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Immunochemical examination of the surface physico-chemical properties of various dicotyledonous and monocotyledonous globulin seed storage proteins

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Immunochemical analysis of the surface properties of seed globulins indicated that the 11S seed globulins of monocotyledonous and dicotyledonous plants possibly share some similarity in their primary structures, i.e. amino acid sequence on their surface topography as well in their structural conformation. Although homologies were found in all globulins, stronger antigenic responses were found to occur between the amaranth globulin antibody and the purified globulins from monocotyledonous than those originating from dicotyledonous seeds. Homologies in primary structure were found to occur almost exclusively in the 'basic' (20–27 000 Da) type subunit of the respective globulins. Data would imply that gene families encoding for these different proteins were derived from a common ancestral gene and must have been present before divergence of the angiosperms into dicotyledonous and monocotyledonous plants. © 1998 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

The uniqueness of the immunochemical approach in the study and comparison of the surface characteristics of globulin storage proteins is based on the specificity of the reaction between the antibodies used and the proteins to be analyzed (Harlow and Lane, 1988). In storage proteins, the parts of the folded polypeptide chains that lie at the surface of the molecule are responsible for its surface topology. The specific patterns that are recognized by antibody molecules on the surface topology of storage proteins are called epitopes or antigenic sites. These surface epitopes of interest usually consist of about 6–10 amino acids that may be arranged in the primary structure either continuously or discontinuously (Hopp, 1986; Harlow and Lane, 1988; Scheidtamann, 1990). In addition, an antigenic epitope may be determined and recognized by its specific sequence or by the conformation in which this sequence is embedded, and in such cases is referred to as either a sequential (amino acid sequence) or a conformational (structural) epitope, respectively. It has also been reported that in some cases, antigenic determinants may depend on both sequence as well as conformation (Harlow and Lane, 1988). In addition, a prerequisite of the antibody/antigen recognition response is the proper geometric exposure and accessibility of the specific antigenic site in question on the surface of the globulin being tested (Hopp, 1986; Harlow and Lane, 1988; Scheidtamann, 1990).

The three known factors that contribute to a high probability of surface exposure of the epitope of all studied antigenic sites are: (1) the degree of hydrophilicity; (2) mobility; and (3) the potential to form β-turns or loop structures (Hopp, 1986; Scheidtamann, 1990). As for hydrophilicity, antigenic sites tend to occur on polar or hydrophilic regions of the polypeptide chain, and therefore, have a propensity to be on the surface, whereas, apolar or hydrophobic regions are normally located in the interior of the protein (Scheidtmann, 1990).

As for mobility, both nuclear magnetic resonance (NMR) and X-ray crystallography studies indicate that the segment of the polypeptide chain containing the epitopes on the surface of a protein have higher mobility than those polypeptide stretches in the interior (Scheidtmann, 1990). As for structural organization, 'rigid' structures such as α-helices and β-sheets are more likely to form the internal core of a protein, whereas β-turn and loop structures have a higher probability of being on the surface and have a higher

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probability of containing the epitope (Hopp, 1986; Scheidtamann, 1990).

Another feature of major antigenic sites is their preponderance to contain charged and polar amino acids (Sela and Mozes, 1966; Hopp, 1986). Some amino acid residues tend to be more immunogenic than others and are found in antigenic epitopes with relatively high frequency, i.e. in decreasing order His, Lys, Ala, Leu, Asp and Arg (Scheidtamann, 1990). Although more charged and polar amino acids are found associated with these sites, this does not preclude hydrophobic interactions from adding to the binding stability between antibodies and antigens. Hydrophobic effects are well established in many antibody binding site interactions (Walter *et al.*, 1980; Hopp and Woods, 1981; Hopp, 1986). However, the highly directional nature of charge–charge and polar interactions (e.g. hydrogen bonds) contribute the critical element of geometric specificity to the binding sites (Hopp, 1986).

In conclusion, the occurrence of epitopes on the surface of proteins are constrained to very small, flexible, hydrophilic, structure specific, exposed patches of polypeptide chain consisting of reoccurring charged and polar amino acids. The specificity of an induced antibody holds great promise for determining the surface characteristics and surface homologies in various purified globulin storage proteins. Immunochemical analysis would provide the protein biochemist with a very fast, efficient and quantitative method of high specificity for the probing and mapping of different and conserved regions on the surface topography of these globulins.

The purpose of this study was, therefore, to determine the level of conservation (if any) between the protein surfaces of monocotyledonous and dicotyledonous seed globulins using antibodies formed against a well studied and understood globulin from amaranth seed (amaranthin). The use of amaranthin, a hetero-oligomeric protein composed of 5 subunits and an overall molecular weight of 337 500 Da (Marcone and Yada, 1991, 1992; Marcone *et al.*, 1994) would provide an appropriate bench mark for comparison between globulins. Since surface properties of proteins are closely linked with functionality, the uniqueness or commonalities in surface properties that amaranth globulin share with other dicotyledonous and monocotyledonous globulins may serve as a bench mark for determining if proteins have a particular physico-chemical property desirable for a particular food or food manufacturing process.

MATERIALS AND METHODS

Amaranth globulin isolation and purification

Non-heat treated milled flour of *Amaranthus hypochondriacus* K343 was purchased from American Amaranth Inc. (Bricelyn, MI) and the globulin was isolated and purified to homogeneity by gel filtration and anion-exchange chromatographies and stored desiccated at −71°C under an argon blanket until further study, as previously described by Marcone and Yada (1991).

Isolation and purification of various monocotyledonous and dicotyledonous derived globulins

Isolation and purification of 11S seed storage globulins from pea (*Pisum sativum* L.), peanut (*Arachis hypogaea* L.), buckwheat (*Fagopyrum esculentum*), barley (*Hordeum vulgare*), corn (*Zea mays*), rye (*Secale cereale*), oat (*Avenua sativa*), coconut (*Cocos mucifera*), soybean (*Glycine* max L.), wheat (*Triticum aestivum*), alfalfa (*Medicago sativa*), caraway (*Carum carvi*), celery (*Apium graveolens*), cumin (*Cuminum cyminum*), dill (*Anethum graveolens*), fennel (*Foeniculum vulgare*), flax (*Linum usitatissimum*), mustard (*Brassica alba*), and pine nut (*Pinus edulis*) were performed as described in Marcone *et al.* (submitted a), i.e. a modification of the Osborne fractionation procedure for globulin proteins (Osborne, 1918). Storage globulins from hemp (*Cannabis sativa*) and pumpkin (*Cucurbita pepo*) were purified by anion exchange chromatography as described in Marcone *et al.* (submitted *a*).

Determination of protein concentration

Protein was measured using the Bio-Rad protein dye-binding assay (modified Bradford) with bovine serum albumin (BSA) as the standard (Bio-Rad Laboratories, 1989). Analyses were performed in triplicate. In addition, a spectrophotometric method was used based on the difference in absorbance of proteins at 235 and 280 nm. (Whitaker and Granum, 1980).

Production of polyclonal antibodies

Two-2.0 kg leghorn chickens were obtained from the Arkell Research Poultry Station and maintained at the Central Animal Care Facility Building, University of Guelph, Guelph, Ontario. Animal housing and immunization were carried out in accordance with the regulations set forth by the University of Guelph Animal Care Committee and the Canadian Council on Animal Care (1984). Immunization was begun two weeks after the chickens were received. Chickens were then injected once a week for six consecutive weeks with a 0.4 ml solution containing (0.2 ml Freund's incomplete adjuvant, 0.2 ml sterile water, 400 μg purified amaranth globulin); with 0.20 ml being injected into each breast muscle per chicken. Eggs were collected each day between weeks 2 and 7. Eggs were also collected prior to immunization and constituted the pre-immune material.

Isolation of egg yolk IgG

Yolks were diluted with 9 %vol of distilled water and frozen and thawed after adjusting the pH to 7.0 with

0.1 N NaOH as described by Jensenius *et al.* (1981). Lipoproteins were then removed by centrifugation at 2000 *g*. The sediment, containing lipid and lipoproteins, was discarded and 1 ml of a 0.40 M phosphate buffer, pH 7.6, and 1.0 ml of a tris-hydroxymethylamino-methane-buffered saline (0.14 M NaCl, 0.01 M tris/HCl, pH 7.4, 0.1% (w/v) NaN_3) was added to the supernatant. Twenty grams of Na_2SO_4 was then slowly added with constant stirring and incubated for 0.5 h. This solution was centrifuged at 2000 *g* and the supernatant discarded. The sediment contained the IgG. This IgG was made up to a concentration of 1.57 mg ml^{-1} with the addition of a 0.010 M tris-HCl buffer (pH 7.5).

Determination of IgG purity

IgG purity was determined by SDS-PAGE (under non-reducing conditions) using precast 8–25 gradient SDS-PAGE gels as described in the manufacturer's instructions (Pharmacia LKB) and described in Marcone *et al.* (submitted *a*).

Immunochemical methods

Measurement of antibody activity

The Enzyme-linked immuno sorbent assay (ELISA) method was used to measure antibody activity. Purified seed globulins from dicotyledonous and monocotyledonous plants were serially diluted from 10 μg to 1.5 μg ml^{-1} in a coating buffer of pH 9.8 containing 0.3 g Na_2CO_3, 0.6 g $NaHCO_3$ and 0.2 g $MgCl_2 \cdot 6H_2O$ in 200 ml of distilled water. Ninety-six-well Immunlon III ELISA plates (Dynatech, Fisher Scientific, Unionville, Ontario) were coated or sensitized with the addition of 100 μl of antigen solution to each well. Incubation of the plates was performed at 37°C for two hours on an orbital shaker. Plates were then washed five times with phosphate buffer saline (PBS)-Tween prepared with 8.0 g NaCl, 2.9 g $KH_2HPO_4 \cdot 12\ H_2O$, 0.5 g KCl and 1 ml of Tween 20 diluted to 1 l and adjusted to pH 7.4. All unbound sites on wells were blocked with 150 μl of a 3% Difco skimmed milk powder made up in PBS and incubated at 37°C for 30 min as described above. Washing was performed three times as described above with PBS-Tween.

First antibody step

One hundred μl of the chicken IgG produced against the amaranth globulin were diluted to 1:100, 1:500, 1:1000 with PBS and added to each well and incubated for 1 h at 37°C. Washing was again performed with PBS-Tween 5 times.

Second antibody step

One hundred μl of a 1:1500 dilution of rabbit anti-chicken IgG alkaline phosphatase conjugate (A-9171 Lot 041H-4858, Sigma, St. Louis, MO) or a 1:3000 dilution of rabbit anti-chicken IgG horseradish peroxidase conjugate (A-9046 Lot 082H-4849 Sigma, St. Louis, MO) were added to each well. These antibody-enzyme conjugates were diluted in PBS immediately before use. Plates with antibody solutions were incubated at 37°C for 1 h. The plates were then washed 5 times with PBS-Tween buffer. Substrates were made up just as before use. For alkaline phosphatase, 1.0 mg ml^{-1} of para-nitrophenyl phosphate (Sigma Product N-2765) was dissolved in a 10% diethanolamine buffer, pH 9.8, containing 0.01% $MgCl_2$ and 0.02% (w/v) NaN_3 and 100 μl applied to wells and allowed to incubate at 37°C for 30 min. The absorbance was read at 405 nm on a Thermomax Microplate Reader (Molecular Devices, Toronto, ON).

For horseradish peroxidase, the ECl (Western blotting detection reagents) RPN 2109 (Amersham, Buckinghamshire, England) was used. Luminescence (emission of light resulting from the dissipation of energy from the luminal substrate in its excited state) was detected with the use of Biomedical Image Quantifier Bioview (BIQ) and Mitsubishi color video copy processor (Cambridge, UK). Analyses were performed in triplicate.

Ouchterlony double immunodiffusion

Gels were prepared using 1.5% ultra-pure DNA grade agarose (Bio-Rad) in PBS, pH 7.5, and 0.02% (w/v) sodium azide. After gentle heating to 45°C, 3 ml of the melted agarose mixture was applied to glass microscope slides. After cooling, 4 mm wells were punched (with the use of a hollow steel cutter) for the antibody and 2.5 mm wells for the antigens, i.e. the various dicotyledonous and monocotyledonous seed globulins (Ouchterlony, 1968). Twenty μl of antibody and 10 μl of antigen was applied to the appropriate wells. Analyses were performed in duplicate.

Assessment of immunoreaction with unfolded protein

Purified globulin (10 μg ml^{-1}) was dissolved in 1 ml of 8 M urea in PBS, 0.1 ml β-mercaptoethanol and 0.04 g of SDS and allowed to stand at room temperature for 1 h, and then boiled for 10 min as described by Deutsch (1976).

Western blotting

SDS-PAGE was performed as previously described in Marcone *et al.* (submitted *a*). Protein bands were transferred onto 0.45 μ nitrocellulose membrane using the PhastSystem Blotting Unit (Pharmacia, LKB, Montreal, Quebec) according to manufacturer's instructions. Corresponding SDS-PAGE gels were also run with the purified globulins and the following molecular weight markers, phosphorylase b (94 000 Da), bovine serum albumin (67 000 Da), ovalbumin (43 000 Da), carbonic anhydrase (30 000 Da), soybean

trypsin inhibitor (20 100 Da), and α-lactalbumin (14 000 Da).

Membranes were blocked overnight at 4°C with a 3% (w/v) fish gelatin solution, followed by 5-3 minute washings with PBS-Tween. Membranes were then incubated with a 1:100 dilution of primary antibody raised against the purified *Amaranthus hypochondriacus* K343 globulin at room temperature for 1 hour followed by 5-3 minute washing with PBS-Tween. Membranes were then incubated in a 1:1500 dilution of secondary antibody (rabbit anti-chicken IgG alkaline phosphatase conjugate, A-9171 Lot 041H-4858, Sigma, St. Louis, MO) followed by 5-3 min washings with PBS-Tween. Membranes were subsequently washed 2-2 min in PBS, and then 2-2 min in 0.1 M tris buffer, pH 9.0.

Enzymatic substrate was made up in 0.1 M tris buffer, pH 9.0 and contained both 1 mg ml^{-1} of fast red (Sigma, St. Louis, MO) and 1 mg ml^{-1} naphthol AS-BI phosphate (Kodak, Rochester, NY). Membranes were incubated in this solution for 2 min.

Isolation of subunits of *Amaranthus hypochondriacus* globulin K343 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis (SDS-PAGE)

Slab SDS-PAGE was performed according to the method of Laemmli (1970) with 5% β-mercaptoethanol, 6.5% (2% cross-linkage) polyacrylamide running gels using a Protean TM II (Bio-Rad, ON). The bands were carefully excised after visualization with a non-fixative copper chloride staining (Lee *et al.*, 1987) and quantitatively recovered by electroelution in a 0.04/0.004 M Tris-acetate buffer, pH 8.40, using an ISCO Model 1750 concentrator (Canberra Packard, Toronto, ON). Electrophoretic elution was carried out at 12°C for 8 h at 12 W as per the manufacturer's instructions.

RESULTS AND DISCUSSION

Selection of the type of antibody for globulin surface characterization

Although monoclonal antibodies are powerful agents for the testing for the presence of a desired (specific) surface epitope on a protein, they are not necessarily the best choice for all immunochemical techniques (Harlow and Lane, 1988). In practice, producing the right set of monoclonal antibodies is often a difficult and laborious task (Harlow and Lane, 1988). However, polyclonal antibodies are easier to produce although they are usually produced as a heterogeneous population recognizing more than one antigenic determinant (Harlow and Lane, 1988).

The use of hyperimmunization rather than the standard immunization for the production of polyclonal antibodies (as selected and used in this study) results in antibodies having many useful properties such as class shift from predominantly IgMs to IgGs with affinity maturation, i.e. the average affinity of the antibody for an antigen increasing with repeated injections (Harlow and Lane, 1988). Another useful property of antibodies produced by hyperimmunization is clonal dominance such that antibodies to a limited number of epitopes will tend to dominate at the later stages of the hyperimmunization protocols (Harlow and Lane, 1988). Production of antibodies using chickens maximizes the amount of antibody that can be produced and allows for the antibodies to be collected and isolated from eggs which is a less invasive technique than the bleeding of animals (Jensenius *et al.*, 1981).

Polyclonal antibody production and testing

The intramuscular injection of purified amaranth K343 seed globulin into the breast muscles of leghorn chickens was successful in eliciting a satisfactory immune response with the adequate amounts of antibody being produced, i.e. approximately 50 mg IgG per egg. The highest titres were found to occur by the fifth week and then dropped slightly thereafter. Although isolation of antibodies from eggs rather than blood serum involved a more complicated procedure, chicken IgG was successfully isolated and purified i.e. > 90% (as determined by SDS-PAGE) from the other egg yolk proteins. All tested dilutions of the chicken egg yolk IgG containing the expressed antibody, i.e. 1:100, 1:500, 1:1000, were found to produce adequate ELISA responses against antigen (purified amaranth globulin) concentrations between 1.5 to 10 g/ml. No immune response was observed to occur with IgG isolated from eggs of preimmunized chickens and the antigen in question. The best dilution of secondary conjugated antibodies were determined to be 1:1500 for alkaline phosphatase and 1:3000 for horseradish peroxidase linked anti-chicken antibodies. Ouchterlony immune reaction was also noted between antigen and antibody (data not shown).

Immunochemical probing for the existence of surface homologies between dicotyledonous and monocotyledonous seed globulins

In order to determine the surface characteristics and surface homologies that the various purified dicotyledonous and monocotyledonous seed globulins may share in common, tests were performed with the expressed amaranth globulin antibody. The results indicated that substantial cross-reactivity was present between the IgG expressed against the purified amaranth globulin and those seed globulins derived from a variety of genetically different dicotyledonous and monocotyledonous plants. It should be noted that all purified 11S globulins tested cross-reacted with the expressed antibody (Table 1) indicating the presence of some degree of surface homology with all globulins.

Table 1. The percentage cross-reactions of polyclonal antibody with storage proteins from different species (antibody dilution 1×10^{-4})

Purified 11S globulin storage protein	Percentage cross-reactivity*
Amaranth (*Amaranthus hypochondriacus*)	100.0
Dicotyledons	
Soybean (*Glycine max*. Merr)	3.6
Pea (*Pisum Sativum* L.)	9.8
Buckwheat (*Fagopyrum esculentum*)	7.8
Pumpkin (*Cucurbita pepo*)	16.3
Hemp (*Cannabis sativa*)	60.9
Alfalfa (*Medicago sativa*)	15.1
Caraway (*Carum carvi*)	4.4
Celery (*Apium graveolens*)	10.3
Cumin (*Cuminum cyminum*)	11.2
Dill (*Anethum graveolens*)	9.1
Fennel (*Foeniculum vulgare*)	9.3
Flax (*Linum usitatissimum*)	26.1
Mustard (*Brassica alba*)	20.7
Pine Nut (*Pinus edulis*)	16.1
Monocotyledons	
Barley (*Hordeum vulgare*)	46.6
Rye (*Secale cereale*)	50.4
Oat (*Avna sativa*)	58.4
Corn (*Zea mays*)	61.2
Wheat (*Triticum aestivum*)	43.7
Coconut (*Cocos mucifera*)	30.1
(BSA)	≈2.0

*Values corrected for non-specific reaction with BSA. Values shown are represented as a percentage of antibody's overall reactivity to the Amaranth globulin (Amaranthin).
Percent cross-reactivity were linear with antigen concentration.

Such results could very well point to a common ancestral relation or evolution. Closer examination of the data indicated a stronger immunogenicity (on average 3.2 times higher) for the seed globulins from monocotyledonous plants than from those from dicotyledonous plants. These results may suggest that the amaranth globulin (although a dicotyledon) has structurally more in common with the monocotyledonous seed globulins, i.e. wheat, oat, corn, barley, and rye, than those of the dicotyledonous globulins, i.e. pea, peanut, soybean, buckwheat, hemp, pumpkin, alfalfa, caraway, celery, cumin, dill, fennel, flax, and mustard (Table 1).

On denaturation of the dicotyledonous and monocotyledonous globulins, antigenicity was substantially reduced but was still present at a detectable level (data not shown). This would indicate that both sequential epitopes (amino acid sequence) and structural epitopes were being recognized by the antibody. The data indicated that all 11S globulins examined in this study may very well share some similarity in primary structure, i.e. amino acid sequence on their surface topography, as well as in their structural conformation. These conclusions further support the conclusions made in Marcone *et al.* (submitted *a,b*) regarding ancestral origins shared among all 11S seed globulins tested, i.e. secondary structure, subunit compositions, amino acid profiles, etc.

Determination of the exact surface location on globulins where homologies are taking place

Although similarities with regard to amino acid sequence and conformation were observed on all globulin surfaces, information regarding where the homologies were occurring was absent. This information would be particularly useful since all globulins were previously shown to be hetero-oligomeric type proteins composed of various subunits (Marcone *et al.*, submitted *a*). Therefore, the globulins, i.e. the purified amaranth globulin and the purified dicotyledonous and monocotyledonous globulin proteins, were run on SDS-PAGE and Western blotting (Figs 1–5). Results indicated that all subunits of the amaranth globulin were recognized by the expressed antibody whereas only a few of their constituent subunits were recognized by the same antibody for the other purified globulins. More specifically, in the case of the pea, peanut, soybean, corn, oat, hemp, pumpkin, caraway, celery, dill, and fennel globulins, only one subunit was found to be immunogenic whereas in the barley, rye, cumin, flax, mustard, pine nut, alfalfa, and wheat globulins, more than one subunit was found to be recognized (Table 2).

Since the experimental test conditions for SDS-PAGE combined with Western blotting denature the subunits of the globulins being tested, it could be concluded that the antibody recognized a sequence epitope, i.e. a similar amino acid sequence, in a few specific subunits and that the antigenic response was occurring through the 'basic' type subunit (abundant in basic type amino acids) of the purified globulins previously described by Marcone *et al.* (submitted *a*). In the case of the 11S soybean globulin, the antigenic response occurred only through the basic 18 KDa subunit (Kitamura and Shibasaki, 1975). Whereas, in contrast, its 'acidic' (abundant in acidic type amino acids) 34 KDa and 47 KDa subunits showed no reaction. Likewise, in the case of the hemp, oat, pumpkin and pea globulins, the antigenic response occurred through their respective basic subunits, i.e. 22.7, 25.8, 24.3 and 35 KDa, but not through their respective acidic subunits, i.e. 33.7, 37.1, 33.1 and 76.5 or 47.3 (for pea), respectively (Derbyshire *et al.*, 1976; Peterson, 1978; Ohmiya *et al.*, 1980; Casey *et al.*, 1981*a,b*). For wheat globulin the antigenic response was found to occur through both the basic 35 KDa and also the acidic 49 KDa subunits (Singh *et al.*, 1988).

Assuming that the basic type subunits are found in a similar overall ratio to acidic type subunits within their respective proteins, the above results suggest that the surface of the amaranth subunits and those of dicotyledonous and monocotyledonous basic subunits are structurally similar but not identical. From the above information and that in Table 2, the majority of subunits being recognized belong primarily to the 'basic' subunit class and relatively fewer to the 'acidic' subunit classification as proposed earlier Marcone *et al.* (submitted *a*) for subunits of all globulins. It is interesting

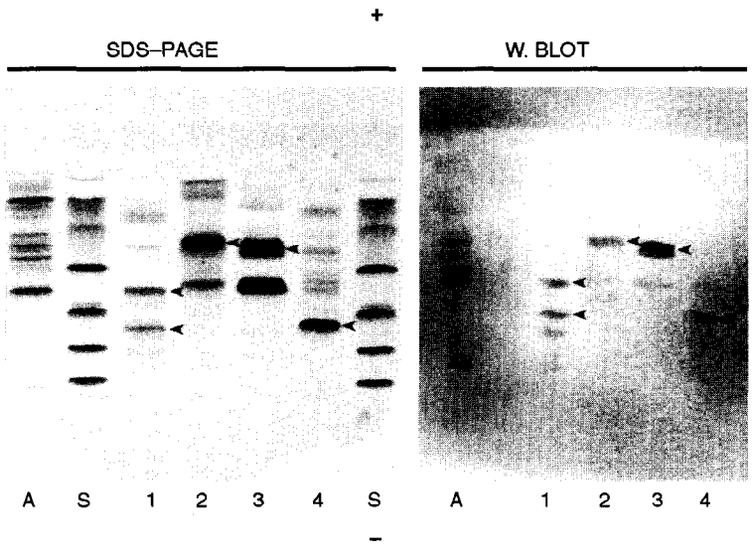

Fig. 1. SDS-PAGE of isolated and purified globulins (3 mg ml^{-1}): Lane A (amaranth), Lane S (standard), Lane 1 (wheat globulin), Lane 2 (hemp globulin), Lane 3 (pumpkin globulin), Lane 4 (coconut globulin) and Lane 5 (standards). Western blot of the purified globulins using a 1:100 dilution of the antibody raised against the purified amaranth globulin.

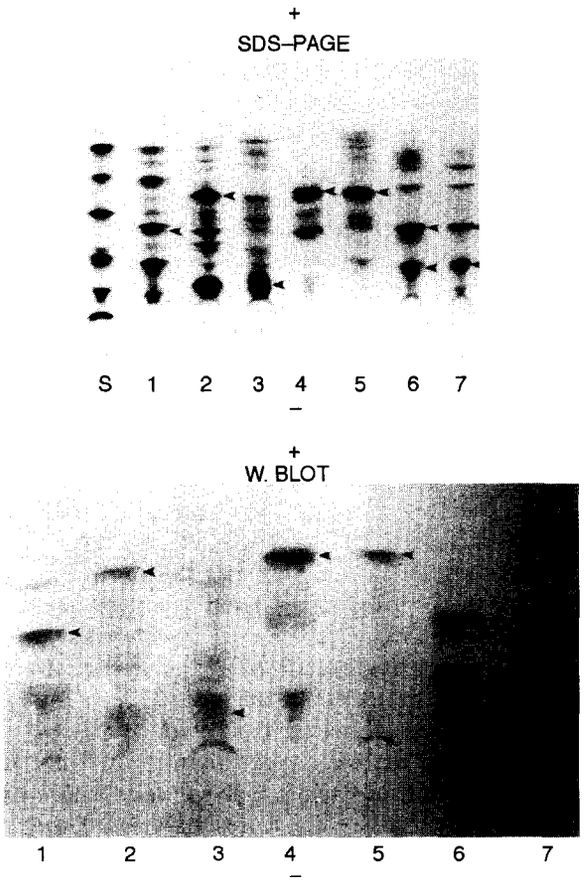

Fig. 2. SDS-PAGE of isolated and purified globulins (3 mg ml^{-1}): Lane S (standard), Lane 1 (pea globulin), Lane 2 (peanut globulin), Lane 3 (corn globulin), Lane 4 (oat globulin), Lane 5 (buckwheat globulin), Lane 6 (rye globulin) and Lane 7 (barley globulin). Western blot of the purified globulins using a 1:100 dilution of the antibody raised against the purified amaranth globulin.

to conclude that these 'basic' subunits which fall into a narrow molecular weight range, (i.e. 20–27 000 Da), are also related with regard to homologies in amino acid sequence which further distinguishes them from those subunits falling in the 'acidic' subunit range, (i.e. 30–39 000 Da) (Marcone *et al.*, submitted 1997*a*).

Since the antigenic response was occurring through the basic subunit, it was important to know which amaranth subunits were responsible for inducing the antibody used in this study. When the isolated subunits from the amaranth globulin were individually tested by the ELISA method, different levels of reactivity were noted. This would indicate that subunits must be distinct from one another. Noteworthy was the fact that isolated subunits gave a much lower reactivity than that produced for the whole oligomer. This may be due to the fact that once the subunits are individually separated, they lose their secondary structure (Marcone *et al.*, submitted *a*) and would indicate that tertiary and quaternary (topography) structures are being recognized by the produced antibody.

In the present study, a stronger antigenic response was found to occur between the anti-amaranth globulin antibody and the purified globulins from monocotyledonous seeds than those originating from dicotyledonous ones. This would indicate that the protein surfaces of the amaranth globulin more closely resemble those of monocotyledonous globulins than those of dicotyledonous ones. Although polyclonal antibodies were used, the expressed antibody was shown to specifically recognize the basic type subunits of all the tested globulins. Therefore, it is conceivable that the noted differences in the level of response in the ELISAs performed in this study could be due to the relative or spatial accessibility of the antibody to the basic type

subunit. With regard to the accessibility of subunits of various dicotyledonous seed globulins, the acidic polypeptides of the 11S soybean (*Glycine max*) and pea (*Pisium sativum*) globulins are found predominantly on the surface, whereas the basic subunits are located in the interior of the native globulin (Bond and Bowles, 1983; Wilson *et al.*, 1986; Gueguen *et al.*, 1988; Plumb *et al.*, 1989). The relative accessibility of acidic/basic subunits was supported by studies in which the 11S globulins of soybean (*Glycine max*), broad bean, (*Vicia sativa*) (Shutov *et al.*, 1981) and pumpkin (*Curcurbita moschata*) (Hara *et al.*, 1976) were hydrolyzed with an endo-peptidase. Results showed that the acidic chains of these globulins were initially degraded with no obvious attack on the basic chains. In all of the above cases, the basic polypeptides were found remaining intact through the limited proteolysis, implying that these chains are situated on the inside of the complex with the acidic chains on the exterior (Plumb *et al.*, 1989). Wilson *et al.* (1986) concluded that the preferential degradation of the acidic chains of 11S globulins was a generalized phenomenon in dicotyledons. The limited amount of sequence data presently available for some 11S globulins is also consistent with the hydrophilic nature of the acidic chains and the general hydrophobicity of the basic chains and their location.

Fig. 3. Western blot of Lane 1 (purified 11S soybean globulin) and Lane 2 (purified amaranth globulin).

Since the antibody in this study recognized the monocotyledenous globulins to a greater extent than the dicotyledonous globulins, it was proposed that the monocotyledonous globulins may have their basic subunits on the exterior while their acidic subunits are found in the interior. The opposite may be true for the dicotyledonous globulins. If this were true, it would constitute a significant finding with regard to differences between monocotyledonous and dicotyledonous globulins.

In an effort to determine if there is a difference in the location of basic and acidic subunits in globulins of dicotyledonous and monocotyledonous globulins (as suggested by the previous antibody work) further investigations into the quaternary structure of these globulins were undertaken.

Plietz *et al.* (1983) suggested that some level of structural homology exists between the dicotyledonous 11S seed globulins from soybean and sunflower and proposed a six-subunit model in which the six (acid-basic (AB)) subunits are arranged in a trigonal antiprism configuration (taking their data, a pictorial representation is represented in Fig. 6). This model complements the theory which suggests that the hydrophobic 'basic' type subunits are located predominately in the interior of the globulin structure whereas the more hydrophilic 'acidic' subunits would remain predominately at the

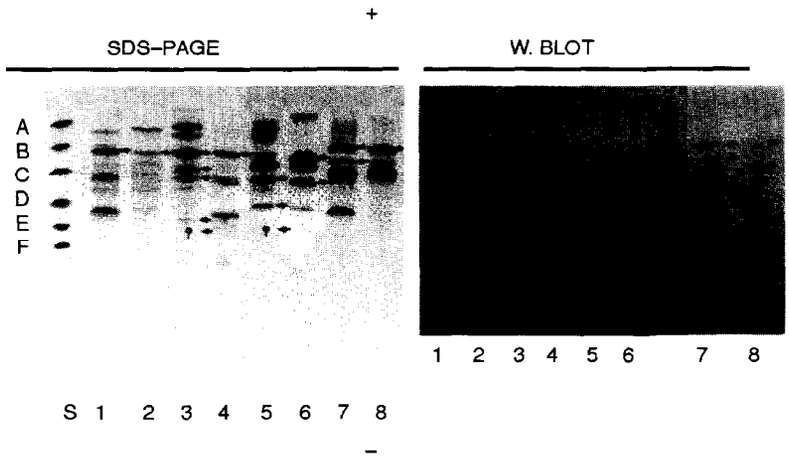

Fig. 4. SDS-PAGE of isolated and purified globulins (3 mg ml^{-1}): Lane S (standard), Lane 1 (dill globulin), Lane 2 (celery globulin), Lane 3 (alfalfa globulin), Lane 4 (fennel globulin), Lane 5 (pine nut globulin), Lane 6 (flax globulin), Lane 7 (cumin globulin), and Lane 8 (caraway globulin). Western blot of the purified globulins using a 1:100 dilution of the antibody raised against the purified amaranth globulin.

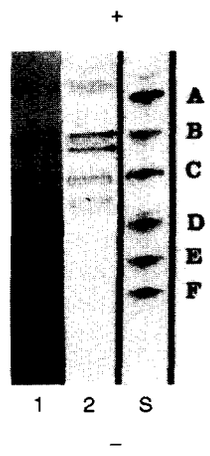

Fig. 5. Western blot of Lane 1 (purified 11S mustard globulin) and Lane 2 (purified mustard globulin), Lane S (standards). Western blot using antibody raised against the purified amaranth globulin.

surface. An alternate theory which has also been suggested (even for the 11S soybean globulin) is a stacked parallel hexagon model. This model, however, would not support the buried nature of basic subunits (Badley *et al.*, 1975).

For the amaranth globulin structure to accommodate more basic subunits on its exterior (which would then in turn help in eliciting antibodies which recognize these particular types of exposed subunits

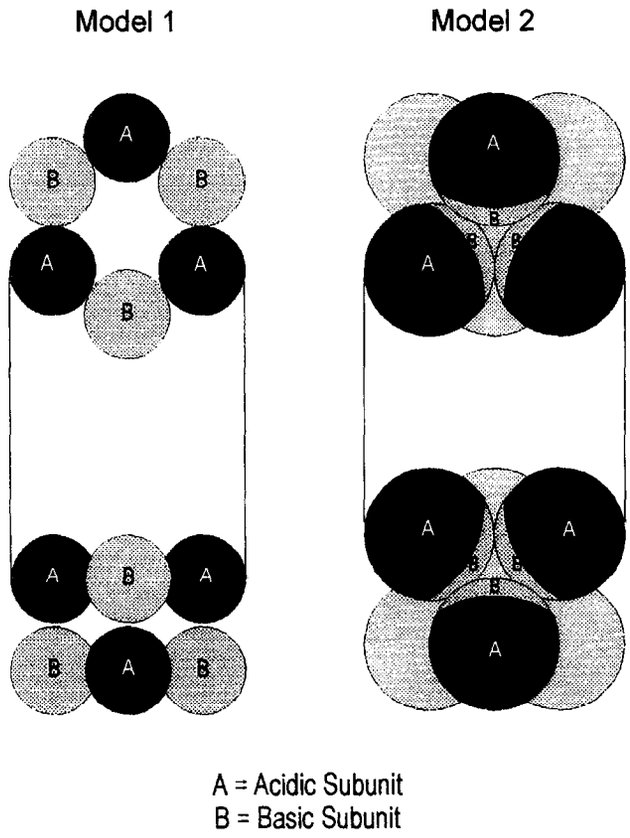

Fig. 6. Two proposed models for the quaternary structure of the soybean globulin. A modification of Plietz *et al.*, 1983.

Table 2. SDS-PAGE molecular mass distribution of subunits from various purified seed storage globulins and their antigenicity to antibody raised against the amaranth globulin

Globulin source	Number of subunits		Molecular mass of subunits	
	Experimental	Recognized by Ab	Experimental[a]	Recognized by Ab
Pea (*Pisum sativum* L.)	5	1	76.5,47.3,35,21.1, 15.5 K	35 K*
Peanut (*Arachis hypogaea* L.)	7	1	63.6,49.9,40.5, 39.9,36.2,30.6, 25.8 K	25.8 K
Soybean (*Glycine max.* Merr.)	3	1	47,34,18 K	18 K*
Corn (*Zea mays*)	3	1	69.7,36.8,33.7 K	69.7 K
Barley (*Hordeum vulgare*)	4	2	60.1,37.1,25.8, 21.1 K	60.1,37.1 K
Rye (*Secale cereale*)	4	2	60.1,37.1,25.8, 21.1 K	60.1,37.1 K
Oat (*Avena sativa*)	2	1	37.1,25.8 K	25.8 K*
Wheat (*Triticum aestivum*)	2	2	35,49 K	35*,49 K
Amaranth (*Amaranthus hypochondriacus*)	5	5	37.5,31.5,26.5, 20.5,14.5	(All)
Buckwheat (*Fagopyrum esculentum*)	1(2)	2	35.3,25.8 K	25.8 K
Hemp (*Cannabis sativa*)	3	1	33.7,22.7,12.3 K	22.7 K*
Pumpkin (*Cucurbita pepo*)	2	1	33.1,24.3 K	24.3 K*
Alfalfa (*Medicago sativa*)	7	3	37.4,35.6,33.3,30.0,22.8,17.4, 15.4 K	33.3,30.0,22.8 K*
Caraway (*Carum carvi*)	3	1	31.9,26.6,21.0 K	21.0 K
Celery (*Apium graveolens*)	5	1	37.6,35.6,31.9, 26.6,22.8 K	22.8 K
Cumin (*Cuminum cyminum*)	5	2	53.9,31.9,26.0, 21.0,16.5 K	26.0,21.0 K
Dill (*Anethum graveolens*)	6	1	53.9,37.8,35.2, 31.9,22.8,16.5 K	22.8 K
Fennel (*Foeniculum vulgare*)	4	1	58.3,35.6,33.3, 22.8 K	22.8 K
Flax (*Linum usitatissimum*)	5	2	50.9,35.2,30.0, 24.6,14.4 K	35.2,24.6 K
Pine Nut (*Pinus edulis*)	8	2	49.5,37.8,33.7, 26.6,22.8,18.0, 16.5,14.9K	49.5,33.7 K
Mustard (*Brassica alba*)	7	2	38.7,32.6,31.4, 25.2,21.8,13.5, 11.6 K	25.2,21.8 K

Ab = Antibody against amaranth globulin.
*Basic subunit.
[a]Marcone *et al.*, submitted 1997*a*.

also in monocotyledonous globulins), it would be reasonable to suggest that the amaranth globulin probably exists in a trigonal antiprism-like structure. If, however, it existed as a stacked parallel hexagon-like structure as suggested for some globulins, its acidic and basic subunits would be equally exposed at the surface and would have been equally accessible during the production of the antibody.

Although the present antibody work would tend to support a trigonal anti-prism-like structure, Marcone *et al.* (1994) observed that the amaranth's quaternary structure approximated a dodecameric structure of two identical parallel hexagonal rings in the form of a hollow oblate cylinder. This quaternary structure model of the amaranth globulin does not support the theory that basic subunits exist at the surface of the molecule and the acidic ones in the interior. Therefore, the results obtained in this present study would suggest that the surfaces of the basic subunits in the monocotyledonous and amaranth globulins are more similar than those of the dicotyledonous globulins.

CONCLUSION

In conclusion, the cross-reaction with all dicotyledonous and monocotyledonous globulins (as evidenced by the recognition of 'basic' type subunits) indicated the existence of regions of surface homology (amino acid sequence) and conformation in all storage globulins. Such results may very well imply that gene families encoding for these different proteins were derived from a common ancestral gene and may have been present before divergence of angiosperms into dicotyledonous and monocotyledonous plants.

In comparison to other studied storage proteins such as prolamins, globulins show much more primary sequence conservation (Pernollet and Mossé, 1983). This may be due to the fact that globulins must first be recognized (probably through amino acid sequence) and transported into protein bodies where they self-assemble into a defined quaternary structure in order to ensure maximal packing. These requirements probably place greater constraints on the divergence of globulin storage proteins (Pernollet and Mossé, 1983). Evidence for the important need for some form of conservation was demonstrated by the observation that both the acidic and basic subunits are initially formed as one large polypeptide chain which is only cleaved post-transitionally hours after it has been recognized, transported, assembled, and packed within the protein body (Shewry and Miflin, 1985). The covalent attachment of both the acidic and the highly conserved basic subunits during the above operations would ensure that some degree of homology is present on each polypeptide to ensure that they can successfully undergo the above operations. With prolamins, the most important requirement may be to maintain a low solubility in aqueous solvents, which would ensure that the newly synthesized polypeptide chains aggregate into deposits that eventually become protein bodies (Shewry and Miflin, 1985).

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