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Enzyme Linked Immunosorbent Assay (ELISA) for Detection of Sulfur-Rich Protein (SRP) in Soybeans (Glycine Max L.) and Certain Other Edible Plant Seeds

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ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DETECTION
OF SULFUR-RICH PROTEIN (SRP) IN SOYBEANS (*GLYCINE MAX* L.) AND
CERTAIN OTHER EDIBLE PLANT SEEDS

By

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List of Abbreviations

Ag – Antigen

AP – Alkaline Phosphatase

Ab - Antibody

β -ME – β -mercaptoethanol

BSA – Bovine Serum Albumin

BSB – Borate Saline Buffer

ELISA – Enzyme Linked Immunosorbent Assay

Glb1 – Globulin-1

Glb2 – Globulin-2

HMW – High Molecular Weight

HPLC – High Pressure Liquid Chromatography

HRP – Horse-Radish Peroxidase

LMW – Low Molecular Weight

MW – Molecular Weight

NFDM – Non-Fat Dry Milk

pAb – Primary Antibody

PBS – Phosphate Buffered Saline

PNPP – p-Nitrophenyl Phosphate

SDS-PAGE – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SRP – Sulfur Rich Protein

TBS-T – Tris Buffered Saline-Tween 20

ABSTRACT

The sulfur-rich protein (SRP) in soybeans is a 7S basic globulin that accounts for up to 5% of the total extractable seed proteins. Sulfur-containing amino acids are the limiting amino acids in most food legumes and therefore there is a continued interest in increasing the expression of sulfur containing proteins in legumes to improve protein quality. The objective of our investigation was to develop a sensitive method for detection of SRP and use the method to detect the presence of SRP or SRP-like proteins in various edible plant seeds.

Rabbit polyclonal antibodies (pAbs) raised against SRP were used in inhibition ELISA, Western blotting and Dot blotting to develop a sensitive assay and to assess cross-reactivity of pAbs to non soybean proteins. Typically, primary and secondary antibody dilutions of 1:10,000 (v/v) and 1:5,000 (v/v) in 0.1% BSA in borate saline buffer (BSB, 0.1M, pH 8.45), respectively, were used. All protein extractions were done in either BSB (pH 8.45), 0.1 M NaOH (pH 12.22) or 70% (v/v) aqueous ethanol and cross reactivity was assessed by comparing sample IC_{50} (50% inhibitory concentration) to IC_{50} value for SRP. Inhibition ELISA assays could detect SRP at concentrations as low as 400 ng/ml in aqueous extracts, Western blotting and Dot blotting could detect SRP at concentrations as low as 50 ng and 1 ng, respectively.

The assays indicated the presence of cross-reactive proteins in several of the BSB (pH 8.45) extracted samples, including soybean, winged bean, black gram, mung bean, navy bean, tepary bean, pinto bean, chickpea, blackeye pea, horse bean, black bean, Great Northern bean, lima bean, small red bean, wheat berries and moth bean. The assays also detected cross-reactive proteins in several of the 0.1 M NaOH (pH 12.22) extracted samples, including soybean, winged bean, black gram, mung bean, navy bean, horse bean, moth bean, black bean, Great Northern bean, tepary bean, pinto bean, chickpea, lima bean, blackeye pea, small red bean. In addition, cross-reactive proteins were also detected in samples extracted with 70% (v/v) aqueous ethanol, including soybean, winged bean, wheat bran and black gram.

Chapter 1

Background

Soybeans (*Glycine max* L.) are believed to have originated in Eastern Asia, probably North and Central China. Soybean has been a staple food in China for thousands of years (Smith and Circle, 1978). Only recently, Western societies have started using soybeans, a major source of edible oil as well as protein, for animal and human food purposes. (Altschul and Wilcke, 1985).

World soybean production has significantly increased in the last half century, leading to the present level of 176,638,828 metric tons per year (FAO, 2001). Such an increase in soybean production is credited to its recent introduction into Western agriculture between 1950 and 1970. Globally, the U.S.A (45%), Brazil (21%), Argentina (15%), and China (9%) are the major producers of soybeans (FAO, 2001). Over 54% of the world production of soybeans (unprocessed) is traded on the international market (FAO, 2000). The U.S.A. is the leading exporter, with approximately 57% of the traded volume, followed by Brazil (24%) and Argentina (9%) (FAO, 2000). The leading importer is China with approximately 26% of the traded global volume, followed by the Netherlands (11%), Japan (10%), Mexico (8%) and Germany (8%) (FAO, 2000). Also, there are considerable quantities of processed soybeans as meal or oil traded among countries.

Soybeans grow best in temperate climates. Almost all types of soils accommodate growth, with optimal soil pH between 6.0 to 6.5 (FAO, 1995). An adequate water supply is essential for good yields, mostly affecting the period of pod and seed development (pod filling stage) (FAO, 1995). Soybeans are normally planted in late spring to early summer and are ready for harvest in early to mid-fall. Ideally, soybeans should be harvested when water content of the seed is between 12-13% (FAO, 1995). If moisture content is higher than 13%, microbial spoilage becomes a problem. In contrast, moisture levels below 12% lead to cracked and/or split beans resulting in increased susceptibility to rancidity during storage.

Soybean shape can vary from almost spherical to elongated and flat depending on the variety. The colors of typical soybeans can also vary widely. Soybean classes for

grading in the U.S.A. are based on color and include yellow, green, brown and black beans (Snyder and Kwon, 1987). Grading standards set by the USDA divide soybeans into two classes based on color: yellow soybeans and mixed soybeans (USDA, 1997). In order to be classified as yellow soybeans a bushel must contain not more than 10% of soybeans of other colors (other than yellow color). If a bushel contains more than 10% it must be classified as mixed soybeans. Yellow soybeans are the standard, so when mixed colors exceed 10% the quality of the bushel decreases. The structure of a soybean consists of an outermost layer called the seed coat or the hull. This layer protects the beans against mechanical stress and insect infestation. The seed coat makes up about 9% of the soybean by dry weight, and contains about 8% protein (Snyder and Kwon, 1987). The seed coat normally is removed from the bean before processing, and may be added back post-processing for additional protein content in animal feed. Soybeans are dicotyledonous. These two cotyledons are held together by the seed coat. The cotyledons represent 90% of the seed weight and contain mainly all the oil and protein in its palisade-like cells (FAO, 1995). The predominant features of these cotyledon cells are the protein bodies and the lipid bodies. Protein bodies or aleurone grains are spherical and range in the size of 1-20 μ m. Tombs (1967) found protein bodies of the soybean cotyledons to be 2-20 μ m in diameter, while Wolf (1970) reported the protein bodies to be no larger than 1-3 μ m. Protein bodies contain approximately 60-70% of the total protein found in soybeans. Lipid bodies are also spherical and range in size between 0.2-0.5 μ m (FAO, 1995).

The chemical composition of soybeans may vary somewhat according to the variety and growing conditions, but on an average consists of 35-40% protein, 20% oil, 35% carbohydrate and about 5% ash on a dry weight basis (Verma and Shoemaker, 1996; FAO, 1995; Altschul and Wilcke, 1985). The two valuable components of the soybean are its oil and protein. The extracted crude oil of soybean contains 96% triglycerides with other minor components, phospholipids and free fatty acids (Synder and Kwon, 1987).

Table 1: Proximate Composition of Soybeans

Nutrient	Units	Value per 100 grams of edible portion
<i>Proximates</i>		
Water	g	8.54
Energy	kcal	416
Energy	kJ	1741
Protein	g	36.49
Total lipid (fat)	g	19.94
Ash	g	4.87
Carbohydrate, excluding fiber	g	30.16
Fiber, total dietary	g	9.3

Compiled from USDA, 2002. Mature and unprocessed soybeans

The protein found in soybeans is the other valuable component. Soybean protein is used mainly as a feed stuff for animals, but a small quantity is used directly in human foods. Because soybean is a rich source of vegetable protein, soy flours are used in a variety of foods to increase protein content and quality. Processing of soybean into food products such as soya flour, soy protein concentrate and isolated soy protein was developed in the 1950's (Wilcke et al., 1979). These forms of soy food products are marketed in a wide variety of forms, including dry powders and texturized products. These products are commonly used in cereals and bakery to increase protein content. Not only do soybeans increase the protein content of various foods, they also have valuable functional properties that are helpful to the food industry. Soybean functional properties include viscosity, emulsification, forming gel and foaming, absorption of fat and/or water and create meat-like texturized structures (FAO, 1995). All of these properties lead to the use of soy products in a large range of foods such as meat and milk substitutes, infant formulas, cereals and baked goods.

A more comprehensive understanding of the total protein content of soybeans must be established, due to the increasing demand for soybeans on a worldwide basis. The amount of protein in soybeans, 35-40%, is larger than the protein content of most other legumes and oil seeds that contain 20-30% protein. The majority of soybean proteins are storage proteins. Attempts to increase the storage protein concentration in

soybeans have been unsuccessful due to a decrease in seed yield typically associated with increased protein content (Verma and Shoemaker, 1996).

Globulins are the most abundant seed storage proteins found in soybeans. Globulins are separated and isolated based on their solubility in salt solutions. Albumins, soluble in water, prolamines, soluble in 50-70% aqueous ethanol and glutelins, soluble in dilute acid or base make up the remaining extractable seed proteins (Synder and Kwon, 1987). The two primary globulin fractions, 11S and 7S, account for 70-80% of the total seed globulins and are referred to as glycinin and β -conglycinin. Since these two proteins represent the major portion of total seed proteins they primarily account for the overall nutritional quality of the soybean protein. The storage proteins of soybean as well as other legumes are nutritionally inadequate due to lack of the sulfur-containing amino acids, methionine and cystine. However, soybeans contain a 7S basic globulin, a sulfur-rich protein (SRP), which accounts for up to 5% of the total extractable soybean proteins. Although a minor protein in soybean, SRP contains a significantly higher proportion of methionine compared to the major proteins, glycinin and β -conglycinin. Sathe et al. (1989) found SRP, β -conglycinin and glycinin to contain 1.83, 1.1 and 0.3 methionine residues/100 residues. SRP is therefore important in improving the nutritional value of soy proteins.

It is therefore important to develop a sensitive and accurate method for detection and quantification of SRP. Upon completion of an acceptable methodology, it will be used to determine if SRP or SRP-like proteins exist in various other edible plant seeds.

Statement of the Problem

1. What is the lowest concentration of SRP that can be detected by using an ELISA assay? Can this method be used to detect cross-reactive proteins in other plant seeds? Can the ELISA be used to quantify SRP?
2. What is the minimum amount of SRP that can be detected by using Western blot analysis? Can the Western blotting method be used to detect cross-reactive proteins in other plant seeds?
3. Can a similar Dot blot analysis be used for detection of cross-reactive proteins in other plant seeds?
4. Do edible plant seeds, other than soybeans, contain SRP or SRP-like proteins? Does SRP still exist in processed soybean products (soy milk, soy tofu and soy nuts)?
5. Do SRP levels vary in soybean cultivars?

Research Hypothesis

- I. Detection of SRP
- II. Immunoreactivity of SRP
 - a. Sulfur rich protein (SRP) will cause antibody binding in an inhibition enzyme-linked immunosorbent assay (ELISA).
 - b. Sulfur rich protein (SRP) will cause antibody binding in a Western blot as well as a Dot blot.
- III. Cross-reactivity with SRP
 - a. There will be cross-reactivity between rabbit anti-SRP Ab and other plant seed proteins assayed.
 - b. An optimized inhibition ELISA will show a substantial increase in binding of test samples containing SRP or SRP-like proteins.
 - c. The closer the IC_{50} value of a test protein is to SRP IC_{50} value, will indicate a high level of cross-reactivity between the two proteins.

- d. The Western blot analysis and Dot blot analysis will indicate cross-reactivity between test proteins and rabbit anti-SRP Ab.

IV. Presence of SRP

- a. Sulfur rich protein (SRP) will be present in various soybean cultivars.
- b. Sulfur rich protein (SRP) will be present in commercial soybean products.

Limitations of the Study

1. Only three different extraction solvents will be used in this study. Therefore, variation in extraction potential may occur depending on concentration and pH differences between the extraction solvents.
2. Only thirty-nine plant seed samples will be investigated. This will not adequately represent a large variety of plant seeds.
3. The structural level similarities and amino acid sequences of cross-reactive proteins will not be investigated.
4. Polyclonal antibodies will be used for cross-reactivity studies; however monoclonal antibodies may show a higher level of specificity.

Significance of the Study

1. Selected soybean cultivars will be examined for SRP content.
2. Plant based protein sources are limited in sulfur containing amino acids (methionine and cysteine), therefore it is valuable to determine the concentration of SRP in different soybean cultivars.
3. Determining the cross-reactivity of various plant seeds with rabbit anti-SRP Ab will be useful to food scientists and geneticists to help improve amino acid balance in seed proteins.
4. This study will help to determine whether an ELISA can be used for detection and quantification of SRP in soybeans, as well as for SRP-like proteins in other plant seeds.

Chapter 2

Review of the Literature

Sulfur-Rich Protein (SRP) Isolation and Characterization:

The two major proteins in soybean are glycinin (11S) and β -conglycinin (7S), which form the major portion of the total soybean proteins (Derbyshire et al., 1976). These two proteins account for up to 70% of the total protein in the seed (Moreira et al., 1979). Therefore, the isolation and purification of these two proteins is imperative to the overall study of soybean proteins and their functions. Although many methods have been developed to isolate glycinin and β -conglycinin (Koshiyama, 1965, 1972; Roberts and Briggs, 1965; Thanh et al., 1975; Thanh and Shibasaki, 1976; Wolf and Briggs, 1959; Wolf et al., 1962) the presence of cross contaminants remain problematic. The complexity of 11S and 7S polypeptides have made it very difficult to separate 7S (conglycinin) from 11S (glycinin).

Three polypeptides (α -, β - and γ -conglycinin) make up the majority of the 7S fraction (Catsimpoolas et al., 1969). β -conglycinin accounts for more than 50% of the 7S fraction, and thus has been studied extensively. β -conglycinin is a trimer and is composed primarily of three subunits (α' , α and β), all of which are glycosylated with mannose and glucosamine (Thanh and Shibasaki, 1976). Glycinin (11S) is a hexamer, where each subunit contains an acidic polypeptide(s) and a basic polypeptide(s) linked by disulfide bonds. A brief summary of β -conglycinin and glycinin biochemical properties is given in Table 2.

Table 2: Properties of the Two Major Soybean Proteins

Protein	Molecular weight (MW)	Subunits	Isoelectric point (pI)	Sedimentation coefficient (S)
β -conglycinin	105-193 kD	3	4.9	7.9
Glycinin	309-393 kD	6	4.6	12.3

Complied from Nielsen (1985) and Altschul and Wilcke (1985)

In an attempt to purify glycinin using ConA-Sepharose 4B followed by Sepharose 6B column chromatography, Kitamura et al. (1974) were able to remove most of the contaminants. Even though a majority of contaminants were removed, still some remained. Another method developed by Thanh and Shibasaki (1976) to also purify glycinin typically yields 90% pure 11S with the remaining 10% contaminants being 7S and other minor proteins.

Hu and Esen (1981) separated soybean seed proteins into six solubility fractions using six different solvents. The water-soluble fraction included the two major soybean protein components, β -conglycinin (7S globulin) and glycinin (11S globulin). The most interesting find occurred in the NaCl-soluble fraction where two major components with the molecular weight around 26,000 and 16,500 dominated the profile of this fraction. The two components referred to as globulin-1 (Glb1) and globulin-2 (Glb2) accounted for 45.8% of the protein in the NaCl-soluble fraction. Taking a closer look at this fraction, Hu and Esen (1982) found Glb1 and Glb2 to contain more methionine (1.2 and 1.4% respectively) and total sulfur amino acids, methionine and cysteine, (2.7 and 3.6% respectively) than β -conglycinin and glycinin. Yamauchi et al. (1984) found the NaCl-soluble fraction contained two main protein bands, the low molecular weight (LMW) and high molecular weight (HMW) subunits together with the subunits of β -conglycinin and glycinin. These HMW and LMW subunits comprised 70% of the NaCl-soluble fraction. Molecular weight of the subunits and the intermediate subunit by SDS-PAGE were estimated to be 16,000 (LMW subunit), 26,000 (HMW subunit) and 42,000 (intermediate subunit). The LMW and HMW subunits found by Yamauchi et al. (1984) were in agreement with Glb1 (MW 26,000) and Glb2 (MW 16,500) reported by Hu and Esen (1982). Also in agreement with Hu and Esen (1982), it was found that the purified protein designated, basic 7S globulin, also contained higher levels of methionine and cysteine compared to β -conglycinin and glycinin. The 7S basic globulin is a tetrameric protein (MW 168,000). Each monomer is composed of a HMW polypeptide linked to a LMW polypeptide via disulfide bond(s).

The majority of soybean protein research has focused mainly on isolation and purification of the two major globulins, glycinin (11S) and β -conglycinin (7S). Sathe (1991) prepared glycinin according to the methods of Thanh and Shibasaki (1976) and

Kitamura et al. (1974), and was able to isolate and identify the major contaminating peptide. Sathe (1991) suspected the contaminating peptide to be the 7S basic globulin first purified by Yamauchi et al. (1984) based on the mobility of these polypeptides in the acrylamide gel. The 7S basic globulin has an alkaline isoelectric point (pH 9.05-9.26), therefore in order to determine whether the contaminating peptide was a basic protein, DEAE DE-53 anion-exchange column was used to further isolate this peptide. The total protein recovered in the flow-through fraction was 5.32% of the total protein recovered from the column. The protein eluted off the anion-exchange column (pH 7.5) before the NaCl gradient development, indicated the protein had an alkaline isoelectric pH (pH > 7.5) The flow through fraction was further analyzed by SDS-PAGE and showed 5 polypeptides with the estimated MW of 45,000 (1 polypeptide), 28,000-29,000 (2 polypeptide) and 17,000-18,000 (2 polypeptide). These findings matched what Sathe et al. (1989) had previously found. To further clarify whether the protein was truly the 7S basic globulin, the protein was subjected to peptide mapping using TPCK-treated trypsin and TLCK-treated chymotrypsin and SDS-PAGE. The peptide maps of both the proteins were identical. These findings indeed indicate the contaminating polypeptide in 11S preparations is the 7S basic globulin, otherwise known as, sulfur rich protein (SRP).

Cross-reactivity:

The question still lingered whether the 7S basic globulin was part of the major soybean globulins, β -conglycinin and glycinin. Sato et al. (1987) using double gel immunodiffusion and immunoelectrophoresis, found that the basic 7S globulin was indeed different from the other soybean globulins. An antiserum to the basic 7S globulin recognized only the purified basic 7S globulin with no recognition to the major soybean globulins (β -conglycinin and glycinin). This concluded the antibody raised strictly against the 7S globulin did not cross-react with any part of β -conglycinin and glycinin indicating a completely separate protein. Hirano et al. (1987) using tryptic peptide mapping by high pressure liquid chromatography (HPLC) and partial N-terminal amino acid sequencing also reported the 7S globulin consisted of a different primary structure compared to the β -conglycinin. Sato et al. (1987) showed that the 7S basic globulin amounted to 3.0% of the total extractable soybean proteins.

Kagawa et al. (1987) found the amino acid sequence of the internal region of the 7S basic globulin, LII subunit, to be homologous to conglutin γ , one of the lupin seed storage proteins. Using anti-serum prepared against soybean basic 7S globulin LII subunit, cross reactivity of seed proteins from various legume species were also found. Using protein blotting, a strong cross reactive reaction was observed with the extracts from azuki bean and mung bean. In contrast, a weak cross reactive reaction was observed with that of winged bean and lupin seed. It was also noted that all of the proteins in the legume species that cross reacted with the antiserum against the LII subunit had almost the same molecular mass as the LII subunit. These findings suggest that the 7S globulin-like protein of soybean may be present in many legume species.

Hirano et al. (1992) found mature soybeans release a large amount of protein into surrounding water when immersed in water at 50-60°C. The protein in the surrounding water was analyzed using SDS-PAGE and revealed three major polypeptides designated as S1, S2, and S4 (~90% of total protein in surrounding water). The water also contained two minor polypeptides, S3 and S5. The polypeptides were electroblotted and sequenced, the N-terminal amino acid sequences of S1 and S2 were identical to the 27,000 subunit of the basic 7S globulin. The amino acid sequence of S4 was also similar to the 16,000 subunit of the basic 7S globulin. In addition, S1 and S2 reacted strongly with anti-serum raised against the 27,000 subunit of the basic 7S globulin, and S4 also reacted with anti-serum raised against the 16,000 subunit. The amino acid sequence of S1 and S4 were also consistent with the 27,000 and 16,000 subunits. Therefore it was concluded that the released polypeptides (S1, S2 and S4) were identical with the 27,000 and 16,000 subunits of the basic 7S globulin. In the same study, azuki-bean, cowpea, mung-bean and winged bean were also shown to release large amounts of protein when immersed in water at 50-60°C. The proteins found in the surrounding water were found to have highly homologous amino acid sequences with the 27,000 and 16,000 subunits of the basic 7S globulin. All of these proteins also strongly reacted with anti-serum raised against the basic 7S globulin. Therefore, the basic 7S globulin (SRP) in soybean and/or SRP-like proteins is found in other plant seeds.

Chapter 3

Materials and Methods

Purification of Soybean Sulfur-Rich Protein.

A schematic diagram for SRP purification is shown in Figure 1 (Sathe et al., 1989). Purified SRP was available in sufficient quantities (mg).

Preparation of Protein Samples.

Thirty-nine unprocessed plant seed samples were obtained from local grocery stores. The plant seeds were ground to approximately 40 mesh in an Osterizer blender and defatted for 6-8 hours in a Soxhlet apparatus using petroleum ether (boiling point range 38.2-54.3°C, Fisher Scientific, Fair Lawn, NJ) as the extraction solvent. The defatted samples were spread in a thin layer on aluminum foil and allowed to dry overnight under a fume hood at 25°C. The dried defatted samples were then ground in an Osterizer blender to make a flour, approximately 40 mesh. The flour samples were stored in tightly capped plastic containers at -20°C until used.

Test proteins were extracted from defatted flour with either BSB buffer (pH 8.4-8.5), 0.1M NaOH (pH 12.22) or 70% (v/v) aqueous ethanol. Albumins and globulins were isolated using BSB buffer (pH 8.4-8.5), glutelins and other aqueous soluble proteins were isolated using 0.1M NaOH (pH 12.22) and prolamines were isolated using 70% (v/v) aqueous ethanol. Flour to solvent ratio of 1:10 w/v was used for each extracted sample (e.g. 100 mg of flour was extracted with 1 ml of solvent). The flour slurries were then vortexed (Vortex Genie 2, setting 8) continuously for 1 hour.

BSB extracted samples. The flour/BSB buffer slurry was vortexed for 1 hour at 25°C. The slurry was then centrifuged (13,600g, 20 min., 25°C) and the supernatant carefully collected. The residue was discarded.

0.1M NaOH extracted samples. The flour/0.1 M NaOH slurry was vortexed for 1 hour at 25°C. The slurry was then centrifuged (13,600g, 20 min., 25°C) and the supernatant carefully collected. The residue was discarded.

70% (v/v) aqueous ethanol extracted samples. The flour/1 M NaCl slurry was vortexed for 1 hour at 25°C. The slurry was then centrifuged (13,600g, 20 min., 25°C) and the

PURIFICATION OF SOYBEAN SULFUR-RICH PROTEIN

Figure 1. Schematic diagram for the SRP purification

supernatant discarded. The residue from the 1 M NaCl extraction was washed 2 times, 10 minutes each, with distilled water. Then the 70% (v/v) aqueous ethanol was added and the flour/70% (v/v) aqueous ethanol slurry was vortexed for 1 hour at 25°C. The slurry was then centrifuged (13,600g, 20 min., 25°C) and the supernatant carefully collected. The residue was discarded.

BSB extracted commercial products (soy milk, soy nuts and soy tofu). The flour/BSB buffer slurry was vortexed for 1 hour at 25°C. The slurry was then centrifuged (13,600g, 20 min., 25°C) and the supernatant carefully collected. The residue was discarded.

Protein concentrations were determined using the Lowry method (Lowry et al. 1951) for all protein samples, with the exception of 70% (v/v) aqueous ethanol extracted sample, which the Bradford method was used (Bradford 1976).

Immunoassay.

A non-competitive enzyme-linked immunosorbent assay (ELISA) was developed and optimized for SRP detection. A schematic diagram of ELISA assay optimization for SRP detection is shown in Figure 2.

The pAb dilutions (ranging between 1:10² and 1:10⁶) were tested to determine the lowest dilution for SRP detection. SRP concentrations tested were 500 ng, 250 ng, 125ng, 62.5 ng, 31.25 ng, 15.625 ng and 7.8125 ng, respectively.

Cross-Reactivity

The cross-reactivity of rabbit anti-SRP Ab with various other plant seed proteins were examined using a competitive inhibition ELISA assay. A modified ELISA protocol previously worked out for detection of almond major protein (Acosta et al., 1999) was followed. The protocol is as follows:

A 96-well polyvinyl microtiter ELISA plate was coated with 250 ng/well of SRP, and incubated for 1 hour at 37°C. After incubation, wells were washed three times with BSB and blocked with 50µl of blocking buffer, then incubated again for 1 hour at 37°C. After incubation, the blocking buffer was discarded and parafilm was placed securely over the plates to protect against contaminants and stored at -20°C until used.

Test proteins were prepared from: almond (*Prunus dulcis* L.), macadamia nut (*Macadamia integrifolia* L.), cashew (*Anacardium occidentale* L.), pistachio (*Pistacia vera* L.), pecan (*Carya illinoensis* L.), sunflower (*Helianthus annuus* L.), pinenut (*Pinus*

monophylla L.), walnut (*Juglans regia* L.), Spanish nut (*Arachis hypogaea* L.), Virginia peanut (*Arachis hypogaea* L.), Brazil nut (*Bertholletia excelsa* L.), soybean (*Glycine max* L.), winged bean (*Psophocarpus tetragonolobus* L.), sesame seed (*Sesamum indicum* L.), split pea (*Pisum sativum* L.), hazelnut (*Corylus avellana* L.), tepary (*Phaseolus acutifolius* L.), lima bean (*Phaseolus lunatus* L.), chickpea (*Cicer arietinum* L.), pinto bean (*Phaseolus vulgaris* L.), black eye pea (*Vigna unguiculata* L.), small red bean (*Phaseolus vulgaris* L.), wheat berries (*Triticum aestivum* L.), pumpkin seed (*Cucurbita moschata* L.), Inca peanut (*Plukenetia volubilis* L.), wheat bran (*Triticum aestivum* L.), moth bean (*Phaseolus aconitifolia* L.), horse bean (*Canavalia ensiformis* L.), whole oat (*Avena sativa* L.), barley (*Hordeum vulgare* L.), Great Northern bean (*Phaseolus vulgaris* L.), black bean (*Phaseolus vulgaris* L.), sorghum (*Sorghum saccharatum* L.), millet (*Panicum milaceum* L.), lentil (*Lens esculenta* L.), basmati rice (*Oryza sativa* var *Basmati* L.), mung bean (*Phaseolus mungo* L.), black gram bean (*Phaseolus aureus* L.) and navy bean (*Phaseolus vulgaris* L.). All defatted test proteins were extracted in either BSB (pH 8.45), 0.1M NaOH (pH 12.22) or 70% (v/v) aqueous ethanol and were normalized to a protein concentration of 1 mg/ml. The normalized (1 mg/ml) test proteins were then combined with pAb (rabbit anti-SRP) (Ag:Ab ratio: 1 to 10) and serially diluted 10 fold in successive wells. Appropriate reagent blanks (pAb, 1:10,000 (v/v) dilution, with no test protein) and SRP (as control) were included on every plate assayed. The assay plates were incubated for 1 hour at 37°C, and then 45µl from each well was transferred to the prepared coated plates (250 ng/well SRP) and incubated for another 1 hour at 37°C. After incubation the assay plates were washed three times with BSB and secondary antibody (goat anti-rabbit; 1:5,000 (v/v) diluted in 0.1% (w/v) BSA in BSB) was added to each well. The plates were incubated for 1 hour at 37°C, and then were washed three times (30 seconds each) with BSB. Fifty µl of alkaline phosphase buffer with p-nitrophenyl phosphate (1 tablet of PNPP dissolved in 5 ml AP buffer) was added to each well, and the color development was allowed to proceed for 7-10 minutes at 25°C. Color development was stopped by adding 50µl/well of 3.0 M NaOH. Each well was read at 405 nm using an ELISA reader (Model EL 307, Bio-Tek Instruments Inc., Riverton NJ). Cross-reactivity was assessed by comparing the IC₅₀ values of test proteins to the IC₅₀ value for SRP. A cross reactive protein contains an IC₅₀ value similar to the

IC₅₀ value of SRP. IC₅₀ is a term that describes the concentration of inhibiting protein that effected a 50% inhibition of binding by pAb (rabbit anti-SRP) with antigen (SRP) in ELISA response.

Polyacrylamide Gel Electrophoresis (PAGE).

Sodium dodecyl sulfate (SDS) with 2% β -mercaptoethanol (β -ME) was preformed on test protein extracts and SRP. SDS-PAGE was done as described by Acosta et al. (1999).

Immunoblotting.

The gels were immunoblotted using the Western blot analysis, as described by Acosta et al. (1999). Proteins from the gels were transferred onto 0.2 μ m nitrocellulose paper using a Hoefer TE22 transverse electrophoresis unit as described by Towbin et al. (1979). The nitrocellulose membrane containing the transferred proteins was stained with 0.1% (v/v) Ponceau S in distilled water to visualize the proteins. The membrane was then blocked with Tris-buffered saline Tween-20 (TBS-T) containing 5% (w/v) NFDM for 1 hour at 25°C. The TBS-T buffer consisted of 10 mM Tris, 0.9% (w/v) NaCl, and 0.05% (v/v) Tween-20. The membrane was washed with 2 changes of fresh TBS-T for 2 minutes each.

The nitrocellulose membrane was then incubated with diluted pAb (1:10,000 (v/v); rabbit anti-SRP) solution for 1 hour at 25°C on a rocker (60 Hz, Rocker II Model 260350, Boekel Scientific, Feasterville, PA). The membrane was then washed once with TBS-T for 15 minutes and again washed 3 times with fresh TBS-T for 5 minutes each. The membrane was then incubated with diluted secondary antibody [1:5,000 (v/v); horse-radish peroxidase-labeled goat anti-rabbit (HRP)] for 1 hour at 25°C on a rocker. The membrane was then washed once with TBS-T for 15 minutes and again washed 3 times with fresh TBS-T for 5 minutes each. The bands that were reactive to the pAb, which is raised against SRP, were visualized by using the luminol/p-coumaric acid system. The luminol and p-coumaric solutions are mixed together and spread evenly to cover the entire area of the nitrocellulose blot. This solution was left on the blot for approximately 5 minutes at 25°C and then blotted dry and the blot placed in a translucent plastic cover and exposed to x-ray film (Kodak X-OMAT AR Film, Eastman Kodak Company, Rochester, NY) for autoradiography visualization.

Dot Blot.

Stenciled circles were etched on to 0.2 μ m nitrocellulose paper using a soft #2 pencil. SRP and original undiluted test protein extracts were pipetted (1-8 μ l) carefully onto the center of each circle. The nitrocellulose paper was allowed to dry at 25°C for about 10-15 minutes. The blots were developed as described under Western blotting.

Statistical Analysis

All experiments were done at least in triplicate. Data is reported as mean $\pm$ standard error of mean (SEM). Data was analyzed for statistical significance ($p=0.05$) using one-way ANOVA procedures provided by SPSS statistical software (SPSS for Windows 2000, Microsoft Corporation, Version 11.0, Chicago, IL).

NON-COMPETITIVE ELISA OPTIMIZATION PROCEDURE FOR SRP DETECTION

Fig 2. Schematic diagram for SRP detection using ELISA

Fig 2. Continued

Chapter 4

Results

Immunoassay for Detection of SRP

A non-competitive enzyme-linked immunosorbent assay (ELISA) for SRP detection was successfully developed and used in this study. The optimized assay gives optimum signal when the pAb dilution is 1:10,000 (v/v), Ag concentration is 250 ng/well and 2^o Ab dilution is 1:5,000 (v/v). This assay can detect SRP as low as 400 ng/ml. An example of an SRP standard curve is shown in Figure 3.

In addition, the SRP detection limit using Western blotting and Dot blotting were investigated (Figure 3). As shown in the figure, Western blotting can detect SRP levels as low as 50 ng and Dot blotting can detect SRP levels as low as 1 ng, respectively.

Figure 3 – ELISA, Western Blotting and Dot blotting detection limits for SRP. **A** - Representative Inhibition ELISA standard curve for SRP. **B** – Western blotting. Detection limit for SRP using Western blotting analysis. **C** – Dot blotting. Detection limit for SRP using Dot blotting analysis.

Cross-Reactivity

The cross-reactivity of rabbit anti-SRP Ab with certain other edible plant seeds was examined using a competitive inhibition ELISA assay. Thirty-nine different edible plant seeds were assayed for cross-reactivity.

The results for the BSB (pH 8.45) extracted test proteins are reported in Table 3. Sixteen samples showed cross-reactivity with rabbit anti-SRP Ab: soybean, winged bean, black gram, mung bean, navy bean, tepary bean, pinto bean, chickpea, blackeye pea, horse bean, black bean, Great Northern bean, lima bean, small red bean, wheat berries and moth bean. All cross-reactive samples were assayed four times, each in duplicate (n=8).

Table 3: Cross-Reactivity of BSB (pH 8.45) Extracted Proteins from Edible Plant Seeds and Rabbit Anti-SRP Ab

Number	Sample	IC ₅₀ (mg/ml) ^a		
1	SRP (control)	0.0004	+	0.0001
2	Soybean (Williams 82)	0.0017	+	0.0005
3	Winged Bean	0.0189	+	0.0146
4	Black Gram	0.0213	+	0.0169
5	Mung Bean	0.0323	+	0.0199
6	Navy Bean	0.2338	+	0.1663
7	Tepary Bean	0.0155	+	0.0001
8	Pinto bean	0.0245	+	0.0133
9	Chickpea	0.0058	+	0.0046
10	Blackeye Pea	0.2691	+	0.2610
11	Horse Bean	0.0087	+	0.0060
12	Black Bean	0.0019	+	0.0008
13	Great Northern Bean	0.1268	+	0.1222
14	Lima Bean	0.0119	+	0.0049
15	Small Red Bean	0.0077	+	0.0040
16	Wheat Berries	0.0511	+	0.0328
17	Moth Bean	0.0223	+	0.0212

^aData are reported as mean $\pm$ standard error of the mean (p=0.05)

The results for the 0.1 M NaOH (pH 12.22) extracted test proteins are reported in Table 4. Fifteen samples showed cross-reactivity with rabbit anti-SRP Ab: soybean, winged bean, black gram, mung bean, navy bean, horse bean, moth bean, black bean, Great Northern bean, tepary bean, pinto bean, chickpea, lima bean, blackeye pea and small red bean. All cross-reactive samples were assayed four times, each in duplicate (n=8).

Table 4: Cross-Reactivity of 0.1 M NaOH (pH 12.22) Extracted Proteins from Edible Plant Seeds and Rabbit Anti-SRP Ab

Number	Sample	IC ₅₀ (mg/ml) ^a		
1	SRP (control)	0.0004	±	0.0001
2	Soybean (Williams 82)	0.0025	±	0.0012
3	Winged Bean	0.0089	±	0.0031
4	Black Gram	0.0621	±	0.0357
5	Mung Bean	0.0338	±	0.0114
6	Navy Bean	0.0239	±	0.0155
7	Horse Bean	0.0078	±	0.0048
8	Moth Bean	0.0077	±	0.0036
9	Black Bean	0.0008	±	0.0001
10	Great Northern Bean	0.0027	±	0.0012
11	Tepary Bean	0.1719	±	0.1531
12	Pinto Bean	0.0041	±	0.0027
13	Chickpea	0.0174	±	0.0121
14	Lima Bean	0.0433	±	0.0326
15	Blackeye Pea	0.0583	±	0.0454
16	Small Red Bean	0.0005	±	0.0003

^aData are reported as mean ± standard error of the mean (p=0.05)

The results for the 70% (v/v) aqueous ethanol extracted test proteins are reported in Table 5. Six samples showed cross-reactivity with rabbit anti-SRP Ab: soybean, winged bean, wheat berries, wheat bran, macadamia nut and black gram. All cross-reactive samples were assayed four times, each in duplicate (n=8).

Table 5: Cross-Reactivity of 70% (v/v) Aqueous Ethanol Extracted Proteins from Edible Plant Seeds and Rabbit Anti-SRP Ab

Number	Sample	IC ₅₀ (mg/ml) ^a		
1	SRP (control)	0.0004	±	0.0001
2	Soybean (Williams 82)	0.0012	±	0.0008
3	Winged Bean	0.0026	±	0.0022
4	Wheat Berries	0.0070	±	0.0066
5	Wheat Bran	0.0937	±	0.0933
6	Macadamia Nut	0.0002	±	0.0001
7	Black Gram	0.0003	±	0.0002

^aData are reported as mean ± standard error of the mean (p=0.05)

The results for the commercial soy products (soy milk, soy nuts and soy tofu) are reported in Table 6. All commercial products showed cross-reactivity with rabbit anti-SRP Ab, and each were assayed three times, each in duplicate (n=6).

Table 6: Cross-Reactivity of Commercial Soy Products Extracted in BSB (pH 8.45) and Rabbit Anti-SRP Ab

Number	Sample	IC ₅₀ (mg/ml) ^a		
1	SRP (control)	0.0004	±	0.0001
2	Soy Milk	0.0005	±	0.0001
3	Soy Nuts	0.0015	±	0.0013
4	Soy Tofu	0.0004	±	0.0002

^aData are reported as mean ± standard error of the mean (p=0.05)

Western Blotting

Western Blotting was performed to further confirm cross-reactivity between rabbit anti-SRP Ab and test proteins observed in ELISAs (Tables 3, 4, 5, 6).

The cross-reactive BSB (pH 8.45) extracted test proteins are shown in Fig. 4. As shown in the figure, polypeptides of soybean, winged bean, black gram, mung bean, navy

bean, tepary bean, pinto bean, chickpea, blackeye pea, horse bean, black bean, Great Northern bean, lima bean, small red bean, wheat berries, moth bean and sunflower were recognized by rabbit anti-SRP Ab, respectively. The results are in agreement with the previously observed ELISA data (Table 3) with the exception of sunflower. Western Blotting reveals a slightly reactive polypeptide in sunflower that was not observed in the ELISAs.

The cross-reactive 0.1 M NaOH (pH 12.22) extracted test proteins are shown in Figure 5. As shown in the figure, polypeptides of soybean, winged bean, black gram, mung bean, navy bean, horse bean, moth bean, black bean, Great Northern bean, tepary bean, pinto bean, chickpea, lima bean, blackeye pea, small red bean, whole oat and sorghum were recognized by rabbit anti-SRP Ab, respectively. The results are in agreement with the ELISA (Table 4) data with the exception of whole oat and sorghum. Western blotting shows slightly reactive polypeptides in whole oat and sorghum that were not observed in ELISAs.

The cross-reactive 70% (v/v) aqueous ethanol extracted test proteins are shown in Figure 6. As shown in the figure, polypeptides of soybean, winged bean, wheat berries, wheat bran and black gram were recognized by rabbit anti-SRP Ab, respectively. The results are in agreement with the ELISA data (Table 5) with the exception of macadamia nut. Western blotting did not detect any cross-reactive polypeptides to rabbit anti-SRP Ab in macadamia nut.

The cross-reactive commercial soy products (soy milk, soy nuts and soy tofu) extracted in BSB (pH 8.45) is shown in Figure 7. As shown in the figure, polypeptides of soy milk, soy nuts and soy tofu were recognized by rabbit anti-SRP Ab, respectively. The results are in agreement with the ELISA data (Table 6).

All soybean cultivars extracted with BSB (pH 8.45) were analyzed for presence of SRP (Figure 8 and 9). As shown in the figure, the presence of SRP is seen throughout each cultivar.

Dot Blots

Dot Blots were conducted to confirm the cross-reactivity between rabbit anti-SRP Ab and test proteins observed in ELISAs (Tables 3, 4, 5, 6) and in Western Blotting (Figures 4, 5, 6, 7, 8, and 9).

The cross-reactive BSB (pH 8.45) test proteins are shown in Figure 10. As shown in the figure, proteins present in soybean, winged bean, black gram, mung bean, navy bean, tepary bean, pinto bean, chickpea, blackeye pea, horse bean, black bean, Great Northern bean, lima bean, small red bean, wheat berries, moth bean and sunflower were recognized by rabbit anti-SRP Ab, respectively. Sunflower was recognized faintly by rabbit anti-SRP Ab, which is in agreement with Western blotting (Figure 4), but in disagreement with ELISA (Table 3).

The cross-reactive 0.1 M NaOH (pH 12.22) extracted test proteins are shown in Figure 10. As shown in the figure, proteins from soybean, winged bean black gram, mung bean, navy bean, horse bean, moth bean, black bean, Great Northern bean, tepary bean, pinto bean, chickpea, lima bean, blackeye pea and small red bean were recognized by rabbit anti-SRP, respectively. Whole oat and sorghum were not recognized as cross-reactive by rabbit anti-SRP Ab. Whole oat and sorghum were only faintly cross-reactive using Western blotting (Figure 5), but not detected as cross-reactive using ELISA (Table 4).

The cross-reactive 70% (v/v) aqueous ethanol extracted test proteins are shown in Figure 10. As shown in the figure, proteins from soybean, winged bean, wheat berries, wheat bran, macadamia nut and black gram were recognized by rabbit anti-SRP Ab, respectively. The results are in agreement with ELISAs (Table 5) and Western Blotting (Figure 6), with the exception of macadamia nut. Macadamia nut was detected as cross-reactive in ELISA, but not cross-reactive in Western Blotting.

The cross-reactive commercial soy products extracted in BSB (pH 8.45) are shown in Figure 11. As shown in the figure, proteins from soy milk, soy nuts and soy tofu were recognized by rabbit anti-SRP Ab, respectively. The results are in agreement with previously observed ELISA (Table 6) and Western blotting (Figure 7).

The soybean cultivars extracted in BSB (pH 8.45) are shown in Figure 11. As shown in the figure, all soybean cultivars are recognized with similar intensity by rabbit anti-SRP Ab, respectively. The results are in agreement with the previously observed Western blotting (Figure 8 and 9).

Figure 4 – Western Blots and SDS-PAGE analysis of cross-reactive BSB (pH 8.45) extracted test proteins. **A** – Western blotting. The protein load for all test samples was 30 μ g and SRP was 2 μ g. **B** – SDS-PAGE (8-25% linear acrylamide gradient) in the presence of 2% β -ME. The protein load for LMW standard 58 μ g, and all test proteins and SRP was 30 μ g.

Figure 5 – Western blots and SDS-PAGE analysis of cross-reactive 0.1 M NaOH (pH 12.22) extracted test proteins. **A** – Western blotting. The protein load for all test samples was 30 μ g and SRP was 2 μ g. **B** – SDS-PAGE (8-25% linear acrylamide gradient) in the presence of 2% β -ME. The protein load for LMW standard 58 μ g, and all test proteins and SRP was 30 μ g.

Figure 6 – Western blots and SDS-PAGE analysis of cross-reactive 70% (v/v) aqueous ethanol extracted test proteins. **A** – Western blotting. The protein load was as follows: SRP 2 μ g, soybean 6 μ g, winged bean 2 μ g, wheat berries 21 μ g, wheat bran 13 μ g, macadamia nut 8 μ g and black gram 3 μ g. **B** – SDS-PAGE (8-25% linear acrylamide gradient) in the presence of 2% β -ME. The protein load was as follows: LMW standard 58 μ g, SRP 6 μ g, soybean 6 μ g, winged bean 2 μ g, wheat berries 21 μ g, wheat bran 13 μ g, macadamia nut 8 μ g and black gram 3 μ g.

Figure 7 – Western blots and SDS-PAGE analysis of cross-reactive commercial soy products extracted in BSB (pH 8.45). **A** – Western blotting. The protein load for all test samples was 10 μ g and SRP was 2 μ g. **B** – SDS-PAGE (12% acrylamide) in the presence of 2% β -ME. The protein load for LMW standards was 58 μ g, all test samples 18 μ g, and SRP 15 μ g.

Figure 8 – Western blots and SDS-PAGE analysis of soybean cultivars. **A** – Western blotting. The protein load for all test samples was 15 μ g and SRP was 2 μ g. **B** – SDS-PAGE (8-25% linear acrylamide gradient) in the presence of 2% β -ME. The protein load for LMW standard was 58 μ g, peptide markers 5 μ l, all test samples and SRP 30 μ g.

Figure 9 - Western blots and SDS-PAGE analysis of soybean cultivars. **A** – Western blotting. The protein load for all test samples was 15µg and SRP was 2µg. **B** – SDS-PAGE (8-25% linear acrylamide gradient) in the presence of 2% β-ME. The protein load for LMW standard was 58µg, all test samples and SRP 30µg.

Figure 10 – Dot blots. **A** – Dot blots of cross-reactive BSB (pH 8.45) extracted test proteins. Test proteins pipetted with 2 μ l of original (undiluted) extracts, SRP pipetted with 1 μ l of 1 mg/ml. **B** – Dot blots for cross-reactive 0.1 M NaOH (pH 12.22) extracted test proteins. Test proteins pipetted with 2 μ l of original (undiluted) extracts, SRP pipetted with 1 μ l of 1 mg/ml. **C** – Dot blots for cross-reactive 70% (v/v) aqueous ethanol extracted test proteins. Test proteins pipetted with 8 μ l of original (undiluted) extracts, SRP pipetted with 1 μ l of 1 mg/ml.

Figure 11 – Dot Blots. **A** – Dot blots for cross-reactive commercial soy products extracted in BSB (pH 8.45). Test proteins pipetted with 2 μ l of original (undiluted) extracts, SRP pipetted with 1 μ l of 1 mg/ml. **B** – Dot blots for soybean cultivars extracted with BSB (pH 8.45). Test proteins pipetted with 2 μ l of original (undiluted) extracts, SRP pipetted with 1 μ l of 1 mg/ml.

Chapter 5

Discussion

It is generally understood that most legumes are limiting in sulfur amino acids. As mentioned before, glycinin (11S) and β -conglycinin (7S) account for up to 70% of the total protein in the soybean seed (Moreira et al., 1979). Therefore, these two proteins are mainly responsible for determining the nutritional quality of the seed protein. Both of these proteins are nutritionally limiting in sulfur amino acids (methionine and cysteine).

Solutions to the nutritional deficiency have been investigated by researchers for many years. Several attempts have been made to detect and increase expression of high sulfur proteins in plant seeds. Plant breeding and genetic manipulation (De Lumen et al., 1997) of protein synthesis are the two most commonly used techniques for such purposes. Youle and Huang (1981) have found 2S albumins in various species to have high levels of cysteine and methionine, including sunflower, mustard, linseed, almond, lupin, cucumber, Brazil nut, hazelnut, yucca, castor bean and cottonseed. In another study, Schroeder (1982, 1984) isolated an albumin fraction with two major polypeptides (MW 8,000 and 22,000 Da) from pea (*Pisum*) seeds which contain a higher sulfur content compared to the other albumin fractions. These two polypeptides were present in all of the 45 lines of peas examined. The present study found similar results in respect to the 31 cultivars of soybeans examined (Figure 8 and 9). Similar levels of SRP were present throughout the soybean cultivars. This may open up new avenues for research on soybean cultivars, since SRP is not restricted to just one particular cultivar. Kortt and Caldwell (1990) isolated the albumin fraction of sunflower seeds and resolved them into eight distinct components by reversed-phased HPLC. Each of the fractions contained a single polypeptide chain with MW ranging between 10,000 and 18,000 Da, and all were basic proteins with distinct amino acid compositions. Two of the sunflower seed albumins with MW 10,000 Da were methionine-rich proteins containing 16 per cent methionine residues and 8 per cent cysteine residues. These sulfur-rich proteins account for 7% of total salt extractable (buffered 0.5 M NaCl, pH 8.0) pea seed proteins.

The present study reports the cross-reactivity of various plant seeds with rabbit anti-SRP Ab. Each plant seed sample was extracted in either BSB (pH 8.45), 0.1 M NaOH (pH 12.22) or 70% (v/v) aqueous ethanol, to isolate the albumins and globulins,

glutelins and prolamins, respectively. The cross-reactivity shown between rabbit anti-SRP Ab and other plant seeds may be due to 1) a similarity in linear and/or structural epitopes between the cross-reactive protein and SRP (an SRP-like protein), 2) the presence of 11S and 7S contaminants in SRP preparation, 3) non-protein components and/or 4) irrelevant proteins (non SRP-like proteins). The methods (ELISA, Western blotting and Dot blotting) used in this study have clarified which plant seed proteins are cross-reactive, with Western blotting supplying a more in depth look at exactly which peptide(s) are being recognized by the antibody (rabbit anti-SRP).

The two major polypeptides of SRP have an estimated MW of 28,000-29,000 Da and 17,000-18,000 Da, with an intermediate polypeptide of MW 45,000 Da. These are the polypeptides of most importance when determining true cross-reactivity with rabbit anti-SRP Ab. The Western blotting for the cross-reactive BSB (pH 8.45) extracted samples is shown in Figure 4 (A). As shown in the figure, soybean, winged bean, black gram, mung bean, navy bean, tepary, pinto bean, blackeye pea, horse bean, black bean, Great Northern bean, lima bean and small red bean all have cross-reactive polypeptides to the two major polypeptides (MW 28-29 and 17-18 kDa) of SRP. In addition, soybean, winged bean black gram, mung bean, chickpea, blackeye pea and horse bean have cross-reactive polypeptides to the intermediate polypeptide (MW 45 kDa) of SRP. Wheat berries, moth bean and sunflower have faintly cross-reactive polypeptides in the 66 kDa region. Removal of minor contaminants from SRP is difficult. However, SRP used in this study (Sathe 1991), was at least 90% pure.

The Western blotting for the cross-reactive 0.1 M NaOH (pH 12.22) extracted samples is shown in Figure 5 (A). As shown in the figure, soybean, winged bean, black gram, mung bean, navy bean, horse bean, moth bean, black bean, Great Northern bean, tepary bean, pinto bean, blackeye pea and small red bean all have cross-reactive polypeptides to the two major polypeptides (MW 28-29 and 17-18 kDa) of SRP. In addition, soybean, winged bean, black gram, mung bean, horse bean and black bean have cross-reactive polypeptides to the intermediate polypeptide (MW 45 kDa) of SRP. Lima bean and chickpea have cross-reactivity to only the HMW subunit (MW 28-29 kDa) of SRP. Whole oat and sorghum show faint polypeptides in the 66 kDa region, and perhaps are not relevant to SRP.

The Western blotting for the cross-reactive 70% (v/v) aqueous ethanol extracted samples is shown in Figure 6 (A). As shown in the figure, soybean, winged bean and black gram show cross-reactive polypeptides to the HMW subunit (MW 28-29 kDa) of SRP. Wheat berries and wheat bran show cross-reactive polypeptides to the intermediate subunit (MW 45 kDa) of SRP. Macadamia nut did not show any polypeptides cross-reactive to SRP on the Western blot, but did show cross-reactivity in ELISA and Dot blotting. This can perhaps be explained by the antibody (rabbit anti-SRP) recognizing a component other than one of SRP major polypeptides, which could be a non-protein component.

The Western blotting for the cross-reactive commercial soy products extracted in BSB (pH 8.45) is shown in Figure 7 (A). As shown in the figure, soy milk, soy nuts and soy tofu all show cross-reactivity to rabbit anti-SRP Ab, but the most interesting factor is the cross-reactive protein is in between the HMW and LMW major polypeptides of SRP. Sathe (1989) found that SRP in soybeans aggregates at $\geq 70^{\circ}\text{C}$ due to thermal denaturation of the protein, which subsequently leads to the formation of soluble and insoluble aggregates. Since commercial soy products are processed at high temperatures, this could perhaps explain the cross-reactive polypeptide. SRP (1 mg/ml) was cooked at 100°C at different time intervals (0, 1, 3, 5, 10, 15, 30 min) and was then subjected to Western blotting and SDS-PAGE analysis both in the presence and absence of 2% β -ME (Figure 13). As shown in the figure, SRP does aggregate at 100°C and with increased exposure time at 100°C , SRP begins to form insoluble aggregates which is demonstrated by the disappearance of the polypeptides.

Lastly, the Western blotting of the soybean cultivars is shown in Figures 8 and 9 (A). Cross-reactivity of all the major polypeptides of SRP is seen throughout the soybean cultivars, in addition all the soybean cultivars show the same level of cross-reactivity, judged subjectively by band width and intensity. This indicates that all the soybean cultivars examined contain similar levels of SRP.

Figure 13 – Western blots and SDS-PAGE analysis of cooked (100°C) SRP at various time intervals in the presence and absence of β -ME. **A** – Western blotting. The protein load for all SRP samples was 4 μ g in the absence of β -ME. **B** – Western blotting. The protein load for all SRP samples was 4 μ g in the presence of 2% β -ME. **C** – SDS-PAGE (8-25% linear acrylamide gradient) in the absence of β -ME. The protein load for LMW standards was 58 μ g and all SRP samples was 28 μ g. **D** – SDS-PAGE (8-25% linear acrylamide gradient) in the presence of 2% β -ME. The protein load for LMW standards was 10 μ l and all SRP samples was 28 μ g.

CONCLUSION

This study sought to answer four questions. The first question was “can a sensitive immunoassay be developed for detection of SRP?” The findings of the study indicate that ELISA, Western blotting and Dot blotting are all sensitive immunoassays that can be used for detection of SRP. The detection limit for ELISA is 400 ng/ml, Western blotting is 50 ng and Dot blotting is 1 ng, respectively.

The second question this study sought to answer was “what other plant proteins are cross-reactive with SRP?” ELISA, Western blotting and Dot blotting data show cross-reactivity in a variety of proteins found in edible plant seeds. These included, soybean, winged bean, black gram, mung bean, navy bean, tepary bean, pinto bean, chickpea, blackeye pea, horse bean, moth bean, black bean, Great Northern bean, lima bean, small red bean, wheat berries and wheat bran.

The third question was “do all soybean cultivars contain SRP?” Western blotting and Dot blotting data indicated similar levels of SRP are found throughout all the soybean cultivars.

Lastly, the fourth question this study sought to answer was “does SRP exist in processed soy products?” ELISA, Western blotting and Dot blotting data show SRP does exist in the commercial soy products (soy milk, soy nuts and soy tofu) examined.

APPENDIX

One-Way Analysis of Variance

Table 7: One-Way ANOVA for Cross-Reactivity between BSB (pH 8.45) Extracted Proteins from Edible Plant Seeds and SRP

ANOVA IC ₅₀					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.425624501	16	0.026601531	0.996184	0.475448
Within Groups	1.361875468	51	0.026703441		
Total	1.787499968	67			

Table 8: One-Way ANOVA for Cross-Reactivity between 0.1 M NaOH (pH 12.22) Extracted Proteins from Edible Plant Seeds and SRP

ANOVA IC ₅₀					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.113985586	15	0.007599039	1.070358	0.406935
Within Groups	0.340777384	48	0.007099529		
Total	0.45476297	63			

Table 9: One-Way ANOVA for Cross-Reactivity between 70% (v/v) Aqueous Ethanol Extracted Proteins from Edible Plant Seeds and SRP

ANOVA IC ₅₀					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.0290	<u>6.00</u>	0.005	0.96702	0.471009
Within Groups	0.1050	<u>21.00</u>	0.005		
Total	0.1340	<u>27.00</u>			

Table 10: One-Way ANOVA for Cross-Reactivity between Commercial Soy Products Extracted in BSB (pH 8.45) and SRP

ANOVA IC ₅₀					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.50815E-06	3	8.36051E-07	0.679051	0.589057
Within Groups	9.84964E-06	8	1.23121E-06		
Total	1.23578E-05	11			

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