjugation reaction with carbohydrates. These results are in accordance with those reported by Jiménez-Saiz et al.⁴² showing a decrease in ovalbumin conjugates' digestibility as a result of glycation. Nevertheless, as compared to PTT/galactose conjugates (29.5% degree of digestion), the higher resistance of PTT/GOS (15.7%) and PTT/galactan (14.2%) conjugates to digestion could be due to the large size of their carbohydrates moieties, shielding PTT proteolytic sites. The results (Figure 4) also show that the digestion resulted in a 6.51-fold decrease in the IgE binding affinity of native PTT as compared to the nondigested one; these experimental findings can be explained by the disruption of both conformational and sequential epitopes. Similarly, a decrease in the IgE binding affinity of PTT/galactose and PTT/GOS conjugates by 3.94- and 7.66-fold, respectively, was obtained upon digestion. In contrast, the IgE binding affinity of digested PTT/galactan conjugates remained unchanged compared to that of the undigested conjugates; this can be due to the large polysaccharide chains that have sterically blocked the disruption of the epitopes.⁴¹ However, compared to the digested native PTT (IC₅₀ of 7.75 µg/mL, $p < 0.01$), a higher retention in IgE binding was exhibited by digested PTT/galactose (IC₅₀ of 2.76 µg/mL), PTT/GOS (IC₅₀ of 2.91 µg/mL), and PTT/galactan conjugates (IC₅₀ of 4.90 µg/mL). These high immunoreactivities of digested conjugates is due to their lower susceptibility to digestion, thereby preserving the integrity of IgE binding sites on conjugated PTT.

■ ASSOCIATED CONTENT

■ Supporting Information

Level of specific IgE found in the serum of donors characterized by ImmunoCAP; SDS-PAGE gel of isolated PTT and PIs through ultrafiltration; Western blot of potato proteins using the serum pool taken from four donors with specific IgE toward potato proteins; peptide composition of the tryptic hydrolysates of PTT and its PTT/galactose conjugates. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

PTT, patatin; PIs, protease inhibitors; GOS, galactooligosaccharides; FTIR, Fourier transform infrared spectroscopy

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**ALLERGENICITY OF POTATO PROTEINS AND OF THEIR CONJUGATES WITH GALACTOSE,
GALACTOOLIGOSACCHARIDES AND GALACTAN IN NATIVE, HEATED AND DIGESTED FORMS**

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Supporting Information 1

The information regarding the donors and the sera was acquired from PlasmaLab. The level of specific IgE was assessed through ImmunoCAP.

Table SI-1. Level of specific IgE found in the serum of donors characterized by ImmunoCAP

Origin of the allergen	Code [*]	Specific IgE from Donor A ^a (kU _A /1)	Specific IgE from Donor B ^b (kU _A /1)	Specific IgE from Donor C ^c (kU _A /1)	Specific IgE from Donor D ^d (kU _A /1)
Potato	F35	8.8	22.4	12.1	17.2
Carrot	F31	6.8	27.1	17.4	14.4
White bean	F15	8.3	—	—	—
Pea	F12	—	3.9	—	—
Peanut	F13	—	32.8	8.0	19.7
Soybean	F14	—	8.9	—	—
Hazelnut	F17	—	—	74.7	—
Almond	F20	—	—	—	10.0
Tomato	F25	—	—	13.5	—
Bermuda grass pollen	G2	—	98.5	25.7	47.1
Common ragweed pollen	W1	—	36.8	—	20.3
Wall pellitory pollen (<i>officinalis</i>)	W19	—	—	—	16.7
White ash tree pollen	T15	—	54.2	—	—
Common silver birch pollen	T3	—	45.0	>100.0	—
Olive tree pollen	T9	—	42.2	—	—
Maple leaf sycamore tree pollen	T11	—	—	—	19.7
Japanese cedar tree pollen	T17	—	—	—	11.8
Latex	K82	—	10.1	—	—

^{*} ImmunoCAP allergen code

^a Male, Caucasian, 28 years old

^b Male, Caucasian, 24 years old

^c Male, Caucasian, 24 years old

^d Male, Native American, 47 years old

Supporting Information 2

The SDS-PAGE of the separated PTT and PIs revealed that the retentate of the ultrafiltration was 90% composed of PTT and that the filtrate was 95% composed of protease inhibitors when analyzed through AlphaView Software (Cell Biosciences, Santa Clara, CA).

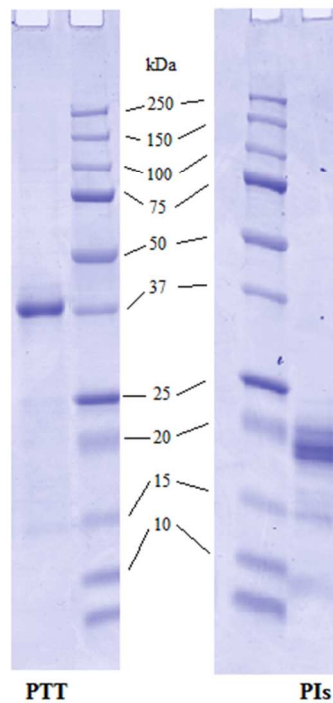


Figure SI-2. SDS-PAGE gel of isolated PTT and PIs through ultrafiltration

Supporting Information 3

Western blot of native potato proteins demonstrates that the serum pool has most immunoreactivity to PTT (Sol t 1 allergen), but also to some protease inhibitors (Sol t 2-4 allergen). The identity of the bands were confirmed by tryptic digestion followed by MS analysis as presented in the Materials section (Protein Primary Sequence Analysis) of the manuscript.

Experimental Method. The native PTT (10 μ l; 1 μ g/ml) were resolved by electrophoresis (Mini-Protean Tetra system, Bio-Rad) on Mini-Protean TGX precast gels (Bio-Rad) and subsequently transferred to immunoblot PVDF membrane (Bio-Rad). Non-specific binding was blocked with 3 % skim milk in PBS-T, and then the membranes were incubated with the serum pool diluted (1:2, v/v) in 3 % skim milk in PBS-T for overnight. After washing for 3 times in PBS-T, the membrane was incubated with mouse anti-human IgE conjugated with horse radish peroxidase (Southern Biotech, Birmingham, AL) diluted (1:2000, v/v) in PBS-T for 1 h. The membrane was again washed three times. The signals were amplified using the Western Blot amplification

module (BioRad) and visualized using the Opti4CN substrate kit (BioRad). The Precision Plus Protein unstained standards (BioRad) were used as molecular weight standards for the gel and their development on the blot was done with Precision Protein StrepTactin-HRP (BioRad) according to the manufacturer's instructions.

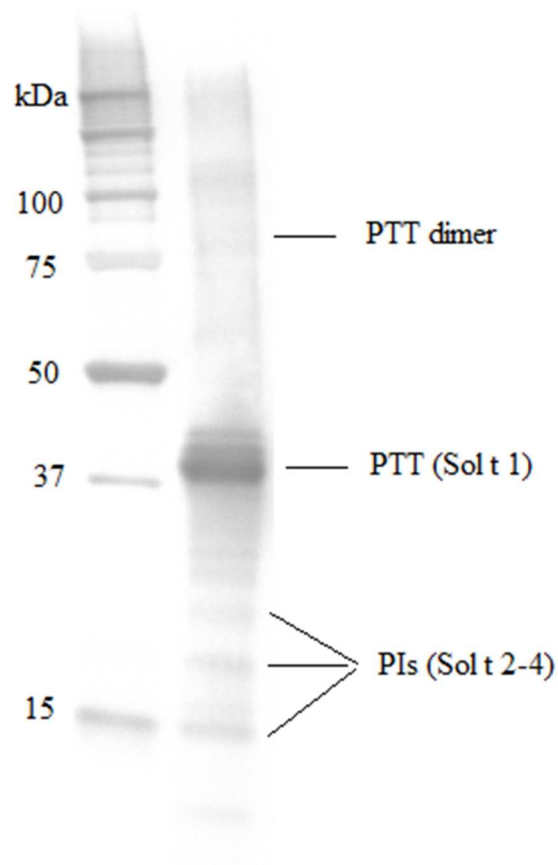


Figure SI-3. Western blot of potato proteins using the serum pool taken from four donors with specific IgE towards potato proteins

Supporting Information 4

The peptide mapping of conjugated PTT allows for the identification of conjugated residues by identifying the skipped cleavage sites due to the glycation of lysine residues and also to the increase in molecular weight of the digested peptides. The glycated residues identified in PTT glycated with galactose are Lys 129, 159, 162, 168, 173, 253, 269, 331, 353, and 357 as demonstrated in Table SI-4. Similar table was obtained for the identification of the glycated residues in PTT:GOS (data not shown).

Table SI-4. Peptide composition of the tryptic hydrolysates of PTT and its PTT:galactose conjugates.

Residues	Sequence	Number of peptides	Actual Mass (amu)
332-352	(K)ADDASEANMELLAQVGENLLK(K)	4	2,230.09
332-357	(K)ADDASEANMELLAQVGENLLKPKVSK(D)	2	2,785.43
237-247	(R)AQEDPAFASIR(S)	24	1,203.58
358-369	(K)DNPETYEEALKR(F)	20	1,463.70
243-252	(A)FASIRSLNYK(K)	1	1,197.63
41-57	(K)GIIPGTILEFLEGQLQK(M)	2	1,855.04
41-65	(K)GIIPGTILEFLEGQLQKMDNNADAR(L)	2	2,758.40
312-319	(L)HSQNNYLR(V)	2	1,030.50
253-269+G	(K)K*MLLLSLGTGTTSEFDK(T)	2	2,002.02
253-278	(K)KMLLLSLGTGTTSEFDKTHTAETAK(W)	4	2,808.44
253-278+G+H ₂ O	(K)K*MLLLSLGTGTTSEFDKTHTAETAK(W)	1	2,986.47
253-278+G	(K)K*MLLLSLGTGTTSEFDKTHTAETAK(W)	2	2,970.48
353-369	(K)KPVSKDNPETYEEALK(R)	9	1,846.94
353-369+G	(K)K*PVSKDNPETYEEALK(R)	4	2,165.09
353-369+G	(K)KPVSK*DNPETYEEALK(R)	5	2,165.05
353-369+2G	(K)K*PVSK*DNPETYEEALK(R)	2	2,327.15
25-40	(S)LEEMVTVL SIDGGGIK(G)	2	1,659.84
139-159	(K)LGETRVHQALTEVAISSFDIK(T)	5	2,313.25
139-169	(K)LGETRVHQALTEVAISSFDIKTNKPVIFTK(S)	2	3,341.85
139-169+G	(K)LGETRVHQALTEVAISSFDIK*TNKPVIFTK(S)	1	3,503.90
254-269	(K)MLLLSLGTGTTSEFDK(T)	9	1,711.86
254-278	(K)MLLLSLGTGTTSEFDKTHTAETAK(W)	17	2,680.35
236-247	(R)RAQEDPAFASIR(S)	15	1,359.69
169-180+G	(K)SNLAK*SPELDAK(M)	2	1,433.73
169-180	(K)SNLAKSPELDAK(M)	4	1,271.67
160-168	(K)TNKPVIFTK(S)	37	1,046.61
160-168+G	(K)TNK*PVIFTK(S)	2	1,208.67
160-173	(K)TNKPVIFTKSNLAK(S)	2	1,559.91
144-159	(R)VHQALTEVAISSFDIK(T)	66	1,756.93
144-168	(R)VHQALTEVAISSFDIKTNKPVIFTK(S)	9	2,785.54
144-168+G	(R)VHQALTEVAISSFDIK*TNKPVIFTK(S)	8	2,947.60
144-173+2G	(R)VHQALTEVAISSFDIKTNKPVIFTK*SNLAK*(S)	1	3,622.96
320-331	(R)VQENALTGTTTK(A)	24	1,261.64
320-352	(R)VQENALTGTTTKADDASEANMELLAQVGENLLK(K)	6	3,473.74
320-352+G+ H ₂ O	(R)VQENALTGTTTK*ADDASEANMELLAQVGENLLK(K)	2	3,651.77
126-138	(K)YDGKYL MQVLQEK(L)	9	1,613.82
126-138+G	(K)YDGK*YLMQVLQEK(L)	1	1,775.87
130-138	(K)YLMQVLQEK(L)	18	1,150.60
130-143	(K)YLMQVLQEKLGETR(V)	2	1,706.90

* Glycated residue