

ANNA HOWARD

Characterisation of allergenic components in soya bean

Master's degree project



UPPSALA
UNIVERSITET

Molecular Biotechnology Programme

Uppsala University School of Engineering

UPTEC X 06 036		Date of issue 2006-08	
Author		Anna Howard	
Title (English)		Characterisation of allergenic components in soya bean	
Title (Swedish)			
Abstract		<p>Many people are affected by allergic diseases, and the incidence of allergy is increasing. In order to obtain the right treatment for patients, it is important that allergy diagnoses are made correctly. Therefore, there is a strong need for accurate allergy tests. The aim of this study was to characterise the allergenic components in soy. Soya bean extract was fractionated, and the fractions were analysed using gel electrophoresis and protein assays. A multiplexed flow immunoassay was set up, and the fractions were analysed with 35 sera from soy-sensitised patients. The results suggested that two soy allergens, glycinin and β-conglycinin, were the most IgE-inducing proteins in soya bean.</p>	
Keywords		Allergy test, IgE, soya bean, flow immunoassay, glycinin, β -conglycinin	
Supervisors		Sigrid Sjölander Phadia AB, Uppsala	
Scientific reviewer		Jörgen Dahlström Phadia AB, Uppsala	
Project name		Sponsors	
Language English		Security	
ISSN 1401-2138		Classification	
Supplementary bibliographical information		Pages 21	
Biology Education Centre Box 592 S-75124 Uppsala		Biomedical Center Tel +46 (0)18 4710000	Husargatan 3 Uppsala Fax +46 (0)18 555217

Characterisation of Allergenic Components in Soya Bean

Anna Howard

Populärvetenskaplig sammanfattning

Miljoner människor i världen lider idag av allergiska besvär. För att dessa människor ska få rätt behandling är det viktigt att diagnostisering av allergi görs på ett korrekt sätt. Principen för de allergitester Phadia AB utvecklar är att man mäter mängden antikroppar riktade mot ett visst allergen (ämnet som orsakar allergi) i patientens blod. Stora mängder antikroppar indikerar starkt att patienten är allergisk mot ämnet ifråga.

Mitt examensarbete var ett led i utvecklandet av sådana tester. Det gick ut på att karakterisera allergena komponenter i sojabönan. I det första steget utvanns proteinerna ur sojabönan, och dessa separerades sedan med avseende på storlek. Därefter analyserade jag de olika sojaproteinproven. Jag mätte varje provs proteinkoncentration och undersökte om det innehöll glykoproteiner, proteiner som har kolhydratkedjor fästa på sig.

I nästa steg sattes små droppar av sojaproven och av renade sojaproteiner på remsor av nitrocellulosa. Sedan lät jag blodprover från sojaallergiker flöda genom nitrocellulosan. Jag kunde då se på vilka droppar som antikroppar från blodproverna fastnade. Mina resultat visade att i många blodprover fanns det antikroppar riktade mot två vanliga proteiner i sojaböna som kallas glycinin och β -conglycinin. Därmed är det troligt att dessa proteiner är inblandade i sojaallergi.

Examensarbete 20 p i Molekylär bioteknikprogrammet

Uppsala universitet augusti 2006

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1. INTRODUCTION

1.1 Allergy

1.1.1 The IgE antibody

Antibodies are a part of the immune system. They are large Y-shaped proteins whose function it is to bind foreign substances (antigens) in the body fluids, thus marking the antigens for destruction. The antibody consists of two parts: the variable region and the constant region (see Fig. 1). These regions have different functions. The variable region binds to the antigen, and the constant region mediates the binding of the immunoglobulin molecule to the host tissue. There are five different classes of antibodies: IgM, IgG, IgA, IgD and IgE. All the antibody classes have a similar structure, consisting of four polypeptide chains. In the IgE molecule, two of these polypeptide chains are identical light chains, each containing two domains, and two are identical heavy chains, each containing five domains (see Fig. 1). The chains are covalently linked to each other by disulphide bonds. The tips of the two arms of the antibody, i.e. the light and the heavy chain domain furthest out on each arm, form the variable region of the antibody. The remaining two light chain domains, as well as the rest of the heavy chain domains, make up the constant region. On the basis of the constant region, the class division of antibodies is made [1].

1.1.2 IgE-mediated allergy

All allergies are due to a deficit in the immune defence. Allergies are hypersensitivity reactions, where the immune system reacts so strongly to a harmless substance that the body's own tissues are damaged. There are four described types of hypersensitivity: immediate hypersensitivity (type I), cytotoxic hypersensitivity (type II), immune complex hypersensitivity (type III) and delayed hypersensitivity (type IV). IgE-mediated allergy is a type I hypersensitivity reaction. This kind

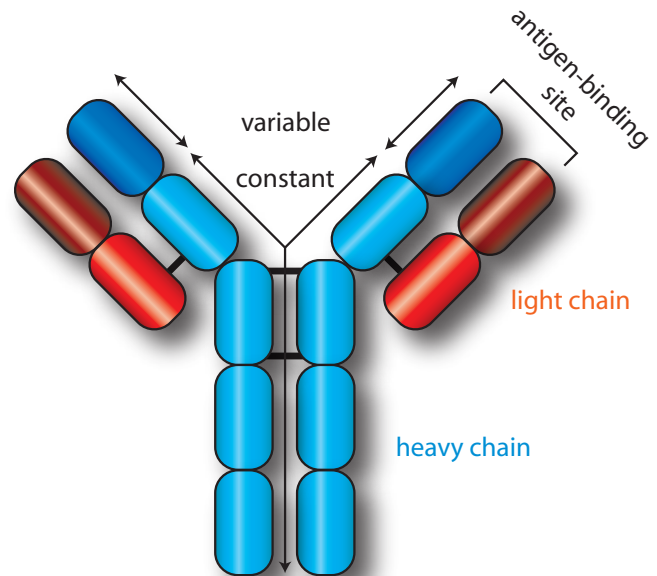


Figure 1. IgE antibody

of hypersensitivity causes classical allergies, like allergies to pollen, pets and different kinds of foods. The basic reason for IgE-mediated allergy is that the body produces IgE antibodies towards an allergen. The allergic reaction occurs when the antigen cross-links IgE molecules that are bound to basophils' and mast cells' Fc-receptors, thereby making the cells secrete a number of substances, among others histamine.

The reasons why IgE-mediated allergies arise are still poorly understood. When an antigen comes in contact with a B cell, the B cell becomes activated and begins to produce antibodies against the antigen. The B cell begins to divide rapidly, and during this process there will be a class switch of the antibodies. Usually, only a very small fraction of the antibodies will switch to IgE, but if something goes wrong, there might be a switch to IgE even when the B cell encounters a perfectly harmless substance. This can cause an allergy to develop. The people who are affected by allergy are so called atopic individuals, meaning that they have unusually high IgE levels in their blood, and sometimes have uncommonly many basophils and mast

cells [1]. Atopy is hereditary, but so far no specific genetic markers have been identified, making it likely that the disorder is polygenic [2].

1.1.3 Allergens

Allergens are substances that induce a hypersensitivity reaction in an allergic patient. Most allergens are proteins, often with carbohydrate side chains [2]. They are frequently associated with potent biological functions, such as being involved in enzymatic, inhibitory and regulatory pathways in the body [3]. The allergen nomenclature has been established by the World Health Organization (WHO). Allergens are named according to the taxonomy of their source: the first three letters of the genus name and the first letter of the species name, followed by a number, assigned to the allergen when it was identified.

Based on structural and functional properties, plant food allergens are classified into families and superfamilies. The cupin and prolamin superfamilies, along with the family 10 of pathogenesis-related proteins (PRs), are the three dominating allergen groups [4].

The cupins have a β -barrel structural core domain and two short conserved consensus sequence motifs in common [5]. Globulin seed storage proteins, that are major components in the human diet, belong to the cupin superfamily. The globulins are divided into 7S vicilins and 11S legumins. Both types of globulins are highly thermostable [4]. 7S vicilins are trimeric proteins and have a molecular weight of 150 to 220 kDa [6]. Because they lack cysteines, they contain no disulphide bonds [5]. Mature 11S legumins are hexameric proteins with a molecular weight of between 300 and 380 kDa [7].

The basis of the prolamin superfamily is a conserved sequence of eight cysteine residues. These heat stable proteins have low molecular weight and a similar three-dimensional folding rich in α -helices [4]. Three plant allergen families belong to the prolamins: the 2S albumins, the lipid transfer proteins (LTPs) and the α -amylase and trypsin inhibitors.

The PRs are proteins that plants induce in response to a pathogen infection. The PR-10 family, also known as the Bet v 1 family, comprises of intracellular proteins that are rather unstable to digestion and heating [4, 5].

Apart from these major allergen groups, there are also other allergenic protein families. Examples of such families are the profilin family, which is comprised of actin-binding proteins, and the plant agglutinin family, also known as the lectin family, which is comprised of proteins that bind specifically to sugar determinants on glycoproteins [8].

1.1.4 Cross-reactivity

The phenomenon that occurs when an antibody binds not only to the antigen it was made against, but also to one or more other substances, is called cross-reactivity. The molecular basis of this reaction is that proteins from different sources can have structural similarities [9]. Cross-reactions can cause severe problems for allergic patients. IgE antibodies, raised against one allergen, can cause an allergic reaction when cross-reacting with another allergen. A classical example of this is patients allergic to birch pollen who develop allergic symptoms after ingestion of apples, carrots and kiwi fruits [10].

1.1.5 Prevalence, clinical symptoms and treatment of allergy

Hypersensitivity reactions can give rise to a broad range of clinical symptoms. The symptoms can be gastrointestinal, cutaneous or respiratory, or a combination of these. Gastrointestinal symptoms include vomiting, diarrhoea, cramping and nausea. Cutaneous symptoms can be urticaria (widespread, itchy wheals) and angioedema (deep mucocutaneous swelling). Patients with respiratory symptoms may experience coughing, wheezing, sneezing, rhinorrhea and nasal obstruction. Allergic patients may also experience anaphylaxis, which is a severe, sometimes fatal, systemic reaction [11].

The true prevalence of allergy is unknown, but evidence suggest that the incidence of allergy is increasing [12]. Allergic diseases can arise any time in life, but generally begin early in childhood. In the westernised world, the number of people affected by food allergy is suggested to be 2-8% in children and 1% in adults [11].

Currently, the main treatment of allergic diseases consists of allergen avoidance. There are also a number of drugs available, for example antihistamines, anticholinergic agents, which give relief of symptoms, and topical corticosteroids, which suppress allergic inflammation. However, no drugs affect the underlying cause of the disease, and they are therefore not able to cure a patient of allergy. The only treatment available today that may cure an allergic patient is immunotherapy for specific allergens, which is also known as hyposensitisation. This therapy is carried out by administering the patient increasing doses of allergen extracts over a long period, usually several years [13, 14].

1.1.6 Allergy tests

The golden standard of food allergy tests is the double-blind placebo-controlled food challenge (DBPCFC), where the allergen is administered orally to the patient, with neither the doctor nor the patient knowing the content in the challenge. However, DBPCFC, as well as being costly and time-consuming, can also induce potentially severe clinical symptoms. It is therefore desirable to have other ways of diagnosing allergy.

Today, there are two major tests for detecting sensitisation to an allergen: skin-prick tests (SPT) and measurement of allergen specific IgE in serum. SPT is an *in vivo* test, where the suspected allergy-causing substance is pricked into the skin, and the diameter of the resulting wheal is measured and compared to a negative control.

An example of an *in vitro* test is the ImmunoCAP™ product from Phadia AB, which measures allergen specific IgE levels in blood serum from the patient. The principle for ImmunoCAP™ is shown in Fig. 2.

The IgE level in the serum sample is measured in kilo-units per litre (kU/l), which is a measurement of the concentration of IgE antibodies against a specific allergen in the serum.

1.2 The soya bean

The soya bean (*Glycine max*) is a legume that belongs to the Fabaceae family (see Fig. 3). It comes originally from the eastern part of Asia, where it has been used for 5,000 years. Today, the United States is the world's largest producer of soya beans, followed by Brazil and China. Economically, it is the most important bean in the world, because of its versatile use [14]. It provides nourishment for millions of people, the nutritional advantage of the soya bean being its high protein content (40%) and fat content (20%) [15]. The beans can be eaten as they are, or used in many different foods, for example soymilk, tofu, soy sauce, margarine and as an additive in, for example, bread and meat products [16]. The soya bean also serves as an ingredient for many different chemical products, among others paint, fertilisers, insect sprays and fire extinguisher fluids [14].

The soya bean is an annual plant that grows erect, sometimes reaching a height of more than two metres. The flowers of the soya bean are self-fertilising, and are shades of either white or purple. The edible seeds range in colours from black, brown and green to yellow, and there are usually one to four seeds per pod. The crop is planted when there is no longer any danger of frost, and harvested, usually mechanically, in the autumn when the leaves of the plant have fallen off. The moisture content of the soya bean has then dropped to around 13%, which makes storage of the seeds safe [14]. Out of the total protein content of the seed, 70-80% is made up of two soya bean globulins: glycinin and β -conglycinin [17].

1.2.1 Soya bean allergens

The prevalence of soy allergy has not been established, but a study in the UK showed that 0.3% of the population were intolerant to soy [18]. There have been at

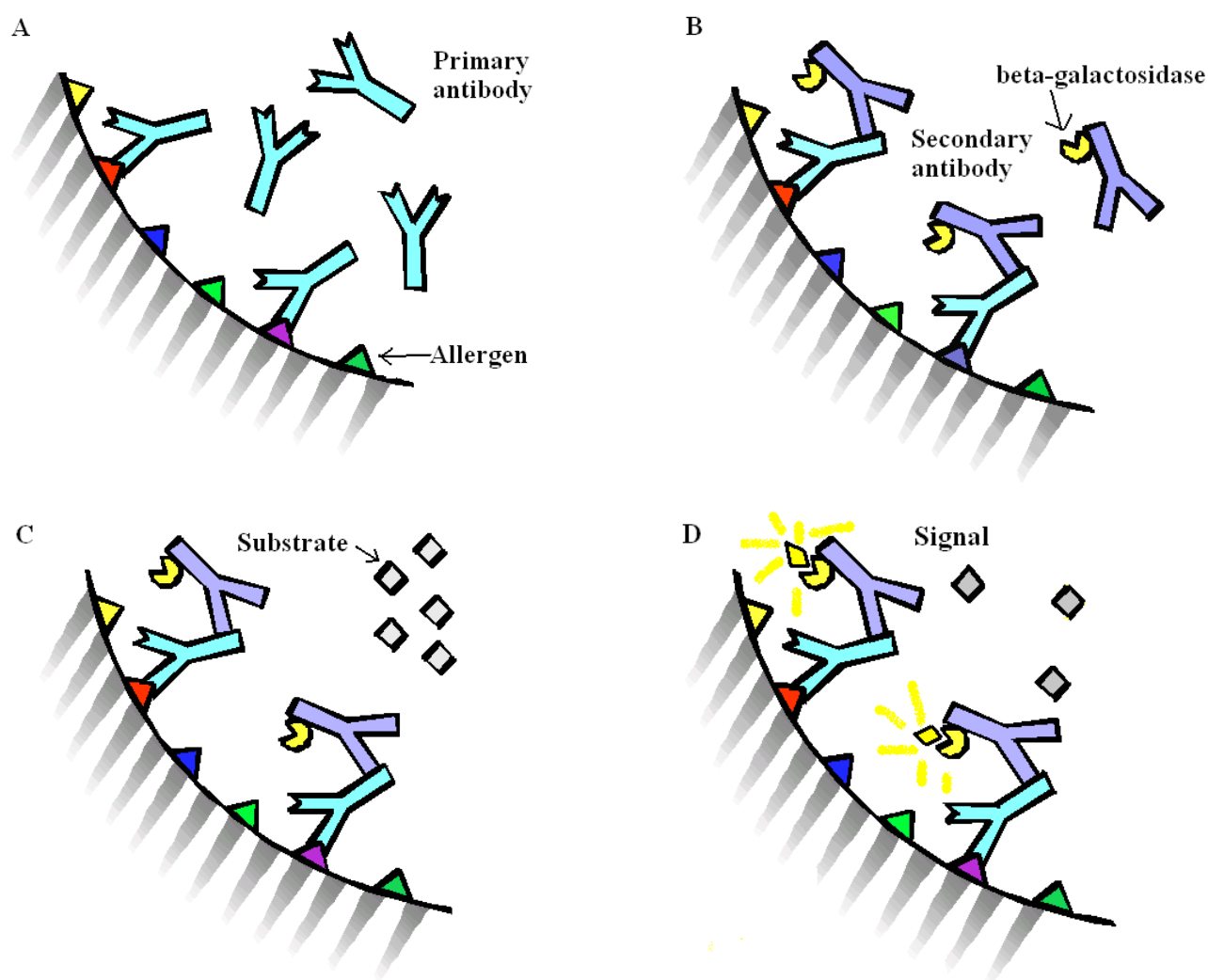


Figure 2. Principle of the ImmunoCAP™ assay

A) The allergen is covalently bound to the ImmunoCAP™ solid phase. The patient sample is incubated on the solid phase, allowing specific IgE in the sample to bind to the allergen. **B)** Non-specific IgE is washed away, and antibodies labelled with β -galactosidase are added, forming a complex with the primary antibodies. **C)** Unbound secondary antibodies are washed away, and the bound complex is incubated with a substrate to β -galactosidase. **D)** The result is a chemiluminescent signal, which is measured and evaluated against a calibration curve.

least 16 different soya bean allergens described in the literature [16]. Three of these have been reported as major allergens: Gly m Bd 60K, Gly m Bd 30K and Gly m Bd 28K [19]. Gly m Bd 60K belongs to the 7S vicilin family. It is the α -subunit of β -conglycinin, a principal seed storage protein in soy consisting in total of three subunits, α , α' and β . β -conglycinin is a glycoprotein, and its molecular mass is around 180 kDa [8]. Gly m Bd 28K is a 7S vicilin-like glycoprotein, with a molecular mass of 26 kDa [20]. Gly m Bd 30K, also known as P34, is a thiol protease, belonging to the papain superfamily [21].

Another important storage protein in soya bean is glycinin, which makes up 35-40% of the total protein content of the bean. It belongs to the 11S legumin family. The molecular mass of glycinin is 320 kDa, and it is made up of six heterogeneous subunits. Two of these subunits, G1 and G2, have been described as allergens [22, 23].

Other allergens that have been identified in soya bean include the Kunitz soya bean trypsin inhibitor, also known as Gly m TI [24], and Gly m 4, a protein belonging to the PR-10 family [25].



Figure 3. Soya bean (*Glycine max*)

Several soya bean allergens have been shown to cross-react with other legume family members. Soy allergic patients have been reported to have had adverse reactions to peanuts, peas, lentils, and kidney, navy and lima beans [16].

1.3 Aim of the study

Allergy is a growing problem in the entire westernised world. Atopic diseases affect millions of patients, and constitute a considerable percentage of the total health care cost. The soya bean is becoming an increasingly popular addition to our diet, but although soy is recognised as one of the most allergenic foods worldwide, the soya bean allergens have been studied relatively little, compared to allergens from other allergy-causing foods such as peanuts.

Today, the soy ImmunoCAP™ product from Phadia AB uses the whole soya bean extract to detect soy allergy in patients. The aim of this study was to make a broad characterisation of the allergenic components of soya beans, by fractionating the soya bean extract through gel filtration, and then studying the separate fractions by setting up a multiplexed flow immunoassay.

2. MATERIALS AND METHODS

2.1 Extraction of soya bean proteins

Soya bean (*Glycine max*) from Allergon was extracted using a phosphate buffer at neutral pH. The solution was shaken at 4°C on a shake board. The extract was then centrifuged and the protein-containing supernatant was retained. The supernatant was filtered through a 0.45 µm filter. Next, PD-10 desalting columns (GE Healthcare) equilibrated with phosphate buffer were each loaded with 2.5 ml of extract solution. The soy proteins were eluted using 3.5 ml of phosphate buffer per column.

2.2 Gel filtration

The soya bean extract was fractionated (Peter Brostedt, Phadia AB) by gel filtration on a Sephadex 200 column (GE Healthcare). Twenty fractions were obtained.

2.3 Gel electrophoresis

The soy fractions were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoresis was performed in a PhastSystem™ apparatus (GE Healthcare). The gels used were PhastGels (GE Healthcare) with a continuous polyacrylamide gradient of 8-25% or 10-15%. The test samples were mixed with dithiothreitol (DTT) and heated to 95°C for 5 min. Approximately 1 µl of test sample was loaded onto each lane. The separation was done in four steps. In the first step, the electrophoresis conditions were 250 V, 10 mA, 3 W, in the second step, 50 V, 10 mA, 1 W, in the third step, 250 V, 10 mA, 3 W, and, in the final step, 25 V, 1 mA, 1 W. The temperature was set to 15°C in all steps.

The soy fractions were also separated using native gel electrophoresis. This electrophoresis was likewise performed in a PhastSystem™ apparatus (GE Healthcare). The gels used were PhastGels (GE Healthcare) with a continuous polyacrylamide gradient of 10-15%. Approximately 1.5 µl of test sample was loaded onto each lane. The separation was done in four steps. In the first step, the electrophoresis conditions were 400 V, 10 mA, 2.5 W, in the second step, 400 V, 2 mA, 2.5 W, in the third step, 400 V, 10 mA, 2.5 W, and, in the final step, 25 V, 1 mA, 1 W. The temperature was set to 15°C in all steps.

2.4 Gel staining

After gel electrophoresis separation, the SDS-PAGE and native gels were stained with Coomassie Brilliant Blue. The staining was performed in a PhastSystem Developing Unit apparatus (GE Healthcare). At 50°C, the gels were stained once with staining solution (0.1% Pharmacia PhastGel™ Blue R, 30% methanol, 10% acetic acid), then destained three times with destaining solution (30% methanol, 10% acetic acid) and finally conserved with storage solution (5% glycerol, 10% acetic acid).

The SDS-PAGE gels with a continuous polyacrylamide gradient of 8-25% were silver stained using a PhastSystem Developing Unit apparatus (GE Healthcare). At 50°C, the gels were washed twice with fixing solution (40% ethanol, 10% acetic acid), then once in 20% ethanol, and once in water. The gels were sensitised in 0.02% $\text{Na}_2\text{S}_2\text{O}_3$ for 24 seconds, washed quickly twice with water and, at 40°C, impregnated with a solution of 0.2% AgNO_3 and 0.00925% formalin for 5 min. At 30°C, excess silver was washed away with water, and the staining was developed with a solution of 6% Na_2CO_3 , 0.01% formaldehyde and 0.001% $\text{Na}_2\text{S}_2\text{O}_3$ for 10 min. Next, at 50°C, the reaction was stopped with a solution of 5% Tris and 2% acetic acid for 4 min, before the gels were washed with water. The gels

were then conserved in storage solution (10% glycerol, 10% acetic acid), and then finally washed once again quickly with water.

2.5 BCA protein assay

The BCA (bicinchoninic acid) protein assay is used to measure the total protein concentration in a solution. The method is based on a reaction in two steps. The first step makes use of the proteins' ability to reduce Cu^{2+} to Cu^{+} in an alkaline environment. This is referred to as the biuret reaction. In the second step, one cuprous cation (Cu^{+}) forms a complex with two BCA molecules. The formation of this complex results in a visible colour change, from green to purple. The purple-coloured complex exhibits a strong absorbance at 562 nm, which is nearly linear with increasing protein concentrations.

The BCA protein assay was performed on the soy fractions to establish the total protein concentration of the samples. The protocol for the BCATM Protein Assay Kit (Pierce) was followed. The soya bean fraction samples were diluted 3:50 in 0.9% NaCl, and were measured in duplicate. The absorbance was measured in a Wallac Victor 2 1420 Multilabel Counter spectrophotometer at 560 nm.

2.6 Glycoprotein carbohydrate detection assay

The glycoprotein carbohydrate estimation assay measures the amount of carbohydrates present on the protein in a solution. The carbohydrates on the glycoproteins are first oxidised with sodium *meta*-periodate. In the next step, the aldehydes that are formed in the oxidation reaction react with a hydrazide-based conjugate, forming a hydrazone bond. This results in a purple reaction, with an absorbance maximum at 550 nm, which is proportional to the amount of carbohydrate in the solution. Non-glycosylated proteins produce only a low absorbance at this wavelength.

The glycoprotein detection assay was performed on the soy fraction samples to detect glycoproteins and estimate the carbohydrate content of each sample. The protocol for the Glycoprotein Carbohydrate Estimation Kit (Pierce) was followed. The absorbance was measured in a Wallac Victor 2 1420 Multilabel Counter spectrophotometer at 550 nm. Each sample was measured in triplicate.

2.7 Thiol protease detection assay

The thiol protease detection assay is used to determine if a sample contains thiol proteases [26]. The colourless, chromogenic substrate N_α -benzoyl-L-arginine 4-nitroanilide hydrochloride (L-BAPA) is hydrolysed by the thiol protease at the bond between the *para*-nitroaniline and the arginine moieties. This releases the yellow chromophore product *para*-nitroaniline, which has a strong absorbance at 410 nm.

To find out which soy fractions contained the soy thiol protease Gly m Bd 30K, a thiol protease detection assay was performed. The experiment was carried out according to a previously published protocol [26], with slight modifications. The soy fractions were diluted 1:10 in Tris buffer (0.1 M Tris, 1 mM CaCl_2 , 1 mM MgCl_2 , 1 M glycerol, pH 8). Then, 100 μl of the fraction solution was mixed with 100 μl of 0.8 mM L-BAPA (Sigma-Aldrich) diluted in Tris buffer. The mixture was incubated in 37°C for 26 hours before the absorbance was measured at 405 nm in a Wallac Victor 2 1420 Multilabel Counter spectrophotometer.

2.8 Multiplexed flow immunoassay

The multiplexed flow immunoassay developed by Phadia AB is used to screen sera for IgE antibodies against many different allergens simultaneously using only a small volume of sample. It is a protein microarray assay, where microspots containing natural allergen extract, purified and recombinant allergens are covalently immobilised on a capillary flow membrane. Since this membrane has an inherent three-di-

mensional structure, it enables a liquid flow from the sample application site through the reaction zone to take place, driven by capillary force.

Soya bean extract, fractions and components (glycinin and β -conglycinin, purified from soya bean extract (Roland Thunberg, Phadia AB), rGly m 4, recombinantly produced in *Escherichia coli* (Lars Mattsson, Phadia AB), soya bean agglutinin (Vector laboratories), Gly m TI (Sigma-Aldrich)) were spotted in duplicates onto a nitrocellulose membrane in an array format, and an absorbent pad was placed on the upper end of the membrane. The flow immunoassay was performed by sequentially applying 20 μ l of assay buffer, 30 μ l of serum, 20 μ l of assay buffer, 20 μ l of fluorophore-labelled anti-IgE conjugate (containing 50 μ g of antibody/ml) and 2×20 μ l of assay buffer to the lower end of the membrane. After the assay had been finished, the arrays were left to dry in darkness over night. The fluorescence intensity of each spot was measured at 670 nm using a Genepix 4000B fluorometer (Axon Instruments Inc), and the obtained data were analysed with the software program JMP 5.1 (Sas).

2.9 Sera

Obtained from an in-house serum bank, 35 sera with specific IgE response to the soy ImmunoCAP™ (kU/l > 0.35), and most of whom had a positive skin-prick test against soy, were used in the multiplexed flow immunoassay.

3. RESULTS AND DISCUSSION

3.1 Protein concentration

The soya bean extract was gel filtered and divided into 20 fractions. The protein concentration for each fraction was measured using a BCA protein quantitation assay. The results are illustrated in Fig. 4. There was a considerable variation in the protein concentration in different fractions. The first two fractions, fractions A1 and A2, as well as the last six fractions, fractions A15-A20, contained practically no protein. In the rest of the fractions, the protein concentration varied from quite low amounts of protein (fraction A4) to rather high amounts (fractions A5 and A6).

3.2 SDS-PAGE

The soy fractions were run on SDS-PAGE gels with different gradients to explore the protein pattern of the different fractions. Fig. 5 shows an SDS-PAGE gel with a continuous polyacrylamide gradient of 8-25% stained with Coomassie Brilliant Blue. The figure shows that the first fraction, A1, contained no measurable amount of protein, whereas the second fraction A2 contained slight amounts of protein. The next six fractions, A3-A8, which all showed a similar pattern, gave several strong bands of protein, ranging in size from approximately 22 to 96 kDa. The next fraction, A9, showed the same band pattern as the previous fractions, but in this fraction a shift in protein content began to take place, meaning that there were less high-molecular weight proteins than before. In

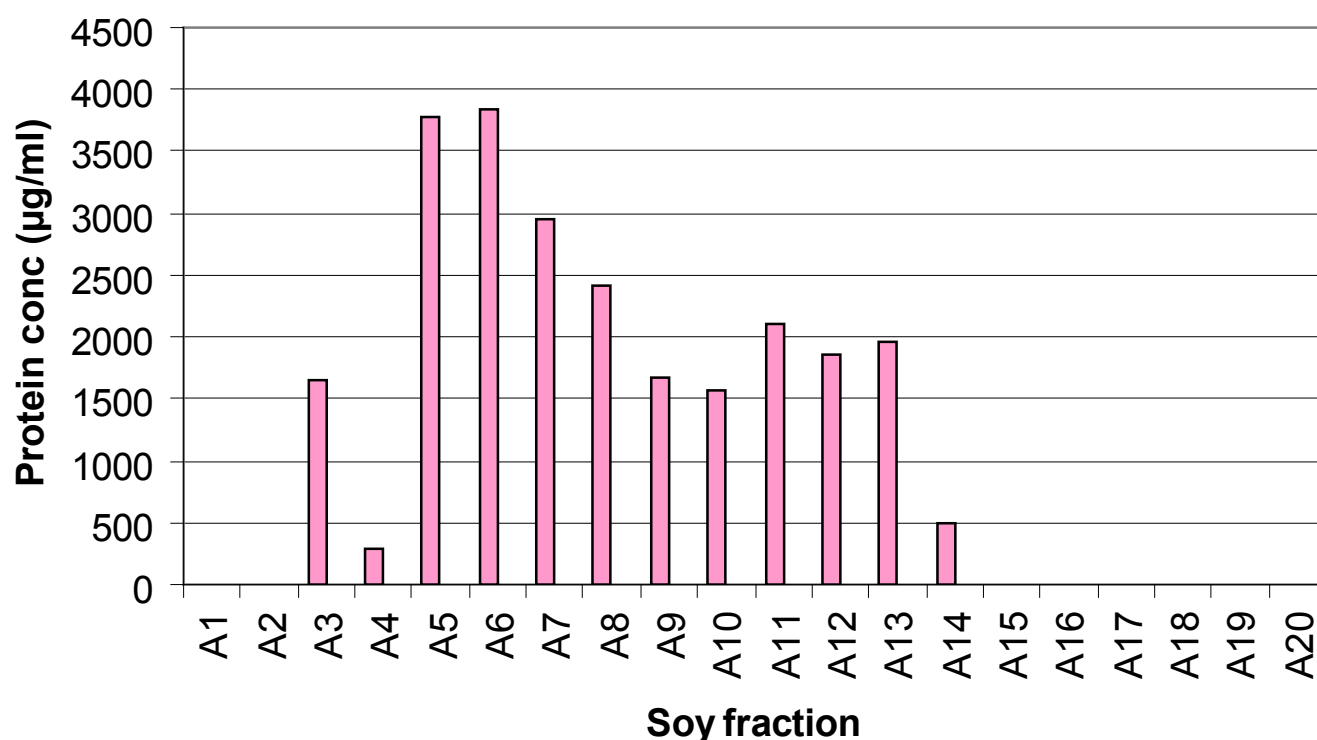


Figure 4. Protein concentration in soy fractions

The protein concentration was measured using a BCA protein quantitation assay.

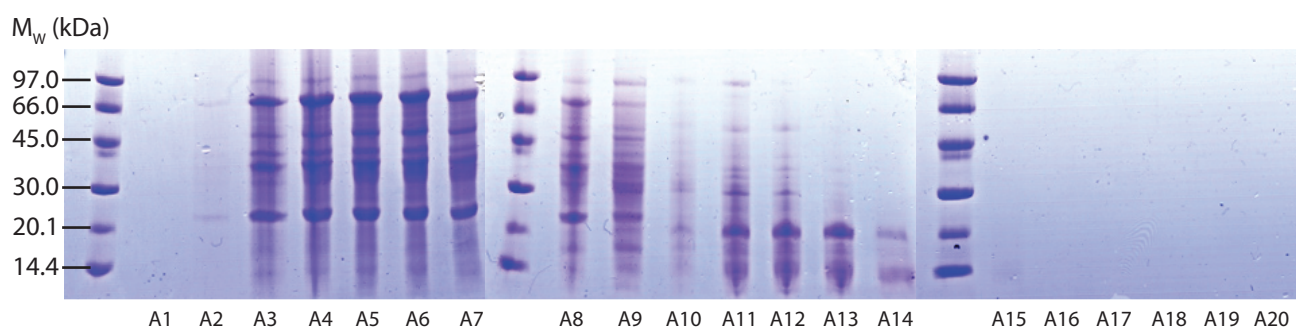


Figure 5. Gel analysis of soy fractions

The soy fractions were analysed on a SDS-PAGE gel with a continuous polyacrylamide gradient of 8-25% and stained with Coomassie Brilliant Blue. The marker used was LMW (GE Healthcare) and was added to the left of fractions A1, A8 and A15.

fraction A10, the bands were quite weak, showing that this fraction contained less protein than the fractions before and after it, which indicated that it was in this fraction that the shift in protein content mainly took place. Although the protein band patterns of the next four fractions, A11-A14, were similar to one another, the high-molecular weight bands practically disappeared in fractions A13 and A14, and in the latter, all the bands were also fairly weak. In the last six fractions, A15-A20, all bands had disappeared, so no protein could be detected with Coomassie Brilliant Blue staining. Silver staining is about a hundred times more sensitive than staining with Coomassie Brilliant Blue, and was therefore used to stain the gels to see if any new bands would appear (data not shown). However, no new bands could be detected. Thus, the conclusion was drawn that these fractions contained very little protein.

3.3 Native gel electrophoresis

To investigate whether the low-molecular weight protein bands that existed in the early soy fractions on the SDS-PAGE gel were due to these proteins belonging to a protein complex under non-denaturing conditions, the soy fractions were run on native gels. Most of the protein in the samples did not leave the wells, indicating that the proteins in these fractions formed complexes that were too large to enter the gel (data not shown). To see if the protein complexes could be dis-

solved, without completely denaturing the proteins, 0.05% Tween20 was added to all samples before they were run on native gels. However, the results showed no change in band pattern (or lack thereof) compared to the native gel electrophoresis without Tween20 (data not shown).

3.4 Glycoprotein carbohydrate estimation assay

To assess the amount of glycoprotein in each soy fraction, a carbohydrate estimation assay was performed (see Fig. 6). This assay shows the amount of carbohydrates present in a sample. The object was to see if there were glycoproteins in all the fractions, or if they were concentrated somewhere in the gradient. The amount of glycoprotein was comparatively small in the first three fractions. Then, the amount stabilised at an intermediate level for fractions A4-A8. It made sense that the glycoprotein level was stable in these fractions, since the SDS-PAGE experiment showed that these fractions seemed to contain roughly the same proteins in equal quantities. In the next fractions, the amount of glycoprotein increased, coming to a peak in fraction A12. The last few fractions showed only traces of glycoprotein.

The glycoprotein carbohydrate estimation assay measured the total amount of carbohydrate in a sam-

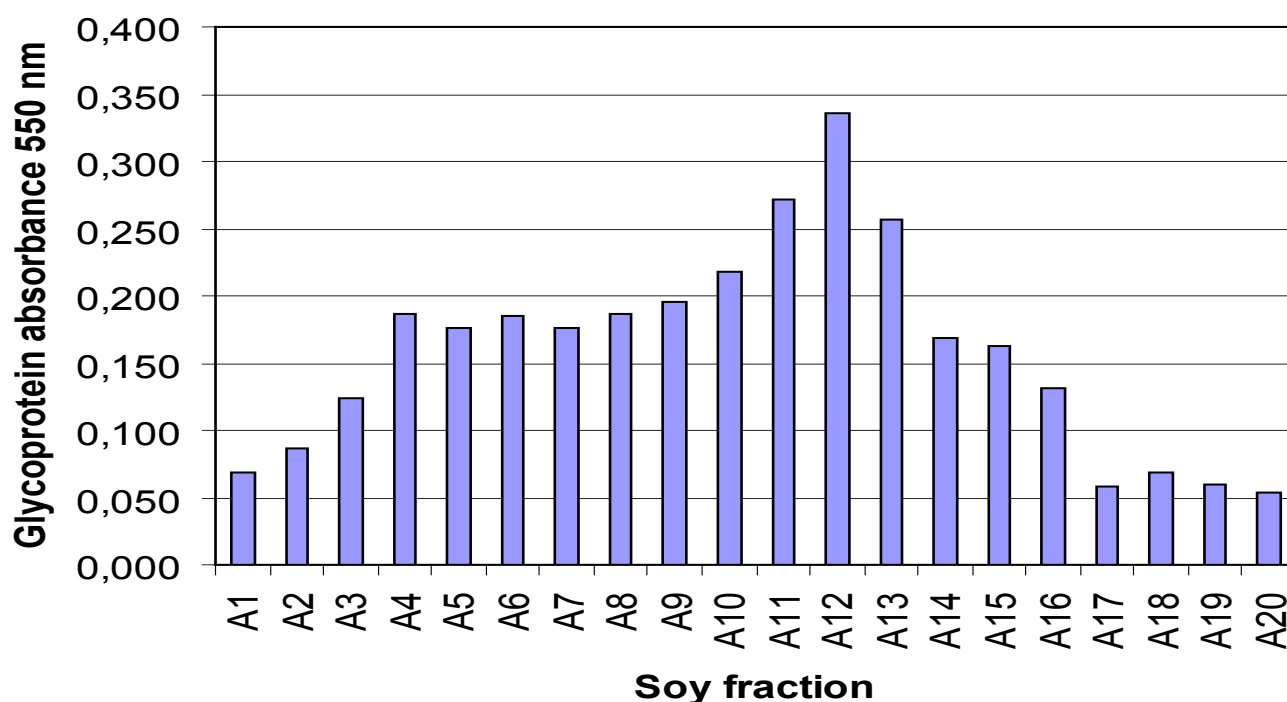


Figure 6. Glycoprotein content of soy fractions

The glycoprotein content was assessed by performing a carbohydrate estimation assay.

ple. Here, I had assumed that all the carbohydrates measured belong to glycoproteins, but, of course, this did not have to be the case. It was also possible, even probable, that there were some free carbohydrates in the sample, and these free carbohydrates could explain why the carbohydrate levels were not zero in the fractions that seemed to contain hardly any proteins (fractions A1 and A15-A20). Assuming, however, that the amount of free carbohydrate was roughly the same in each fraction, this assay still gave a good indication as to the correlation between the amount of glycoproteins and the total amount of proteins in each fraction, but, to confirm these results, further experiments would need to be done.

action's by-product *para*-nitroaniline by spectrophotometry. The results can be seen in Fig. 7. There was a big peak in the absorbance around fractions A7-A8, and a smaller peak around fractions A10-A11. The high absorbance in these peaks suggested the presence of Gly m Bd 30K in one or more of these fractions.

The soya bean contains a wide range of different proteases, and in this assay there was no way to be completely sure that the enzymatic reaction with L-BAPA was specific to only the thiol protease Gly m Bd 30K. Still, even though it could not be concluded for certain that Gly m Bd 30K existed in these fractions, the results pointed in that direction.

3.5 Thiol protease detection assay

A thiol protease, Gly m Bd 30K, has previously been reported as an allergen present in soya bean [9, 21]. To see if this enzyme could be localised in one or a few specific fractions, an assay was performed in which I incubated the fractions with a substrate, L-BAPA, and then measured the presence of the enzymatic re-

3.6 Multiplexed flow immunoassay

An array containing soya bean extract, fractions and components was set up (see Fig. 8A). In the next step, 35 sera were run through the assay. The sera came from patients that were IgE-sensitised against soya bean,

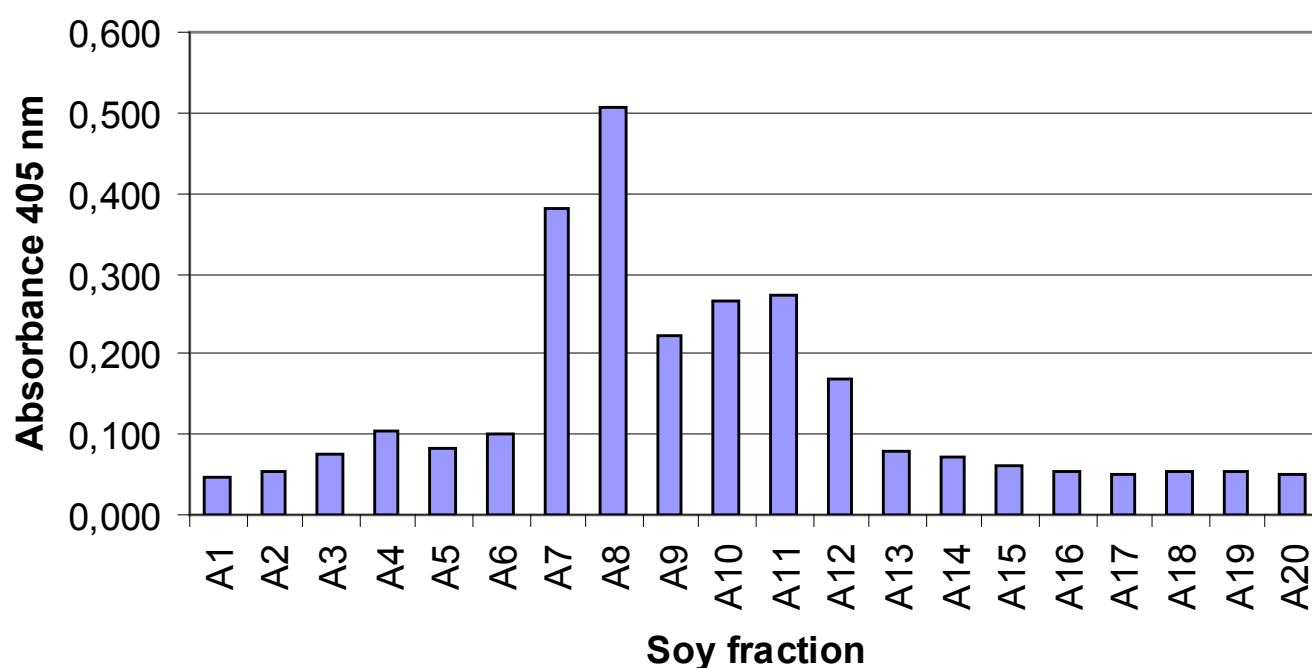


Figure 7. Presence of the thiol protease Gly m Bd 30K in soy fractions

The presence of Gly m Bd 30K was studied by incubating the soy fractions with L-BAPA, a substrate to thiol proteases, and then measuring the enzymatic reaction's by-product *para*-nitroaniline by spectrophotometry.

and most of whom had positive skin-prick tests. The objective was to try to identify which components in soy that caused allergic reactions, and to see in which fractions these components could be found. Examples of the obtained results can be seen in Fig. 8B-D. Fig. 8B exemplifies a pattern of signals that was expressed in 11 of the sera. The array showed a strong signal from fraction A4, and medium-strong signals from fractions A5 to A9. This corresponded with a strong signal from glycinin and a medium-strong signal from β -conglycinin. These results strongly indicated that glycinin and β -conglycinin could be found in these fractions. This conclusion is supported by two-dimensional gel electrophoresis results [27], which show that glycinin and β -conglycinin are found in fractions A5-A8 and A5-A7, respectively (fraction A4 was not run).

There were also two sera where β -conglycinin gave a medium-strong signal, but where glycinin gave no signal at all (see Fig. 8C). In these arrays, there was a signal from fraction A4, but the rest of the fractions were silent. This indicated that β -conglycinin existed in fraction A4, but that it was mainly glycinin that gave the signals in fractions A5-A8.

Fig. 8C also showed a strong signal from Gly m 4. When looking at only this array, it could easily be believed that the signal from A4 could just as well come from Gly m 4 as from β -conglycinin, but when Fig. 8C was compared to Fig. 8D, where Gly m 4, but not β -conglycinin, showed a signal, it could be seen that no fraction gave any signal in Fig. 8D. There was also no signal for the soya bean extract. The coupled component Gly m 4 is a recombinant protein. The fact that there was no fraction signal when there was a medium-strong signal from the Gly m 4 component suggested that Gly m 4 did not exist in the soya bean extract or in the fractions. I could only speculate in the reason for this; maybe Gly m 4 had not yet developed in the soya beans that were used, or perhaps it had not survived intact in the extraction process.

It has been shown that the Kunitz soya bean trypsin inhibitor, Gly m TI, can be found in fractions A10-A13 [27]. Two of the selected sera in this study showed weak signals from Gly m TI. These sera also showed signals from almost all of the fractions, including fractions A10-A12 (data not shown). However, since glycinin and β -conglycinin, as well as the extract, gave strong

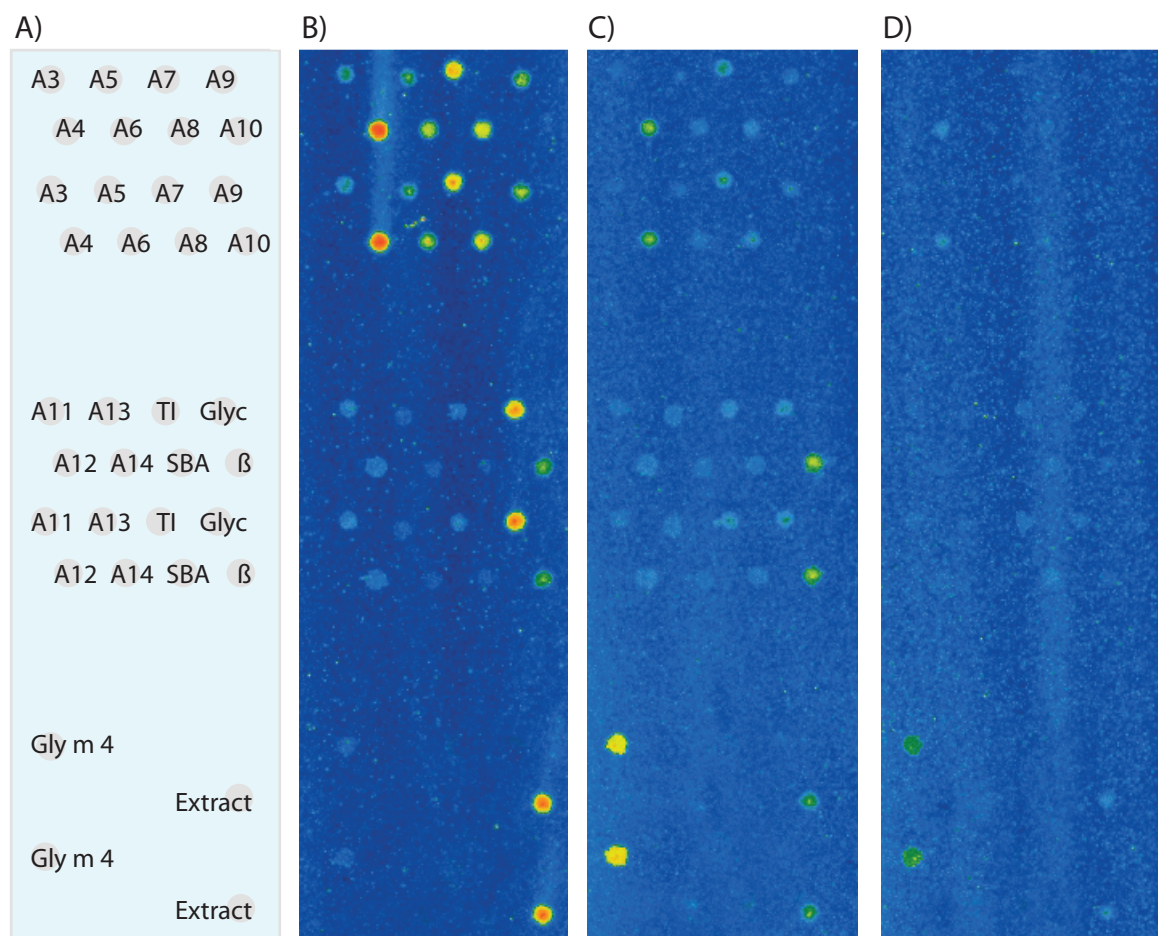


Figure 8. IgE responses to soy fractions, components and extract

Multiplexed flow immunoassay analysis of soy sensitised individuals. **A)** Array set-up. **B-D)** IgE responses from different serum samples. Abbreviations: A3-A14: soy fractions; TI: Gly m TI; SBA: soya bean agglutinin; Glyc: glycinin; β: β-conglycinin.

signals in these arrays, this indicated that none of the sera in this study showed a mono-specific response to Gly m TI.

Finally, the soya bean agglutinin (SBA) component did not give any signals at all in the arrays. Neither was the protein reported to be found in any of the fractions using the two-dimensional gel electrophoresis [27]. This could be due to SBA not existing in the soya bean extract or fractions, just as was thought for Gly m 4. It could also be that none of the sera used in this study contained IgE antibodies against SBA.

Glycinin and β-conglycinin have been reported to make up a large portion of the soya bean extract [27], and, overall, my results showed that glycinin and β-conglycinin were the soy proteins that generated

the highest IgE response. These responses are illustrated in Fig. 9. Here, it is shown that out of 35 sera, 16 showed IgE responses towards glycinin, and 15 towards β-conglycinin, which must be considered a frequent response. My results, therefore, strongly suggested that glycinin and β-conglycinin were the most IgE-inducing agents in soya bean.

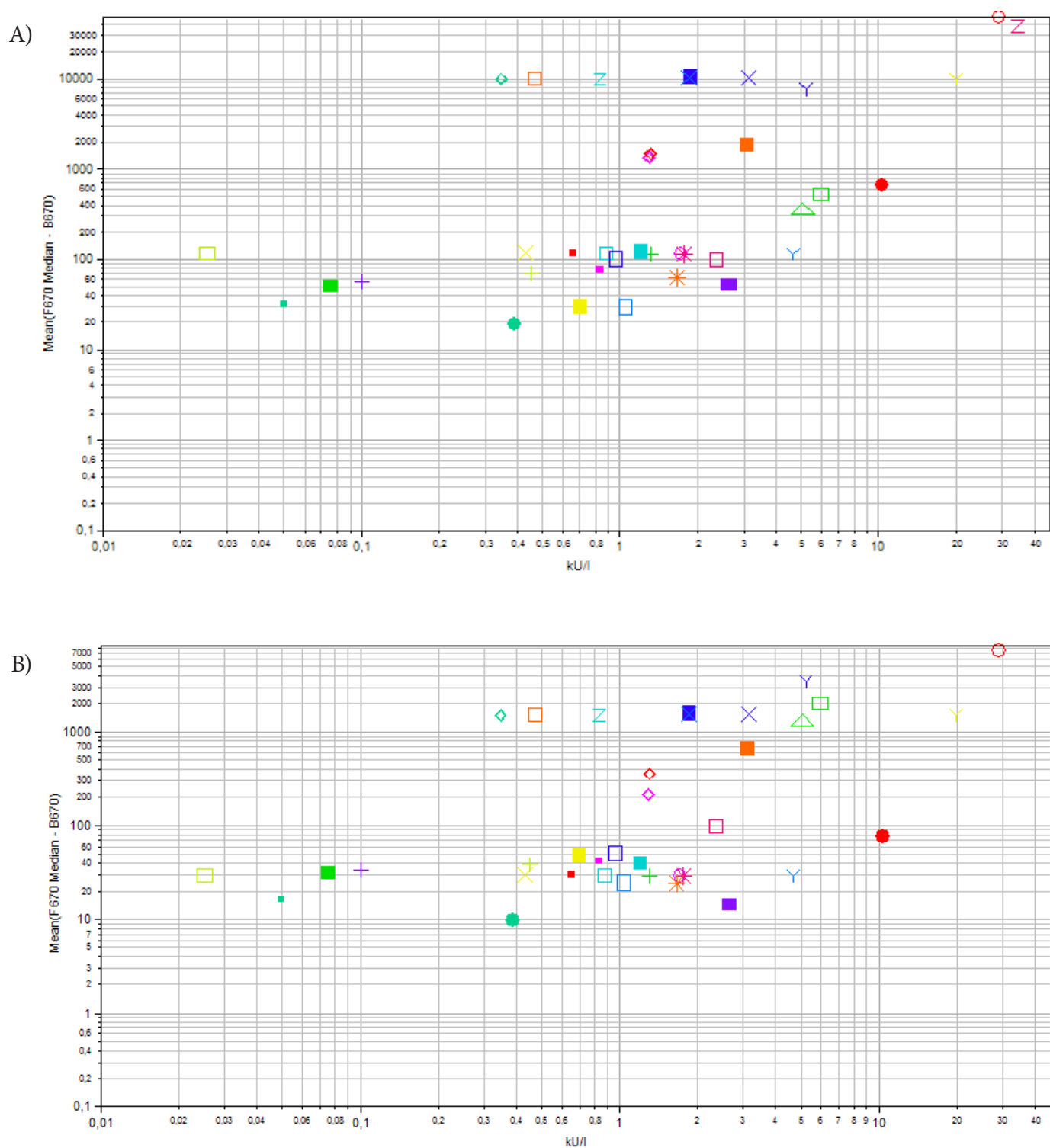


Figure 9. IgE responses to glycinin and β -conglycinin

IgE responses from multiplexed flow immunoassay analysis of soy allergens **A)** glycinin and **B)** β -conglycinin, using serum samples from soy sensitised patients ($\text{kU/l} > 0.35$). The vertical axis shows the signal strength from the glycinin and β -conglycinin spots, respectively. The horizontal axis shows the concentration of IgE directed to soy in the serum samples after analysis using ImmunoCAPTM (kU/l). Each dot represents one serum. The four sera furthest to the left are negative controls. A signal three times higher than the highest negative control was considered positive.

4. CONCLUSIONS

The multiplexed flow immunoassay showed that glycinin and β -conglycinin generated a strong or medium-strong IgE response in the array with almost half of the sera. These responses corresponded with strong and medium-strong signals from the fourth to the ninth soy fractions, strongly indicating that glycinin and β -conglycinin could be found in these fractions. In the future, therefore, I would like to further investigate the allergenicity of these proteins. Since the multiplexed flow immunoassay is a strongly suitable tool for more advanced screenings of larger amounts of sera, I would like to apply this technique to do additional screenings of the soy allergens, using sera with more defined clinical data.

5. ACKNOWLEDGEMENTS

I would like to thank the Allergens group at Phadia AB for giving me the opportunity to do my master's degree project there, and for taking good care of me in the lab. Especially, I would like to thank my excellent supervisor Sigrid Sjölander for her guidance, her help and her encouragement.

Big thanks to Peter Brostedt for performing gel filtration of the soya bean extract, and thanks also to Lars Mattsson and Roland Thunberg for providing soya bean components. A special thanks to Camilla Eriksson for answering all my questions regarding the multiplexed flow immunoassay.

Thank you to all the people who critically read this report and gave me valuable comments and suggestions: Ingrid Holmquist, Karl-Johan Leuchowius, Rick Howard and, especially, my scientific reviewer Jörgen Dahlström.

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