

CHARACTERIZATION OF POTATO ALLERGENS

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Academic Dissertation

To be presented for public criticism, with the permission of the Faculty of Science,

University of Helsinki

In the auditorium 1041 at Viikki Biocenter, Viikinkaari 5, Helsinki

On March 9, 2001, at 12 noon

Helsinki 2001

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ISSN 0359-3584
ISBN 951-740-199-X (NID)
ISBN 951-740-204-X (PDF)
<http://ethesis.helsinki.fi>
Yliopistopaino
Helsinki 2001

Go on reading

If you do not understand a particular word in a piece of technical writing, ignore it. The piece will make perfect sense without it.

Arthur Bloch

Murphy's Law Book

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based upon the following original papers, referred to in the text by Roman numerals (I-IV)

- I Seppälä U, Alenius 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;103:165-71.
- II Seppälä U, Ylitalo L, Reunala T, Turjanmaa K, Kalkkinen N, Palosuo T. IgE reactivity to patatin-like allergen, Hev b 7, and to patatin of potato tuber, Sol t 1, in adults and children allergic to natural rubber latex. *Allergy* 2000;55:266-73.
- III 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. (submitted)
- IV Seppälä U, Majamaa H, Turjanmaa K, Helin J, Reunala T, Kalkkinen N, Palosuo T. Identification of new potato (*Solanum tuberosum*) allergens belonging to the family of soybean trypsin inhibitors. (in press)

ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
AD	atopic dermatitis
APC	antigen presenting cell
CBB	coomassie brilliant blue
CCD	cross-reactive carbohydrate determinant
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CLA	cutaneous lymphocyte-associated antigen
CTLA-4	cytolytic T lymphocyte-associated antigen-4
DBPCFC	double-blind placebo-controlled food challenge
ELISA	enzyme-linked immunosorbent assay
Fuc	fucose
Gal	galactose
GALT	gut-associated lymphoid tissue
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetyl glucosamine
Hev b 7	<i>Hevea brasiliensis</i> 7
HIC	hydrophobic interaction chromatography
HLA	human leucocyte antigen
HSA	human serum albumin
IL	interleukin
KSTI	Kunitz-type soybean trypsin inhibitor
MALDI-TOF MS	matrix-assisted laser desorption ionization-time of flight mass spectrometry
Man	mannose
MC	mast cell
MHC	major histocompatibility complex
NRL	natural rubber latex
PBS	phosphate buffered saline
pI	isoelectric point
PR	pathogenesis-related
PVDF	polyvinylidene difluoride
Q-TOF	quadrupole-time of flight
RAST	radioallergosorbent test
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sol t 1-4	<i>Solanum tuberosum</i> 1-4
SPT	skin prick test
TCR	T-cell receptor
TFA	trifluoroacetic acid
Tc	cytotoxic T-cell
Th	helper T-cell
Xyl	xylose

INTRODUCTION

An allergy, said Mekori in 1996, "is the immune-mediated state of hypersensitivity that results from exposure to allergens". Allergens, in turn, are proteins or chemical substances, which are able to induce the allergic reactions. Earlier, Coombs and Gell (1963) classified the immunopathological mechanisms of hypersensitivity into four types (I-IV) of distinct reactions. However, in practice these reactions may not occur in isolation from each other (Mekori 1996, Kay 1997).

Almost 20% of people in western populations suffer from IgE-mediated allergies, and individuals with familial atopic background are susceptible to development of allergic reactions to food and/or environmental allergens (Valenta et al. 1999). The prevalence of food allergy has been considered to be between 2 and 8% for infants and children, decreasing to 1% for the adult population (Stanley and Bannon 1999). However, of the individuals suffering from atopic dermatitis, approximately 20% of the children and 10% of the adults experience clinically significant food allergies (Metcalf 1998).

Hypersensitivity reactions against raw fruits and vegetables have been associated with individuals suffering from pollinosis, and the immunological basis for these reactions has been considered to be IgE-mediated cross-reactivity (Breiteneder and Ebner 2000). Particularly, birch pollen-allergic individuals are known to experience hypersensitivity reactions when handling raw potatoes (*Solanum tuberosum*) (Hannuksela and Lahti 1977, Dreborg and Foucard 1983, Sampson 1999), and heat-labile potato proteins have been thought to be responsible for these reactions (Nater and Zwart 1967, Hannuksela and Lahti 1977, Larkö et al. 1983, Quirce et al. 1989).

In atopic children, the most common foods causing allergic reactions are eggs, cow's milk, soy, wheat, tree nuts, and fish (Sampson 1983, Sicherer and Sampson 1999), while allergy to potato has been considered uncommon (Castells et al. 1986). Hannuksela (1987) first reported that cooked potato could cause atopic eczema in infants suffering from atopic dermatitis. A few years later, using sera of atopic children in immunoblotting, Wahl et al. (1990) detected IgE-binding to potato proteins ranging from 16 to 65 kDa in size. However, the identity and the clinical relevance of these IgE-binding potato proteins remained to be identified.

The aim of the present study was to characterize potato allergens by use of sera of atopic children suspected of having allergy to cooked potato. In addition, IgE cross-reactivity was investigated between potato and a natural rubber latex (NRL) allergen having significant amino acid sequence similarity.

REVIEW OF THE LITERATURE

1. IgE-mediated allergy

1.1 Allergens

Antigens able to elicit the production of IgE antibodies are called allergens. During the last ten years, identification and purification of allergens have been important for structural and immunological studies in order to discover how these molecules stimulate IgE antibody production (Stanley and Bannon 1999). Currently, the primary structure of some 200 allergens is known; however, as yet only a relatively small number of plant food allergens have been characterized. All the known allergens have been registered in the Allergen Database in which are also compiled data on their known biochemical and immunological properties (World Health Organization (WHO)/IUIS´allergen list:<http://www.allergen.org>). Allergens are designated according to the accepted taxonomic name. The first three letters describe the genus, followed by the first letter of the species and an Arabic number. The Arabic numbers are assigned to the allergens in the order of their identification. Furthermore, isoallergens and variants are characterized by additional numbers, the first two identifying the isoallergen and the second two the variant (WHO/IUIS´allergen list: <http://www.allergen.org>, Cromwell 1997) (Table 1).

Table 1. Major plant food allergens*

Common name	Species	Allergen	Synonym/function
Apple	<i>Malus domestica</i>	Mal d 1	pathogenesis-related protein
Avocado	<i>Persea americana</i>	Pers a 1	endochitinase
Celery	<i>Apium graveolens</i>	Api g 1	pathogenesis-related protein
Barley	<i>Hordeum vulgare</i>	Hor v 1	α -amylase inhibitor
Brazil nut	<i>Bertholletia excelsa</i>	Ber m 1	2S albumin
English walnut	<i>Juglans regia</i>	Jug r 1	2S albumin
Mustard	<i>Sinapis alba</i>	Sin a 1 (yellow)	2S albumin
	<i>Brassica juncea</i>	Bra j 1 (oriental)	
Peanut	<i>Arachis hypogea</i>	Ara h 1	vincilin
		Ara h 2	conglycinin
		Ara h 3	parvalbumin
Rice	<i>Oryza sativa</i>	Ory s 1	α -amylase inhibitor
Soybean	<i>Glycine max</i>	Gly m 1	conglycinin

*from allergen data based on the Sampson (1999) and WHO/IUIS list of allergens

Most of the known plant food allergens have been shown to be storage proteins (soybean, peanuts) and proteinase inhibitors (wheat, rice, soybean) that are present in large amounts in these foods (Lehrer et al. 1996). In addition, a number of pathogenesis-related (PR) proteins occurring in fresh fruits and vegetables have been reported to function as food allergens (Breiteneder and Ebner 2000).

1.2 Immunoglobulin E (IgE)

Prausnitz and Küstner (1921) were the first to demonstrate a factor in the serum of allergic individuals, which was directly involved in the allergic reactions. Much later, Ishizaka and Ishizaka (1967), and Johansson together with Bennich (1967) identified the factor inducing the allergic reactions as a novel immunoglobulin E (IgE). IgE has originally been thought to have evolved as a defence molecule against parasitic infections. Nowadays, however, high serum IgE levels are primarily considered as a marker for an atopic disease (Kay 1997).

IgE is one of the five classes (IgA, IgD, IgE, IgG, IgM) of immunoglobulins consisting of two identical light-chains (κ or λ) and two identical ϵ heavy-chains folded into constant (C) and variable (V) domains. Both the heavy- and the light-chain V-domains and the IgE light-chains are conducted from the same gene segments as the other immunoglobulin molecules. The heavy-chain C-domains instead are encoded by the ϵ gene located in the heavy-chain gene cluster, and the production of IgE is a result of isotype switching (Sutton and Gould 1993, Mekori 1996).

The IgE molecule shares the overall Y-shaped structure of the other classes of immunoglobulins, but it contains an additional C-chain domain (C ϵ 2). Both the C ϵ 2 and the IgE-receptor binding site are located in the C-region fragment (Fc) of the molecule. The antigen-binding specificity of all the five classes of immunoglobulin molecules is determined by special segments in the V-chain domains referred to as complementary determining regions (Sutton and Gould, 1993, Mekori 1996).

1.3 Synthesis of IgE

For B-lymphocytes to differentiate into IgE-producing plasma cells the B-cell receptor on the cell surfaces must recognize specific B-cell epitopes on the antigen surface (Huby et al.

2000). Furthermore, the isotype switching for the IgE-secreting B-cells requires help from T-lymphocytes (Vercelli and Geha 1992, Corry and Kheradmand 1999). T-cells, on the other hand, are matured into helper (Th) and cytotoxic (Tc) T-cells in the thymus. The circulating T-cells are further induced to differentiate into subsets of Th1/Th2- and Tc1/Tc2 -cells and distinguished from one another by the cluster of differentiation (CD) markers (CD4+/CD8+) (Alam 1998, Kemeny 1998, Corry and Kheradmand 1999).

IgE response has traditionally been dominated by Th2/CD4+-cells, and the role of Tc/CD8+-cells has instead been associated with regulation and inhibition of the IgE response. However, recent studies have shown that in some cases, *e.g.*, in chronic asthma, Tc2-subtypes are also involved in inducing the IgE response (Kemeny 1998).

T-cells recognize antigens as short peptide fragments through the specific cell-surface T-cell antigen receptors (TCRs). The fragments are bound to the self-major histocompatibility complex (MHC I/II) molecules on the surface of antigen presenting cells (APCs). Especially MHC II, also known as human leukocyte antigen (HLA II), is responsible for the presentation of antigenic fragments to the Th2-cells. In addition to HLA II-TCR interaction, for the activation of T-cells it is crucial that they receive multiple signals from what are termed accessory molecules. Most important among the signals is, however, the “second signal” delivered through CD80/86 - CD28/CTLA-4 (cytolytic T-lymphocyte-associated antigen-4). Without this “confirming activation signal,” T-cells become unresponsive to antigen stimulation (Umetsu and DeKruyff 1997, Alam 1998).

Conditions that favor Th2-recognition between Th2 and antigen-specific B-cells result in the release of cytokines in particular IL-4, IL-5, and IL-13 from Th2-cells. Concomitantly, the Th2/CD4+/CD45RA+ cells turn into activated memory Th2/CD4+/CD45RO+ cells (Alam 1998). The antigen recognition in turn leads to B-cell proliferation and differentiation into plasma cells, and in the end production of allergen-specific circulating IgE antibodies (Umetsu and DeKruyff, 1997, Corry and Kheradmand, 1999) (Fig 1).

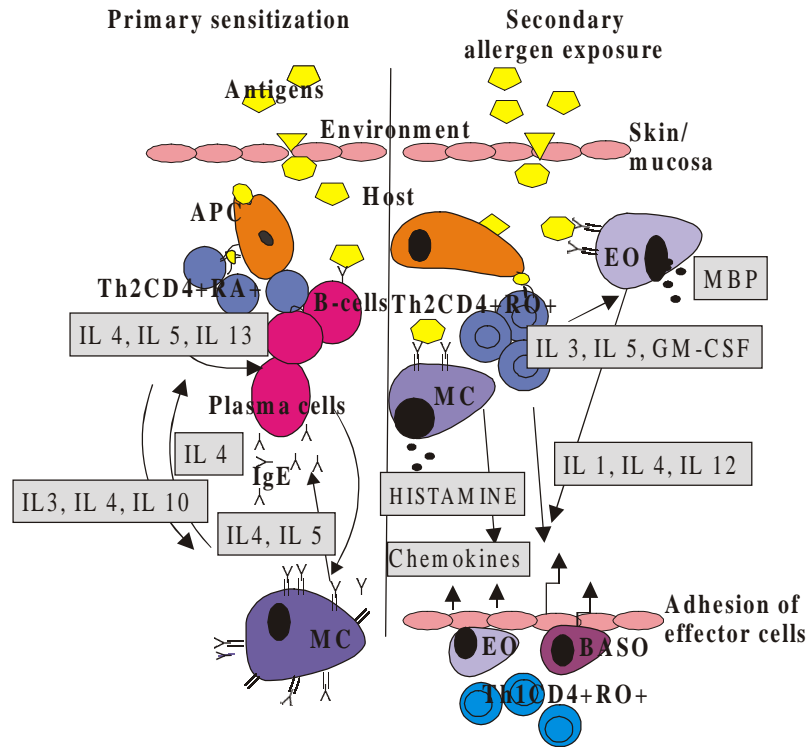


Figure 1. Simplified scheme of basic immunopathogenic steps in development and expression of IgE-mediated hypersensitivity reactions, modified from Umetsu and Dekruyff (1997), and Galli (2000). Antigen presenting cells (APCs), mast cells, (MC), basophils (BASO), eosinophils (EO), major basic protein (MBP), granulocyte-macrophage colony-stimulating factor (GM-CSF).

1.4 Mechanisms of IgE-mediated reactions

IgE-mediated allergic reactions typically begin with an immediate phase response lasting up to 30 min. Upon antigen challenge, the immediate-phase responses may be followed (4-6 hours later) by a late-phase response (Umetsu and DeKruyff 1997), and the more prolonged allergic responses can lead to chronic inflammation (Umetsu and DeKruyff 1997, Tsicopoulos et al. 1999, Galli 2000).

IgE is central in eliciting the immediate-phase responses through its binding to high-affinity receptors (FcεRI) on the surface of the effector cells, such as mast cells (MCs), Langerhans cells, and eosinophils (Corry and Kheradmand 1999, Wollenberg and Bieber 2000). Binding of a multivalent antigen to FcεRI-bound IgE cross-links the receptor molecules on the effector cell membrane and leads to the cell activation. The cross-linking of FcεRIs results in signal cascades that resemble those observed in T-cell activation (Mekori 1996, Reischl et al. 1999). The activation triggers degranulation of the tissue-

specific MCs, thus releasing histamine and other biochemical mediators which attract and activate other inflammatory cells responsible for the clinical manifestations of allergic diseases (Sutton and Gould 1993, Tsicopoulos et al 1999).

Another type of receptor involved in the allergic reactions, the low-affinity IgE receptor (FcεRII), was characterized as a marker of activated B-cells and named CD23. It was later found also on various inflammatory cells, T-cells, follicular dendritic cells (FDCs), and Langerhans cells induced by interleukin-4 (IL-4). In addition, CD23 has several roles in IgE-dependent antigen presentation to T-cells and in adhesion of B-cells to each other, and it may also be involved in homing of B-cells to lymphatic organs (Sutton and Gould 1993, Sayers and Helm, 1999) (Fig 1).

2. Atopy

2.1. Definition and genetic background

The term “atopy” (atopos = unusual, strange) was first coined by Coca and Cooke (1923) to describe a skin disorder associated with a familial tendency toward both hay fever and asthma (Charlesworth 1998). In general, atopy refers to a hereditary predisposition to produce large amounts of IgE antibodies to common environmental allergens (Wahn 2000). Several candidate genes have been identified and suggested to associate with asthma and atopy. In addition, association between IgE-responsiveness to specific allergens and the HLA II-subtypes has been surveyed in several studies (Kay 1997). However, all the findings still suggest that mixed combinations of several genes are involved in the development of atopy (Galli 2000, Wahn 2000).

2.2 Atopic diseases

In the industrialized western world, about 15 to 20% suffer from atopic diseases, *e.g.*, atopic dermatitis (AD) (also known as atopic eczema), allergic rhinitis, and/or asthma (Mygind et al. 1996a). Symptoms of these atopic diseases typically appear early in life, showing a tendency for remission with age (Wahn 2000). The manifestations of atopic disorders are often a mixed picture, involving several target organs such as gut, skin, and lungs (Sampson 1989, Metcalfe 1998). Furthermore, at cellular level, the pathogenesis of atopic diseases involves a number of specific cell types ranging from cutaneous

Langerhans cells (Sampson 1989, Wollenberg and Bieber 2000) and skin-associated CLA-expressing T-cells (Abernathy-Carver et al. 1995) to gut-associated lymphoid tissue (GALT) (Strobel and Mowat 1998, MacDonald 1998, Sampson 1999).

Most children with AD develop allergic rhinitis and/or asthma, and approximately one-third experience food-related hypersensitivity reactions (Myding et al. 1996a) of which the role of IgE-mediated food allergy has been disputed for decades (Sicherer and Sampson 1999).

3. General characteristics of plant food allergens

3.1 Stability of plant food allergens

Walzer (1927) suggested, based on the Prausnitz-Küstner tests, that the ability of food allergens to reach the intestinal mucosa is prerequisite to allergenicity. Because of this, the stability of an allergen has become an important parameter in distinguishing food allergens from “nonallergens” (Astwood et al. 1996). Most known plant food allergens, such as allergens in soybeans and peanuts, are stable molecules that resist the effects of food processing and digestive processes, and remain immunologically active through the intestinal tract (Kimber et al. 1999). In regard to the effects of the digestive processes, it has been questioned whether antigens can be altered, resulting in the formation of neo-epitopes that can elicit allergic reactions (Lehrer et al. 1997).

3.2 Structure and cross-reactivity of allergens

Why some proteins are allergens is not known. One survey has implied that allergens tend to be ovoid in shape, and others have suggested various structural motifs as being important for allergenicity (Huby et al. 2000). The protein structure of an allergen can be described at different levels known as primary (amino acid sequence), secondary, tertiary, and quaternary structures (Creighton 1997). However, from the immunological point of view, most important are the structures defined as epitopes (also known as antigenic determinants), through which allergens are recognized by the T- and B-cells. Those that interact with T-cells are called T-cell epitopes, and the ones that interact with antibodies or B-cells are called B-cell epitopes. In general, B-cell epitopes are considered to be

discontinuous, conformational epitopes, and T-cell epitopes “linear” continuous epitopes (Lehrer et al. 1996). The term "cross-reactive B-cell epitope" is used when the antibodies are able to recognize similar structures in at least two different antigens. This may occur, for instance, between botanically related species or between conserved proteins having sequence similarity. On the other hand, the clinical significance of the cross-reacting allergens typically seen in connection with *in vitro* tests is, however, not very clear and remains to be assessed (Lehrer et al. 1996).

3.3 Carbohydrate determinants

The presence of IgE antibodies against cross-reactive carbohydrate determinants (CCDs) was first reported in the early 1980s (Aalberse et al. 1981). Most studies concerning CCDs have dealt with the cross-reactivity between grass and/or tree pollens and plant food allergens (Aalberse and van Ree 1997), but more recently also with natural rubber latex (NRL) (Fuchs et al. 1997, Levy et al. 2000).

The CCDs have been suggested to be responsible for the false-positive IgE responses in *in vitro* tests, *e.g.*, in solid-phase radioallergosorbent tests (RASTs). For example, patients allergic to pollen demonstrated IgE-reactivity with vegetable foods *in vitro*, but were reported to be free of clinical food allergy (van Ree 1999). Thus, IgE-binding to CCDs between structurally unrelated and glycosylated allergens have become an important issue. Oligosaccharides are added to many proteins during or shortly after their synthesis in the endoplasmic reticulum. Glycosylation involves the covalent attachment of oligosaccharides either to asparagine (N-linked) or to serine/threonine (O-linked) amino acid residues within the primary amino acid sequence (Huby et al 2000).

Typical plant N-glycans are of the high-mannose type (ranging from Man₉GlcNAc₂ to Man₅GlcNAc₂), complex type, paucimannosidic type, and/or hybrid type (Lerouge et al 1998, van Ree et al 2000) (Fig 2). It is important to note that the $\alpha(1,3)$ -fucosylation and $\beta(1,2)$ -xylosylation of N-glycan core structures are typical for plant-glycoproteins but are not found in mammals. In addition, both the $\alpha(1,3)$ -fucose and $\beta(1,2)$ -xylose units have been found partly responsible for the immunogenicity of plant-glycoproteins in mammals (Garcia-Casado et al. 1996, Wilson and Altmann 1998).

In a recent study, van Ree et al. (2000) determined the primary structures of two major pollen allergens and a major peanut allergen. Both the major olive tree (*Olea europaea*) pollen allergen, Ole e 1, and the peanut (*Arachia hypogea*) allergen, Ara h 1, were found to

carry one high-mannose N-glycan and one major complex type N-glycan. In contrast, the major rye grass (*Lolium perenne*) pollen allergen, Lol p 11, was N-glycosylated exclusively by $\alpha(1,3)$ -fucose and/or $\beta(1,2)$ -xylose-containing N-glycans (Fig. 2).

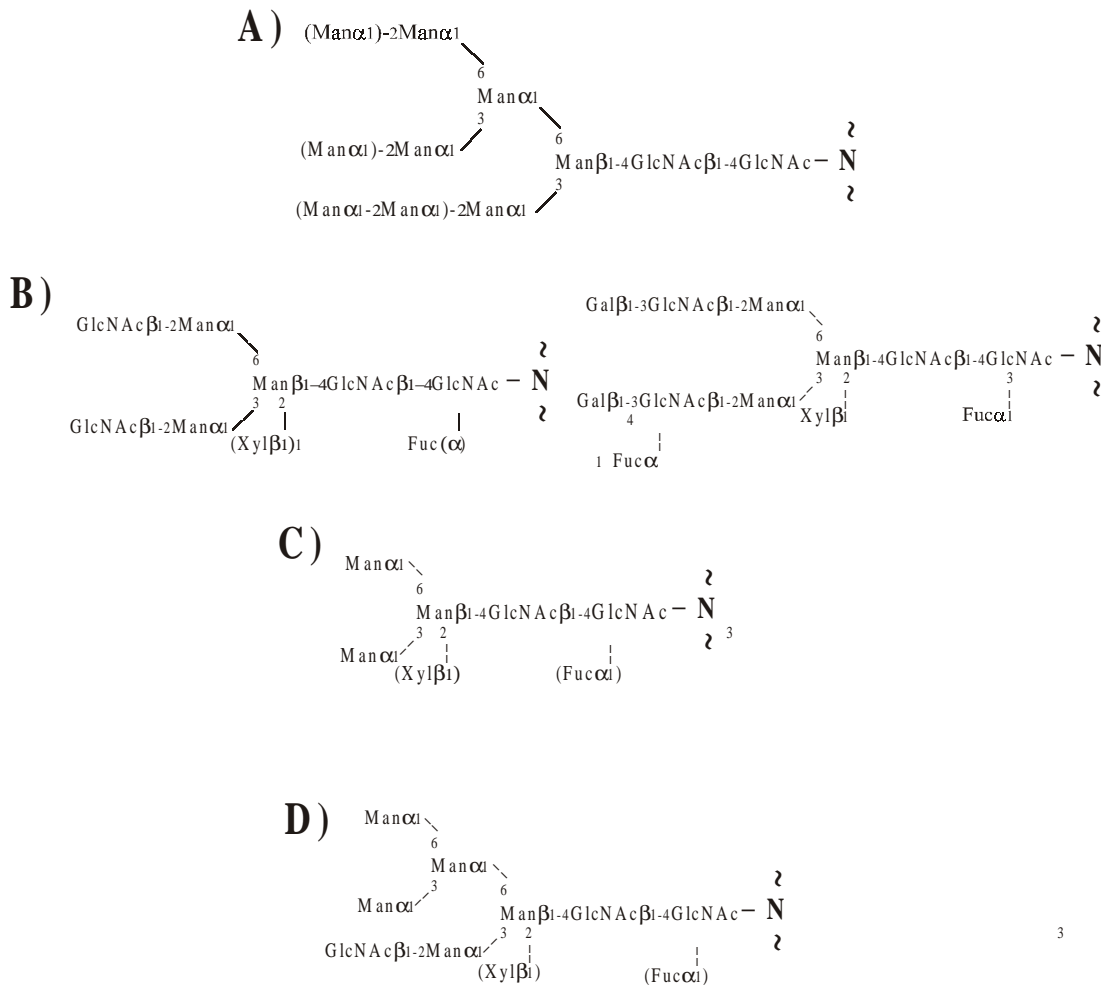


Figure 2. The four typical plant N-glycan structures: A) high-mannose type, B) complex type(s), C) paucimannosidic type, and D) hybrid type (Lerouge et al 1998). Mannose (Man), Xylose, (Xyl), N-acetylglucosamine (GlcNAc), Fucose (Fuc), N-acetylgalactosamine (GalNAc), galactose (Gal). Units of the N-glycan structures which may occur are in parentheses.

To elucidate the structural basis for the IgE recognition of plant N-glycans, van Ree et al. (2000) used RAST analysis with protease digests of these three allergens and a panel of glycoproteins with known N-glycan structures. The authors were able to confirm that both $\alpha(1,3)$ -fucose and $\beta(1,2)$ -xylose are necessary for IgE-binding, although the IgE-binding epitope is not dependent on the CCDs only (Garcia-Casado et al. 1996, Wilson and Altmann 1998, van Ree et al. 2000).

Interestingly, van Die et al. (1999) reported that parasitic helminths (*Haemonchus contortus*) synthesize N-glycans carrying core $\alpha(1,3)$ -fucosylation and $(\beta 1,2)$ -xylosylation similar to those of plant N-glycans. In that study, core $\alpha(1,3)$ -fucosylated N-glycans bound a substantial part of the parasite-specific IgE in serum of *H. contortus*-infected sheep. Based on their results, the authors suggested that these naturally occurring conserved and originally parasitic IgE epitopes could partly explain the IgE-reactivity to these types of glycan structures in the process of sensitization to plant allergens.

3.4 Recombinant allergens

In recent years, cDNA clones have been isolated corresponding to several important inhalant, food, and NRL allergens (Lehrer et al. 1996, Yip et al. 2000). Molecular cloning and expression of allergens have been thought to provide means for precise definition of the allergens, including their primary amino-acid sequences and their potential to produce large quantities of clonally pure or modified allergens (Thomas 1997). Molecular sequencing of allergens has, however, revealed that most allergens show minor or major polymorphisms that can prove important, for example in the development of immunotherapy (Thomas 1997) and in clinical diagnostics (Platts-Mills 1999, Breiteneder and Ebner 2000, Yip et al. 2000).

Traditionally, the expression of recombinant allergens has been carried out in microbial (*Escherichia coli*) and/or yeast (*Pichia pastoris*) systems (Thomas 1997). However, these systems are not able to mimic such processes as N-glycosylation of plant proteins (Huby et al. 2000). Correct glycosylation of allergens has been shown to be important for such processes as the stabilized folding of polypeptide chains (Huby et al. 2000), in addition to protecting these molecules from the actions of proteases (Cerriotti et al. 1998, Lerouge et al. 1998). Incorrect folding may in turn lead to a lack of immunological identity between the sensitizing allergen (native, glycosylated protein) and the recombinant allergen, which then is more likely to display only limited cross-reactivity (Bush and Helge 1996, Soldatova et al. 1998, Huby et al. 2000). However, production of foreign proteins with plants used as bioreactors has become an alternative production system for recombinant proteins (Sonnewald et al. 1990, Lerouge et al. 1998, Herbers and Sonnewald 1999), including plant allergens.

4. Hypersensitivity to potato

4.1 History of hypersensitivity reactions to potato

Sensitivity to potato was studied for the first time in 1966 as an occupational hazard for housewives (Pearson 1966). A year later, Nater and Zwartz (1967) demonstrated that hypersensitivity reactions to raw potato were caused by proteins in the potato sap. Using both heated and unheated potato juice in skin testing, the authors showed that the protein(s) causing the allergic symptoms were heat-labile. Larkö et al. (1983) and Quirce et al. (1989) later confirmed these results. Recently, a case report of Jeannet-Peter et al. (1999) described a middle-aged female patient in whom facial dermatitis developed after handling of raw potatoes.

Along with the studies on IgE-mediated hypersensitivity against potato proteins, Zock et al. (1996) studied airborne dust antigen exposure and specific IgG responses in 131 potato-processing workers. The authors were able to detect specific IgG₄ antibodies against heat-labile molecules in potato extract, but the clinical data suggested that the IgG₄ response probably had no relevance to the occurrence of the respiratory symptoms.

The published history of hypersensitivity to potato in children is short and ambiguous. Castells et al. (1986) were the first to describe the potato as an allergenic food. In that study, the authors described a child who developed anaphylactic symptoms to cooked potatoes after eating them for the first time at 5 months of age. The following year, Hannuksela (1987) reported that cooked potatoes were able to elicit atopic eczema in infants under one year of age, whereas older children appeared to be asymptomatic. Wahl et al. (1990) investigated sera of 12 children (mean age 6.5 years) with suspected IgE-mediated hypersensitivity reactions to potato. Using Western blot analysis, the authors were able to detect specific IgE-binding to potato proteins ranging from 16 to 65 kDa in size. More recently, Delgado et al. (1996) described a 4-year-old boy who exhibited symptoms after contact with raw potato pulp but was able to eat cooked potatoes without symptoms (Table 2).

Table 2. Clinical hypersensitivity to raw and cooked potato

Author(s)	Year	Patients Adults/Children	Symptoms	Potato Raw/Cooked	Diagnostic methods
Pearson	1966	Adults	Rhinitis, asthma	Raw+cooked	Prausnitz-Kustner test
Nater and Zwartz	1967	Adult	Rhinitis, contact urticaria	Raw	Scratch/intradermal, Prausnitz-Kustner test
Larkö et al.	1983	Adult	Rhinitis, contact urticaria	Raw	Prausnitz-Kustner test
Castells et al.	1986	Child	Anaphylaxis, asthma, angioedema	Cooked	SPT, RAST, RAST-inhibition histamine release assay, immunoblotting
Hannuksela	1987	Children	Atopic eczema	Cooked	Scratch chamber test, oral potato-challenge
Quirce et al.	1989	Adults	Rhinitis, asthma, contact urticaria	Raw	RAST, histamine release test, bronchial provocation test
Wahl et al.	1990	Children	Urticaria, gastrointestinal	Cooked/raw	RAST, RAST-inhibition, histamine- release test , immunoblotting
Zock et al.	1996	Adults	Rhinitis	Raw+cooked	SPT,IgG1/IgG4
Delgado et al.	1996	Child	Contact urticaria, erythema	Raw	SPT atopy patch test
Jeannet-Peter et al.	1999	Adult	Contact urticaria, facial dermatitis, rhinitis, asthma	Raw	SPT, atopy patch test

4.2 Hypersensitivity to potato in association with pollinosis

In 1977 Hannuksela and Lahti demonstrated that 23% of their patients allergic to birch (*Betula verrucosa*) pollen had positive skin prick tests (SPTs) to raw potato. The authors also reported that positive SPTs to fruits and vegetables were common in atopic patients allergic to birch pollen. Furthermore, the results of an inquiry they made into 152 subjects suffering from birch pollen allergy revealed that peeling of potatoes caused symptoms in 13% of the cases. Andersen and Løwenstein (1978) and Eriksson et al. (1982) confirmed these results, and a few years later, Eriksson described associations between pollen-related hypersensitivity to various fruits and vegetables, establishing the "clusters" of hypersensitivity (Eriksson 1984).

In recent years, in patients suffering from birch pollen allergy, protein homologues of the birch (*Betula verrucosa*) pollen allergens Bet v 1 (Breiteneder et al. 1989) and Bet v 2 (Valenta et al. 1991) in fruits and vegetables have been suggested to be responsible for their hypersensitivity to potato (Ebner et al. 1995, Breiteneder and Ebner 2000).

Løwenstein and Eriksson (1983) demonstrated cross-reactivity between potato and grass pollen allergens, in which, by RAST, potato and apple extracts inhibited IgE-binding to grass pollen. Calkhoven et al. (1987) and Bircher et al. (1994) later confirmed these findings. Immunoblotting has revealed IgE-binding to two proteins with molecular masses of 20 and 18 kDa, and immunoblot inhibition has shown that the 20 kDa molecule appears to be responsible for the co-occurring IgE-binding to fruits, whereas the 18 kDa molecule appears to be cross-reactive with grass pollen, potato, and fruits (Calkhoven et al. 1987). Later, the 18-kDa glycoprotein of rye grass (*Lolium perenne*) was identified as a major grass-pollen allergen and named Lol p 11 (Van Ree et al. 1995).

In 1983, Dreborg and Foucard investigated skin sensitivity to apple, carrot, and potato in 174 children (mean age 12 years) of whom 128 suffered from birch and/or grass pollen allergy. Only four of the 128 reported clinical symptoms on contact with raw potato, although between 80 and 85% of the birch pollen-allergic and 19% of the grass pollen-allergic children had positive skin test responses to raw potato. However, those four children were reported to have significantly larger SPT responses to potato than did the children without notable clinical symptoms. Furthermore, a case report of Szépfalusi et al. (1995) suggested that association with birch pollen-related foods was responsible for early sensitization to airborne allergens.

5. NRL allergy in sensitization to plant foods

IgE-mediated reactions to NRL have become a widely recognized medical problem among individuals who are frequently in contact with NRL products, such as surgical gloves, catheters, condoms, balloons, and baby pacifiers (Turjanmaa 1988, Turjanmaa et al. 1996). These individuals include not only adult health care-workers or patients with congenital malformations and histories of multiple surgical operations, but also children (Alenius 1995, Ylitalo 2000). Moreover, the majority, both children and adults, who suffer from clinical NRL allergy are atopics and frequently show positive SPTs to various foods of plant origin (Ylitalo 2000).

Blanco et al. (1994) suggested the term “latex-fruit” syndrome for the simultaneous occurrence of allergic reactions to NRL and various fruits. In the past few years, several studies have demonstrated that “latex-fruit” syndrome is at least partly caused by class I endochitinases or their hevein-like domain, which is cross-reactive with the major NRL allergen, hevein (Hev b 6.02) (Mikkola et al. 1998, Chen et al. 1998, Posch et al. 1999, Diaz-Perales et al. 1998, 1999, Blanco et al. 1999, Sánchez-Monge et al. 1999). However, in contrast to fruits, NRL allergy in relation to birch and grass pollen allergy, as well as co-occurring clinical hypersensitivity to raw vegetables, has not been well documented (Turjanmaa et al. 1996, Fuchs et al. 1997, Bernardini et al. 1998, Nel and Gujuluva, 1998, Levy et al. 2000).

6. Principles in the diagnosis of plant food allergy

The first step in the diagnosis of allergy is to obtain an adequate clinical history. Equally important is to rule out other possible immunological mechanisms that may lead to symptoms similar to IgE-mediated reactions (Sicherer 1999, Ylitalo 2000). Some patients show only mild symptoms which may pass unnoticed or be confused with other allergies (Ylitalo 2000). Moreover, diagnosis of food allergy may be difficult when multiple foods are suspected and the patients show a clinical history of atopic dermatitis (AD). It is also important to note that patients sensitized to NRL frequently show positive SPTs to various foods of plant origin (Ylitalo 2000), which may be due to cross-reactive allergens. Furthermore, children with AD may continue to show positive SPTs to a food, although they have already grown out of a clinical food allergy (Sampson 1999, Sicherer 1999). In conclusion, the diagnosis of IgE-mediated allergies should be based on the combination of

both *in vitro* and *in vivo* allergy tests, preferably using purified natural and/or recombinant allergens in addition to oral food challenges.

6.1 Skin prick test

Skin prick tests are routinely utilized in assessment of allergy in all age-groups (Turjanmaa et al. 1996, Eigenmann and Sampson 1998). Multiple SPTs can be performed rapidly, and standardization of skin prick testing methods (*e.g.*, skin prick and prick-prick methods) has contributed to the increased reliability of this diagnostic procedure (Burks et al. 1998). However, in diagnosis the precision of SPT is limited because of apparent variability in the sensitivity and specificity to different foods (Eigenmann and Sampson 1998, Ylitalo 2000). The result of SPTs is generally recorded as positive when the diameter of the wheal is equal to or greater than 3 mm or than the negative control (Isolauri and Turjanmaa 1996). Although methods for SPTs are standardized, most commercial SPT reagents are not. However, both commercial glycerinated food extracts, (Eigenmann and Sampson 1998) and the prick-prick method (Dreborg and Foucard 1983) to test fresh plant foods, *e.g.*, apple, potato, and tomato can serve in diagnosis of food allergy.

6.2 RAST and other tests measuring specific IgE antibodies

The most common *in vitro* measurement for specific serum IgE is the radioallergosorbent test (RAST). However, RAST has increasingly been replaced by assays, which, for labeling of anti-IgE, use fluorescence or enzymes instead of the traditional γ -emitting isotope (Mygind et al. 1996b). Unlike SPTs, RASTs can be used while the patient is taking anti-histamines, and like SPTs, a negative result is very reliable in ruling out any IgE-mediated reaction to a particular food. Although the RAST method seems to be rather sensitive, a positive RAST may not necessarily imply clinical sensitivity to NRL or to the foods in question (Turjanmaa et al. 1996, Sicherer 1999). A positive RAST may also result from *in vitro* cross-reactivity between members of a plant family or animal species (Aalberse and van Ree 1997, Sicherer 1999).

Other solid-phase techniques comparable with RAST include enzyme-linked immunosorbent assay (ELISA) and a commercial AlaSTAT immunoassay system (Diagnostic Products Corporation, DCP, Los Angeles, CA, USA). In addition, a histamine-release assay is used mainly for research purposes. Immunoblotting and other

immuno-electrophoretic methods like cross-lined immunoelectrophoresis and crossed-radioimmuno-electrophoresis can also be used in research laboratories; these methods are, however, at present primarily used in direct identification of IgE-binding proteins (Alenius 1995, Mäkinen-Kiljunen et al 1992, Mygind et al. 1996b).

6.3 Oral food challenges

The oral food challenges have proven to be the gold standard for the diagnosis of adverse reactions to foods. Although the oral food challenge may be turn out to be laborious, it clearly tells when the diagnosis is correct and when the treatment of food allergy is succeeding (Bock 2000). In addition to the various serological laboratory tests, oral food challenges are an integral part of diagnostic procedures, especially in children, who are likely to lose in time their clinical reactivity to the food(s) in question (Sicherer 1999).

There are three ways to perform oral food challenges: 1) Single-blinded with the food masked and patients unaware whether they are eating the test food or the food under suspicion, 2) Double-blinded in a placebo-controlled food challenge (DBPCFC) where neither the patient nor physician knows which challenges contain the food being tested (Bock et al. 1988), 3) Open and performed partly on the ward and/or in the patients' home. This last type of challenge is best performed when there is little psychological interplay to bias results, *e.g.*, in infants (Mygind et al. 1996c) or when the challenged food has no potential for causing severe allergic symptoms (Sicherer 1999, Bock 2000). In all oral challenges, the food is given in gradually increasing amounts and, during challenges, symptoms are recorded and frequent assessments made of the symptoms affecting the skin, gastrointestinal, and/or respiratory tract. When the patient is suffering from AD, a special severity scoring (SCORAD) of AD can be used before and after oral food challenges to illustrate the severity of late-phase and delayed-type reactions (European Task Force on AD).

7. Allergen purification and characterization

Characterization of both natural and recombinant allergens is based on various immunochemical and biochemical techniques, and ultimately on detailed protein chemistry and molecular biology. Furthermore, it is important to remember that the strategies applied to allergen purification are the same as those for protein purification in general, and

therefore each allergen is purified according to its biochemical properties (Cromwell 1997). The most common methods for characterization of the unknown proteins are amino-terminal sequencing and mass spectrometry. The acquired amino acid sequence is then compared by computer-assisted search to the ones reported for various databases.

The methods commonly used for protein purification and characterization are described in Table 3.

Table 3. Purification and characterization of allergens [⌘]

Method	Principle and example
<u>Electrophoresis/blotting</u>	
SDS-PAGE/electroblotting	Separates proteins based on size /electroblotting onto PVDF membrane for amino-terminal sequencing
Isoelectric focusing	Migration of a biomolecule to isoelectric point in a pH gradient
<u>Chromatography</u>	
Gel filtration	Separates molecules on the basis of their size, desalt/buffer exchange
Anion and cation exchange	Separates molecules on the basis of charged groups; elution with ionic or pH gradients
Chromatofocusing	Separates molecules on the basis of isoelectric point (pI); elution by a polyampholyte buffer system
Hydrophobic interaction	Based on hydrophobic interactions with matrix and surface of the biomolecule
Reversed-phase	Hydrophobicity; elution from hydrophobic stationary phase in water / water-soluble solvent gradient
Affinity	Protein interacts with specific ligand on a column matrix; e.g., purification of recombinant allergens
<u>Amino-terminal sequencing</u>	Edman degradation; identifies the amino-terminal sequence of the protein or peptide
<u>Mass spectrometry</u>	Molecular mass of a protein or its identification by mass-fingerprinting or sequence tagging

AIMS OF THE STUDY

The purpose of the present study was:

1. To identify previously unknown allergens of potato tubers with sera of atopic children having positive SPTs to raw potato
2. To purify these IgE-binding potato proteins, identify and characterize them by partial amino acid sequencing, and to study their allergenicity by *in vitro* and *in vivo* tests
3. To study potential cross-reactivity between patatin of potato tuber (Sol t 1) and patatin-like NRL allergen (Hev b 7) by use of sera of NRL-allergic children and adults
4. To evaluate the significance of potato as an allergenic food in atopic infants in relation to IgE antibodies to Sol t 1

MATERIALS AND METHODS

1. Subjects of the study

1.1 Infants and children with positive SPT to raw potato (I, III, IV)

A total of 39 atopic children (mean age 3 years, range 4 months to 10 years) were studied because of suspicions of allergy to potato. Of these 39 children, 95% had atopic dermatitis in addition to suffering from conditions ranging from asthma and allergic rhinitis to various gastrointestinal symptoms. All of the children, including 12 infants (age < 2 years) (III), had positive SPTs to raw potato. In addition, these children had positive SPTs to several common food allergens, 58% to egg, 67% to cow's milk, and 56% to wheat and other cereals. Ten of the 39 (26%) children had a positive SPT to birch pollen.

Of the 39 sera, 26 had positive and 3 had negative potato RAST; in 10 of the children potato RAST was not performed. Total serum IgE was measured for 87% of the children (mean 508 kU/L, ranging from < 5 to 2324 kU/L).

1.2 NRL-allergic children (II)

Sera came from 35 NRL-allergic children, including seven children with spina bifida or other congenital malformations (mean age 9.5 years, range 1-18 years, mean number of surgical operations 12, range 5-21 operations). All of the children met three diagnostic criteria for NRL allergy: 1) a positive SPT to standardized commercial NRL allergen (Stallergènes SPT reagent, Antony Cedex, France) and/or to a NRL glove extract (Triflex, Baxter, Valencia, CA, USA), 2) a positive latex RAST, and 3) a positive NRL glove (Triflex) -use test. In addition, all the 35 children were prick-prick tested with raw potato and 27/35 with purified potato allergen Sol t 1 (30 and 200 µg/ml) (Ylitalo 2000).

1.3 NRL-allergic adults (II)

Sera were obtained from 35 NRL-allergic adults (mean age 28 years, range 19 to 65 years). Of these 35 patients, 23% were health-care workers. All of the patients showed positive SPTs to a NRL glove extract (Triflex) and a positive RAST and/or a Triflex glove-use test.

Total serum IgE was measured for all of the adult patients (results ranging from 19 to 54 600 kU/L, mean value 4477 kU/L) Of these 35 NRL-allergic patients, 33 had been prick-prick tested for allergy to raw potato, and 73% of them showed a positive response.

1.4 Controls (I-IV)

Control sera for children came from 44 randomly chosen healthy children (range 7-36 months of age) and 10 atopic children with negative SPT to raw potato (I). Sera of 11 atopic children (mean age 2.5 years, range 5 months to 6 years) with negative SPT to potato, but positive SPTs to common food allergens served as controls for NRL-allergic children (II), and atopic infants (III). Control sera for NRL-allergic adults were obtained from 25 atopic adults with negative SPT to raw potato and NRL glove extract but positive SPTs to various inhalant allergens such as grass pollen, animal dander, or house dust mites (II). Control sera from 30 atopic children (mean age 7.8 months, range 2 to 26 months) having negative SPTs to potato, birch pollen (ALK a/s, Hørsholm, Denmark), hazelnut, and commercial NRL extract (Stallergènes SPT reagent) and positive SPTs to cow's milk, egg, and animal dander were used in Study IV.

2. Total serum IgE and RAST (I-IV)

A commercial latex and potato RAST and total serum IgE assay were performed by the immunoCAP method (Pharmacia UpJohn, Uppsala, Sweden). Measurements were performed in the routine clinical laboratory, with RAST levels >0.4 kU/L considered positive (I-IV).

3. Extraction of potato proteins and isolation of NRL C-serum

Potato tubers (*Solanum tuberosum* cv. Bintje) were first peeled and the pulp homogenized in 150 ml 50 mM Tris-Cl, 150 mM NaCl, pH 8.0. The homogenate was filtered through a filter paper (Whatman 3M/M) at 4°C. Potato proteins were precipitated with 60% saturated ammonium sulfate on ice. The precipitate was collected by centrifugation, resolved in 50 mM Tris-Cl, 150 mM NaCl, pH 8.0, and stored at -20°C (I-IV).

The NRL C-serum fraction used in the present study was obtained as a gift from Dr. Esah Yip, Rubber Research Institute of Malaysia, Kuala Lumpur. Fresh NRL extract from the rubber tree, *Hevea brasiliensis*, had been collected in Malaysia, deep-frozen, and stored at -70°C. By ultracentrifugation, the NRL extract was fractionated into three separate (top, central, and bottom) fractions which were then stored at -70°C (Alenius et al 1992). Because the central fraction, called “C-serum,” is known to contain the majority of soluble rubber proteins, this particular fraction was used in Study II to isolate patatin-like NRL allergen (Hev b 7).

4. Identification of IgE-binding proteins from potato and NRL C-serum

Proteins were separated according to their molecular weights by SDS-PAGE in 12 or 15% acrylamide gels by the method of Laemmli (1970) at a constant current of 25 mA/gel, in a Mini Protean II device (Bio Rad Laboratories, Hercules, CA, USA). Electroblothing was carried out at 100 V on a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore Corporation, Bedford, MA, USA) with Mini Trans-blot cells (Bio Rad Laboratories) according to the method of Matsudaira (1988). After electroblotting, the transferred proteins on a blotted membrane were incubated for 45 min in a blocking buffer: 10 mM Tris-Cl, 9% NaCl, pH 7.5 containing 5%-non-fat milk powder.

For immunodetection, the electroblotted PVDF membrane was incubated with patient and control sera (diluted 1:5) in a Miniblotter (Biometra, Göttingen, Germany). IgE-binding was investigated by use of biotinylated epsilon chain-specific anti-human IgE (Vector, Burlingame, CA, USA) together with streptavidin-conjugated alkaline phosphatase (Bio Rad Laboratories). Finally, IgE-binding protein bands were revealed with either color development (Bio Rad Laboratories) (I-III) or a chemiluminescence solution (Immune-StarTM 8*substrate, Bio Rad Laboratories). With the chemiluminescence solution, bound IgE was visualized on film (HyperfilmTMECLTM, Amersham International plc, Buckinghamshire, UK) (IV). Sera from two patients who exhibited strong IgE-binding to potato proteins served as references in immunoblot experiments (I). In Study II, sera of adult patients having IgE-binding to 43 kDa potato protein (patatin of potato tuber) were used to identify 46 kDa patatin-like NRL allergen. Twelve potato-challenged infants were used to identify IgE-binding to a total potato protein extract (III, IV).

5. Purification of IgE-binding allergens

5.1. Purification of the 43 kDa potato allergen (I-IV)

The ammonium sulphate-precipitated potato proteins were subjected to gel filtration on a 1.6x60 cm Superdex 75 Fast Flow column (Pharmacia Biotech, Uppsala, Sweden) in 50 mM Tris-Cl, 75 mM NaCl, pH 8.0, buffer. The flow rate was 2.0 ml/min, and elution was monitored at 214 nm. The chosen fractions from pool I containing the IgE-binding 43 kDa protein were subjected to anion exchange chromatography, which was performed with a Mono Q HR5/5 column (Pharmacia Biotech). The column was equilibrated with 50 mM Tris-Cl, pH 8.0, and elution was performed with a linear gradient of NaCl (0 to 0.5 M in 20 min) in the equilibration buffer. The flow rate was 1.0 ml/min, and chromatography was monitored at 214 nm. Hydrophobic interaction chromatography (HIC) was performed on a 0.5x5.0 cm column packed with Octyl Sepharose 4 Fast Flow (Pharmacia Biotech). The column was equilibrated with 10 mM sodium phosphate, 1.0 M (NH₄)₂SO₄, pH 7.0. The fractions containing the 43 kDa protein were brought to approximately 1.0 M (NH₄)₂SO₄ and applied to the column. The elution was performed with a decreasing linear gradient (1.0 to 0 M (NH₄)₂SO₄ in 20 min) followed by H₂O in the end of the gradient. The flow rate was 1.0 ml/min, and elution was monitored at 280 nm. Reversed-phase chromatography was performed in a 0.5x5.0 cm POROS 20 R2 (PerSeptive Biosystems, Framingham, MA, USA) column and in a 0.21x10 cm TSK TMS 250 (TosoHaas Corporation, Japan) column. Columns were equilibrated with 0.1% trifluoroacetic acid (TFA) and eluted with a linear gradient of acetonitrile (3-100% in 60 min) in 0.1% TFA. The flow rate for the POROS R2 column was 1.0 ml/min and for the TSK TMS 250 column 0.2 ml/min. Elution from both columns was monitored at 214 nm.

5.2 Purification of the 43 kDa patatin-like NRL allergen, (Hev b 7) (II)

Hev b 7 was purified from NRL C-serum. NRL C-serum was diluted to 10 mM sodium phosphate 150 mM NaCl, pH 7.0, gel filtration running buffer, and the sample was placed in a 1.6x60 cm Superdex 75 Fast Flow column (Pharmacia Biotech). The flow rate was 2.0 ml/min, and the chromatography was monitored at 280 nm. Fractions containing the IgE-binding 43 kDa NRL allergen were chosen for hydrophobic interaction chromatography

(HIC). HIC was performed in a 1 ml RESOURCE™ PHE (phenyl) column (Pharmacia Biotech). The column was equilibrated with 10 mM sodium phosphate, 1.5 M (NH₄)₂SO₄, pH 7.0. The chosen fractions from the gel filtration were brought to approximately 1.5 M (NH₄)₂SO₄ and applied to the column. Elution was performed with a decreasing linear gradient of (NH₄)₂SO₄ (1.5–0 M in 20 min). The flow rate was 2.0 ml/min, and elution was monitored at 280 nm. After that, the fractions of interest were pooled and desalted. Anion exchange chromatography of the desalted sample was performed on a Mono Q HR5/5 column (Pharmacia Biotech) equilibrated with 50 mM Tris-HCl, pH 7.0. Elution was performed with a linear gradient of NaCl (0–0.5 M in 20 min) in the equilibration buffer. The flow rate was 1.0 ml/min, and the chromatography was monitored at 214 nm.

5.3 Purification of the 16 to 20 kDa potato allergens (IV)

Gel filtration chromatography on a 1.6 x 60 cm Superdex 75 Fast Flow column (Pharmacia Biotech) in 20 mM sodium phosphate buffer, 150 mM NaCl, pH 6.0, was first used to separate the potato proteins according to molecular size. The flow rate was 2.0 ml/min, and chromatography was monitored at 214 nm. According to SDS-PAGE and immunoblotting analyses, fractions containing < 30 kDa IgE-binding proteins were chosen for further purification steps. 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, pH 6.0. The fractions chosen from the gel filtration chromatography were pooled, brought to 1.5 M (NH₄)₂SO₄, and applied to the column. Elution from the column 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 in the end of the gradient. Flow rate was 0.5 ml/min, and elution was monitored at 214 nm. Cation-exchange chromatography was then performed in a Mono S HR5/5 column (Pharmacia Biotech) which was equilibrated with 50 mM sodium acetate buffer, pH 3.8. Fractions chosen from HIC were pooled, the buffer changed to 50 mM sodium acetate buffer pH 3.8, with pressure microfiltration (3K Omegacell™, Filtron Technology Corporation, Northborough, MA, USA), and the sample was applied to the column. Elution from the cation-exchange column was performed with a linear gradient of NaCl (0 to 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 chromatography was performed in a 0.21 x 10 cm TSK TMS 250 (TosoHaas Corporation, Japan) and/or 0.21 x 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% to 100% in 60 min or 30% to 100% in 120 min) in 0.1% TFA. In both columns the flow rate was 0.2 ml/min and the chromatography monitored at 214 nm.

6. Protein quantification and staining

Reversed-phase chromatography was used to estimate the quantity of the purified proteins for ELISA and SPT assays. Quantification was performed either in a 0.21 x 10 cm TSK TMS 250 (TosoHaas Corporation, Japan) and/or 0.21 x 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% to 100% in 60 min or 30% to 100% in 120 min) in 0.1% TFA. In both columns, the flow rate was 0.2 ml/min and the chromatography monitored at 214 nm. The protein peaks observed were integrated, and the protein amounts calculated by comparison of the peak areas obtained to those areas obtained with known amounts of bovine serum albumin and ovalbumin (I-IV).

Proteins were stained either with Coomassie Brilliant Blue (CBB) (I-IV), by the silver staining method (I, II, IV) of O'Connell et al. (1997) or with SYPRO Orange (II) (Bio Rad Laboratories). Glycoproteins were visualized with PAS (periodic acid-Schiff)-staining, with 5 µg of transferrin as a positive control (II, IV). For amino terminal sequencing from the PVDF membrane, protein bands were visualized with CCB (0.1% PhastGel Blue R-250 in 1% acetic acid/40% MeOH).

7. Identification of purified allergens

7.1 Amino-terminal sequencing

For amino-terminal sequencing, proteins were transferred to a ProBlott (Applied Biosystems, Perkin Elmer, Foster City, CA, USA) PVDF membrane (Matsudaira 1998) or adsorbed onto a ProSorbTM membrane (Applied Biosystems, Perkin Elmer). Amino-terminal sequencing of the proteins was performed with an Applied Biosystems 494 A

ProCise™ sequencer (Applied Biosystems, Perkin Elmer). The N-terminal sequences obtained were compared by computer-assisted search against known databases (I-IV).

7.2 Mass spectrometric analysis

The molecular masses of the proteins were determined by mass spectrometry using either matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) with a Biflex II instrument (Bruker-Franzen Analytik, Bremen, Germany) (I) or a quadrupole-time of flight (Q-TOF) instrument (Micromass Co., Manchester, UK) (IV). When the MALDI-TOF MS was used to determine the molecular weights, sinapic acid (saturated solution in 33% ACN in 0.1% TFA) was used as the matrix and bovine serum albumin as an external standard. Electrospray MS with the Q-TOF instrument was performed using reversed phase purified proteins and was 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 fingerprinting analysis of the protein digest was performed by the MALDI-TOF MS instrument. Angiotensin II and ACTH (adenocorticotrophic hormone) fragment 18-39 (Sigma, A 0673, St Louis, MO, USA) were included in the peptide samples as internal standards. The peptide data obtained was then compared by computer-assisted search against known databases (II, IV).

7.3 Digestion of the purified proteins and separation of the peptides

For peptide mass fingerprinting or internal sequencing, the protein to be identified was reduced and alkylated and digested with modified trypsin (Promega Corporation, Madison, WI, USA) or endoproteinase Lys-C by the methods described by Saarinen et al.(1999) and Nyman et al. (1998). The protein digest was then desalted by microtip reversed phase chromatography for mass fingerprinting analyses (Saarinen et al. 1999) (II, IV). The peptide mixture was separated on a 1.0 mm x 150 mm Vydac C8 column (MIC-15-05-C8, LC-Packings, Amsterdam, The Netherlands) with a linear gradient of acetonitrile (0-40% in 120 min) in 0.1% TFA. The chromatography was performed at a flow rate of 50µl/min and monitored at 214 nm. Separated peptides were automatically collected (SMART™ Systems, Pharmacia Biotech) (II).

8. Occurrence of IgE antibodies to purified allergens

8.1 IgE-ELISA to potato and NRL-allergens

For ELISA studies, the concentrations of the purified proteins were adjusted to 1.5 to 2.0 µg/ml in 50 mM sodium carbonate buffer, pH 9.6. The samples were applied on 96-well polystyrene microtiter plates (100 µl/well) (Nunc, Roskilde, Denmark) and incubated for 3 hours at room temperature and then overnight at 4°C. The wells were then post-coated for 1 h with 1% human serum albumin (HSA) in 100 µl of 50 mM carbonate buffer, pH 9.6. The patient and control sera were diluted 1:10 with phosphate-buffered saline (PBS) containing 0.05% Tween (T), and 0.2% human serum albumin (HSA) was added to the wells and incubated for 2 h at room temperature. After each step, the wells were washed three times with PBS-T. Biotinylated goat epsilon chain-specific anti-human IgE (Vector) diluted 1:1000 with PBS-T-HSA was added and incubated for 1 h on the plates. Wells were again washed three times with PBS-T and incubated for 1 h with streptavidin-conjugated alkaline phosphatase (Bio-Rad Laboratories) diluted 1:3000 with PBS-T-HSA. After three washes with PBS-T, the substrate development solution, containing para-nitrophenyl phosphate (104 Sigma, St Louis) 1 mg/ml in 50 mM carbonate buffer, pH 9.8, was added, and the developed color read at 405 nm wavelength with an ELISA-reader (Titertek Multiskan, Eflab, Espoo, Finland) (I-IV).

8.2 ELISA inhibition

For competitive ELISA inhibition studies, the coating concentration of the potato protein extract was adjusted for ~50 µg/ml (I, III-IV) and for Sol t 1 and Hev b 7 (II) 2.0 µg/ml. Individual patient or pooled sera (diluted 1:20) were first incubated for 1 hour at room temperature with an equal volume of the inhibitor solution. The mixtures consisting of the sera and the inhibitor (or sera without inhibitor as a positive control) were incubated in the antigen-coated microtiter wells (Nunc, Roskilde, Denmark) for 2 hours at room temperature. After this, the assay was continued as described for IgE-ELISA.

In the first study (I), the serum of a reference patient and pooled sera from 6 other patients were chosen for inhibition studies. Competitive ELISA inhibition was performed against

potato protein extract, and the final concentrations of the purified 43 kDa potato protein used for inhibition ranged from 10 to 0.01 µg/ml.

In the second study (II), two pools were made from the sera of 10 NRL-allergic children and of 10 NRL-allergic adults. In addition, the serum of one patient having high IgE binding against the 43 kDa NRL-allergen Hev b 7 served as a reference. The final concentrations of the purified proteins Sol t 1 and Hev b 7 in crosswise inhibition experiments ranged from 280 to 0.1 µg/ml.

In the third study (III), ELISA inhibition was performed with sera of four infants showing a positive oral challenge response to cooked potato. Sol t 1 was used as an inhibitor, at concentrations ranging from 200 to 0.001 µg/ml against potato protein extract.

In the fourth study (IV), the final concentrations of the purified potato allergens (Sol t 2-4) against potato protein extract ranged from 200 to 0.001 µg/ml. Pooled sera came from 10 children previously shown to have high levels of IgE antibodies in ELISA against these purified proteins.

9. *In vivo* tests

9.1 SPT to raw potato and NRL

All SPTs were performed on the forearm with a commercial one-peak lancet (ALK a/s) as described by Turjanmaa (1988). Potato SPTs were performed by a prick-prick method with raw potato (I-IV). SPTs to NRL were done with standardized commercial NRL allergen (Stallergènes SPT reagent, Antony, Cedex, France) and/or with a NRL glove extract (Triflex) prepared as described by Turjanmaa (1988) (II, III). SPTs with purified potato allergens (diluted in PBS) were performed in concentrations ranging from 20 to 200 µg/ml (I-IV). Histamine dihydrochloride (10 mg/ml ALK a/s) served as a positive control and physiological saline as a negative control. The mean diameter was measured after 15 min, and a wheal half the size of that of histamine and at least 3 mm was considered positive (I-IV).

9.2 Challenge tests to cooked and raw potato (III)

At this examination, potato had been eliminated from the patient's diet for a mean of 8.5 months (range 2-18 months). The open potato challenge was started by rubbing both raw and cooked potato (*Solanum tuberosum* CV Bintje) for about half a minute on the volar aspect of the forearm; any skin reaction was sought for 20 min. An oral potato challenge was performed with cooked potato by giving increasing servings at 30-min intervals until one medium-size potato was consumed. If no reaction occurred within 4 hours, the challenge was continued at home with the consumption of one to two cooked potatoes a day. If any abnormal reaction developed, the child was brought back to the hospital, and if the reactions were considered to be due to potatoes, the challenge was discontinued. If no reaction occurred, the final examination was performed on day 7. Seven of the children exhibiting positive oral challenge responses to potato were called for a follow-up visit after spending 6 months on a potato-free diet.

The cutaneous potato challenge response was considered positive if at least two to three wheals were visible on the exposed skin. An immediate oral challenge response was regarded as positive if symptoms such as generalized urticaria or acute gastro-intestinal symptoms appeared within the first 2 hours. The severity of atopic dermatitis was scored (SCORAD) at the beginning and at the end of the oral challenge. A delayed oral challenge response was considered positive if the score increased >50%.

10. Ethics

Examination of the children and adults with SPTs, open oral challenges, and blood sampling (I-IV) were approved by the Ethics Committee of Tampere University Hospital. Informed consent was obtained from the adults and parents and/or their children.

RESULTS

1. Purification and identification of IgE-binding potato proteins and patatin-like NRL allergen Hev b 7 (I, II, III, IV)

1.1 Purification and identification of patatin of potato tuber (Sol t 1) (I-IV)

First, immunoblotting with patient sera was used to detect the IgE-binding proteins from potato extract. IgE-binding to potato protein extract was observed to vary widely between individuals, the apparent molecular weight of the target proteins ranging from 16 to 70 kDa bands. However, the most frequent reaction was to a band with an apparent molecular weight of 43 kDa. In the first study (I), sera of two patients were used as references in immunoblotting to follow the purification step procedure of the 43 kDa protein. Purification was performed by the least denaturing methods in order to retain the native structure of the 43 kDa protein. The methods used in Study I ranged from size exclusion to hydrophobic interaction chromatography as depicted in Figure 1. Although the IgE binding was observed to follow the 43 kDa band in immunoblotting, based on the gel filtration chromatography (exclusion limit of the column 100 000 kDa) results, the early eluting IgE-binding protein seemed to be a dimer ~80 kDa. Amino-terminal sequencing of the purified 43 kDa protein from the PVDF membrane gave a sequence of KLEDMVTVLS IDGGGIKGIIPAIL, which could be identified as the amino terminal sequence of the mature form of patatin of potato tuber (AC. P15476; 96% identity in a 25 aa overlap). The MS analysis of the peak from the reversed-phase chromatography gave an average molecular mass of 40729 Da, compatible with that reported for the multigene family of patatins of potato tuber (Pots et al. 1998). Glycosylation of patatin was demonstrated by 12% SDS-PAGE analysis and the Schiff base reaction involving oxidation of carbohydrates by periodic acid, following staining with Schiff's reagent (PAS) (II).

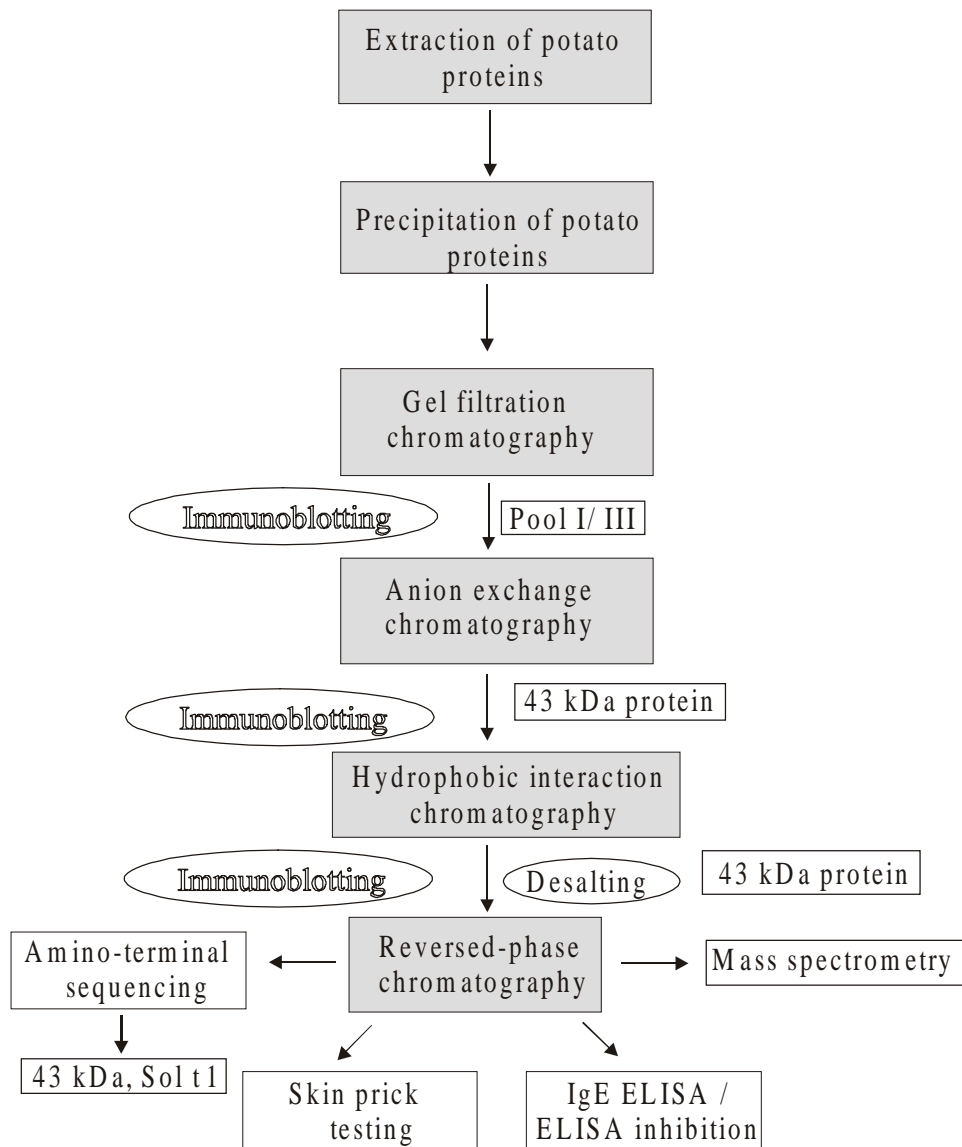


Figure 1. Purification scheme of patatin of potato tuber (Sol t 1)

1.2 Purification and characterization of the 43 kDa patatin-like NRL allergen (Hev b 7) (II)

A patatin-like NRL allergen appearing as a 43 kDa band in immunoblotting was purified from NRL C-serum by gel filtration, HIC, and anion-exchange chromatography (Fig. 2). Amino-terminal sequencing of this 43 kDa-allergen gave no result, suggesting that the amino terminus was blocked. Therefore, the identity of the protein was further investigated by mass fingerprinting and internal sequence analysis. Mass fingerprinting of the 43 kDa NRL-protein after reduction, alkylation, and digestion by endoproteinase Lys-C identified it as Hev b 7 (EMBL AJ223039/ EMBL AJ223038) (8 peptides covering 30% of the protein sequence) (Table 1). The information obtained from the internal sequencing of the four peptides gave 100% sequence identity with Hev b 7 (EMBL AJ223039) with a calculated molecular mass of 42735 kDa. Use of 12% SDS-PAGE analysis and the PAS-staining method tended to show natural Hev b 7 not to be glycosylated.

Table 1. Identification of patatin-like NRL allergen (Hev 7) by mass fingerprinting and comparison of peptides to a database*

Measured Mass (m/z)#	Computed Mass	Matching peptides to Hev b 7 (1-388 aa) (Residues)
847.53	846.45	78- 84
1130.71	1129.68	242- 252
1233.57	1232.55	88- 96
1417.81	1416.79	150-162
1560.91	1559.85	136-149
1590.92 ^a	1589.86	136-149
2851.44	2850.49	347- 373
2871.43 ^a	2870.48	347- 373
3147.51	3146.57	168- 196

* NCBI's nr database/ ProFound,

#[M+H]⁺, monoisotopic,

aa= amino acid,

^a = matching sequence to an isoform (AJ223038)

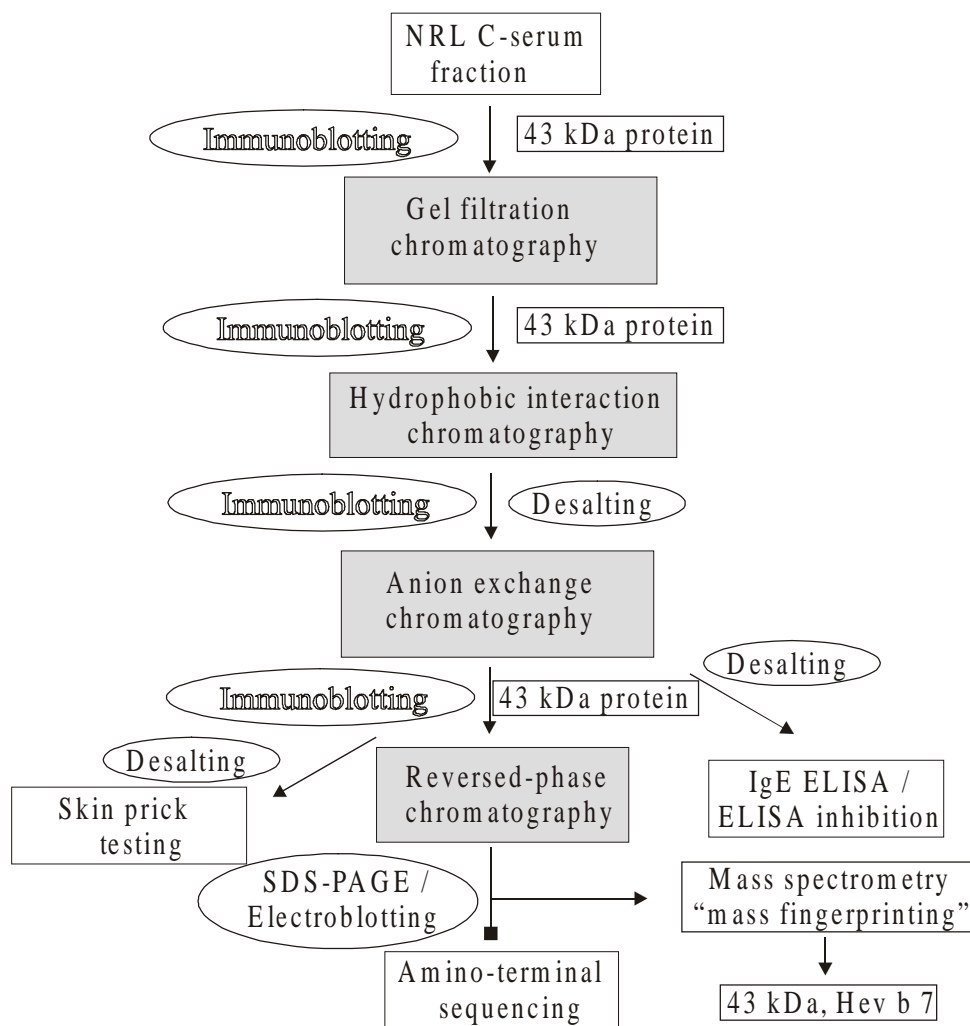


Figure 2. Purification scheme of patatin-like NRL allergen (Hev b 7)

1.3 Purification of the 16 and 20 kDa potato allergens (IV)

IgE-binding of patient sera to 16 and 20 kDa potato proteins was first observed by immunoblotting experiments. The fractions containing < 30 kDa potato proteins from gel filtration chromatography were pooled and subjected to reversed-phase chromatography. The eluted fractions were again pooled in six fractions and analyzed by immunoblotting. IgE-binding to proteins with molecular weights around 20 kDa was observed in most of the fractions.

1.3.1 Purification of cathepsin D proteinase inhibitor (Sol t 2)

A fraction containing a 20 kDa-protein from reversed-phase chromatography was identified by immunoblotting and further characterized by amino-terminal sequencing and mass spectrometry. The amino-terminal sequencing resulted in the sequence ESPLPKPVLDTNGKELNPXSSYRII, which could be identified as the cathepsin D proteinase inhibitor (PDI) (AC P16348; 100% identity in 24 aa overlap). In addition to amino acid sequencing, mass fingerprinting analysis with MALDI-TOF MS gave (with 3 peptides covering 18% of the protein sequence) the highest score for cathepsin D proteinase inhibitor of potato tuber (Fig. 3a). Based on the previous studies, the inhibitor was known to have a potential glycosylation site at Asn 19. For further information, the molecule was subjected to electrospray MS analysis which gave molecular masses of 21626, 21755, and 21958 Da. Considering the calculated molecular weight of the PDI amino acid sequence (20589 Da), the signals were tentatively assigned to glycoforms carrying (Hex)₃(HexNAc)₂(dHex)₁ (+1039 Da), (Hex)₃(HexNAc)₂(dHex)(Pen)₁ (+1171 Da), and (Hex)₃(HexNAc)₃(dHex)(Pen)₁ (+1374 Da), respectively. The major signal may thus carry a typical plant N-glycan designated the paucimannosidic type (Lerouge et al. 1998) (Fig. 3a). To further verify the glycosylation of the cathepsin D proteinase inhibitor, 2.0 µg of the purified allergen was analyzed in 15% SDS-PAGE, and PAS-staining was used to visualize the glycosylated protein (unpublished data).

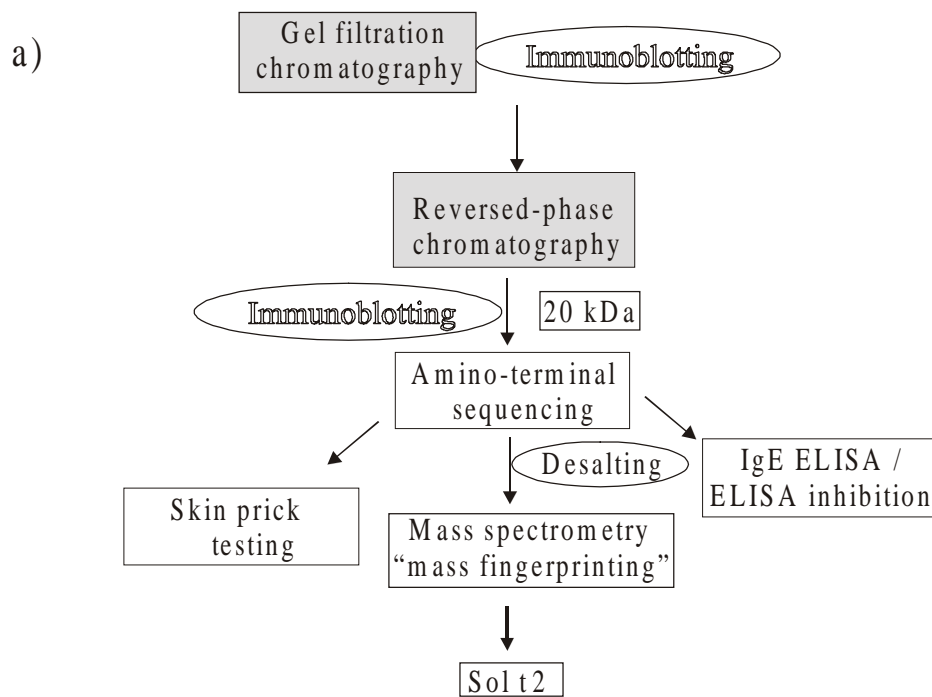


Figure 3a. Purification scheme for cathepsin D proteinase inhibitor, (Sol t 2)

1.3.2 Purification of cysteine proteinase inhibitors (Sol t 3) and aspartic proteinase inhibitor (Sol t 4)

To purify and identify the remaining 20 kDa IgE-binding potato proteins, the previously analyzed fractions from gel filtration chromatography were subjected to HIC. Eluted fractions were combined in five pools (1 to 5), desalted, and analyzed by SDS-PAGE. The purification procedure was followed by immunoblotting, and IgE-binding was observed in all the pools (1 to 5) containing 20 kDa-proteins, and in pool 4, both 20 and 16 kDa proteins (Fig. 3b). In order to identify the IgE-binding proteins, the pools (1 to 5) were electroblotted after SDS-PAGE on a PVDF membrane and subjected to amino-terminal sequencing. The amino-terminal sequence obtained from all of the 20 kDa-proteins showed the closest match with a sequence corresponding to cysteine proteinase inhibitors of potato tuber (95-100% identity in 10 aa overlap).

Pools 2, containing 20 kDa, and pool 4, containing both 20 and 16 kDa IgE-binding proteins, were then chosen for further analysis (Fig. 3b). First, pool 2 was subjected to cation-exchange chromatography, resulting in a purified 20 kDa protein with IgE-binding ability. Amino-terminal sequencing of this protein gave the result LVLPEVYDQDN PLRIGERYIIKNP, based on which it could be identified as mature cysteine proteinase

inhibitor 10-potato (AC gi|7489251| pir| T07746|, 100% identity in a 25 aa overlap). Although the calculated molecular mass of this cysteine proteinase inhibitor was 20 148 Da, the MS analysis of the present study gave a molecular mass of 20114 Da for this IgE-binding mature cysteine proteinase inhibitor designated as Sol t 3.0101.

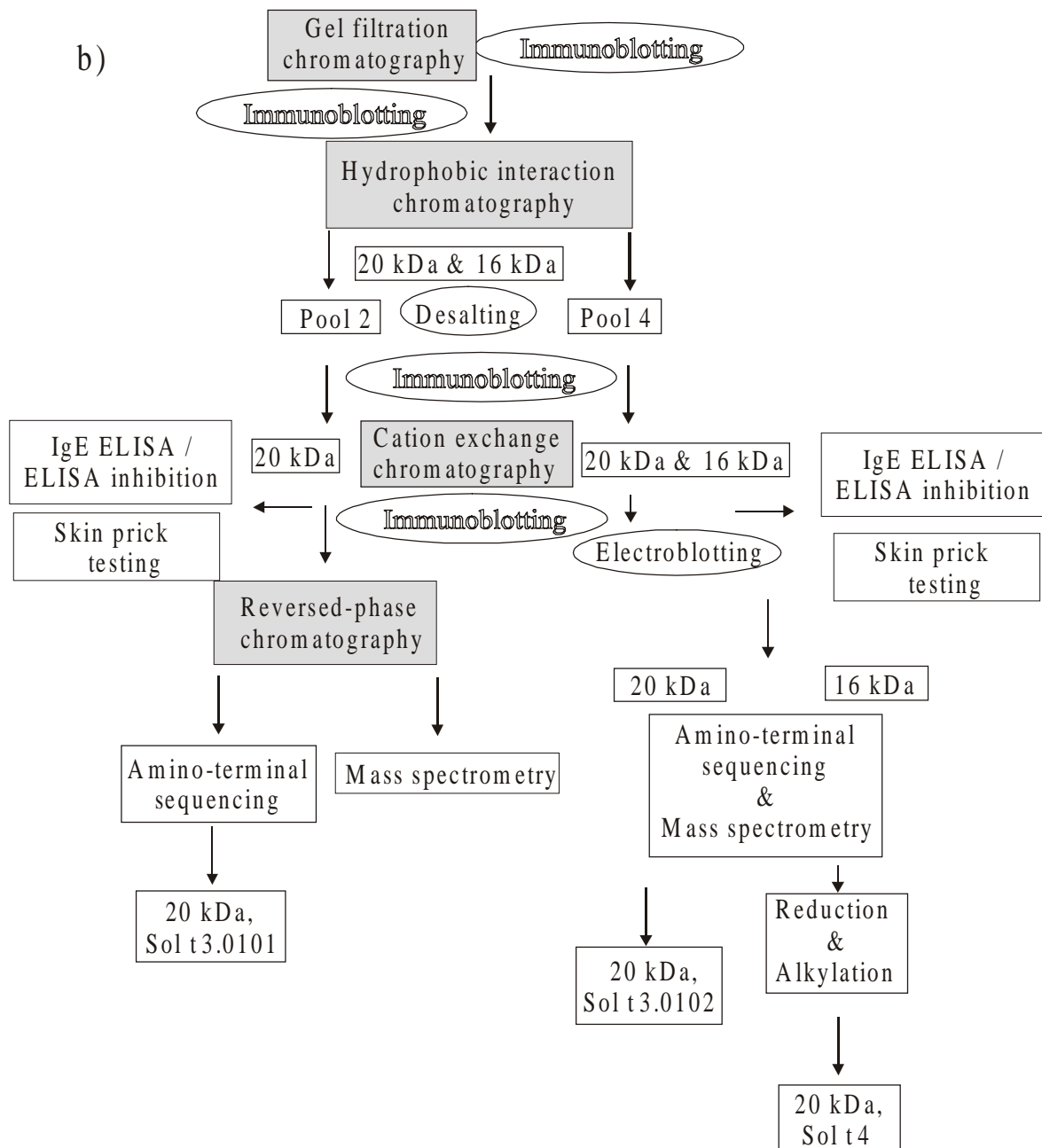


Figure 3b. Purification scheme for the cysteine proteinase inhibitors (Sol t 3.0101, 3.0102) and the aspartic proteinase inhibitor (Sol t 4).

To further analyze the IgE-binding proteins from pool 4, this pool was subjected to cation-exchange chromatography. Two major peaks eluting from the column were analyzed by SDS-PAGE and immunoblotting and found to correspond to the 16 and 20 kDa proteins. Together with the 16 kDa band, a low molecular weight protein was detected from the CBB-stained PVDF-membrane. The amino-terminal sequence of the 20 kDa-protein from the PVDF membrane gave a sequence of LVLPEVYDQDGDPLRIGERY IINNPLIGAGAVYLYNIGNLQCPNAV LQHMSIPQFLGKGPVV, based on which it was identified as the mature cysteine proteinase inhibitor PCPI 8.3 of potato tuber (AC P20347, 95% identity in a 64 aa overlap) with a molecular mass calculated as 20076 Da. MS analysis of the present study, however, gave a molecular mass of 20193 Da for this mature cysteine proteinase inhibitor, and it was designated as Sol t 3.0102 (Fig. 3b).

Amino-terminal sequencing of the 16 kDa band from the PVDF membrane gave a sequence of LPSDATPVLDVAGKELDSRLSYRII, based on which it could be identified as the amino-terminus of mature aspartic proteinase inhibitor of potato tuber (AC P30941, with 100% identity in a 25 aa overlap) with a calculated molecular mass of 20556 Da. The electrospray MS analysis of this mature molecule, however, gave a molecular mass of 20302 Da, which did not correspond to the size observed from SDS-PAGE and electroblotting. To further investigate the mass difference observed, the aspartic proteinase inhibitor was reduced, alkylated, and subjected to reversed-phase chromatography. Now, a 4274 Da peptide corresponding to the C-terminal part of the aspartic proteinase inhibitor could be isolated. This peptide was then further subjected to amino-terminal sequencing, which gave a sequence of SDDQFCLKVGVVHQN with 100% identity with the C-terminal part of the aspartic proteinase inhibitor of potato tuber in a 15 aa overlap. These results were later confirmed by subjecting the intact 20 kDa protein to amino-terminal sequencing. Amino-terminal sequencing of the intact 20 kDa aspartic proteinase inhibitor from the ProsorbTM membrane resulted in the previously observed amino-terminal sequences for both of the ~16 and ~4 kDa polypeptide chains. Although further characterization is needed, based on the present results, the purified aspartic proteinase inhibitor of potato tuber seems to consist of two polypeptides (~4 kDa and ~16 kDa), joined by a disulphide bond. This aspartic proteinase inhibitor appearing major as the 16 kDa band in immunoblotting was designated Sol t 4 (Fig. 3b).

2. IgE binding to potato and NRL allergens in immunoblotting

2.1 IgE binding to Sol t 1 and Hev b 7

In the second study (II), sera from NRL-allergic adult patients showing IgE binding to a 43 kDa potato allergen patatin of potato tuber (Sol t 1) were used to identify and follow the purification of the 43 kDa patatin-like NRL-allergen (Hev b 7). IgE cross-reactivity in immunoblotting between purified Hev b 7 and Sol t 1 was studied with pooled sera of 10 NRL-allergic adults recognizing both Hev b 7 and Sol t 1. Crosswise-immunoblot inhibition studies with purified Sol t 1 and Hev b 7, respectively, showed that Hev b 7 at 50 µg/ml was able to inhibit all the IgE binding to Sol t 1, whereas 100 µg/ml of Sol t 1 was not seen to diminish IgE binding to Hev b 7.

Using sera of 7/35 (20%) NRL-allergic adults showing specific IgE binding to Hev b 7 in ELISA, 5/7 patients showed IgE binding in immunoblotting. Specific IgE binding to Sol t 1 was also observed in 5/35 (14%) NRL-allergic adults. Of these five adults, four showed IgE binding by immunoblotting. When sera of NRL-allergic children were tested against Hev b 7 and Sol t 1 in immunoblotting, the children did not show IgE binding to Hev b 7, instead, they showed IgE antibodies to Sol t 1.

In the third study (III), sera of 12 oral-challenged infants were used to study IgE binding to Sol t 1. Of these infants, 7/12 (58%) showed IgE binding to Sol t 1.

2.2 IgE binding to Sol t 2-4

Sera of the 12 potato-challenged infants were also used to identify other IgE-binding potato proteins with molecular weights ranging from 16 to 20 kDa (IV). Of the 12 infants, 7 (58%) showed IgE-binding to the 20 kDa-band and 3 (25%) to the bands in the 16 and 18 kDa regions. To study immunological cross-reactivity, inhibition was assessed of the binding of IgE from pooled sera of 10 children to total potato protein extract by purified Sol t 2-4. After a preincubation of Sol t 2-4 (1.0 and 10 µg/ml) with the serum pool, IgE binding to Sol t 1 was not diminished. However, Sol t 2 (10 µg/ml) was able to block all the IgE binding to proteins from the 16 to 20 kDa area containing Sol t 2-4. IgE binding to the 20 kDa area seemed not to be affected when it was inhibited with either Sol t 3.0101 or

Sol t 3.0102. Furthermore, Sol t 4 (as the native, 20 kDa form), was seen to diminish the IgE binding to the 20 kDa region and, abolish all binding to the 16 kDa region (IV).

3. Occurrence of IgE antibodies to potato and NRL allergens in ELISA (I-IV)

3.1 IgE antibodies to Sol t 1

In the first study (I), when ELISA assays were performed against both denatured (after reversed-phase chromatography) and native forms of Sol t 1, reaction patterns were essentially similar. Of 27 atopic children, 20 (74%) showed ELISA values that exceeded the mean +3SD of the 44 control children. When IgE-binding antibodies of 35 atopic NRL-allergic children were analyzed against Sol t 1, 29/35 (83%) showed positive responses exceeding the mean + 3SD of the 11 control children. A total of 15 (43%) of the NRL-allergic adults showed IgE antibodies to Sol t 1 (II). In Study III, of 12 oral-challenged infants, 9 (75%) showed IgE antibodies to Sol t 1. This study also included an experiment in which Sol t 1 retained its IgE-binding ability after being boiled for 5 min before the assay (unpublished data).

3.2. IgE antibodies to Hev b 7 and cross-reactivity with Sol t 1 (II)

Of the 35 NRL-allergic adults, 17 (49%) showed IgE-binding to Hev b 7. In contrast, sera of 35 NRL-allergic children did not recognize Hev b 7, except for one borderline case. Of the 35 NRL-allergic adult patients' sera, a total of 10 (29%) showed IgE antibodies to both Hev b 7 and Sol t 1 at levels that exceeded the mean +3SD of the 30 atopic adult controls. Results of crosswise ELISA-inhibition studies with Sol t 1 and Hev b 7 showed that at a concentration of 60 µg/ml Sol t 1 was able to inhibit 50% of IgE binding to solid-phase Hev b 7. Conversely, Hev b 7 (10 µg/ml) inhibited 50% of IgE binding to solid-phase Sol t 1 when adult or children's sera were used. Serum of one reference patient having specific IgE binding to Hev b 7 served as a reference.

3.3 IgE antibodies to Sol t 2-4

When assayed against purified Sol t 2, 20 (51%) of 39 children showed ELISA values that exceeded the mean +3SD of the 30 control children. Against Sol t 3.0101 and Sol t 3.0102, 17 (43%) and 23 (58%) of the 39 children, respectively, had IgE antibodies. Increased IgE binding to native (20 kDa) Sol t 4 was evident in 26 (67%) of the 39 atopic children.

3.4 ELISA inhibition assays with purified potato allergens

ELISA inhibition assays were carried out to measure the contribution of each of the new allergens to the patients' total IgE-binding ability against potato proteins and to study the immunological cross-reactivity. In the first study (I), purified patatin (Sol t 1) at a final concentration of 10 µg/ml inhibited 75% of the binding of IgE from the pooled sera to solid-phase potato proteins. With sera of four oral potato-challenge positive infants in Study III, 0.1 to 10 µg/ml was needed to inhibit 50% of IgE binding against solid-phase total potato protein extract. Sol t 2 (20µg/ml), Sol t 3.0101 (10µg/ml), Sol t 3.0102 (3µg/ml), and Sol t 4 (25µg/ml) in Study IV were able to inhibit 50% of the binding of IgE from the pooled sera against potato protein extract. In this study, Sol t 1 (3µg/ml), used as a reference, inhibited 50% of IgE binding against the solid-phase potato proteins.

4. *In vivo* reactivity of raw potato, Sol t 1-4 and Hev b 7

Of 39 atopic children with positive SPT to raw potato, 26 (67%) were skin prick tested with purified Sol t 1 (concentration ranging from 100 to 200 µg/ml). Of these 26 children, 19 (53%) showed a positive response (I, III). All of the 35 NRL-allergic children were prick-prick tested with raw potato and 27 of them with Sol t 1. Of these 35, 22 (63%) had a positive SPT response to raw potato, and 30% of the 27 showed positive responses to Sol t 1 (II).

In adult patients, Sol t 1 and Hev b 7 were proven to be reactive *in vivo*, as well (II). When the four infants and four older children were skin prick tested with Sol t 2-4, all showed a positive response to at least one of the four allergens (IV).

5. Skin and oral challenge responses to potato (III)

Of the 12 infants, 7 had positive skin challenge responses to raw potato and one infant to cooked potato. A total of 8 (67%) oral potato challenges with cooked potato were positive, and 4 of the 12 infants showed positive responses both to raw and to cooked potato. However, only one immediate-type response was observed. A positive delayed challenge response, *i.e.*, increased eczema (SCORAD >50%), occurred in 7 infants. Of the 8 infants with challenge-proven potato allergy, 7 had elevated total IgE levels and 6 a positive potato RAST.

6. Allergen nomenclature

All of the four potato proteins have been accepted by the WHO/IUIS Allergen Nomenclature Sub-Committee as Sol t 1- Sol t 4.

DISCUSSION

1. General characteristics of potato allergens

Most food allergens appear to be 10 to 70 kDa water-soluble glycoproteins relatively resistant to acids and proteases (Lehrer et al. 1996, Metcalfe 1998, Sampson 1999). Considering this criterion, patatin (Sol t 1) and the proteinase inhibitors (Sol t 2-4) of potato tuber identified in the present study seem to qualify. Moreover, all of the four novel potato allergens full fill the description and criteria of the “complete allergen” of Aalberse and van Ree (1997), and can therefore be considered as “true” food allergens.

Patatin (Sol t 1) is encoded by two multigene families and appear as acidic isoproteins that account for 40% of the total soluble tuber proteins. Sol t 1 is mainly known as a storage protein showing phospholipase A₂ (PLA₂) activity in defending the host (Sonnewald et al. 1990, Senda et al. 1996). According to MS and SDS-PAGE analyses, the molecular weight of Sol t 1 ranges from 40 to 45kDa (I). It is important to note, however, that native Sol t 1 has a dimeric structure (~80 kDa) (Racusen and Foote 1980, Pots et al. 1998) which may influence the IgE-binding *in vitro* and *in vivo* (I-IV).

Plant proteinase inhibitors are widely distributed in nature and are present in many common foods (*e.g.*, legumes, cereal grains, potatoes, and other tubers) of the human diet (Liener 1986). Several proteinase inhibitors, such as the Kunitz-type soybean trypsin inhibitor (KSTI) (Moroz and Yang 1980) and α -amylase trypsin inhibitors of rice and wheat have already been identified as food allergens (Metcalfe et al. 1996). Moreover, proteinase inhibitors are known to be extremely stable molecules being, for example, involved in interfering with the digestion of food proteins in the gut (Liener 1986, Astwood et al 1996).

Potato tuber is by nature a rich source of proteinase inhibitors whose primary role is in defending the host from pathogen attacks (Laskowski and Kato 1980). The amino acid sequences of various potato proteinase inhibitors have, however, merely been deduced from the cDNA clones, and little is thus known about their exact biological roles or about the appearance of the mature protein structures (Štrukelj et al. 1992). Based on the results of the present study, however, Sol t 2-4 are members of a structurally related family of KSTI (IV). The isoelectric points of Sol t 2-4 calculated in the present study are overlapping, most variation being between the numerous subtypes of cysteine proteinase inhibitors (Gruden et al 1997). Interestingly, purified aspartic proteinase inhibitor (Sol t 4)

was demonstrated to be post-translationally processed to result in ~4 and ~16 kDa polypeptides held together by a disulfide bond. Furthermore, IgE from these children's sera bound more effectively to the native 20 kDa Sol t 4 in ELISA than to the reduced 16 kDa protein in immunoblotting. This suggests that conformational epitopes are important for the IgE binding to Sol t 4.

Before Sol t 1-4 were identified, hypersensitivity reactions between pollen and vegetables in individuals suffering from pollinosis were suggested to result from profilin as the potential cross-reactive allergen (Ebner et al. 1995, Breiteneder and Ebner 2000). In the present study, however, when sera of the 10 atopic children having positive SPT to birch pollen were used, IgE binding to the 14 kDa area corresponding to the size of plant profilins was not observed.

2. IgE binding to potato and NRL allergens in vitro / in vivo

In immunoblotting, Sol t 1 appeared to be the major IgE-binding potato protein for atopic food-allergic children having positive SPTs to raw potato (I-III). This finding was later confirmed by Tücke et al. (1999) and Astwood et al. (2000) using sera of potato-allergic patients who had been positive in DBPCFCs.

In the present study, the frequency of IgE binding in ELISA to native Sol t 1 was observed to range from 74 to 83% (I-II). Surprisingly, however, the highest frequency of IgE binding to Sol t 1 was seen in the sera of NRL-allergic children (II), of whom 63% showed positive SPTs to raw potato and 30% to Sol t 1. Regarding these results, it seems that in addition to Sol t 1, the NRL-allergic children have been co-sensitized to other potato proteins. This suggestion is also in line with the results of Tücke et al. (1999), although these other potato proteins remain to be identified (Table 2).

In contrast to NRL-allergic children, NRL-allergic adults showed IgE binding both to patatin (Sol t 1) and to the patatin-like NRL allergen, Hev b 7 (Table 2). It is also of interest that there were subgroups of NRL-allergic adults showing specific IgE antibodies either to Sol t 1 or Hev b 7 (Table 2). However, the difference between IgE binding to Hev b 7 in children and in adults could not be explained, even after a clinical background analysis of both the groups. Considering this, the present study suggests that Hev b 7 is not an important allergen for NRL-allergic children. Furthermore, the clinical relevance and the occurrence of specific IgE antibodies in adult patients to Sol t 1 and/or Hev b 7 require further work.

In ELISA, IgE antibodies to Sol t 2-4 were detected in 43 to 67% of atopic children (IV) (Table 2). The frequency of specific IgE antibodies suggests that, in addition to Sol t 1, Sol t 2-4 are important potato allergens, as well. Due to their stability and resistance against proteases, Sol t 2-4 may be able to interfere with the functions of digestive enzymes in the gut, which could in turn lead to enhancement of the stability and allergenic properties of other food proteins (Astwood et al 1996), *e.g.*, Sol t 1.

To evaluate the *in vivo* reactivity of Sol t 1-4, four infants and four older children were skin prick tested with purified allergens (Table 2) (IV). As expected, the infants had positive SPTs to Sol t 1-4 more frequently than did the older children, many of whom had most likely already grown out of their allergy to potato (IV). This suggestion is in accordance with the previous observations, since SPTs to specific foods have been reported to remain positive for years following the achievement of clinical tolerance (Sicherer 1999).

Table 2. Summary of potato and NRL allergens

Allergen	Function	MW (kDa)	Subjects children/adults	%SPT (+) #	%ELISA (+) #
Sol t 1	Storage protein, host-defence	40729	children (I)	57% (14)	74% (27)
			children (II)	30% (27)	83% (35)
			adults (II)	25% (4)	43% (35)
			children III	50% (12)	75% (12)
Sol t 2	Cathepsin D proteinase inhibitor	20589*	children (IV)	38% (8)	51% (39)
Sol t 3.0101	Cysteine proteinase inhibitor	20114	children (IV)	75% (8)	43% (39)
Sol t 3.0102			children (IV)	63% (8)	58% (39)
Sol t 4	Aspartic proteinase inhibitor	20302	children (IV)	50% (8)	67% (39)
Hev b 7	Host-defence	42870*	children (II)	nd	3% (35)
			adults (II)	50% (4)	49% (35)

positivity/number of tested subjects,

* calculated molecular weight,

nd, not done

3. Clinical findings of hypersensitivity to potato

Although the present study identified four new potato allergens for young atopic children, allergic reactions to potato can still be considered a dilemma, because responses to raw and cooked potato are thoroughly different (Castells et al. 1986, Delgado et al 1996). Adults and older children allergic to birch pollen commonly experience immediate-type reactions

to raw potato (Hannuksela and Lahti 1977, Dreborg and Foucard 1983 Eriksson 1984, Ortolani et al. 1993 and Sampson 1999). Several studies have also demonstrated that hypersensitivity to potato in these patients normally appears as contact urticaria after handling of raw potatoes and that these reactions are usually followed by symptoms of rhinoconjunctivitis and asthma, leading in some cases even to anaphylaxis (Pearson 1966, Nater and Zwartz 1967, Delgado et al 1996).

Rather than suffering contact urticaria, the atopic children in the present study had clinical histories of symptoms more characteristic of IgE-mediated food allergy (Castells et al. 1986, Hannuksela 1987, Wahl et al. 1990) (I, III, IV). Moreover, infants and young children are rarely in contact with raw potatoes (Dreborg and Foucard 1983). Instead, in northern Europe and Scandinavian countries, cooked potato is one of the first solid foods that infants encounter.

Hannuksela (1987) was the first to report that cooked potatoes are able to cause infantile eczema in atopic infants under one year of age, and after that age, children appeared to be asymptomatic. This finding is in accordance with the results, of the present study since most of the older children having positive SPTs to raw potato were able to consume potato as food (I, II, IV). The diagnosis of potato allergy has, however, been considered rather difficult, since most of the atopic children simultaneously experience multiple allergies to common foods such as cow's milk, eggs, cereals, and wheat and also suffer from AD (I-IV).

In the present oral potato-challenge study, all of the atopic infants suffered from multiple food allergies and all had experienced clinical symptoms from cooked potato (III). Before the oral challenges, potato had been eliminated from the diet (range 2-18 months, mean 8.5 months) and the 7-day challenge with cooked potato was started when the skin seemed asymptomatic. In addition to oral challenges, when the 12 infants were challenged on the skin with both raw and cooked potato, 7 (58%) had positive responses to raw potato but only one to cooked potato. This finding suggests that these infants may have also been co-sensitized to heat-labile potato allergens, which in turn have been connected to hypersensitivity reactions to raw potato and to birch pollen allergy (Nater and Zwartz 1967, Delgado et al. 1996, Hannuksela and Lahti 1977, Ebner et al. 1995).

Interestingly, only one infant had an immediate-type oral response to cooked potato, whereas two-thirds showed delayed-type responses. One explanation for the low number of the former was probably the relatively long elimination period of potato from the diet. It can also be the case that some infants were already growing out of their potato allergy (Sicherer 1999), since all except one child one year later were able to consume potato.

In concordance with the results of the present study, the immediate, late-onset, and delayed types of responses against cooked potatoes were recently observed in seven infants (mean age 11.5 months, range 5-25 months) by De Swert et al. (2000). The authors also suggested that heat-stable allergens in potatoes were responsible for the continuation of eczema in young atopic children. In sum, these results suggest that allergic reactions to cooked potato may vary greatly between individuals suffering from AD.

4. Cross-reactive carbohydrate determinants

Several plant allergens are known to be glycosylated (Ebner et al. 1995). In addition, the $\alpha(1,3)$ -fucosylated and $\beta(1,2)$ -xylosylated glycoproteins have been suggested to be responsible for false-positive reactions *in vitro* (Aalberse et al. 1981, Aalberse and van Ree 1997). However, according to the recent study by van Ree et al. (1999), IgE binding to these plant glycoproteins seems not to be dependent on CCDs only.

Like several other plant food allergens, Sol t 1 is glycosylated (II) and known to carry complex-type N-glycans with fucose and/or xylose units (Racusen and Foote 1980, Pots et al. 1998). In addition to Sol t 1, Sol t 2 was observed to be N-glycosylated. Based on the crosswise immunoblotting results of the present study, Sol t 1 and Sol t 2 are not cross-reactive; however, further studies with a higher concentration of Sol t 2 are needed to verify this result also in ELISA. (IV).

In theory, both of these potato allergens may be responsible for such phenomena as *in vitro* cross-reactivity between potato and tree/grass pollen allergens (Aalberse and van Ree 1997). Furthermore, according to Ebner et al. (1995), the IgE-binding potato proteins may also be relevant allergens for patients suffering from birch pollen allergy.

Interestingly, the proposed N-glycan of Sol t 2 may be of a type similar to the one described for the major rye grass (*Lolium perenne*) pollen allergen, Lol p 11 (van Ree et al. 2000). Lol p 11 has also been reported to have sequence similarity to the KSTI-family (van Ree et al. 1995). These two allergens may thus be responsible for the *in vitro* cross-reactivity between grass pollen and potato (Løwenstein and Eriksson 1983, Calkhoven et al. 1987, Bircher et al. 1994). This reasoning is also accordant with the recent findings of De Swert et al. (2000), who reported that both birch and grass pollen extracts were able to inhibit the IgE-binding of pollen- and potato-allergic patients' sera to potato proteins. Considering the evidence currently available, it seems that conserved N-glycan structures (van Die et al. 1999) may be relevant for the allergenicity of the plant proteins.

5. Cross-reactivity of potato and NRL allergens

Although several potato and NRL proteins have sequence similarity (Breiteneder and Ebner 2000), Sol t 1 has been the most frequently suggested candidate to explain the clinical cross-reactivity between potato and NRL allergens. Beezhold et al. (1996) reported that 36% of adult NRL-allergic subjects complained of local reactions to potato and tomato. Earlier, the same group (1994) had reported, based on immunoblotting, that 9/40 (23%) of NRL-allergic patients recognized a 46 kDa NRL protein, designated later Hev b 7, having sequence similarity to patatin of potato tubers. To demonstrate potential cross-reactivity between potato proteins and the 46 kDa NRL allergen in NRL extract by immunoblot inhibition, the authors incubated potato extract with rabbit antiserum to NRL. Consequently, antibody reactivity to several NRL proteins was blocked, and reactivity to the bands at 16 and 46 kDa was diminished but not completely abolished (Beezhold et al. 1996). This finding of Beezhold et al. (1996) is, in essence, in line with the crosswise immunoblot inhibition finding of the present study (II), in which purified Sol t 1 was unable to inhibit the IgE binding of NRL-allergic patient sera against Hev b 7. The most likely reason for this is that the cross-reactive IgE-binding epitope(s) was hidden inside the dimeric structure of native Sol t 1.

The patatin-like NRL allergen (Hev b 7) has more recently been cloned and expressed using *E coli* and *Pichia pastoris*, by Kostyal et al. (1998), and Sowka et al. (1998). According to Sowka et al (1998), only 4/36 (11%) of NRL-allergic individuals in Austria showed IgE binding to natural and/or recombinant Hev b 7 (rHev b 7). Interestingly, one year later, the same authors reported that among their Hev b 7-sensitized population, Hev b 7 represented the only NRL allergen for 47% and the major allergen for 80% of the 15 patients. The authors had also investigated cross-reactivity between deglycosylated rHev b 7 and Sol t 1 from potato extract, using immunoblotting and sera of patients having specific IgE binding to Hev b 7 but no IgE binding antibodies to Sol t 1. Using this population of NRL-allergic subjects, the authors failed to demonstrate cross-reactivity between these two allergens (Sowka et al. 1999). Yet, the present study, using IgE-ELISA and sera of 10 patients with IgE to both natural Hev b 7 and Sol t 1, showed that these two allergens exhibit distinct cross-reactivity. These results also suggest that although these two allergens are cross-reactive *in vitro*, this cross-reactivity may not be clinically relevant (II).

FUTURE ASPECTS

Years before patatin (Sol t 1) was identified as an allergen, Sonnewald et al. (1990) studied the effect of glycosylation on molecular stability in resistance against protease digestion using modified and wild-type patatins of potato tubers as model molecules. According to their results, a trypsin-resistant peptide derived from the patatins including the potential glycosylation sites of the molecule was left to accumulate *in vitro*. Obviously, this accumulating peptide should be identified and further characterized. This type of protease-resistant peptide has recently been reported to contain important IgE-binding sites, *e.g.*, in the trimeric major peanut allergen Ara h 1 (Maleki et al. 2000). In addition, using synthesized overlapping peptides, Astwood et al. (2000) reported having identified both major and minor IgE-binding epitopes in Sol t 1. The same group also reported, based on results in immunoblotting, that the IgE binding to deglycosylated recombinant Sol t 1-variants (rSol t 1) was reduced (Alibhai et al. 2000). The effect of glycosylation for IgE binding obviously needs to be further studied by use of both natural dimeric and modified recombinant Sol t 1s. In addition, the significance of the modified Sol t 1(s) in transgenic potato if, for instance, for use in immunotherapy should be elucidated (Platts-Mills et al. 1999).

Instead of developing immediate-type allergic reactions to cooked potato, most of the challenge-proven potato-allergic infants having specific IgE antibodies to heat-stable Sol t 1-4 experienced delayed-type responses. Furthermore, the findings suggest that a number of infants with a positive skin-challenge response to raw potato have been co-sensitized to heat-labile potato allergens (Nater and Zwart 1967, Szépfalusi et al. 1995). Considering that the mechanisms of food-induced hypersensitivity reactions and the development of oral tolerance are still largely undiscovered (Sampson 1999), further studies are needed to understand the various immunopathological mechanisms of food-induced hypersensitivity reaction (Wollenberg and Bieber 2000). In addition, the heat-labile potato allergens responsible for the hypersensitivity reactions to raw potatoes remain to be identified.

It has been suggested that birch pollen allergy-related foods, such as apple, potato, and tomato in the diet of young atopic children may predispose them to pollen allergy (Szépfalusi et al. 1995, De Swert et al. 2000). Interestingly, the study of Ebner et al. (1995) demonstrated that the sera of birch pollen-allergic individuals recognized in immunoblotting several IgE-binding proteins from potato extract. To confirm these

observations, IgE-binding and cross-reactivity between these potato and pollen allergens, in particular between Sol t 2 and Lol p 11, should be assessed and their clinical relevance carefully examined. Furthermore, the cross-reactivity between potato and botanically related species among the family of *Solanacea* should be further studied to identify other potential patatin-like food allergens.

In addition to characterizing potato allergens, the present study also assessed cross-reactivity with botanically unrelated allergens of potato and NRL. Although specific IgE antibodies to the patatin-like NRL-allergen (Hev b 7) were detected *in vitro* and *in vivo*, Hev b 7-specific epitopes were not found in various manufactured NRL products. Further studies are thus warranted to identify the molecular structures of clinically significant allergens and the basis of cross-reactivity between plant food, pollen, and NRL allergens (Turjanmaa et al. 1996, Fuchs et al 1997, Levy et al. 2000).

Diagnosis of sensitization is currently performed with commercially available or in-house protein extracts that are derived from natural source materials and thus may contain a variety of allergic and non-allergic components (Platts-Mills et al. 1998, Valenta et al. 1999). The use of purified or recombinant allergens as test reagents may overcome many of the shortcomings related to uncharacterized or unstandardized mixtures of proteins. The present study characterized four new potato allergens and now provides prospects for developing specific new tools for *in vivo* and *in vitro* diagnostics of food allergy.

SUMMARY AND CONCLUSIONS

Four heat-stable potato proteins (Sol t 1-4) were characterized as novel food allergens for atopic children. All 39 children invited to the study showed positive SPT responses to potato and had a clinical history of symptoms from eating cooked potato. Like many plant-food allergens currently known, potato allergens were identified as storage proteins (Sol t 1) and proteinase inhibitors (Sol t 2-4). Two of these four potato allergens, Sol t 1 and Sol t 2, were identified as glycoproteins and were most likely carrying typical $\alpha(1,3)$ -fucosylated and/or $\beta(1,2)$ -xylosylated glycan units.

The majority of atopic children showed specific IgE antibodies by ELISA to Sol t 1, and, according to ELISA inhibition results with pooled sera of atopic children, Sol t 1 appeared to be the major potato allergen. Importantly, 75% of the challenge-proven potato-allergic infants showed IgE antibodies in ELISA to purified Sol t 1. However, instead of developing immediate-type reactions to cooked potato, 67% of these infants exhibited delayed-type responses. Allergic reactions to cooked potato may thus vary between individuals, and further studies are needed to better understand the immunopathological mechanisms behind the various types of responses.

The frequency of IgE antibodies to Sol t 2-4 ranged between 43 and 67%. With regard to these results, Sol t 2-4 seem to be important potato allergens; however, based on the results of immunoblot inhibition experiments, Sol t 2-4 are not cross-reactive with Sol t 1. As far as *in vivo* reactivity is concerned, all of the purified potato allergens showed positive wheal-and-flare responses.

In addition to the IgE-binding ability, the immunological cross-reactivity between the botanically unrelated allergens of potato (*Solanum tuberosum*) and rubber tree (*Hevea brasiliensis*) was assessed. Of the NRL-allergic adults, 43% showed IgE binding to Sol t 1 and 49% to Hev b 7. In addition, 29% of these adult subjects showed concurrent IgE binding to both Sol t 1 and Hev b 7.

In contrast to NRL-allergic adults, NRL-allergic children showed no IgE binding to Hev b 7, implying that Hev b 7 is not an important NRL allergen for children. Furthermore, the cross-reactivity observed between Sol t 1 and Hev b 7 in NRL-allergic adults seems not to be clinically relevant.

In conclusion, the present study demonstrates that even potato, which has been considered to be a safe food, can induce allergic reactions at least for a subgroup of atopic children suffering from atopic dermatitis. Considering these findings, potato should probably be avoided for young atopic children showing a positive SPT to raw potato until the final diagnosis of clinical allergy has been made with a challenge test.

ACKNOWLEDGEMENTS

The laboratory work of this study was carried out at the Department of Immunobiology, National Public Health Institute, Helsinki, and at the Laboratory of Protein Chemistry, Institute of Biotechnology, University of Helsinki. The clinical parts of this study were carried out at the Department of Dermatology, Tampere University Hospital.

I wish to thank Professor Jussi Huttunen, Head of the National Public Health Institute, and Professor Mart Saarma, Head of the Institute of Biotechnology, for providing the splendid research facilities needed to complete this work.

I wish to express my deepest gratitude-

to my supervisor, Professor Timo Palosuo, for patience and excellent guidance. He has been the key person keeping all the strings concerning this project in his hands, managing to find time to listen to, value, and support my ideas, for which I will be forever grateful. Most of all, he has a great sense of humor, and I want to thank him for all the laughs we shared when writing the manuscripts, something I will never forget.

to my other supervisor, Nisse Kalkkinen, PhD, for his valuable guidance into the world of protein chemistry. He is a true wizard in fixing instruments and keeping everything in order. It has been a great honor to be able to work in his laboratory. I want to thank him also for keeping my feet on the ground, and teaching me to finish up one thing before starting another, something which a young scientific mind tends to forget.

I wish to express my warmest thanks-

to Professor Timo Reunala, for his enormous support and constructive guidance in carrying out this study. He has great enthusiasm both for science and for nature, and his spirit tends to capture everyone around him. It has been a thrill to work with him.

to Docent Kristiina Turjanmaa, who originally came up with the idea to study potato allergy in atopic children. I am grateful for her friendly support and useful comments when writing my thesis and performing this study. She has taught me to speak up, something which I do value.

to Heli Majamaa, MD who has become my efficient right arm at the Department of Dermatology, my co-author, and a good friend. She has a great ability to get things and people moving, something which I admire a lot.

to Leea Ylitalo, MD, for patience and friendly support. She completed her thesis on the most tight schedule I have ever seen: an amazing performance indeed. Winter Greetings to Klaara!

My warmest thanks-

to Docent Harri Alenius, our man from overseas, for valuable opinions and constructive comments on this study. In the beginning of this project, I could not understand how he was able to fit so many words and ideas into the same sentence. Now I do understand, I have found myself in the same situation.

to Docent Jari Helin, PhD, for his friendly support, advice, and discussions concerning this study. He introduced to me and taught me the little that I now know of glycobiology and mass spectrometry. I am ever grateful for that. I will never forget his *quality jokes* that have lifted our laboratory spirits during the years, one of the many things I will miss in the future.

I wish to thank the referees of my thesis, Professor Matti Vuento, and Docent Kirsti Kalimo, for their most valuable and constructive criticism.

My deepest thanks I owe to all the children and adult patients who took part in this study.

I would like to thank Professor Markku Kulomaa, and Piia Karisola MSc, my collaborators from the University of Jyväskylä, for the discussions and cheerful moments we have shared during these years. I also wish to express my thanks to the “girls” of the Laboratory of Immunobiology, National Public Health Institute. It has been fun to work beside you. I have enjoyed our lively discussions of everyday life.

I also wish to express my sincere thanks to my colleagues and friends at the Laboratory of Protein Chemistry, Institute of Biotechnology, at the Division of Biochemistry, at the Department of Occupational Health, and at the Institute of Biomedicine. My special thanks to Docent Jaana Tyynelä, Ragna Rönnholm, PhD, Tuula Nyman, MSc, Anne Olonen, MSc, and Riikka Nissinen, MSc, for their professional support and the hours of ongoing "girl-talk".

I would also like to thank Carolyn Norris, PhD, for the careful language editing of my thesis, in addition to hugging and playing with my dog.

I wish to express my sincere thanks to my mother and father, for their endless love and support. My warmest thanks to Iikka Saarelainen, for his love and encouragement to finish up my study. His help and knowledge were crucial in keeping our “home office” up-dated and in shape.

This study was supported by the Academy of Finland (Grant No.37852), the Finnish Foundation for Allergy Research, Ansell International, Melbourne, Australia, and Danisco Cultor, Kantvik, Finland.

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