Analysis of soybean-proteins (*Glycine max)* by two-dimensional electrophoresis and MALDI-TOF

Lena Olken

Supervisors:

Sigrid Sjölander

Research and Development

Department of Immunoassay Development

Phadia AB, Uppsala

Pia Larsson

Department of Biomedical Science and Veterinary Public Health

Division of Pathology, Pharmacology and Toxicology

Swedish University of Agricultural Science, Uppsala

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ABSTRACT

Food allergy is a growing problem in the world today, which makes it important to find out which proteins in food that causes allergic reactions. Soybeans are one of the most common food allergens in many countries and little is known about it allergenic properties. There are about 15 known soybean-proteins that have been shown to be allergenic. Most of these are seed proteins and the most common ones are Globulines called Glycinin and β-conglycinin. Other proteins known to be allergenic are Gly m Bd 28, soybean agglutinin (SBA), Kunitz trypsin inhibitor (TI), Gly m 4 and Gly m Bd30.

The main aim of this study was to set up a two-dimensional (2-D) electrophoresis method for separation of proteins in soybean extract. The 2-D technique was used to identify soybean components and especially allergenic components in the soybean extract.

The 2-D electrophoresis method was very useful for separation of proteins. First the proteins were separated during isoelectric focusing according to the proteins isoelectric point. In a second step they were separated in an electrophoresis according to their molecular weight. The gels were then stained with Coomassie brilliant blue (CBB) and proteins could be seen as blue spots on a clear background. To identify the proteins in the soybean extract known soybean components were run separately on 2-D electrophoresis and compared to the gel over the whole soybean extract. To further confirm the identity of allergic components in the soybean extract matrix-assisted laser desorption ionization (MALDI) was used. The soybean extract was also gel filtrated through a Sephadex gel column and collected in 20 different fractions. From these fractions some were chosen for 2-D electrophoresis, these were also compared to the previously gels over the extract.

Results from 2-D electrophoresis showed that glycinin was a very common protein in the soybean extract. The Trypsin inhibitor was also relatively common. SBA, β-conglycinin and Gly m 4 gave rise to fewer spots. In the gel filtrated fractions several of the Glycinin and β-conglycinin were successfully separated. All proteins identified through 2-D electrophoresis were summarized on a gel with the whole soybean extract. To confirm the identity of some of the proteins MALDI-TOF was used. Several Glycinin and β-conglycinin spots could be confirmed and also three other proteins with no known allergenic properties.

The 2-D electrophoresis method in combination with MALDI-TOF was shown to be a very useful combination for identification of proteins.

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ABBREVIATIONS

2-D	Two-dimensional
BFB	Bromphenol blue
CBB	Coomassie brilliant blue
DTT	Dithiothreitol
ESI	Electro spray ionozation
IAA	Iodoacetamid
IEF	Isoelectric focusing
IgE	Immunoglobulin E
IPG	Immobilized pH gradient
kDa	kilo Dalton
LMW	Low molecular weight
MALDI	Matrix assisted laser desorption ionization
MS	Mass spectrometry
Mw	Molecular weight
pI	Iso-electric point
PMF	Peptide mass fingerprint
PR-10	Pathogenesis related 10 proteins
SBA	Soybean agglutinin
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TI	Trypsin inhibitor
TOF	Time of flight

PREFACE

This study is made at the Department of Immunoassay Development, Research and Development, Phadia AB, Uppsala. The study is my graduate project for finishing my studies at the Swedish University of Agricultural Science and to get the degree for Master of Science in Biology. The study is a part of a project at Phadia AB to find out more about proteins with allergenic properties in soybean. Examination of this project was performed at Department of Biomedical Science and Veterinary Public Health, Division of Pathology, Pharmacology and Toxicology at the Swedish University of Agricultural Science in Uppsala.

1. INTRODUCTION

1.1 HYPERSENSITIVITY

Hypersensitive reactions are the results from an inappropriate immune response to harmless substances (antigens). Normally the immune system removes these antigens from the body by various mechanisms without damaging the tissue. For example, different effector molecules create an inflammation response to get rid of the antigen. Sometimes this does not work the right way and the immune response leads to tissue damage or even worse. These reactions to an antigen are called hypersensitivity *(Goldsby el al., 2003).*

There are four types of hypersensitivity responses, the first three types are immediate hypersensitivity witch are caused by antibody or antigen-antibody complexes. In these types the symptoms comes within minutes or hours after contact with the allergen. The fourth type is a delayed hypersensitivity reaction and is first shown days after exposure. The first type is IgE-mediated and includes hay fever, asthma, food allergies and eczema. The second type is IgG-mediated and includes blood transfusion reactions among others. The third type is immune complex-mediated (antigens + antibodies) and among these reactions rheumatoid arthritis is found. The fourth type witch is a delayed reaction includes contact dermatitis and graft reactions *(Goldsby el al, 2003).*

1.1.1 Allergy

When certain types of antigens, referred to as allergens causes hypersensitivity or allergy it induces the first type of response, the IgE-mediated one. It is the IgE-mediated type that is of greatest interest in this work and it will be further described. When an individual is first exposed to the antigen, IgE antibodies are formed and secreted by plasma cells. These antibodies bind to receptors on tissue mast cells and blood basophils. After a second exposure to the same antigen the allergen cross-binds to the IgE-antibodies on the cells. This causes degranulation and histamine release and the tissue are damaged. Normally, the IgE-mediated response is specific towards parasitic infections. However, some people have an inherited tendency that allows IgE to respond towards non-parasitic antigens. These people have a higher amount of circulating IgE-antibodies in their system, making them more vulnerable to allergies such as hay fever and asthma. Common allergens are rye grass, ragweed, penicillin, nuts, beans and seafood, among others *(Goldsby el al, 2003).*

1.1.2 Food allergens

Intolerance to food is a common disorder and is often referred to as food allergy by lay people. In medical terms, food allergy means an immunological disorder involving hypersensitivity to some food. IgE-mediated food allergy is most common among children and young people. Studies show that 6% of young children and around 1-2% of the adult population suffer from food allergy. The most common food allergens are egg, milk, fish, peanuts, tree nuts and soybean (*Björkstén, 2004).* In the United States seafood is the most common cause of allergy reactions to food, and peanut causes most death cases (*Nieuwenhuizen, 2005).* All proteins in a certain food are not allergenic; often only a few proteins give rise to an allergic reaction (*Björkstén, 2004).* Symptoms associated with food allergy range from gastrointestinal discomfort to anaphylactic shock and in the worst cases death (*Nieuwenhuizen, 2005).* It is difficult to treat food allergy and the most useful way of treatment today is avoidance of the food causing the allergy. Avoidance may be easy for some foods but some food proteins are used as additives in different foods where they are not expected. For example milk and egg proteins can be found in sausages and bread, without the food being labelled with either egg or milk (*Björkstén, 2004)*

1.1.2.1 Soybean and its allergenic proteins

Soybean is of great importance in human and animal nutrition. Soybeans contain high amounts of proteins and fatty acids (*Hajduch M et al. 2005*). Soy proteins can for example be used to stabilize oil in water emulsions, for example in soups and sausages. It can also be used for its gel forming functional properties (*Walsh, 2002).*

Among the soy proteins around 15 seed proteins have been shown to be allergenic (*Magni C et al. 2005*). Most seed proteins from soybean that are used in the food industry are classified as globulins or albumins. The globulins have a storage function in the bean (*Walsh, 2002*) and almost half the dry weight of a mature soybean seed is constituted by storage proteins. Two of the most important ones are Glycinin and β-conglycinin (*Hajduch M et al. 2005*). Glycinin and β-conglycinin belongs to the Cupin superfamily of plant food allergens (*Shewry P.R. et al. 2004*). Glycinin can be divided into three types after its different subunits, Glycinin G1 (*Zeece M.G. et al. 1999*), Glycinin G2 (*Helm R.M. et al. 2000*) and last identified Glycinin G4 (*Magni C. et al. 2005*). The Cupin superfamily includes two groups of seed storage proteins called 7S and 11s globulins. Among the Cupin superfamily allergenicity seems to be

restricted to these two components. The Glycinins belongs to the 11S globulins and β-conglycinin to the 7S vicilines (*Magni C et al. 2005*). Also a soy protein called Gly m Bd 28K (*Tsuji H. 1997*) belong to this family. These types of storage proteins are not exclusive for soybean but can also found in peanut and cashew nuts among others.

Another family witch contains several plant food allergens is the prolamin superfamily first identified by Kreis et al. In this family Kunitz soybean TI (*Moroz L.A et al. 1980*) (and Ara h 3 in peanut) can be found (*Shewry P.R. et al. 2004*). It also contains the soy allergens Gly m 2S albumin (*Gu X. 2001*) and Gly m 1 (*Gonzalez R. 1995*).

Gly m 4 (*Kleine-Tebbe J. 2002*), another soy allergen belongs to a group called the pathogenesis related 10 proteins (PR-10). The homolog proteins of this family can be found in a wide range of flowering plants, for example apple, birch and apricot. Most of them have been found to be allergenic (*Breiteneder H. 2000*).

Other proteins in soy recognized as allergens are; Gly m 3 which is a profilin and an actin-binding protein (*Rihs H.P. 1999*), Gly m 2 (*Hoffman D.R. 1993*), a protease called Gly m Bd 30K (*Ogawa T. 1991*) and a lectin called soy bean agglutinin (SBA) (*Baur X. 1996*).

1.2 METHOD BACKGROUND

1.2.1 2-D electrophoresis

The 2-dimensional electrophoresis (2-D), which is a combination of isoelectric focusing (*Bjellqvist B, 1982; O´Farell PH, 1975)* and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (*O´Farell PH, 1975; Laemmli UK, 1970)* is a very useful method for analyses of protein mixtures. It combines separation of proteins according to their isoelectric point (pI) and their molecular weight. Each spot on the 2-D gel corresponds to a protein/protein subunit in the sample. In several studies, for example in one by C. Magni *et al.* the two-dimensional electrophoresis were shown to be a very powerful method for detection of cross-reacting proteins and mapping of potential food allergens in peanut, soybean and lupin.

In the first dimension isoelectric focusing (IEF) separates proteins according to their isoelectric point. All proteins have a net charge which is the sum of all their negative and positive charged amino acid side chains and amino- and carboxyl-terminals. The pI of a

protein is at the specific pH where the net charge is equal to zero. At pH values below a proteins pI the net charge is positive and at values above its pI the net charge is negative. During the isoelectric focusing proteins will migrate towards the position in the pH gradient where their net charge is zero. This leads to a separation of the proteins due to their differences in charge. In the second dimension, SDS-PAGE, proteins are separated according to their molecular weight *(Bjellqvist B, 1982; O´Farell PH, 1975; Laemmli UK, 1970)*. SDS is added to the equilibration buffer to break down the protein structure. The protein chain becomes unfolded and surrounded by negatively charged SDS molecules; this makes the proteins own charge insignificant. Since the protein is elongated it will migrate through the electrophoresis gel depending only of its length. Under influence of an electric field, small proteins run faster than big proteins towards the positive side of the gel. This results in a separation according to molecular weight. Some proteins contain multiple subunits and more than one band can then be seen on the gel (*Biochemistry, 1990)*.

1.2.2 MALDI-TOF

Mass spectrometry (MS) is used in both industry and academia and can provide information, for example about molecular weight, amino acid sequence and oligonucleotide sequence for proteins (*www.astbury.leeds.ac.uk/facil/MStut/mstutorial.htm)*. A mass spectrometry system is built up of an ion source and an analyser with a detector, and a computer to control the equipment.

For analyses of proteins there are two main approaches, the electro spray ionization (ESI) and matrix assisted laser desorption ionization (MALDI). There are also other types of instruments but these two are the most used ones *(Walsh, 2002)*.

The MALDI technique is quite robust and has some tolerance towards buffers and other additives (*www.astbury.leeds.ac.uk/facil/MStut/mstutorial.htm)*.

In a MALDI-TOF system, the MALDI is the ion source and the Time of Flight (TOF) is the analyzer, the whole system is under vacuum. Matrix Assisted means that the sample is co crystallized with a matrix, which is a low mass organic acid, on a MALDI target plate. Laser Desorption Ionization means that a pulsed laser converts the crystallized sample into a high-energy gas phase where the sample molecules are ionized by protons from the matrix. After a few nano seconds the ions are accelerated by an electric potential into the TOF analyzer. In

the TOF the ions fly towards the detector in a field free drift. When ions with the same charge (number of protons) but with different molecular mass is accelerated by the same potential a large ion is going to get a longer flight time than a small one. The time of flight is proportional to the molecular mass.

Peptide mass fingerprinting (PMF) is a method used to identify proteins through a unique fingerprint. A protease that cleaves proteins on specific sites is used to digest proteins into smaller peptides. When the digestion is completed the protein has been cleaved and a set of peptides are produced. These peptides have varying masses and the fingerprint is characteristic of that protein. The fingerprint can then be used to identify the protein by searching data already present in different databases (http://www.aber.ac.uk/~mpgwww/Proteome/MS_Tut.html).

MS/MS is a method that can be used to decide the amino acid sequence of a protein. A peptide mass from the first PMF-spectra is chosen and the fragments from only that peptide are analysed. The MS/MS technique used in MALDI-TOF is called Post Source Decay (PSD).

The mascot search engine searches among sequence databases, for example, NCBInr which maintains composite, non-identical protein and nucleic acid databases for their search tools BLAST and Entrez, SwissProt which give information about the function of a protein, its domains structure, post-translational modifications, variants, etc, and MSBD which is a non-identical protein sequence database *(www.matrixscience.com*).

Mass spectrometry can also be used to generate protein sequence data; such as in MS/MS. Mass analysis of protein fragments can yield a nearly complete sequence *(Walsh, 2002).* The sequence can then be used to identify the protein in the Mascot search engine *(www.matrixscience.com).*

2. AIM OF THE STUDY

The major aim of this study is to set up a 2-D electrophoresis method to separate the proteins in the soybean extract. The 2-D technique will then be used to identify selected soybean components and specifically soybean allergenic components in the soybean extract. The second purpose is to use MALDI-TOF for confirmation of the identity of selected proteins.

Finally the technique will be used for identification of soybean allergenic components in soybean extract.

3. MATERIAL AND METHODS

3.1 MATERIAL

Soybeans were purchased from Allergon AB, Ängelholm, Sweden, where they had been grounded and freeze dried. Glycinin and β-conglycinin purified from soybean were kindly provided by R. Thunberg on Phadia AB in Uppsala, Sweden. Soybean agglutinin was bought from Vector laboratories (distributed by Immunkemi F&D AB, Järfälla, Sweden) and trypsin inhibitor from Sigma Aldrich, Stockholm, Sweden. Gly m 4 and Gly m Bd 30 recombinantly produced in *Escherichia coli* were kindly provided by Jonas Lidholm and Lars Mattsson, Phadia AB. A RAST-buffer nr5/tw and a radio labelled tracer was also obtained from Phadia AB. PD-10 columns, a Sephadex 200 gel column, NAP columns, PhastGel Blue R and low molecular marker (LMW) were purchased from GE Healthcare, Uppsala, Sweden. Slide-a-lyzer dialysis cassettes were bought from Pierce (distributed by Nordic biolabs AB, Täby, Sweden), filtration centrifugal devices from Pall (distributed by Colly Filtreringsteknik, Kista, Sweden) and BCA protein assay kit from Pierce (distributed by Nordic biolabs AB, Täby, Sweden). Human sera with known IgE-reactivity to soybean were obtained from the Phadia sample unit (DSU) at Phadia AB (Uppsala, Sweden).

Other chemicals in the study were of analytical grade and obtained from regular commercial sources.

3.2 EXTRACTION OF SOYBEAN

Soybean was extracted in a phosphate based extraction buffer with neutral pH. The solution was then centrifuged at 5700 rpm for 30 minutes followed by filtration and buffer exchange. The protein concentration was determined by using a BCA protein assay kit. After buffer exchange into water on PD-10 columns the extract was freeze-dried.

3.3 GEL FILTRATION OF SOYBEAN EXTRACT

Extracted, centrifuged and filtrated soybean was run on a Sephadex 200 gel column and the extract was separated into twenty different fractions (kindly provided by P. Brostedt, Phadia AB). Some fractions were selected for analysis in two-dimensional electrophoresis. The fractions used in this study were named A5-A13.

3.4 TWO-DIMENSIONAL ELECTROPHORESIS

3.4.1 Sample preparation for 2-D separation

It is necessary for the samples to be diluted in rehydration buffer (see table 1) for more accurate results in the isoelectric focusing (table 1). Buffer exchanges were done using three different methods. On Glycinin and fraction A5 and A6 the exchange were done using gel filtration on NAP columns. The β-conglycinin buffer was exchanged to rehydration solution using a slide-a-lyzer dialysis cassette. Filtration through centrifugal devices was used to change the buffer in Gly m 4, Gly m BD30K and the rest of the fractions. All the buffer exchanges were done according to the manufactures instructions. The freeze-dried soybean extracts was dissolved in rehydration solution. SBA was dissolved in milli-Q water before diluted in rehydration buffer.

Table 1. Dilutions schedule. The dilutions were made before run on the first dimension in the 2-D electrophoresis.

Sample	**Concentration after dilution (mg/ml)**
Soybean	0.48
Glycinin	0.40
TI	0.40
β -conglycinin	0.24
SBA	0.24
Gly m 4	0.24
Gly m BD30K	0.24
A4	0.08
A5	0.25
A6	0.25
A7	0.24
A8	0.24
A9	0.24
A10	0.24
A11	0.24
A12	0.24
A13	0.24

3.4.2 First dimension

According to the manufactures standard method *(GE Healthcare)* for 2-dimensional electrophoresis the Immobiline dry strips (pH 3-10) were soaked in rehydration solution (8% Urea, 2% Triton X-100 and 0.002% Bromophenol Blue (BFB)) before isoelectric focusing. Right before start of the focusing 0.5% immobilized pH gradient (IPG) and 0.2% Dithiothreitol (DTT) was added to the rehydration solution. IPG was added to eliminate potential background staining and DTT is a reducing agent. To this rehydration mixture the prepared sample (see above) was added. The rehydration mixture with sample was loaded into a strip holder on an Ettan IPGphor II Isoelectric Focusing System (*GE Healthcar)*. On the 7 centimetres strip holder used in these studies, 125 µl samples were loaded. The IPG strips were added to the holder with the anodic part (+) towards the pointing edge. The strips were then covered with Dry Strip Cover Fluid (*GE Healthcare)* to avoid dehydration during the rehydration period (10-20 hours). The 7 centimetres strips were then focused in three steps; step 1; 500V, 0.5h, 0.25kVh, step 2; 1000V, 0.5h, 0.5kVh and step 3; 5000V, 1h and 40min, 7.5kVh. After the isoelectric focusing the strips were equilibrated for 2x15 minutes in an equilibrium buffer which consist of 75mM Tris-HCl, 6M Urea, 30% Glycerol, 2% SDS and

0.002% BFB. During the first equilibrium step 100 mg DTT was added to the buffer and in the second equilibrium step 250 mg Iodoacetamid (IAA) was added.

3.4.3 Second dimension

In the second dimension the 1-dimensional electrofocused strips were placed on a SDS 2-D gel placed on a Multiphor II Electrophoresis system (*GE Healthcare)*. Each strip has a small sample application piece under both its ends. These pieces collect water during the electrophoresis. The LMW was loaded on application pieces. On top of the gel, buffer strips were placed. The gel was first run for 40 minutes at 600V; 20mA; 30W, then the strips and sample application pieces were removed and the cationic buffer strip was placed over the area where the strips were placed. During the second part the gel was run for one and a half hour at 600v; 40mA; 30W. After the electrophoresis the gel was ready for detection by staining or blotting.

3.5 IMMUNOBLOTTING

Immunoblotting was performed according to the standard method at Phadia. After 2-D separation of the proteins they can be transferred to a nitrocellulose membrane. It is easier to handle a membrane than a gel and small amount of samples are more easily detected by antibodies on a membrane than on a thick gel. To avoid non-specific binding of antibodies to the membrane the free protein-binding sites of the gel is usually blocked. Human sera with a known reactivity to soybean were added to the blot, IgE antibodies in the sera were bound to antigen on the membrane. Secondary I^{125} labelled antibodies specific for the IgE antibodies were added and the antigen-IgE complexes were visualized on a film.

3.5.1 Transfer of protein onto nitrocellulose

It takes several steps to transfer the proteins from the gel into a nitrocellulose membrane. First anionic and cationic plates were soaked in process water before placed on a Multiphor II unit. Six electrode papers were wet in anionic I solution (18.17g Tris, 100ml methanol Milli-Q water up to 500 ml) and placed on the anionic plate. Air was removed by rolling a glass tube over the papers. At the site were the samples were loaded, one centimetre of the 2-dimensional gel was cut of. The gel was then equilibrated in anionic I solution for seven

minutes and removed from its support film with help from a filmremover. Nitrocellulose membrane was cut into proper size and wet in anionic II solution (1.52 g Tris, 100 ml Methanol and Milli-Q water up to 500 ml). The membrane was placed on top of the gel; the frontline from the samples was marked on the membrane. Three more electrode papers were wet in anionic II solution and placed on the "sandwich". To this sandwich the gel and nitrocellulose membrane were added with the membrane placed down towards the anionic plate. The support film was carefully removed from the gel and six electrode papers wet in cationic solution (2.6 g 6-aminohexanoic acid, 100 ml Methanol and Milli-Q water up to 500 ml) were placed on top of the gel. Further, three more electrode papers were wet in cationic solution and placed on the sandwich. Finally, air was removed and the cationic plate placed on top of the sandwich. The blotting was run for one hour at 10V; 200mA; 5W. After the blotting the membrane was removed from the gel and electrode papers. If LMW was used, that part of the gel was cut of and stained with amidoblack. The rest of the membrane was blocked in a RAST buffer number 5/tween solution for 0.5-3 hours.

3.5.2 Binding of human samples to soybean proteins

Human serum samples were diluted at least 1.25 times in RAST buffer nr 5/tw. The nitrocellulose membranes were cut down as much as possible. For incubation with serum, small plastic bags were made in proper sizes. The membranes were placed in the plastic bags with the serum and the bags were sealed. The bags were placed on a shaking table in room temperature over night. Next day the membranes were washed 3x5 minutes in washing solution (9 g NaCl, 1000 ml Milli-Q water and 5 ml Tween20). Antigen-IgE complexes were detected after four hour incubation with anti-IgE (CAP RAST RIA- I^{125} labelled tracer, *Phadia AB*) diluted 1:4 in RAST buffer nr 5/tw. After incubation the membranes were further washed (3x5 minutes in washing solution and 3x5 minutes in water) before drying and detection on film.

3.5.3 Detection of IgE-binding protein

The dry membranes were put onto a paper that was marked after the size of the membranes and the LMW. The paper with the membrane was then put into a cassette with the sample side up. In a dark room a film was put on top of the membrane and the cassette was incubated in a -70^{o}C freezer for about 72 hours. After incubation the film was developed in dark room.

3.6 COOMASSIE BLUE STAINING

Coomassie Brilliant Blue (CBB) staining is a method for detection of proteins (*Shevchenko et al. 2006)*. CBB binds non-specifically to almost all proteins. During the staining the gel is soaked in CBB solution, and after the destaining step, proteins can bee seen as blue bands on a clear background *(GE Healthcare).*

Staining was performed using Phadia standard method and a staining/destaining machine (*GE Healthcare*), see table 2. The following solutions for the staining and destaining was used; Coomassie stem solution (one tablet PhastGel Blue R), 80 ml Milli-Q water and 120 ml ethanol), Coomassie working solution (135 ml fix solution, 5 ml HAc, 20 ml Coomassie stem solution and 40 ml Milli-Q water), Fix solution (400 ml ethanol, 100 ml HAc up to 1000 ml with Milli-Q water), Destaining (250 ml ethanol and 80 ml HAc up to 1000 ml with Milli-Q water) and Storage solution (250 ml ethanol, 80 ml HAc and 153 ml glycerol (86%) up to 1000 ml with Milli-Q water). Tubes for input and output were placed according to the protocol for the automatically Coomassie Blue staining/destaining protocol.

Table 2. Protocol for CBB staining performed using a Phadia standard method.

Step	Time (min)	In-prot	Out-prot	Solution	Amount of solution (ml)
1	20	1	9	Fix	175
2	2.0	2	9	Destain	700
3	60	3	3	CBB	175
4	10	2	9	Destain	175
5	30	2	9	Destain	175
6	30	2	9	Destain	175
7	30	4	4	Storage	175

3.7 CALCULATIONS OF THE 2-D RESULTS

To calculate the isoelectric point (pI) of each spot on the gels, a diagram from GE Healthcare was used (*www.gehealthcare.com)*. The distance (in centimetres) from the left side of the gel to the spot was measured. The measured distance was then divided with 1/7 = 0.143 (the length of the gel was 7 cm). This calculation gives a percent value. The percent value can then be compared with the diagram and the pI of that spot can be read in the diagram.

3.8 MALDI-TOF

2-D gel with the proteins of interest was stained with CBB. Spots, which identity had to be further confirmed were the ones of interest. These were picked one and one and put into an eppendorf tube. To every tube 30 µl of 50 mM NH_4HCO_3/ Acetonitrile (1+1) was added. The gel pieces were washed for 15 minutes in this solution. The liquid was then removed and 10 µl of acetonitrile was added, and then removed after the gelpieces had whitened. To rehydrate the gel pieces, 10 µl 50 mM NH_4HCO_3 was added and after 5 minutes 10 µl acetonitrile was added to a total volume of 20 µl. After 15 minutes of incubation the liquid was removed and 10 µl of acetonitrile was added. When the gel pieces had shrunk the acetonitrile was removed and all the washing steps were repeated. After the second time of washing the gel pieces were dried in a vacuum centrifuge. Thereafter, an enzyme solution of 50mM NH_4HCO_3 with 20 ng/µl of trypsin was added to every tube. The gel pieces in enzyme solution were then incubated over night at 37^{o}C.

The next day the peptides were extracted by sonification for 10 minutes. The peptides were then mixed with a matrix (a-cyano-4-hydroxycinnamic acid + (0.1% TFA, AcN/10mM $NH_4H_2PO_4$)) and put on a special MALDI target plate. For calibration a peptide calibration standard was used. The sample plate with the peptides and matrix was then loaded into the MALDI TOF. In some cases the MS/MS method was used to further identify the proteins according to their sequence. The results were then compared to databases with the Mascot search engine. In this case protein scores above 65 were significant on a PMF search and in a MS/MS search individual ions scores above 34 indicate identity or extensive homology. Theese values can vary, depending on how the search is performed.

4 RESULTS

4.1 OPTIMIZATION OF EXTRACT CONCENTRATION

To optimize the 2-D electrophoresis for the soybean-extract it was necessary to determine the optimal sample concentration. Two-dimensional electrophoresis was performed using different sample concentrations. Concentrations of 0.48 mg/ml, 0.4 mg/ml and 0.32 mg/ml were tested. The gel in figure 1 show that there were no big differences between the used concentrations, but the 0.4 mg/ml concentration had some sharper spots than the other

concentrations. Therefore most samples used in this study were diluted to a concentration of around 0.4 mg/ml. However some samples having a concentration below 0.4 mg/ml were diluted with rehydration buffer, but as little as possible.

Also a 2-D electrophoresis was done to compare if age of urea had some effect on the results. Both new urea (Sigma) and old urea (Merck) were diluted to the same concentrations. No differences, such as repeated spots, could be seen on the two gels.

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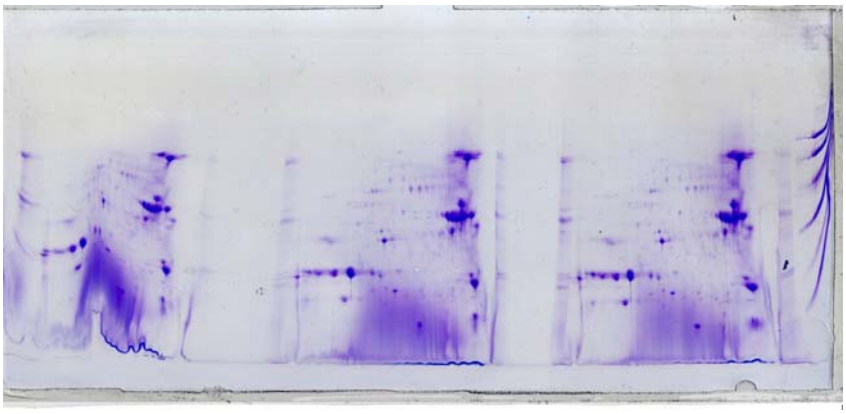


Figure 1. CBB stained 2-D electrophoresis of three different concentrations of the soybean extract. To the left 0.32 mg/ml, in the middle 0.4 mg/ml and to the right 0.48 mg/ml. PH gradient from right to the left (10-3) on each concentration and the molecular weight is decreasing down in the picture.

4.2 TWO-DIMENSIONAL ELECTROPHORESIS OF SOYBEAN EXTRACT AND SOYBEAN PROTEINS

The concentration optimization in figure 1 resulted in that 0.4 mg/ml was chosen to be the optimal concentrations. At this concentration several soybean proteins can be seen.

4.2.1 Trypsin inhibitor

The soybean specific TI purchased from Sigma was run at 0.4 mg/ml and the spots were compared to the spots from the soybean extract. Spots representing TI were concentrated to four areas, one at 40-45 kDa and pI at 5.5-7.2. The second areas were at 22 kDa with pI at 4.3 and 8.3-9.1, the third area was found at 16 kDa with a pI at 4.2-4.8 and a spot at 50 kDa with pI 4.8. Altogether eight TI corresponding spots could be found on the 2-D electrophoresis from the whole soybean extract (figure 2 and table 3). However, one of the spots had no corresponding spot on the 2-D gel of the whole soybean extract (figure 5 and table 8).

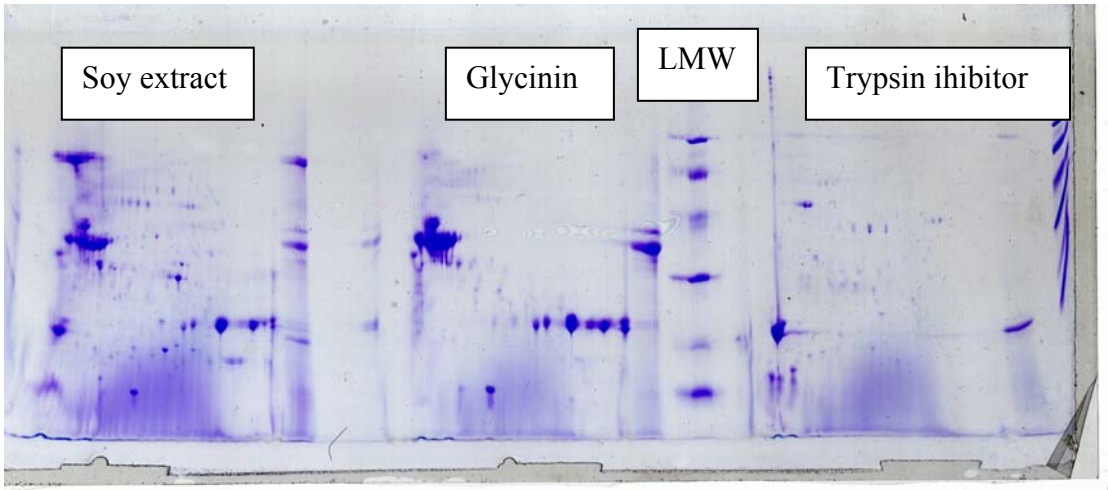


Figure 2. CBB stained 2-D electrophoresis. To the left the whole soybean extract, in the middle the glycinin sample, then LMW and to the right Trypsin inhibitor. The pH gradient from left to the right (3-10) for each sample and the molecular weight is decreasing down in the figure.

Table 3. The pI-values and molecular weights (Mw) of the spots from the TI. The right column in the table is the number of the corresponding spot on the gel over the whole soybean extract in figure 5 and table 8.

Estimated pI	Estimated Mw (kDa)	Spot Id
8.3-9.6	22	24
7.1	45	16
7.2	45	16
5.5	40-45	17
5.8	40-45	17
6.0	40-45	17
4.3	22	18
4.2-4.8	16	19
4.8	50	-

4.2.2 Glycinin

Glycinin purified from soybean extract were run at 0.4 mg/ml and the spots were compared to the spots from the soybean extract. The spots representing Glycinin proteins are mainly concentrated to three areas on the gel. One area with a molecular weight around 40-45 kDa and pI around 4.6 and 8.7. The second area is between 23-25 kDa with pI values ranging from 6.2-8.1. There are also one spot at 15 kDa with a pI at 5.6. Altogether, fourteen spots were found on the gel (figure 2 and table 4) and all had corresponding spots on the 2-D gel from the whole soybean extract (figure 5 and table 8).

Table 4. The pI-values and molecular weight of the spots from the glycinin gel. The column on the right represents the number of the corresponding spot on the gel over the whole soybean extract, figure 5 and table 8.

Estimated pI	Estimated Mw (kDa)	Spot Id
4.8	45	1
4.6	40	2
4.6	37	3
4.7-5.2	40	4
5.2	32	5
8.7-9.6	42	6
8.7-9.6	40	7
6.5	23-25	8
6.9	23-25	9
7.3	23-25	10
7.9	23-25	11
8.1	23-25	13
5.6	15	14
6.2	23-25	15

4.2.3 Soybean agglutinin

Soybean agglutinin purchased from Vector was run at 0.4 mg/ml and the spots were compared to the spots from the soybean extract. SBA proteinspecific spots are mainly concentrated in one area at 40 kda and pI at 4.7-5.7. There is also one spot at 20 kDa with a pI at 7.3. Two spots were found on the gel (figure 3 and table 5) and both had corresponding spots on the 2-D gel from the whole soybean extract (figure 5 and table 8).

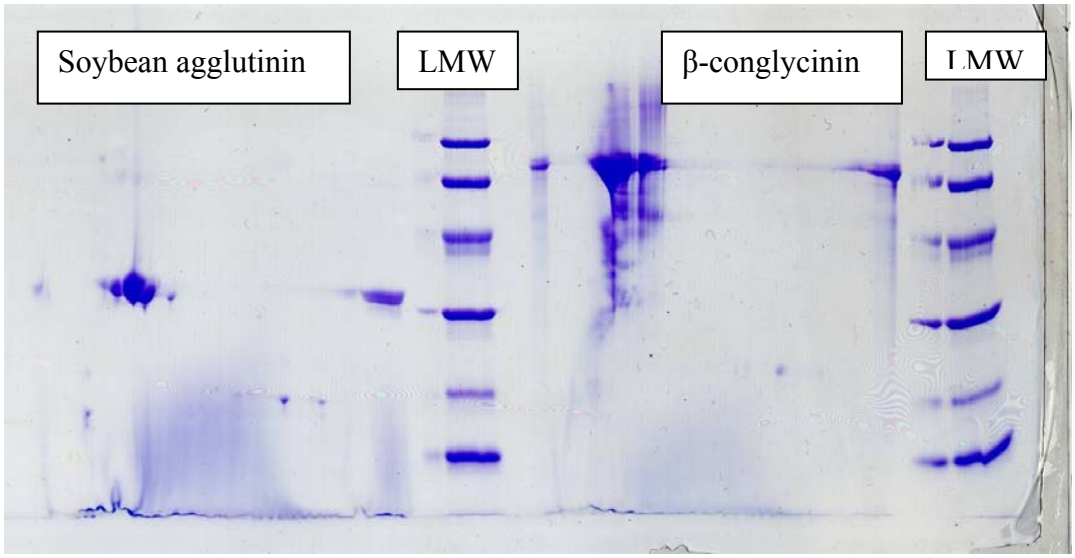


Figure 3. To the left SBA is loaded, then LMW, β-conglycinin and to the right LMW. The pH range from left to the right (3-10) and the molecular weight decreases down in the picture.

Table 5. The pI-values and molecular weight of the spots from SBA. The right column is the number on the spot that represent it on the gel over the whole soybean extract, figure 5 and table 8.

Estimated pI	Estimated Mw (kDa)	Spot Id
4.7-5.7	40	20
7.3	20	21

4.2.4 β-conglycinin

Beta-conglycinin purified from soybean extract was run at 0.4 mg/ml and the spots were compared to the spots from the soybean extract. β-conglycinin specific spots were found at 80-90 kDa with pI at 4.5-5.3 and 8.1-8.8, see figure 3 and table 6. Both spots had corresponding spots on the 2-D gel over the whole soybean extract (figure 5 and table 8).

Table 6. The pI-values and molecular weights of the spots from β-conglycinin. The right column is the number of the corresponding spot on the gel over the whole soybean extract, figure 5 and table 8.

Estimated pI	Estimated Mw (kDa)	Spot Id
4.5-5.3	80-90	22
8.1-8.8	80-90	23

4.2.5 Gly m 4

Recombinantly produced Gly m 4 (rGly m4) were run at 0.4 mg/ml and the spots were compared to the spots from the soybean extract. The spots on the rGly m 4 gel were found at molecular weights ranging from 14 kDa to above 20 kDa with pI between 4.9-6.3, see figure 4 and table 7. No corresponding spots were found on the 2-D gel representing the whole soybean extract (figure 5).

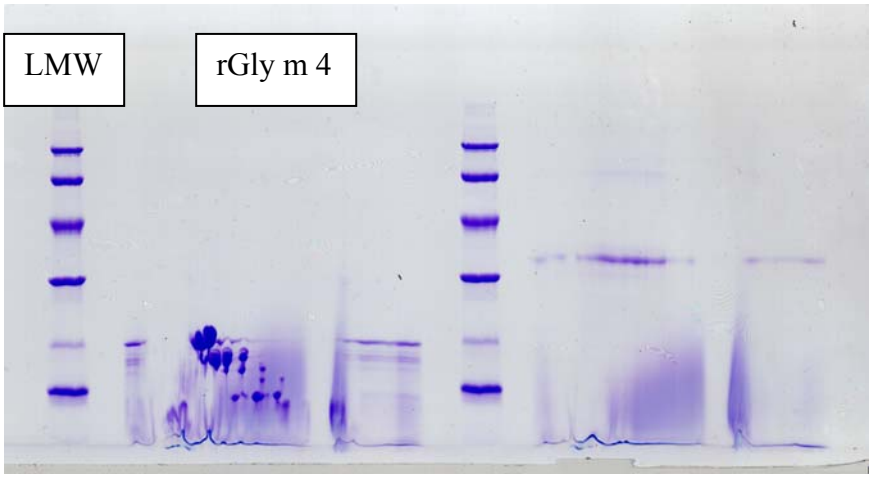


Figure 4. CBB stained 2-D electrophoresis. To the left LMW and then rGly m 4. The pH gradient from the left to the right (3-10) for each protein and the molecular weight decreases down in the picture.

Table 7. The pI-values and molecular weight of the spots from rGly m 4.

Estimated pI	Estimated Mw (kDa)
4.9-5.1	20
5.1	16-18
5.4	16-18
5.8	16-18
6.0	15-16
5.5	14
6.0	14
6.3	14

4.3 IDENTIFIED PROTEINS ON A SOY-MAP

A summary of the proteins identified with 2-D electrophoresis is marked with numbers on a gel-map over the whole soybean protein and shown in figure 5 and table 8. The colours on the arrows are specific for each protein group and the numbers correspond to a specific protein spot that can bee found in table 3-6.

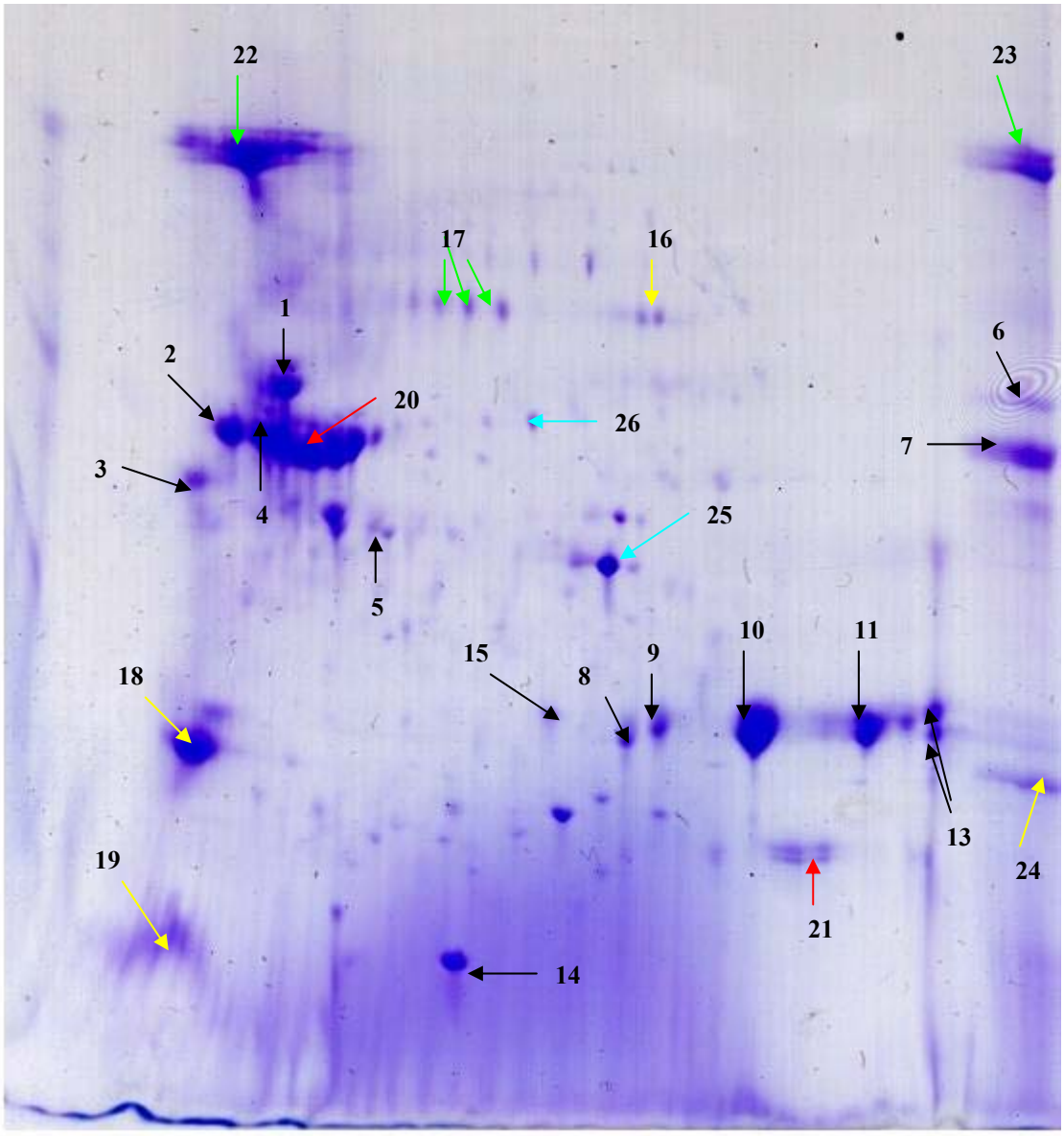


Figure 5. CBB stained 2-D electrophoresis over the whole soybean extract. The identified proteins from 2-D electrophoresis are marked with arrows and numbers. The colour of the arrow represents a specific group of proteins. Black = glycinin, Yellow = TI, Read = SBA and Green = β-conglycinin. The blue arrows represent spots that were picked for identification with MALDI-TOF.

Table 8. Summary of the spots identified through 2-D electrophoresis.

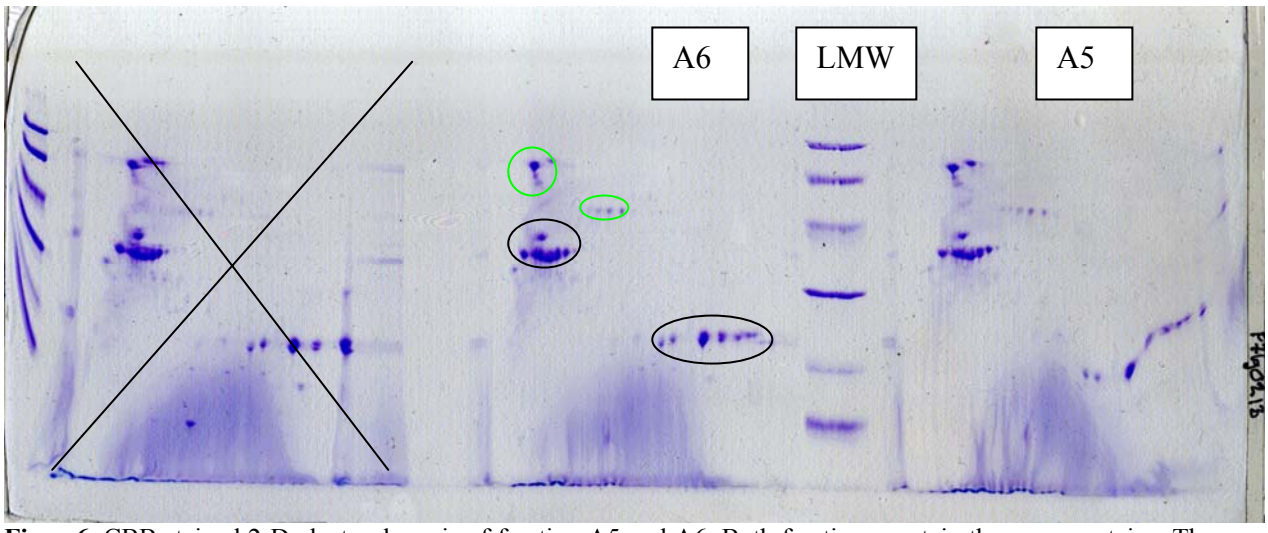
Protein	Spot Id
Glycinin	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 14, 15
β-conglycinin	22, 23
TI	16, 17[a], 18, 19
SBA	20, 21

[a] Identification by 2-D showed that the spots numbered with 17 were TI, further analysis thorough MALDI-TOF identified these spots as β-conglycinin.

4.4 GEL FILTRATED SOYBEAN EXTRACT

Fractions from gel filtrated soybean extract were run on 2-D electrophoresis to identify which protein each fraction contained. In a gel filtration the biggest proteins comes out first, while smaller proteins get stucked in the gel-beads and come out later. The results for the fractions were compared with the results for the whole extract (figure 2).

When results shown in figure 6 were compared with the whole soybean extract it could be seen that fraction A5 and A6 contained Glycinin and β-conglycinin, which both have a high molecular weight.



Figur 6. CBB stained 2-D electrophoresis of fraction A5 and A6. Both fractions contain the same proteins. The gel on fraction A5 has a "smiling effect" which means that the proteins have not migrated propertely. Both fractions contain glycinin (black) and β-conglycinin (green).

Fraction A7 and A8 (figure 7) contained both Glycinin (black). Beta-conglycinin (green), which was clearly seen in fraction A6 was also found in less amounts in fraction A7 but decreases further and could not be found in fraction A8.

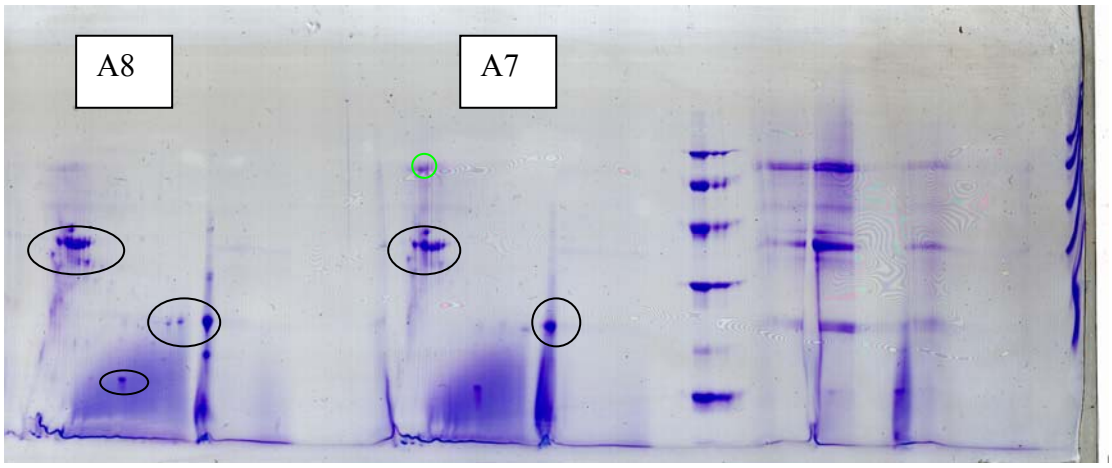


Figure 7. CBB stained 2-D electrophoresis of fraction A7 and A8. Fraction A7 and A8 are a lot like each other, but β-conglycinin can bee found in fraction A7 and not in A8. Some Glycinin spots can bee seen first in fraction A8.

The 2-D gels representing fraction A9 and A10 showed weak staining and few spots, see figure 8. In fraction A10 one spot can be seen, maybe representing the Trypsin inhibitor.

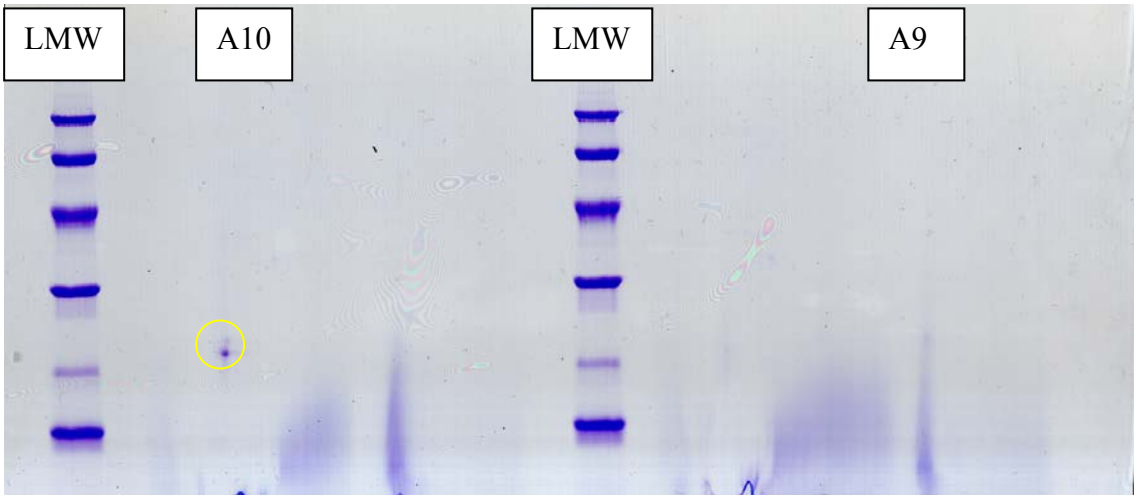


Figure 8. CBB stained 2-D electrophoresis of fraction A9 and A10. Only one spot can be seen in fraction A10, the values of the spot corresponds with Trypsin inhibitor.

Fraction A11, A12 and A13 can bee seen in figure 9, A11 and A12 are a lot like each other and contain Trypsin inhibitor and some unidentified proteins. In A13 further spots of TI can

bee found (figure 9). The unidentified spot in A11 and A12 was identified using MALDI-TOF analysis (see section MALDI-TOF).

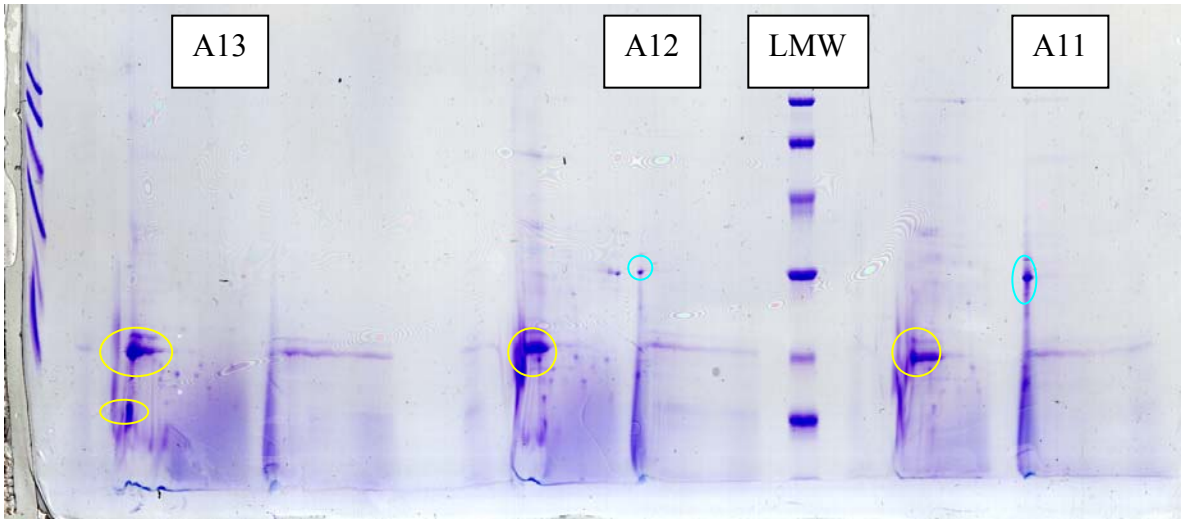


Figure 9. CBB stained 2-D electrophoresis of fraction A11, A12 and A13. All three fractions contain different amount of TI (yellow). Fraction A11 and A12 also contain one sharp spot that was unidentified.

The results from the 2-D electrophoresis on the fractions are summarized in table 9. SBA could not bee found in any fraction.

Table 9. Summarize of soybean allergenic proteins found by 2-D electrophoresises in fractions A4-A13.

Fraktion	Glycinin	TI	SBA	β-conglycinin
A5	x			x
A6	x			x
A7	x			x
A8	x			
A9				
A10		x		
A11		x		
A12		x		
A13		x		

4.5 IDENTITY CONFIRMATION OF 2-D IDENTIFIED PROTEINS

MALDI-TOF was used for confirmation of the identity of proteins identified with 2-D electrophoresis from the whole soybean extract (figure 5 and table 8). There was not enough time to confirm the identity of all spots so twelve spots were chosen. The peptide mass fingerprint (PMF) method confirmed the identity on some proteins. On others MALDI-

MS/MS had to be performed and some spots did not show any match. Table 10 show the results from the MALDI experiments.

Table 10. Selected spots on the soy-map were picked for MALDI analysis. The table summarize which MALDI method that was chosen for identification of the spots as well as values and score for each identified protein.

Spot on the gel-map (figure 5)	**Method for identification**	**Identified protein with soybean origin**	**pI/Mw (kDa) (MALDI)**	**Score[a]**
1	PMF	Glycinin	5.4/58.5	124
2	PMF	Glycinin	4.3/24.3	206
10	PMF MALDI-MS/MS	Glycinin	5.8/56	80
14	PMF MALDI-MS/MS	Glycinin	-/64.2?	65
16	-	-	-	-
17	PMF	β-conglycinin	-	>180
18	PMF	Trypsin inhibitor	-	174
19	-	-	-	-
21	PMF MALDI-MS/MS	AB0084260 NID	9.8/47.9	-
22	PMF	β-conglycinin	4.8/63.2	262
25	PMF	Maturation-associated protein MAT9 alt. Dehydrin-like protein	6.1/ 23.7	170
26	PMF	35 kDa seed maturation protein	5.9/ 35	145

[a] Scores above 65 are significant in PMF, in MS/MS scores above 34 indicate identity or extensive homology.

4.5 IMMUNOBLOTTING

Immunoblotting of 2-D gels was used to show that the technique could be used for identification of soybean specific allergens. Human sera sensitized to soybean proteins (IgE) were used for the identification. The results showed IgE binding to β-conglycinin with serum 27702, see figure 10. Serum 26540 did also show IgE binding but the protein could not be identified.

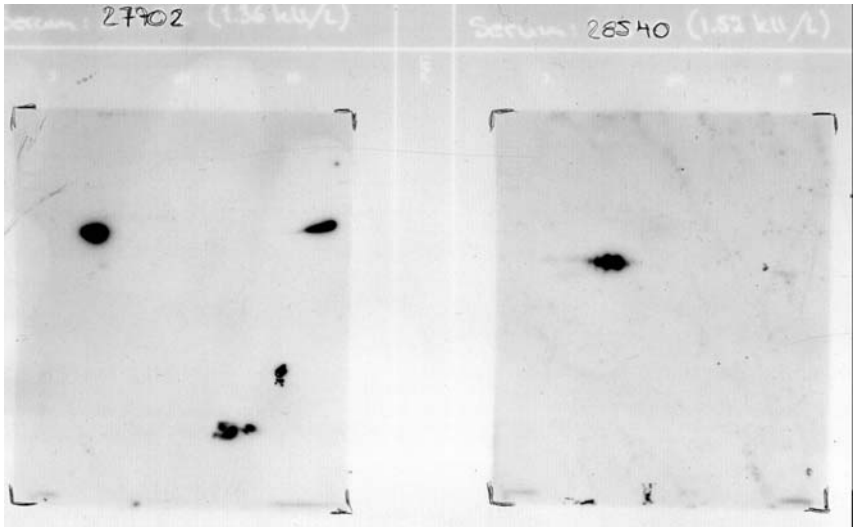


Figure 10. Immunoblotts incubated with human sera 27702 and 26540 containing IgE. The black spots represent the IgE binding to soybean protein.

5 DISCUSSION

The main aim of the study was to set up a method for two-dimensional electrophoresis to separate proteins in soybean extract. In developing the method different soybean sample concentrations and components were tested. While testing different sample concentrations it could be seen that by using concentrations between 0.12-0.48 mg/ml it was possible to run 2-D electrophoresis with clear visible results. However, 0.4 mg/ml was chosen to be the standard concentration in further studies. The 2-D gels that were not used for immunoblotting were stained with CBB. It is possible that more spots could have been found on the gels if stained with silver instead.

In previous studies on Phadia AB it has been found that some spots on 2-D gels were repetitions of the same protein. One possible explanation to this could be the Urea component in the rehydration buffer. If the Urea is old or heated above 30^{o}C a Urea breakdown product (iso-cyanate) can covalently modify the protein and change its isoelectric point. This is why the comparison of the old and new Urea was done. The results showed no visible effect on the spots in this study.

After the 2-D electrophoresis was set up and the sample concentrations decided, the already identified soybean allergenic proteins Glycinin, β-conglycinin, TI, SBA and Gly m 4 were run. The pI-values and molecular weights in the results were compared with results from the whole soybean extract and in some cases with published data found by Hajdush *et al*.

In this study the pI-values for Glycinin were 4.6-5.6 and 6.2-9.6 and the molecular weight 23-25 kDa and around 40 kDa, one spot could be found at as low as 15 kDa. From the purified Glycinin altogether fourteen spots could be identified on the whole soybean extract by 2-D electrophoresis. Four of these spots were also identified as Glycinin by MALDI-TOF (PMF and MS/MS). Glycinin is a storage protein in soybean and can therefore be expected in great amounts.

Soybean agglutinin could only be found as two spots. One spot was found at pI 4.7-5.7 with a molecular weight at 40 kDa and one spot with pI 7.3 with a molecular weight at 20 kDa. Both spots were found in the whole soybean extract. Compared with published values the SBA in this study had a much lower molecular weight but corresponding pI (*Hajduch et al.)*. Spot 21 identified as SBA by 2-D electrophoresis was picked for MALDI-TOF but showed to be another soybean protein, not recognized as a soybean allergen.

Subunits of Trypsin inhibitor was found at pI 4.2-4.8 and 5.5-9.6 with molecular weights around 20 kDa and 45 kDa. Eight spots had corresponding spots in the whole soybean extract. One spot at 50 kDa could not be found in the whole soybean extract. The molecular weight at 20 kDa corresponded well with published values by Hajduch but not the pI or the higher molecular weights. MALDI-TOF (PMF) confirmed the identity of spot 18 to be TI. The spots numbered 17 were also picked for MALDI-TOF. Two-dimensional electrophoresis identified these spots as TI but MALDI-TOF identified them as β-conglycinin. The origin of the spot that could not be found in the soybean extract and the three spots numbered 17 could be TI subunits not present in the soybean extract or, probably more likely, impurities in the TI product from Sigma.

In the 2-D electrophoresis for β-conglycinin two spots could be identified on the 2-D from the whole soybean extract, one spot with pI 4.5-5.3 and one with 8.1-8.8. Both spots had molecular weights at 80-90 kDa. MALDI-TOF (PMF) identified spot 22 as β-conglycinin. However, the three spots (17) initially thought to be of TI origin but later on identified as β-conglycinin using MALDI-TOF were missing in the purified protein. If these subunits are of importance for the structure and/or function for the protein needs to be further evaluated.

The gel over recombinant produced Gly m 4 showed several spots with molecular weights ranging from 14-20 kDa. The correct molecular weight of biologically active Gly m 4 is around 17 kDa (*Klein-Tebbe et al., 2002)* indicating that the spots were found in the right area of the gel. No corresponding spots were found on the 2-D gel from the whole soybean extract.

This may either indicate that Gly m 4 is not present in the soybean extract or that the native form of Gly m 4 differs from the recombinant one.

The results from the studies of the fractionated soybean extract show that the proteins have been successfully separated. The large storage proteins Glycinin and β-conglycinin are built up by several subunits with protein masses in the range 150-450 kDa and came out early in the fractions A5-A7. Glycinin can also be found in fraction A8. The smaller protein TI came in the later fractions A10-A13. In fraction A9 no spots could be seen and in A10 only one spot (TI). These two fractions had a much lower protein concentration than other samples (personal communication with Anna Howard, Phadia AB), so that is most likely the explanation why no proteins could be found. Perhaps silver staining could have visualized some more spots. There are also some spots in the fractions that have not been identified as soybean allergens. In fraction A11 and A12 especially one spot was very sharp, this spot was identified with MALDI to be a maturation-associated protein (MAT9) alternatively a dehydrin-like protein (neither known to be allergenic) in the whole soybean extract (spot 25). Both matches had identical PMF score.

MALDI was used to identify several spots from the gel with the whole soybean extract. It was shown to be a very useful tool and almost all spots tested could be identified.

Finally one of the aims of this study was to use the two-dimensional electrophoresis method to identify soybean allergenic components. This was done by immunoblotting of 2-D gels followed by incubation with human sera containing IgE antibodies from two individuals having specific IgE directed to soybean proteins. The soy specific IgE was bound to allergenic components in the soybean extract. Immunoblotted soybean extracts were compared with the gel of the whole soybean extract. On the immunoblot with the first human sample, binding to one protein identified as β-conglycinin was confirmed. Beta-conglycinin is probably one of the most common allergenic components in soybean. In the second human serum sample the IgE-binding protein was not identified. Further studies needs to be performed to identify this protein.

6. CONCLUSIONS

Two-dimensional electrophoresis is a very useful method for separation of proteins. With the right sample concentrations the gels can be very sharp and it was found to give a good picture over the proteins in the soybean extract. It was also found to be a useful method for identification of proteins in size separated soybean extract. To confirm the identity of proteins found in the 2-D electrophoresis MALDI-TOF and MALDI MS/MS were used and found to be a very useful and powerful tool in combination with the results from the 2-D electrophoresis.

In this study we have used a combination of 2-D electrophoresis and MALDI-TOF for identification of several known soybean allergens in soybean extract as well as in gel filtrated soybean extract. We have also demonstrated that 2-D electrophoresis and immunoblotting in combination with MALDI-TOF is a powerful tool for identification of allergenic proteins in human sera. By combining the three methods and the use of human sera from soy allergenic individuals possibly new allergenic protein can be identified.

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