

Original article

Identification of four novel potato (*Solanum tuberosum*) allergens belonging to the family of soybean trypsin inhibitors

Background: We have previously identified patatin (Sol t 1) of potato tubers as a major food allergen among atopic children. In addition to Sol t 1, concomitant IgE binding to other, then unidentified, potato proteins was observed.

Methods: Purification and identification of the putative allergens were done by both standard and advanced methods of protein chemistry. The patient series comprised 39 children with positive skin prick test (SPT) to raw potato.

Immunoblotting and ELISA were used to examine IgE-binding ability and skin prick testing to assess *in vivo* reactivity of the purified potato proteins.

Results: Four IgE-binding potato proteins with molecular masses ranging from 16 to 20 kDa were purified and identified as cathepsin D-, cysteine-, and aspartic protease inhibitors belonging to the family of soybean trypsin inhibitors (Kunitz type). The proteins were designated Sol t 2, Sol t 3.0101, Sol t 3.0102, and Sol t 4. In ELISA, 51% of the sera of the 39 atopic children showed specific IgE to Sol t 2, 43% to Sol t 3.0101, 58% to Sol t 3.0102, and 67% to Sol t 4, respectively. All these four allergens were able to produce positive wheal-and-flare responses in SPT.

Conclusions: In addition to Sol t 1, potato tubers contain several proteins belonging to the family of soybean trypsin inhibitors against which atopic children with positive SPT responses to raw potato have *in vitro* and *in vivo* reactive IgE antibodies.

U. Seppälä^{1,2}, H. Majamaa³,
K. Turjanmaa³, J. Helin¹,
T. Reunala³, N. Kalkkinen¹,
T. Palosuo²

¹Institute of Biotechnology, University of Helsinki, Helsinki; ²National Public Health Institute, Helsinki;

³Department of Dermatology, Tampere University Hospital, Finland

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Ulla Seppälä, MSc
Laboratory of Protein Chemistry, Institute of
Biotechnology
University of Helsinki
PO Box 56
FIN-00014 Helsinki
Finland

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Several reports have shown that eating potatoes or peeling raw potatoes may elicit allergic reactions in children and adults (1–6). Patatin (Sol t 1) was the first allergen of potato to be identified (7–10). Sol t 1 is known as a defense-related major storage protein of potato tubers (11). Defense-related proteins play an important role in resistance to pathogens. Some defense-related proteins have been associated with allergy, and a few of them have been reported to show cross-reactivity among plant-derived food allergens (12). However, hypersensitivity reactions to cross-reactive allergens are common in patients allergic to birch and grass pollen and to various plant foods (13–15).

Abbreviations. CEC: cation-exchange chromatography; dHex: deoxy-hexose; GFC: gel filtration chromatography; Hex: hexose; HexNAc: *N*-acetylhexosamine; HIC: hydrophobic interaction chromatography; ELISA: enzyme-linked immunosorbent assay; MALDI-TOF: matrix-assisted laser desorption ionization time-of-flight; Pen: pentose; Q-TOF: quadrupole-time-of-flight; SPT: skin prick test; STI: soybean trypsin inhibitor.

*The cathepsin D-, cysteine, and aspartic proteinase inhibitors have been recognized by the WHO/IUIS Allergen Nomenclature Subcommittee as Sol t 2 (AC P16348), Sol t 3.0101 (AC gi|7489251|pir|T07746), Sol t 3.0102 (AC P20347), and Sol t 4 (AC P30941).

Several food allergens have been shown to be storage proteins or protease inhibitors, i.e., molecules that are relatively stable and resist heating and digestive proteases. One of the best-known protease inhibitors is the family of Kunitz type soybean trypsin inhibitors (STI), which includes a 20-kDa allergen of soybean (16–19).

Here we show that cathepsin D-, cysteine-, and aspartic protease inhibitors* of potato (*Solanum tuberosum*) that have sequence similarity and belong to the family of Kunitz type STI bind IgE from sera of atopic infants and children with suspected food allergy and positive skin prick test (SPT) responses to raw potato. These protease inhibitors also cause distinct wheal-and-flare responses in SPT.

Material and methods

Patients and controls

Altogether, 39 children examined for food allergy and all showing positive SPT to raw potato were included in the study. The patient series comprised a subgroup of 12 infants (mean age 13 months, range 10–24 months; total serum IgE, mean 508, range <5–2324 kU/l). For all 12 infants, potato had been eliminated from the diet for 2–18 months, after which all 12 had undergone a 7-day open challenge. When challenged, the skin response to raw potato was positive in seven (58%), the oral response to cooked potato in eight (67%), and both

types of responses in four (33%) infants, implying that challenge-proven potato allergy was demonstrated in eight of the infants. All of the infants had symptoms and signs of atopic dermatitis and were considered to have multiple food allergies. All 12 had positive SPTs to eggs, 10 to cow's milk and cereals (ALK a/s, Hørsholm, Denmark), and one also to birch pollen (9).

The other subgroup comprised 27 children (mean age 4 years; range 4 months to 10 years; total IgE, range 13–5193 kU/l) with symptoms and signs of atopic dermatitis ($n=25$), asthma ($n=8$), or allergic rhinitis ($n=9$). Besides positive SPT to raw potato, these children also had other positive SPTs; 11 to eggs, 16 to cow's milk, 12 to wheat, and nine to birch pollen (ALK a/s) (7). Control sera were obtained from 30 atopic children (mean age 7.8 months, range 2–26 months, total IgE range <5–197 kU/l) having negative SPTs to potato, hazelnut, birch pollen, and commercial NRL (natural rubber latex) allergen extract (Stallergènes SPT reagent, Antony, Cedex, France) and positive SPTs to cow's milk, eggs, and various animal danders. The study was approved by the ethics committee of Tampere University Hospital (Finland).

Electrophoresis and immunoblotting

Proteins were separated by SDS-PAGE on 15% gels, according to Laemmli (20), under reducing conditions. Separations on a Mini-Protein II device (Bio-Rad Laboratories, Hercules, CA, USA) were performed at a constant current of 25 mA/gel. Molecular weight standards were either low molecular weight (LMW) (Pharmacia Biotech, Uppsala, Sweden) or biotinylated SDS-PAGE standards, broad range (Bio-Rad Laboratories). Proteins were stained either with Coomassie brilliant blue (CBB) (PhastGel Blue R, Pharmacia Biotech) or silver (21).

For amino-terminal sequencing, the proteins were transferred to a ProBlott (Applied Biosystems, Perkin Elmer, Foster City, CA, USA) PVDF (polyvinylidene difluoride) membrane or adsorbed to a ProSorbTM PDVF membrane (Applied Biosystems, Perkin Elmer). Electroblooming to the ProBlott was carried out at 50 V for 2.45 h, and protein bands were visualized by staining with CBB (0.1% PhastGel Blue R-250 in 1% acetic acid/40% MeOH) (Pharmacia Biotech).

For immunoblotting, SDS-PAGE-separated proteins were transferred to a PVDF membrane (Immobilon-P, Millipore Corporation, Bedford, MA, USA). The proteins were transferred at 100 V for 75 min in a Mini Trans-Blot Cell (Bio-Rad Laboratories) in 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid), pH 11, 10% methanol (22). Transferred proteins on the PVDF membrane were incubated for 45 min in a blocking buffer containing 5% nonfat milk powder in 10 mM Tris-Cl, 0.9% NaCl, pH 7.5 (TBS), and then placed into a Miniblotter (Biotra, Göttingen, Germany). The sera of patients (diluted 1:5) exhibiting strong IgE binding to several potato proteins were used as references in immunoblotting. The assay was otherwise continued as described elsewhere (7). For detection of IgE-binding, the membrane was placed in Immune-StarTM Substrate (Bio-Rad Laboratories) development solution, and bound IgE was visualized using HyperfilmTM ECLTM, high performance chemiluminescence film (Amersham International, Buckinghamshire, UK). The ability of the purified proteins (1 and 10 µg/ml) to inhibit the binding of IgE of pooled sera from 10 children against total protein extract of potato was assessed by immunoblot inhibition.

Methods in protein purification

The extraction of potato proteins was performed as described earlier (7). Gel filtration chromatography (GFC) on a 1.6 × 60 cm Superdex 75 Fast Flow column (Pharmacia Biotech) in 20 mM sodium phosphate buffer, 150 mM NaCl, pH 6.0, was used to separate the proteins by molecular size. The flow rate was 2.0 ml/min, and GFC was monitored at 214 nm. Hydrophobic interaction chromatogra-

phy (HIC) was then performed on a Phenyl Superose HR 5/5 column (Pharmacia Biotech) equilibrated with 20 mM sodium phosphate buffer and 1.5 M (NH₄)₂SO₄. The fractions chosen from the GFC were pooled, brought to 1.5 M (NH₄)₂SO₄, and applied to the column. The elution was performed with a decreasing linear gradient of (NH₄)₂SO₄ (1.5–0 M (NH₄)₂SO₄ in 30 min) followed by 2.0 ml H₂O. The flow rate was 0.5 ml/min, and elution was monitored at 214 nm. Cation-exchange chromatography (CEC) was then performed in a Mono S HR5/5 column (Pharmacia Biotech) equilibrated with 50 mM sodium acetate buffer, pH 3.8. Fractions chosen from HIC were pooled, the buffer was changed to 50 mM sodium acetate buffer, pH 3.8, with pressure microfiltration (3K OmegacellTM, Filtron Technology Corporation, Northborough, MA, US), and the sample was applied to the column. Elution was performed with a linear gradient of NaCl (0–0.5 M in 30 min) in the equilibration buffer. The flow rate was 1.0 ml/min, and chromatography was monitored at 280 nm.

Reversed-phase (RP) chromatography was performed in a 0.21 × 10 cm TSK TMS 250 (TosoHaas Corporation, Japan) and/or a 0.21 × 15 cm Rexchrom Silica C8 Fec (Regis Technologies, Inc., Morton Grove, IL, USA) column. Columns were equilibrated with 0.1% trifluoroacetic acid (TFA) and eluted with a linear gradient of acetonitrile (3–100% in 60 min or 30–100% in 120 min) in 0.1% TFA. In both columns, the flow rate was 0.2 ml/min, and the chromatography was monitored at 214 nm. The protein peaks observed were integrated and the protein amounts calculated by comparison of the obtained peak areas to the areas obtained with known amounts of bovine serum albumin and ovalbumin.

The buffer exchanges/desalting for ELISA (50 mM sodium carbonate buffer, pH 9.6) and SPT (10 mM sodium phosphate buffer 0.9% NaCl, pH 7.5) were performed by GFC in a 1 × 10 cm BioGel P-6DG column (Bio-Rad Laboratories) and/or pressure microfiltration (3K OmegacellTM, Filtron Technology Corporation).

Amino-terminal sequencing and mass spectrometry

Amino-terminal sequencing of the proteins was performed with an Applied Biosystems 494 A Procise sequencer (Applied Biosystems, Perkin Elmer, Foster City, CA, USA). The molecular masses of all proteins were determined by electrospray ionization mass spectrometry (MS) using a quadrupole time-of-flight (Q-TOF) instrument (Micromass Co., Manchester, UK). RP-purified proteins were directly injected into the MS with a syringe pump at a flow rate of 0.5 µl/min through a 20-µm inner diameter-silica capillary (New Objective, Inc., Cambridge, MA, USA). The mass analyses of peptides were performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) with a Biflex II instrument (Bruker-Franzen Analytik, Bremen, Germany), as described elsewhere (23). For peptide mass fingerprinting or internal sequencing, the protein to be identified was alkylated and digested with modified trypsin (Promega Corporation, Madison, WI, USA). For mass fingerprinting, the protein digest was desalted by microtip RP chromatography (23) and analyzed by MALDI-TOF MS. Angiotensin II and ACTH fragment 18–39 (Sigma, A0673, St Louis, MO, USA) were included in the peptide samples as internal standards.

IgE ELISA

The concentration of the purified potato proteins was adjusted to 1.5–2.0 µg/ml in 50 mM sodium carbonate buffer, pH 9.6, applied to 96-well polystyrene microtiter plates (100 µl/well) (Nunc, Roskilde, Denmark), and incubated for 3 h at room temperature and then overnight at 4°C. The wells were then emptied and postcoated for 1 h with 1% human serum albumin (HSA) in 100 µl of 50 mM sodium carbonate buffer, pH 9.6. After rinsing with 0.05% Tween 20 in PBS (PBS-T), 100 µl of patient and control sera, diluted (1:10) with PBS-T containing 0.2% HSA (PBS-T-HSA), was added to the wells and

incubated for 2 h at room temperature. The procedure was then continued as described earlier (7). The cutoff level for positivity was defined as the mean OD value + 3 SD of the 30 control children.

In inhibition experiments, coating and postcoating of the microtiter plates were performed as described above for ELISA. The pooled sera of 10 children (diluted 1:20 with PBS-HSA), each serum previously shown in ELISA to contain IgE antibodies against all the purified proteins, were chosen for inhibition studies. Sera were first incubated for 1 h at room temperature with an equal volume of the inhibitor solution. The final concentrations of the purified proteins used as inhibitors ranged from 200 µl/ml to 0.001 µg/ml. Mixtures containing the sera and the inhibitor (or serum without inhibitor as a control) were incubated in the wells for 2 h at room temperature. After this, the assay was continued as earlier described (7).

SPT and RAST

The ordinary skin prick technique, as earlier described (24), was used to test purified Sol t 1 (200 µg/ml), Sol t 2 (20 µg/ml), Sol t 3.0101 (20 µg/ml), Sol t 3.0102 (40 µg/ml), and Sol t 4 (20 µg/ml) on the volar aspect of the forearm. The concentrations chosen were based on preliminary results with Sol t 2–4 and results from a previous study for Sol t 1 (7). A wheal with a mean diameter of ≥ 3 mm was considered a positive response. Histamine dihydrochloride (10 mg/ml, ALK a/s) was used as the positive control and physiologic saline as the negative control. Total serum IgE was measured, and potato RAST (Pharmacia UpJohn, Uppsala, Sweden) was performed as previously described (24). RAST values greater than 0.4 kU/l were considered positive.

Results

Detection of IgE-binding potato proteins by immunoblotting

Selected GFC fractions were first analyzed by SDS-PAGE (Fig. 1A), and then pooled and subjected to immunoblot analysis with sera of the 12 infants (Fig. 1B). Seven (58%) infants showed IgE binding to a 20-kDa band and three (25%) to bands in the 16- and 18-kDa regions.

Purification and molecular characterization of IgE-binding potato proteins

The pooled GFC fractions were subjected to RP chromatography (C8 column). Eluted fractions were then combined into six pools, subjected to SDS-PAGE, and analyzed by immunoblotting. In most of the fractions, IgE-binding to potato proteins with molecular masses around 20 kDa was observed. A late eluting 20-kDa protein, having IgE-binding ability, was further analyzed by SDS-PAGE, and subjected to amino-terminal sequencing and mass spectrometry. Amino-terminal sequencing yielded the sequence ESPLPKPVLDTNGKELNPXSSYRII, which could be identified as the cathepsin D protease inhibitor (PDI) (AC P16348) (Table 1) (25). That the residue no. 19 (X) gave no signal (N) in the sequence analysis indicates that the asparagine (N) at the potential glycosylation site (XSS) was glycosylated (Table 1). Mass fingerprinting analysis with MALDI-TOF MS yielded (with three peptides covering 18% of the protein

sequence) the highest score for the PDI (Table 1). The analysis with electrospray MS of PDI yielded molecular masses of 21 626, 21 755, and 21 958 Da. Considering the calculated molecular mass of the PDI protein sequence (20 589 Da), the signals were tentatively assigned to glycoforms carrying (Hex)₃(HexNAc)₂(dHex)₁ (+1039 Da), (Hex)₃(HexNAc)₂(dHex)₁(Pen)₁ (+1171 Da), and (Hex)₃(HexNAc)₃(dHex)₁(Pen)₁ (+1374 Da), respectively. The major signal may thus carry a typical plant N-glycan, as depicted in Fig. 2 (26). To purify and identify the other IgE-binding potato proteins, pooled fractions from GFC were subjected to HIC. Eluted fractions were combined into five pools (I–V), desalted, and analyzed by SDS-PAGE. Binding of IgE to the proteins (I–V) was again assessed by immunoblotting, and IgE-binding to proteins in the 20-kDa region was observed in all the pools (I–V). Concomitant IgE-binding in pool IV to a 16-kDa protein was observed. For further analysis, the pools (I–V) were subjected to SDS-PAGE and electroblotted on a PVDF membrane. Protein bands of interest were subjected to amino-terminal sequencing. The obtained amino-terminal sequence of all of the 20-kDa proteins corresponded to cysteine protease inhibitors of potato tuber (90–100% identity in 10-aa overlap), after which pools II and IV were chosen for further analysis.

Pool II was subjected to CEC and resulted in a single peak of a 20-kDa protein with IgE-binding ability. Amino-terminal sequencing of this protein yielded

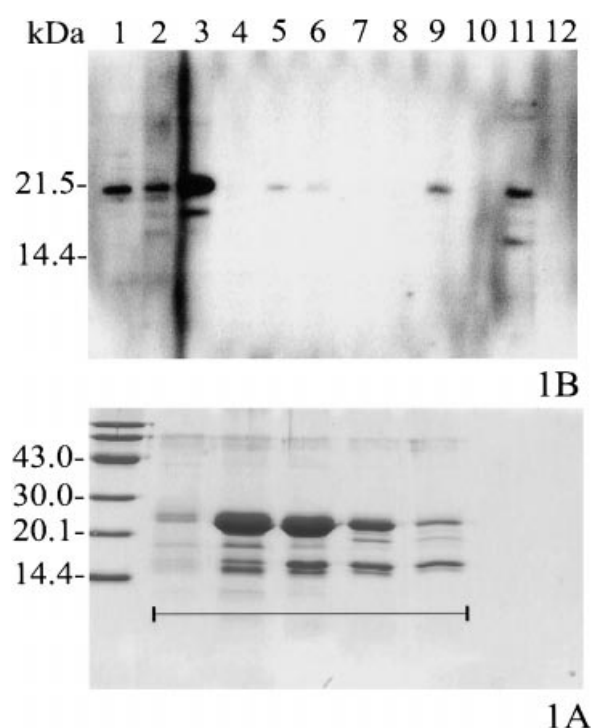


Figure 1. A) Selected gel-filtration fractions of potato tuber analyzed by SDS-PAGE. B) Immunoblot analysis of IgE-binding to pooled GFC fractions with sera of 12 infants with suspected potato allergy and positive SPTs to raw potato.

Sol t 2	sp P16348 ICTD_SOLTU	-----	-----	-----ESPLPKP	VLDTNGKELNFXSSY	RIISIGRGALGGDVY	LGKSPNSDAPCPDV
Sol t 3.0101	gi 7489251 pir T07746			-----TCHDDDNVLPE	VYDQDGNPLRIGERY	IIKNPLLGA--AVYLDNI	GNLQCPNAVLQ
Sol t 3.0102	sp P20347 PC83_SOLTU	SINILSFLLLSSTLS	LVAFARSFTSENPIV	LPTTCHDDDNVLPE	VYDQDGPLRIGERY	IINNPLIGAG--AVY	LYNIGNLQCPNAVLQ
Sol t 4	sp P30941 IAP2_SOLTU	-----MKCLF	LLCLCLVPIVVSST	FTSKNPINLPSDATP	VLDVAGKELDSRLSY	RIISTFWGALGGDVY	LGKSPNSDAPCANGI
Sol t 2	sp P16348 ICTD_SOLTU	FRYNSDVGPSGTPVR	FIPLSGG----IFE	DQLNLIQFNIAIVKL	CVSYTIWKVGNLNAY	FRTMLLETGGTIGQA	DSSYFKIVKLSNFGY
Sol t 3.0101	gi 7489251 pir T07746	HMSIPQFLGKGTPEV	FIRKSESDDYGDVRL	MTAVYIKFFVKTKTKL	CVDETVWKVNNEQLV	VTGGNVGNENDIFKI	KKTDLVIRG-MKNVY
Sol t 3.0102	sp P20347 PC83_SOLTU	HMSIPQFLGKGTPEV	FVRKSESDDYGDVVR	MTVVYIKFFVKTKTKL	CVDQTVWKVNDQQLV	VTGGKVGNDENDIFKI	MKTDLVTPGGSKYFY
Sol t 4	sp P30941 IAP2_SOLTU	FRYNSDVGPSGTPVR	FSFHQGQ----IFE	NELLNLIQFAISTSKL	CVSYTIWKVGDYDAS	LGTMLLETGGTIGQA	DSSWFKIVKSSQFGY
Sol t 2	sp P16348 ICTD_SOLTU	NLLYCPITPPFLCPF	CRDDNFCAKVGVIQ	NGKRRALVNNENPLD	VLFQEV--		
Sol t 3.0101	gi 7489251 pir T07746	KLLHCPs-----	---HLECKNIGSNFK	NGYPRLVTVNDEKDF	IPFVFIIKA		
Sol t 3.0102	sp P20347 PC83_SOLTU	KLLHCPs-----	---HLGCKNIGSNFK	NGYPRLVTVDDKDF	IPFVFIIKA		
Sol t 4	sp P30941 IAP2_SOLTU	NLLYCPVTSMTSCPF	SSDDQFCCLKVGVVHQ	NGKRRALVKNNDPLD	VSFQKQVO-		

LVLPEVYDQDNPLRIGERYIIKNP, identifying it as the probable cysteine protease inhibitor 10 of potato (AC gi|7489251| pir|T07746|, 100% identity in a 25-aa overlap) (27). MS analysis gave a molecular mass of 20 114 Da.

Pool IV was also subjected to CEC. Two peaks eluting from the column were subjected to SDS-PAGE and analyzed by immunoblotting. The bands of 16 and 20 kDa corresponded to the eluted peaks and showed IgE-binding ability by immunoblotting. Together with the 16-kDa band, a low-molecular-mass protein was detected from the CCB-stained PVDF-membrane. However, at this point, only the IgE-binding 16- and 20-kDa bands were subjected to amino-terminal sequencing. The amino-terminal sequencing of the 16-kDa band

yielded LPSDATPVLDVAGKELDSRLSYRII, which could be identified as the aspartic protease inhibitor of potato tuber (AC P30941, with 100% identity in a 25-aa overlap) (Table 1) (28). However, the electrospray MS analysis of the intact protein gave a molecular mass of 20 302 Da, which did not correspond to the one observed in SDS-PAGE and electroblotting. The intact protein was then alkylated and subjected to RP chromatography. Afterward, a 4274-Da fragment with the amino-terminal sequence SDDQFCLKVGVVHQN (with 100% identity with the C-terminal part of the aspartic protease inhibitor of potato tuber in a 15-aa overlap) could be separated from the 16-kDa part of the molecule. This resulting sequence was later confirmed by subjecting the low-molecular mass band from the PVDF membrane to

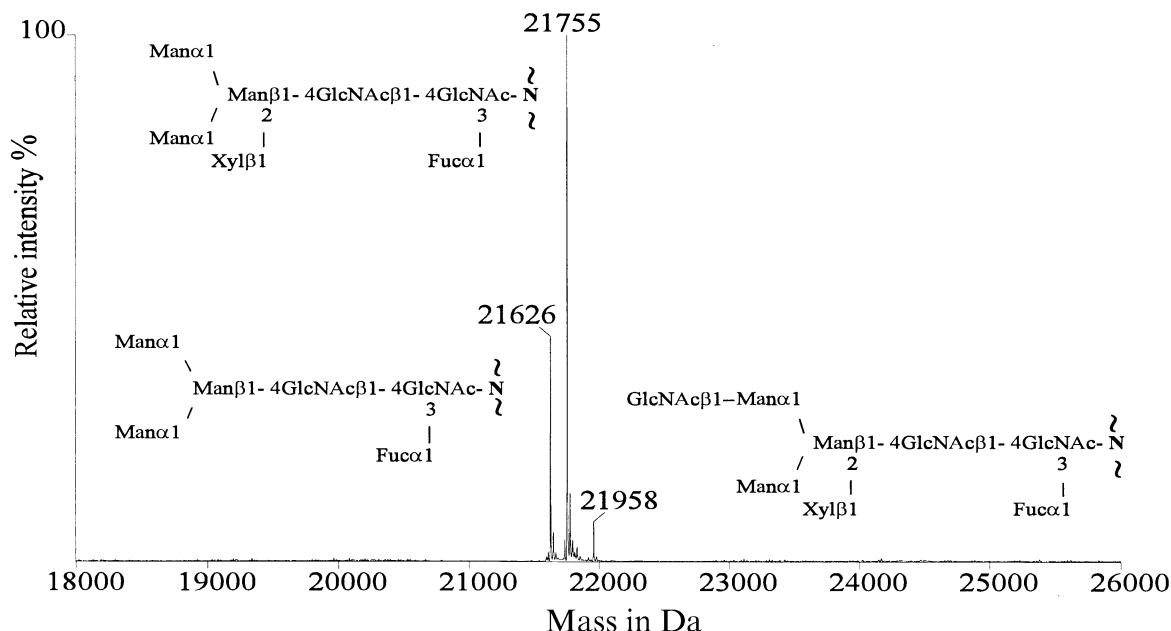


Figure 2. Deconvoluted electrospray MS data of cathepsin D proteinase inhibitor (PDI) Sol t 2. Heterogeneity of purified PDI can be ascribed to N-glycosylation. Tentative glycan structures for different glycoforms of PDI, (Hex)₃(HexNAc)₂(dHex)₁, (Hex)₃(HexNAc)₂(dHex)₁(Pen)₁, and (Hex)₃(HexNAc)₃(dHex)₁(Pen)₁ are shown.

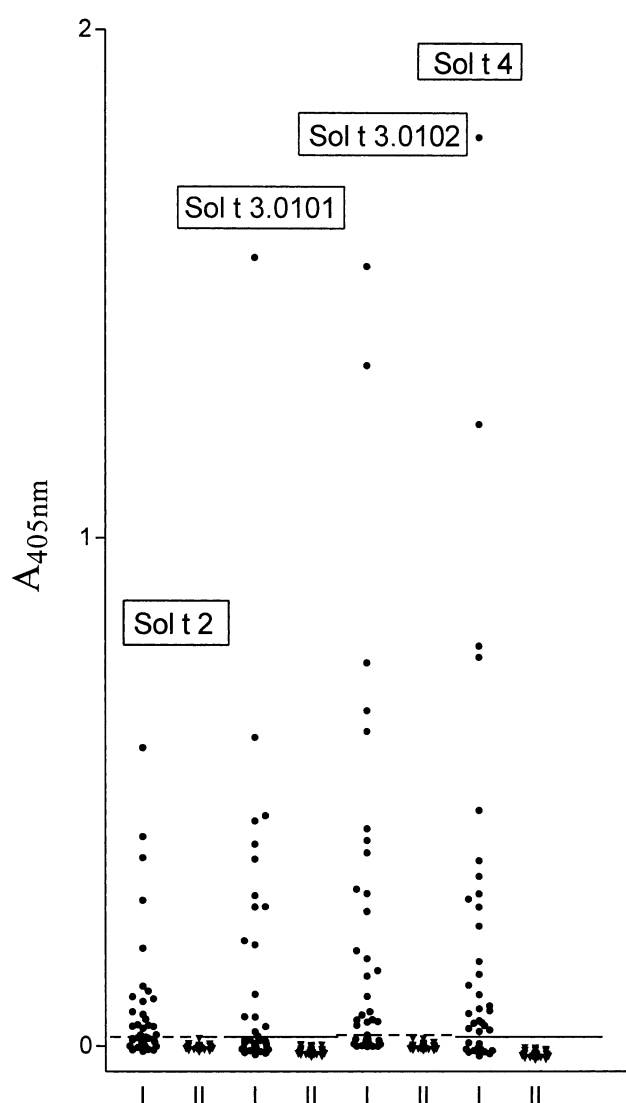


Figure 3. Occurrence of IgE antibodies to purified Sol t 2, Sol t 3.0101, Sol t 3.0102, and Sol t 4. IgE-binding of sera (diluted 1:10) from atopic children ($n=39$) with positive SPT to raw potato (I) and control children ($n=30$) (II) are shown. Cutoff levels for positive response (mean OD value + 3 SD of 30 controls) are indicated by horizontal lines.

amino-terminal sequencing. According to the present results, the aspartic protease inhibitor of potato tuber seems to consist of ~4-kDa and ~16-kDa fragments held together by a disulfide bond.

Amino-terminal sequencing the 20-kDa protein in fraction IV, after absorption to a ProSorbTM PDVF membrane (Applied Biosystems, Perkin Elmer), yielded LVLPEVYDQDGDPLRIGERYIINNPLIGAGAVY-LYNIGNLQCPNAVLQHMSIPQFLGKGTPVV. The protein was identified as the cysteine protease inhibitor (PCI) 8.3 of potato tuber (AC P20347, with 95% identity in a 64-aa overlap) (29). MS analysis of the purified protein gave a molecular mass of 20 193 D (Table 1).

Occurrence of IgE antibodies to purified cathepsin D-, cysteine-, and aspartic protease inhibitors in ELISA

Increased binding of IgE to the purified cathepsin D protease inhibitor (PDI) was seen in 20/39 sera (51%) (Fig. 3). Against the two isoforms of cysteine protease inhibitors, 17 (43%) and 23 (58%) children, respectively, had increased levels of IgE antibodies (Fig. 3). Increased IgE-binding to the aspartic protease inhibitor was seen in 26/39 sera (67%) (Fig. 3). According to these results, we designated the PDI Sol t 2, the cysteine protease inhibitors Sol t 3.0101 and Sol t 3.0102, and the aspartic protease inhibitor Sol t 4 (Table 2). In ELISA inhibition, low to moderate concentrations of Sol t 2 (20 µg/ml), Sol t 3.0101 (10 µg/ml), Sol t 3.0102 (3 µg/ml), and Sol t 4 (25 µg/ml) inhibited at least 50% of the binding of IgE from the pooled sera to solid-phase potato proteins (Fig. 4). Sol t 1 was studied for comparison; at a concentration of 3 µg/ml, it was able to inhibit 50% of the binding of IgE from the pooled sera against the solid-phase potato protein extract (Fig. 4).

Ability of purified Sol t 2–4 to inhibit IgE-binding from pooled sera to blotted potato protein extract

After preincubation of Sol t 2–4 (0, 1.0, and 10 µg/ml) with pooled sera of the 10 children (used in ELISA inhibition), Sol t 2 (10 µg/ml) was able to block the IgE-binding against potato proteins in the 16–20-kDa area, containing Sol t 3.0101, Sol t 3.0102, and Sol t 4. IgE-binding to the 20-kDa area seemed not to be affected when either Sol t 3.0101 or Sol t 3.0102 was used as inhibitor. In addition, Sol t 4 was able to decrease significantly the IgE-binding to the 20-kDa region and completely inhibit the binding to the 16-kDa region (Fig. 5).

SPT results for Sol t 2–4

Four older children, four challenge proven potato-allergic infants, and six control children (age range 6–14 months) were chosen for SPT with Sol t 2, Sol t 3.0101, Sol t 3.0102, and Sol t 4 (Table 3). All eight children were retested at the same time with raw potato and Sol t 1 (200 µg/ml). Potato RAST and total serum IgE were also retested in this context (Table 3). Two of four

Table 2. Summary of *in vitro* IgE reactivity to newly described potato allergens

Allergen	kDa	Positive in IgE ELISA (%)			Putative function
		Infants $n=12$	Children $n=27$	Total $n=39$	
Sol t 2	20 589*	46%	56%	51%	Cathepsin D protease inhibitor
Sol t 3.0101	20 114	38%	48%	43%	Cysteine protease inhibitor
Sol t 3.0102	20 193	53%	63%	58%	
Sol t 4	20 302	67%	67%	67%	Aspartic protease inhibitor

* Calculated molecular mass of protein sequence.

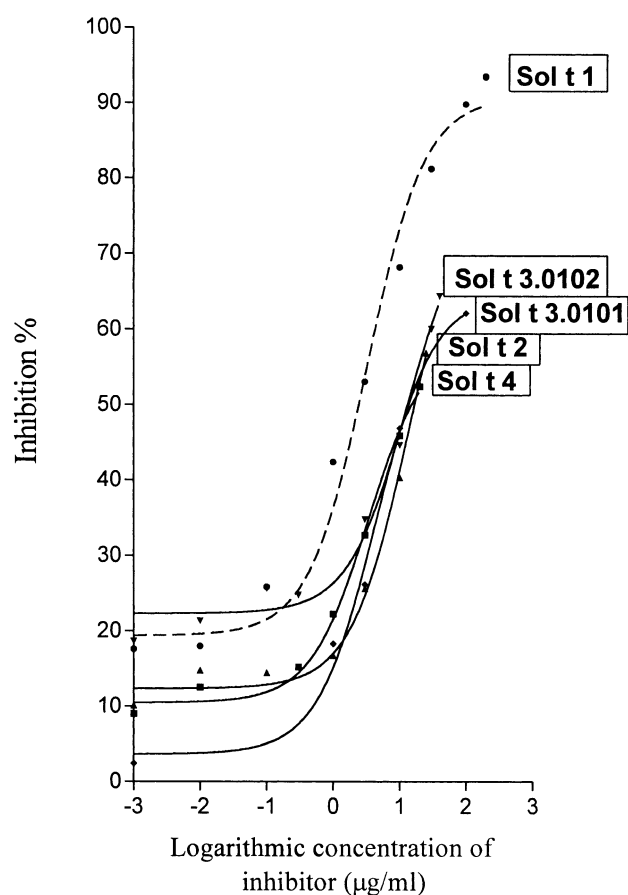


Figure 4. Inhibition of IgE-binding to solid-phase total protein extract of potato by different concentrations of purified Sol t 2–4. Inhibition ability of Sol t 1 is shown for comparison.

infants and one of four older children had a positive SPT response to Sol t 2 (20 µg/ml). All four infants and two of four older children had positive SPT responses to Sol t 3.0101 (40 µg/ml). Positive SPT responses to Sol t 3.0102 (40 µg/ml) were seen in all four infants and one older child. Two infants and two older children had

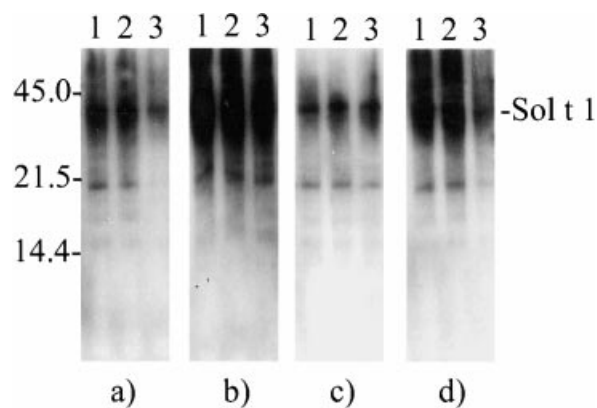


Figure 5. Immunoblot inhibition: analysis of IgE-binding to total potato protein extract by new potato allergens after preincubation with pooled sera from 10 children. a) Sol t 2; b) Sol t 3.0101; c) Sol t 3.0102; d) Sol t 4, in concentrations of 1) 0, 2) 1.0, and 3) 10.0 µg/ml.

positive SPT responses to Sol t 4 (20 µg/ml). All eight children tested had a positive response to raw potato, and six of the eight did so to Sol t 1. The results for the six control children tested remained negative.

Discussion

We have previously identified patatin (Sol t 1) as a major food allergen in potato tubers (7). In the present study, four novel potato allergens were purified and identified as cathepsin D-, cysteine-, and aspartic protease inhibitors of potato tuber. In ELISA, specific IgE-binding to these proteins, designated Sol t 2–4, was seen in 43–67% of atopic children suspected of having allergy to potato (Tables 2–3). In addition, all these new purified allergens were able to produce distinct wheal-and-flare responses in the infants and children tested.

The new potato allergens, cathepsin D-, cysteine-, and aspartic protease inhibitors of potato tuber, have sequence similarity and belong to the family of Kunitz-

Table 3. Skin prick test (SPT) results for raw potato and purified potato allergens

Patient no.	Initials/sex (F/M)	Total IgE (kU/l)	Potato RAST (kU/l)***	SPT to raw* potato	SPT to Sol t 1** (200 µg/ml)	SPT to Sol t 2** (20 µg/ml)	SPT to Sol t 3.0101** (20 µg/ml)	SPT to Sol t 3.0102** (40 µg/ml)	SPT to Sol t 4** (20 µg/ml)
Infants									
1	IK/F	1841	86.5	12/5	7	11	6	8	4
2	AK/M	491	4.0	10/7	5	0	3	5	2
3	MM/M	139	1.3	11/6	4	0	3	10	0
4	OK/F	371	0.8	10/5	4	3	5	7	3
Children									
5	HM/F	879	2.5	10/4	4	5	1	3	5
6	HJ/M	5200	5.4	5/3	3	0	3	0	0
7	KJ/M	1145	0.8	12/6	1	0	3	2	0
8	SA/M	1868	nd	9/5	1	1	1	1	3

* Mean diameter of potato wheal/mean diameter of histamine control in millimeters.

** Mean diameter of Sol t 1–4 wheal in millimeters.

nd: not done.

*** Cutoff limit for positivity > 0.4 kU/l.

type STI. The STI family comprises plant proteins (such as those of soybean, rice, wheat and barley) that inhibit the serine proteases of the trypsin and subtilisin families, the thiol- and aspartic proteases, and some proteins that are probably involved in seed storage. The structural superfamily also contains the interleukin-1 proteins and heparin-binding growth factors, which have similar structures, but share no sequence similarity with the STI family (30).

The cathepsin D protease inhibitor (PDI), designated Sol t 2, is a glycoprotein containing one glycosylation site at Asn-19 (25). The proposed glycan structure (depicted in Fig. 2) may well correspond to the one determined for Lol p 11, the 16-kDa major grass-pollen allergen of ryegrass (*Lolium perenne*) (26). The sequence similarity of Lol p 11 to the STI family (32% homology) (31) and the proposed likelihood of the glycan structures for Sol t 2 and Lol p 11 could be one explanation for the reported cross-reactivity *in vitro* between grass pollen, potato, and fruits (13). However, the effect of glycan structures for the IgE-binding ability between these two allergens needs to be established.

In ELISA, 51% of 39 children showed IgE antibodies to Sol t 2 (Fig. 3). Interestingly, the IgE immunoblot inhibition results using pooled sera binding to denatured total potato protein extract showed that native Sol t 2 (10 µg/ml) could inhibit IgE-binding to all the other new potato allergens except Sol t 1 (Fig. 5). Thus, it seems that there is cross-reactivity among Sol t 2–4. However, further studies with purified potato and pollen allergens are needed to determine whether IgE-binding to protease inhibitors of potato tuber has any association or clinical congruity to other structurally related proteins in birch and grass pollen, fruits, and vegetables (13, 14).

The potato cysteine protease inhibitors (PCPIs) represent a distinct group of proteins, as they show no homology to any other known cysteine protease inhibitor superfamilies but belong to a Kunitz-type STI family (28). PCPIs are single-chain proteins with molecular masses ranging from 20 to 25 kDa with different isoelectric points and functional properties. Amino-acid substitutions occurring on the surface of these proteins suggest the need for further classification into subgroups (28, 29); therefore, these two members of this family were designated Sol t 3.0101 and Sol t 3.0102. In ELISA, 43% of 39 children showed IgE-binding to Sol t 3.0101. In contrast, 58% of them had specific IgE antibodies to Sol t 3.0102 (Fig. 3). These results are in line with the previous findings of different IgE-binding ability between allergen isoforms (32). The occurrence of several isoforms of PCPIs (28, 29) could be one explanation for the observed lack of inhibition ability of both isoforms (10 µg/ml) to inhibit IgE-binding to potato extract in immunoblotting (Fig. 5).

Aspartic protease inhibitors are encoded by a multi-gene family. However, only a few of them have been

isolated and characterized. Moreover, the exact physiologic functions of aspartic protease inhibitors in potato tuber remain to be elucidated (28). In immunoblotting, IgE-binding to the 16-kDa band corresponding to aspartic protease inhibitor of potato tuber was observed (Fig. 1B). Further analysis by mass spectrometry, alkylation, and sequence analysis revealed that the 16-kDa band was part of a larger molecule (20 302 Da) where the 16- and 4-kDa polypeptides were joined together by a disulfide bond. IgE-binding to the 4-kDa fragment was not noticed in immunoblotting (Fig. 1B); however, the native structure seems to be important for IgE-binding in ELISA (Fig. 3). In ELISA, 67% of the children, in contrast to the small number of children reacting in immunoblotting, showed IgE-binding to the aspartic protease inhibitor designated Sol t 4. Furthermore, in immunoblot inhibition, the native Sol t 4 (10 µg/ml) was able to diminish the IgE-binding from pooled sera to the 20-kDa region (Fig. 5). These findings are in line with the view that conformational epitopes are important for the reactivity of IgE antibodies to these allergens.

Protease inhibitors are known to have a particularly stable conformation (30, 33). Resistance to processing (such as cooking) and to digestion by digestive proteases enables the entrance of these immunologically active proteins into the gut (34). This suggests that these protease inhibitors, by interfering with the functions of digestive enzymes, might be able to enhance the stability and allergenic properties of proteins, such as Sol t 1. Such properties have already been linked to the allergenic potential of the cow's milk allergen, β -lactoglobulin (33). These kinds of factors could therefore influence the ability of plant proteins to sensitize individuals (34).

The purified plant protease inhibitors of potato tuber produced positive SPT responses in the eight children tested, so that each subject reacted to at least one of the four new allergens (Table 3). Although all four older children and three infants (nos. 2–4 in Table 3) are now able to eat cooked potatoes without clinical symptoms, they still developed in retesting positive SPT responses to raw potato and to purified potato allergens. This suggests that the majority of these children have already grown out of clinical manifestations of potato allergy (35).

In conclusion, four new potato allergens were identified. In addition, this study presents a new allergen family of proteins (Kunitz-type STI) structurally related to the well-known allergens of soybean (15–18) and to a major grass-pollen allergen, Lol p 11 (31).

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References

- PEARSON RS. Potato sensitivity an occupational allergy in housewives. *Acta Allergol* 1966;**21**:507–514.
- NATER JP, ZWARTZ JA. Atopic allergic reactions due to raw potatoes. *J Allergy* 1967;**40**:202–206.
- CASTELLS MC, PASCUAL C, ESTEBAN M, OJEDA JA. Allergy to white potatoes. *J Allergy Clin Immunol* 1986;**78**:1110–1114.
- QUIRCE S, DIEZ GOMEZ ML, HINOGOSA M, et al. Housewives with raw potato-induced bronchial asthma. *Allergy* 1989;**44**:532–534.
- WAHL R, LAU S, MAASCH HJ, WAHN U. IgE-mediated allergic reactions to potatoes. *Int Arch Allergy Appl Immunol* 1990;**92**:168–174.
- DELGADO J, CASTILLO R, QUILARTE C, BLANCO C, CARRILLO T. Contact urticaria in a child from raw potato. *Contact Dermatitis* 1996;**35**:179.
- SEPPÄLÄ U, ALENIOUS H, TURJANMAA K, REUNALA T, PALOSUO T, KALKKINEN N. Identification of patatin as a novel allergen for children with positive skin prick test responses to raw potato. *J Allergy Clin Immunol* 1999;**103**:165–171.
- TÜCKE J, POSCH A, BAUR X, RIEGER C, RAULF-HEIMSOOTH M. Latex type I sensitization and allergy in children with atopic dermatitis. Evaluation of cross-reactivity to some foods. *Pediatr Allergy Immunol* 1999;**10**:160–167.
- MAJAMAA H, SEPPÄLÄ U, PALOSUO T, TURJANMAA K, KALKKINEN N, REUNALA T. Positive skin and oral challenge responses to potato and occurrence of IgE antibodies to Sol t 1 in infants with atopic dermatitis [Abstract]. *J Allergy Clin Immunol* 2000;**105**:S184.
- ASTWOOD J, ALIBHAI M, LEE T, FUCHS R, SAMPSON HA. Identification and characterization of IgE binding epitopes of patatin, a major food allergen in potato [Abstract]. *J Allergy Clin Immunol* 2000;**105**:S184.
- SENDA K, YOSHIOKA H, DOKE N, KAWAHITA K. A cytosolic phospholipase A₂ from potato tissues appears to be patatin. *Plant Cell Physiol* 1996;**37**:347–353.
- BREITENEDER H, EBNER C. Molecular and biochemical classification of plant-derived food allergens. *J Allergy Clin Immunol* 2000;**106**:27–36.
- CALKHOVEN PG, AALBERS M, KOSHTÉ L, POS O, OEI R, AALBERSE RC. Cross-reactivity among birch pollen, vegetables and fruits detected by IgE antibodies is due to at least three distinct cross-reactive structures. *Allergy* 1987;**42**:382–390.
- EBNER C, HIRSCHWEHR R, BAUER L, et al. Identification of allergens in fruits and vegetables: IgE cross-reactivities with the important birch pollen allergens Bet v 1 and Bet v 2 (birch profilin). *J Allergy Clin Immunol* 1995;**95**:962–969.
- JONES SM, MAGNOLFI CF, COOKE SK, SAMPSON HA. Immunologic cross-reactivity among cereal grains and grasses in children with food hypersensitivity. *J Allergy Clin Immunol* 1995;**96**:341–351.
- MOROZ LA, YANG WH. Kunitz soybean trypsin inhibitor; a specific allergen in food anaphylaxis. *N Engl J Med* 1980;**302**:1126–1128.
- BRANDON DL, HAQUE S, FRIEDMAN M. Antigenicity of native and modified Kunitz soybean trypsin inhibitors. *Adv Exp Med Biol* 1986;**199**:449–467.
- HERIAN AM, TAYLOR SL, BUSH RK. Identification of soybean allergens by immunoblotting with sera from soy-allergic adults. *Int Arch Allergy Appl Immunol* 1990;**92**:193–198.
- SICHERER SH, SAMPSON HA, BURKS AW. Peanut and soy allergy: a clinical and therapeutic dilemma. *Allergy* 2000;**55**:515–521.
- LAEMMLI UK. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 1970;**227**:680–685.
- O'CONNELL KL, STULTS JT. Identification of mouse liver proteins on two-dimensional electrophoresis gels by matrix-assisted laser desorption/ionization mass spectrometry of *in situ* enzymatic digests. *Electrophoresis* 1997;**18**:349–359.
- MATSUDAIRA P. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J Biol Chem* 1988;**262**:10035–10038.
- SAARINEN J, WELGUS HG, FLIZAR CA, KALKKINEN N, HELIN J. N-Glycan structures of matrix metalloproteinase-1 derived from human fibroblasts and from HT-1080 fibrosarcoma cells. *Eur J Biochem* 1999;**259**:829–840.
- ISOLAURI E, TURJANMAA K. Combined skin prick and patch testing enhances identification of food allergy in infants with atopic dermatitis. *J Allergy Clin Immunol* 1996;**97**:9–15.
- MARES M, MELOUN B, PAVLIK M, KOSTKA V, BAUDYS M. Primary structure of cathepsin D inhibitor from potatoes and its structural relationship to soybean trypsin inhibitor family. *FEBS Lett* 1989;**251**:94–98.
- VAN REE R, CABANES-MACHETEAU M, AKKERDAAS J, et al. $\beta(1, 2)$ -xylose and $\alpha(1, 3)$ -fucose residues have a strong contribution in IgE binding to plant glycoallergens. *J Biol Chem* 2000;**275**:11451–11458.
- GRUDEN K, STRUKELJ B, RAVNIKAR M, et al. Potato cysteine proteinase inhibitor gene family: molecular cloning, characterization and immunocytochemical localisation studies. *Plant Mol Biol* 1997;**34**:317–323.
- STRUKELJ B, PUNGERCAR J, MESKO P, et al. Characterization of aspartic proteinase inhibitors from potato at gene, cDNA and protein levels. *Biol Chem Hoppe Seyler* 1992;**373**:477–482.
- KRIZAJ I, DROBNIC-KOSOROK M, BRIZIN J, JERALA R, TURK V. The primary structure of inhibitor of cysteine proteinases from potato. *FEBS Lett* 1993;**333**:15–20.
- LASKOWSKI M, KATO I. Protein inhibitors of proteinases. *Ann Rev Biochem* 1980;**49**:593–626.
- VAN REE R, HOFFMAN DR, VAN DIJK W, et al. Lol p XI, a new major grass pollen allergen, is a member of a family of soybean trypsin inhibitor-related proteins. *J Allergy Clin Immunol* 1995;**95**:970–978.
- FERREIRA F, HIRTENLEHRER K, JILEK A, et al. Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergic isoforms for immunotherapy. *J Exp Med* 1996;**183**:599–609.
- ASTWOOD JD, LEACH JN, FUCHS RL. Stability of food allergens to digestion *in vitro*. *Nat Biotech* 1996;**14**:1269–1273.
- BRANDON DL, BATES AH, FRIEDMAN M. ELISA analysis of soybean trypsin inhibitors in processed foods. *Adv Exp Med Biol* 1991;**289**:321–337.
- SICHERER SH. Food allergy: when and how to perform oral food challenges. *Pediatr Allergy Immunol* 1999;**10**:226–234.