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Salt-soluble seed globulins of various dicotyledonous and monocotyledonous plants—I. Isolation/purification and characterization

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Detailed characterization of 21 purified seed globulins derived from both monocotyledonous and dicotyledonous plants indicated that globulins from both class types (as well as within the same class type) lay within a narrow molecular weight range between 300 000 and 370 000 Da and were composed of multiple subunits. In all cases, purified globulins could be classified as hetero-oligomers being composed of a non-equimolar ratio of various subunits. The vast majority of subunits forming these globulins were shown to be held together by non-covalent bond forces. A small percentage of linkages between subunits were also shown to be disulfide linked, in the case of dicotyledonous seed globulins. It was also found that the majority of subunits composing the dicotyledonous and monocotyledonous seed globulins examined fell within two very narrow molecular weight ranges, i.e. 20 000–27 000 and 30 000–39 000 Da and were believed to correspond to basic and acidic subunits, respectively. Unlike monocotyledonous seed globulins, globulins derived from dicotyledonous plants were found to undergo alkaline-induced dissociation due to electrostatic repulsion between subunits. The amino acid composition of both dicotyledonous and monocotyledonous seed globulins suggests that they have a storage role and may be similar proteins based on a high content of amides (glutamic acids–glutamine and aspartic acid–asparagine and arginine). From the results of the structural and chemical data obtained in this study, it is concluded that the 11S storage globulin, having several similar properties, exists in many leguminous and non-leguminous dicotyledonous plants as well as monocotyledonous plants. This similarity among 11S storage globulins could be due either to convergent evolution in response to a common functional need, or to common ancestry.

INTRODUCTION

It has been estimated that directly or indirectly plants comprise up to 95% of the present world's food supply (Walsh, 1984) with cereal/grains providing in excess of 50% of man's basic protein requirement (Johnson, 1984). Presently, cereal crops still predominate in providing both calories and proteins; however, the utilization of other grains such as the legume family is gaining in overall popularity (Massecaa and Baudet, 1983; Lambert and Yarwood, 1992). This increased popularity in the utilization of non-cereal grains stems not only from the fact that these grains commonly possess two to four times more protein than traditionally used cereal grains but that their proteins are often of higher nutritional quality (Lambert and Yarwood, 1992). Of the three types of protein commonly associated with seeds, i.e. the structural, metabolically active and storage-type proteins, it is the storage-type proteins which are found in most abundance and, therefore, are considered responsible for the nutritional as well as technological properties of the whole grain (Boulter, 1983). In cereal grains the main storage proteins are usually the alcohol-soluble prolamins, whereas in non-cereal grains they are usually predominated by the more nutritiously balanced salt-soluble globulins (Waggle *et al.*, 1989) (Table 1). Although researchers have shown that globulins can be subdivided into two distinct classes, termed 7 and 11 S, on the basis of their sedimentation coefficients (Wright, 1988), the 11S globulin is the predominant protein in dicotyledonous plants (Pernollet and Mossé, 1983; Derbyshire *et al.*, 1976).

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Table 1. Globulin content in various dicotyledonous and monocotyledonous plant seeds (% of total protein)

	Globulin content	Major protein type in seed
Monocotyledonous		
Wheat (Kent, 1983)	5–10	Prolamin/glutelin
Barley (Kent, 1983)	10–20	Prolamin/glutelin
Oat (Kent, 1983)	50–60	Globulin
Rye (Kent, 1983)	5–10	Prolamin/glutelin
Corn (Kent, 1983)	10–20	Prolamin/glutelin
Dicotyledonous		
Soybean (Koshiyama and Fukushima, 1979)	90	Globulin
Peanut (Yamada *et al.*, 1979)	73	Globulin
Pea (Thomson *et al.*, 1978)	(60–80)	Globulin
Amaranth	(~60)	Globulin
Buckwheat	NR	NR
Pumpkin (O'Kennedy *et al.*, 1979)	NR	NR
Hemp	NR	NR
Alfalfa (Krochko and Bewley, 1994)	30–60	Globulin
Caraway	NR	NR
Celery	NR	NR
Cumin	NR	NR
Dill	NR	NR
Fennel	NR	NR
Flax	NR	NR
Mustard (Venkatesh and Rao, 1988)	25 (11 S) + 1.3 S	Globulin
Pine Nut (Allona *et al.*, 1994)	NR	Glutelins

NR, none reported.

Since 11S globulins are such a vital major component of dicotyledonous seeds, their utilization has typically centered around their extraction from the seed meal in such a way as to maintain their functional attributes (Lambert and Yarwood, 1992).

In the past, oil seeds such as soybean and sunflower seed, were basically processed for their edible oils with their protein-rich meals being utilized as animal feed, whereas the globulins were commonly recovered and marketed as food ingredients in developed countries (Wright and Bumstead, 1984; Uzzan, 1988). Over the last 20 years these isolated proteins have become indispensable market commodities for increasing overall protein content and/or imparting functionality and texture as well as enhanced nutritional value in a variety of food products (Wright and Bumstead, 1984; Uzzan, 1988). The largest market for protein isolates (usually derived from soybean) in human foods continues to be as a meat substitute (Uzzan, 1988; Waggle *et al.*, 1989) in products such as burgers, sausages, and cooked pork meat, where the chief advantages are reduced lipid content and increased protein levels, and most importantly reduction in overall unit cost of the product. Other markets include bread and bakery products (mainly for increasing protein levels), dietetic and health foods, pet foods and dairy analogues (e.g. soyamilk, soy cheese, whipped toppings and ice-creams) (Lambert and Yarwood, 1992). Soya isolates have also been used as whipping agents, emulsifiers and binding (lipid or water) agents in many general foodstuffs as a replacement for the more expensive animal-based protein ingredients from egg and milk (Uzzan, 1988; Waggle *et al.*, 1989).

Protein isolates from soybean predominate in the market, although protein isolates from other non-cereal grains, e.g. pea, fava bean, sunflower, cottonseed and rapeseed, are presently being explored with great interest (Sosulski, 1983, 1984; Uzzan, 1988; Lambert and Yarwood, 1992). This shift from soybean to other non-cereal grains has been partly fuelled by the reduction in overall soybean production in the US; and in response to the EEC promotion, use and demand for alternative isolates (Bilidt, 1990; Lambert and Yarwood, 1992). Properly processed, the alternative isolates have been shown to exceed the suggested patterns of amino acid requirements for humans published by the FAO/WHO/UNV (Lambert and Yarwood, 1992).

In order to adequately and effectively respond to the present market trend for alternative protein isolates having similar functional and nutritional properties as soya isolates, an in-depth comparison of the physicochemical properties of the globulins from other isolates needs to be performed. Therefore, the isolation and purification of the storage proteins, which satisfy stringent homogeneity criteria, need to be undertaken (Derbyshire *et al.*, 1976). Unfortunately, to date, no standard method has been developed to purify globulins from different genetic sources. Each research group has optimized their own purification techniques, making the comparison of globulins purified by different laboratories difficult. The purpose of the following study was two-fold. The first objective was to undertake the efficient extraction and purification of dicotyledonous and monocotyledonous seed globulins derived from dicotyledonous plants [i.e. pea (*Pisum sativa*), peanut (*Arachis hypogaea*), soybean (*Glycine max*), amaranth

(*Amaranthus hypochondriacus*), buckwheat (*Fagopyrum esculentum*), pumpkin (*Cucurbita pepo*), hemp (*Cannabis sativa*), alfalfa (*Medicago sativa*), caraway (*Carum carvi*), celery (*Apium graveolens*), cumin (*Luminum cyminum*), dill (*Anethum graveolens*), fennel (*Foeniculum vulgare*), flax (*Linum usitatissimum*), and mustard (*Brassica alba*)] and monocotyledonous plants [i.e. corn (*Zea mays*), barley (*Hordeum vulgare*), rye (*Secale cereale*), oat (*Avena sativa*), and wheat (*Triticum aestivum*). The second objective was to investigate: (i) the subunit composition, (ii) the forces responsible for holding the subunits together, and (iii) amino acid compositions of the above seed globulins, in order to determine if any common physical or structural features exist.

MATERIALS AND METHODS

Materials

Non-heat treated milled seeds of *Amaranthus hypochondriacus* K343 (the major raw source of the amaranth globulin used throughout the studies) marketed under the trade name Ambake Lot No. 1010 were purchased from American Amaranth Inc. (Bricelyn, MI). In the case of soybean, cultivar Corsoy 79 was obtained from the Harrow Research Station (Agriculture Canada, Harrow, Ontario). Pea (*Pisum sativum* L.), peanut (*Arachis hypogaea* L.), wheat (*Triticum aestivum*), buckwheat (*Fagopyrum esculentum*), barley (*Hordeum vulgare*), corn (*Zea mays*), rye (*Secale cereale*), oat (*Avena sativa*), 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 obtained from the 1993 or 1994 growing seasons (University of Guelph, Department of Crop Science). Seeds were ground with a mechanical grinder and material passed through a 60 mesh screen was used for further study. The pumpkin globulin (*Cucurbitapepo*) and the hemp globulin (*Cannabis sativa*) were obtained twice crystallized from Sigma Chemical Co. (St Louis, MO).

All seed meal materials were mixed and quartered in order to ensure homogeneous/representative composite samples. The samples were stored in sealed polycarbonate containers at room temperature throughout the study. Chromatographic materials, i.e. a Separon Hema-Bio 1000 DEAE anion-exchange column and Concanavalin A–Sepharose 4B affinity chromatography media were both obtained from Fisher Scientific (Toronto, ON), while Sephacryl S-300 superfine gel permeation media, Mono-Q 5/5 anion-exchange column and high molecular weight calibration proteins were products of Pharmacia LKB (Montreal, PQ).

All other reagents and chemicals were of AR or HPLC grade from Sigma Chemical Co. (St Louis, MO), Aldrich (Milwaukee, WI) or Fisher Scientific (Toronto, ON).

Proximate analysis of dicotyledonous and monocotyledonous plant seeds

Proximate analysis was performed as per official standard methods of the American Association of Cereal Chemists, Inc. (1983). Analyses were performed in duplicate.

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). Crude protein was also measured by a modified semi-micro Kjeldahl procedure (American Association of Cereal Chemists, Inc., 1983). A nitrogen to protein conversion factor of 5.85 was used in the calculation (Becker *et al.*, 1981) in the case of amaranth and 5.70 in the case of soybean, wheat and rye and 6.25 in the case of all other cereals (Kent, 1983). For the proteins of all other dicotyledonous seeds a factor of 6.25 was used (Kent, 1983).

Isolation and purification of a variety of salt-soluble seed storage globulins from dicotyledonous and monocotyledonous plants

Sample preparation

All seed meals were defatted with either cold HPLC grade acetone or hexane (serially extracted) in a ratio of 1:10 (w/v, flour/solvent) essentially performed as described by Marcone and Yada (1991). These meals were used to determine the contents of crude albumin, globulin and total soluble solids (not proteins) from each seed material used.

Protein extraction

The salt-soluble proteins from each grain were exhaustively extracted from the defatted meal by a modification of the method of Konishi *et al.* (1985). The total salt-soluble protein, i.e. albumins and globulins, were determined from the salt extractions; whereas total albumin content was determined after dialysis (precipitation of the globulin) by Kjeldahl. Globulin content was determined gravimetrically after dialysis. Total soluble solids (not protein) was determined after dialysis (removal of globulins) as the difference between total albumin content and material remaining after freeze drying.

Gel filtration chromatography (GFC)

A Sephacryl™ S-300 Superfine (2.5×95 cm) gel filtration column with a molecular weight fractionation range of 10 000–500 000 was packed and equilibrated with three bed volumes of the above extraction buffer.

Blue dextran was used to measure the void volume. A standard curve was generated using the following proteins: ferritin (440 000 Da), catalase (232 000 Da), aldolase (158 000 Da), and cytochrome c (12 400 Da) as described by Marcone and Yada (1995). Gel filtration was also performed on treated and nontreated globulin extracts (alkylation of sulfhydryls as described in the appropriate section) of amaranth, soybean and wheat globulins.

Anion-exchange chromatography
Anion-exchange chromatography was performed using a Fast Protein Liquid Chromatograph (FPLC) equipped with two P-500 pumps, LCC-500 PLUS/CI control unit and UV-280 detector (Pharmacia LKB, Montreal, PQ) (Marcone and Yada, 1995).

Evaluation of purity/characterization of isolated globulin proteins

Sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE)
The purified globulins were reconstituted to 3 mg ml^{-1} in a 10 mM Tris–HCl buffer, pH 8.0, containing 1 mM EDTA, 2.5% (w/v) SDS, 5.0% (v/v) β-mercaptoethanol, and 0.1% (w/v) bromophenol blue. Samples (1 μl) were put on PhastGel gradient polyacrylamide (PAA) 8–25% SDS–PAGE gels (Pharmacia LKB) (Marcone and Yada, 1995).

Native–PAGE electrophoresis
The purified globulins were reconstituted to 3 mg ml^{-1} in a 20 mM phosphate buffer, pH 8.3, and 1 μl was loaded on PhastGel 7.5% polyacrylamide (PAA) gels with native buffer strips (Marcone and Yada, 1997).

Amino acid analysis

Samples (200 μl) containing 0.4 mg ml^{-1} of purified globulin were lyophilized and then hydrolyzed in 200 μl of 5.7 M HCl for 24, 48, and 72 h at 108°C *in vacuo*, followed by HPLC determination of amino acids (Marcone and Yada, 1997).

Alkylation of sulfhydryl groups

The sulfhydryl groups were alkylated with *N*-ethylmaleimide (NEM), as required, both during and subsequent to protein fractionation, using a modification of the method of Wolf and Briggs (1958).

Circular dichroism spectroscopy (far-UV) (CD)

Circular dichroism measurements were performed in the far-UV (190–250 nm) on 0.1 mg ml^{-1} globulin/buffer solutions (32.5/2.6 mM K_2HPO_4/KH_2PO_4), pH 7.5, using a Jasco J-600 Spectropolarimeter (Japan Spectroscopic Co. Ltd., Tokyo, Japan) with a cell pathlength of 0.1 cm (Marcone and Yada, 1997).

Carbohydrate and phosphate determination

Carbohydrate and phosphate determination were performed essentially as described by Marcone and Yada (1997).

RESULTS AND DISCUSSION

Optimization of extraction and purification protocols

Since proper isolation, purification and characterization of seed globulins is often complicated by the effects of pH and ionic strength (Naismith, 1955; Nash and Wolf, 1967; Gueguen *et al.*, 1988), conditions developed in a previous study (Marcone *et al.*, 1997) were used. Extraction solutions of ionic strength $I = 0.5$–1.0 and pH 6–9 were utilized to ensure the most reproducible and stable secondary, tertiary and surface properties for seed globulins from dicotyledonous and monocotyledonous plants. In addition, such conditions would allow for valid comparisons to be made without danger of experimentally produced structural/chemical artifacts in globulins.

Characterization of starting materials

Prior to the described comparative study among seed globulins from dicotyledonous and monocotyledonous plants, representative samples of their raw seed material were subjected to proximate analysis. Values obtained from these tests (Table 2) were found to be in general agreement with results reported by Saunders and Becker (1984). The only exceptions were those values for oat and soybean meals which were lower in fat, and oat which was lower in protein. Genetic differences between species used in this study and those reported in the literature could have accounted for any observed differences.

Extraction of crude seed globulins from monocotyledonous and dicotyledonous plants

The relative recovered amounts of the albumins and globulins are reported in Table 3. Dicotyledonous seeds possessed higher amounts of globulin proteins than monocotyledonous seeds which was consistent with results reported in the literature.

Extraction of the globulin fraction from monocotyledonous and dicotyledonous seed meals was found to be complete when compared to those theoretical globulin contents reported in the literature (Kent, 1983) (Tables 1–3) except for oat and pea, peanut and soybean, respectively. In the case of pea and peanut meals, globulin extraction was found to be in excess of 60%.

Table 2. Proximate analysis of various dicotyledonous and monocotyledonous seeds used in the study

Seed	Moisture[a] (%)	Ash[a] (%)	Protein[a] (%)	Fat[a] (%)	CHO[ab] (%)
Pea (*Pisum Sativum* L.)	10.47	2.6	18.83	1.03	67.07
Peanut (*Arachis hypogaea* L.)	5.27	2.33	25.97	15.82	50.61
Soybean (*Glycine max* Merr.)	8.96	4.73	34.35	5.33	46.63
Corn (*Zea mays*)	11.67	1.29	9.97	1.02	76.05
Barley (*Hordeum vulgare*)	11.48	1.60	10.49	0.96	75.47
Rye (*Secale cereale*)	11.14	1.71	12.41	1.12	73.62
Oat (*Avena sativa*)	11.48	0.81	7.27	2.18	78.26
Wheat (*Triticum aestivum*)	8.12	1.76	14.65	1.52	73.95
Amaranth (*Amaranthus hypochondriacus*)	9.60	3.10	16.40	9.60	61.30
Buckwheat (*Fagopyrum esculentum*)	12.41	1.92	9.63	1.87	74.17
Pumpkin (*Cucurbita pepo*)	7.14	12.22	22.23	9.62	48.79
Hemp (*Cannabis sativa*)	N/A	N/A	N/A	N/A	N/A
Alfalfa (*Medicago sativa*)	7.09	3.68	34.71	6.49	48.03
Caraway (*Carum carvi*)	10.17	5.43	12.31	6.27	65.82
Celery (*Apium graveolens*)	8.72	9.96	18.58	10.12	52.62
Cumin (*Cuminum cyminum*)	8.79	6.78	18.87	11.50	54.06
Dill (*Anethumgraveolens*)	10.47	7.86	20.63	4.81	56.23
Fennel (*Foeniculum vulgare*)	7.81	11.41	22.86	6.05	51.87
Flax (*Linum usitatissimum*)	6.39	3.11	15.85	31.09	43.56
Pine Nut (*Pinus edulis*)	2.70	2.83	18.92	67.80	7.75
Mustard (*Brassica alba*)	8.43	4.24	23.78	26.63	36.92

N/A, data not available. All analyses performed according to standard AOAC methods.
[a]The average of two replicates.
[b]Carbohydrate (CHO) by difference.

It is well known that total extraction of globulin from these high globulin-containing meals, i.e. greater than 50%, is very difficult (Derbyshire *et al.*, 1976) and may explain the lower amounts of extractable globulin obtained in the present research.

The addition of β-mercaptoethanol/dithiothreitol in the extraction media for globulins has been reported to increase the amount of protein extracted (Nash *et al.*, 1974); however, it was found for three of the plant species tested, i.e. amaranth, soybean and wheat, that their extractability was not appreciably increased when performed in the presence of any of these reducing agents (data not shown). A common practice is to permanently block (alkylate) any free sulfhydryl groups to prevent disulfide bridge formation in crude globulin preparations. However, modifications of this kind would then preclude their subsequent use in some physical and biological studies (Derbyshire *et al.*, 1976).

Purification and characterization of seed globulins

Gel filtration chromatography

Figures 1 and 2 illustrate the typical Sephacryl™ S-300 superfine gel filtration chromatograms obtained from each of the various isolated crude globulin preparations, i.e. from pea, peanut, corn, barley, rye, oat, buckwheat, alfalfa, caraway, celery, cumin, dill, fennel, flax, and pine nut. The gel filtration chromatograms for amaranth, soybean, wheat and mustard are reported in Marcone and Yada (1991), Marcone *et al.* (1994), Marcone and Yada (1995), and Marcone *et al.* (1997), respectively. Typical of all globulin preparations was a strong UV-absorbing, turbid fraction (labelled fraction 1) which appeared at or near the void volume, $K_{av} = 0$. This turbid fraction, although demonstrating strong absorptivity in the ultraviolet spectral range, typically contained very little proteinaceous material as determined by the Bradford dye binding test. In each case, this fraction was found to contain high levels of both phosphorus and carbohydrate (approx. 30–40%), possibly indicating the presence of nucleic acids as well as other non-identifiable high molecular weight components. The presence of this turbid peak in gel filtration studies has previously been reported: in soybean by Okubo and Shibasakai (1967) and Marcone *et al.* (1994); in linseed by Madhusudhan and Singh (1983); in amaranth by Konishi *et al.* (1985) and Marcone and Yada (1991); and in mustard by Marcone *et al.* (1996). The existence of this peak appears to be a common observation in all studied crude seed globulin preparations and may serve as a means of identifying globulin protein preparations.

The major peak in all gel filtration chromatographs (labelled peak 2) was identified as the 11S seed storage globulin. The K_{av}'s for the respective globulins (i.e. from peak 2) were: 0.318 for wheat (Marcone and Yada, 1995); 0.432 for oat and dill; 0.480 for soybean (Marcone *et al.*, 1994); 0.500 for alfalfa, caraway and pine nut; 0.520 for amaranth (Marcone and Yada, 1991); 0.523 for cumin, celery, peanut, and buckwheat; 0.545 for fennel and flax, and 0.568 for pea, corn, barley, rye, and mustard. The corresponding derived molecular weights for each of these purified globulins are listed in Table 4. Also listed are the molecular weights of

Table 3. Extractability of soluble proteins (globulins and albumins) from various seed sources

	Crude albumin and globulin[a] ($mg\,g^{-1}$ defatted meal)	Crude globulin[a] ($mg\,g^{-1}$ defatted meal)	Crude albumin[a] ($mg\,g^{-1}$ defatted meal)	Soluble solids[a] (not protein) ($mg\,g^{-1}$ defatted meal)	Total protein (in seed meal) which is globulin
Pea (*Pisum sativum* L.)	83.4	67.8 (53.0)	15.6	14.1	36.0
Peanut (*Arachis hypogaea* L.)	168.7	114.3 (113.7)	54.5	0	44.0
Soybean (*Glycine max* Merr.)	98.8	68.6 (54.0)	30.1	46.4	20.0
Corn (*Zea mays*)	11.2	8.2 (9.1)	3.1	12.6	8.2
Barley (*Hordeum vulgare*)	15.5	8.6 (5.3)	6.8	35.0	8.2
Rye (*Secale cereale*)	28.0	17.1 (13.6)	10.9	27.6	13.8
Oat (*Avena sativa*)	7.0	6.0 (6.0)	1.0	11.4	8.3
Wheat (*Triticum aestivum*)	24.6	16.5 (14.0)	8.1	14.3	11.3
Amaranth (*Amaranthus hypochondriacus*)	113.3	98.4 (96.8)	4.9	10.0	60.0
Buckwheat (*Fagopyrum esculentum*)	37.4	26.5 (20.6)	10.9	11.5	27.5
Pumpkin (*Cucurbita pepo*)	136.3	127.6 (118.6)	8.7	17.7	57.4
Hemp (*Cannabis sativa*)	N/A	N/A	N/A	N/A	N/A
Alfalfa (*Medicago sativa*)	37.5	24.9 (35.0)	12.7	59.7	7.2
Caraway (*Carum carvi*)	18.3	6.2 (5.2)	12.2	33.4	5.0
Celery (*Apium graveolens*)	16.9	7.1 (7.1)	9.8	29.0	3.8
Cumin (*Cuminum cyminum*)	20.5	15.5 (16.2)	5.0	29.1	8.2
Dill (*Anethum graveolens*)	5.9	2.3 (3.0)	3.6	23.2	1.1
Fennel (*Foeniculum vulgare*)	18.1	9.1 (9.6)	9.0	27.3	4.0
Flax (*Linum usitatissimum*)	55.7	40.9 (41.2)	14.8	76.1	25.8
Pine Nut (*Pinus edulis*)	52.2	42.4 (42.1)	9.8	31.2	22.4
Mustard (*Brassica alba*)	87.1	73.4 (73.4)	13.7	24.3	30.9

N/A, data not available. Values in parentheses are values obtained by gravitometric analysis of crude globulin.
[a]The average of two replicates.

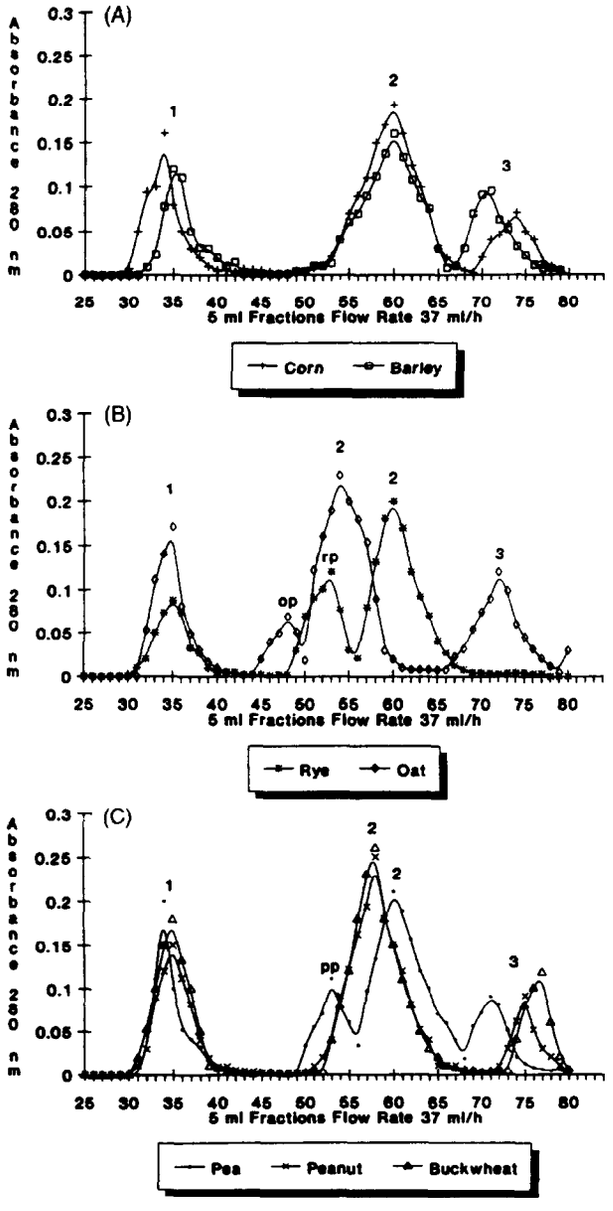

Fig. 1. Sephacryl™ S-300 gel filtration chromatogram of crude globulin preparations (30-mg injections). Flow rate was 37 ml h^{-1} with a 32.5/2.6 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.5) containing 0.4 M NaCl. (2.5 ml containing 35 mg). (A) Corn and barley, (B) rye and oat, and (C) pea, peanut and buckwheat.

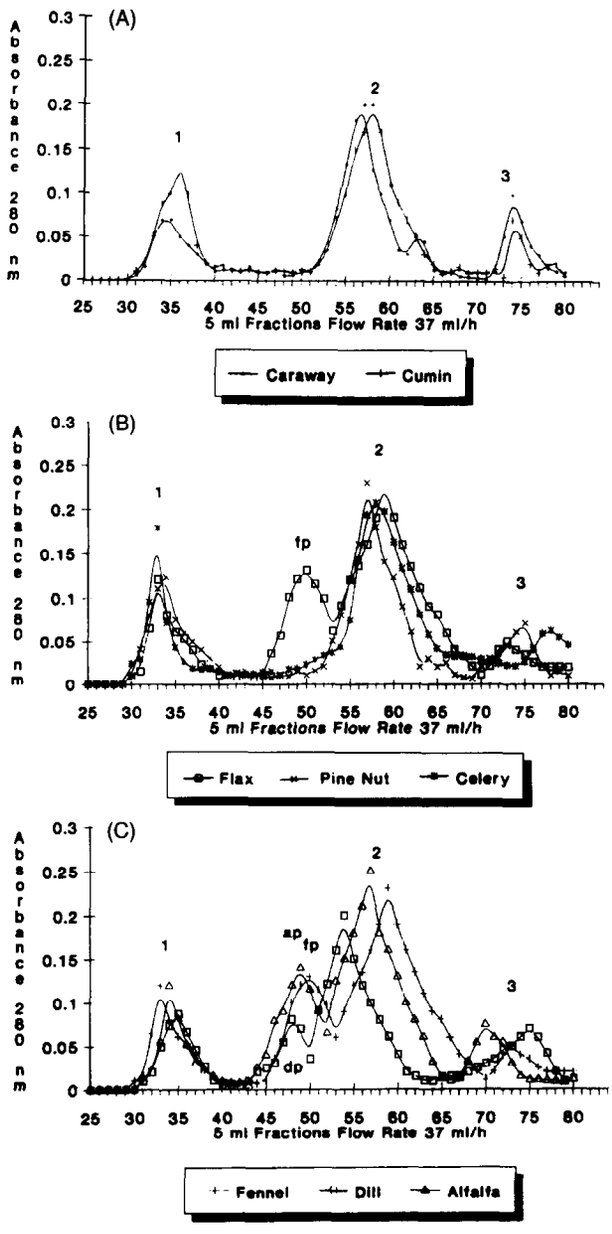

Fig. 2. Sephacryl™ S-300 gel filtration chromatogram of crude globulin preparations (30-mg injections). Flow rate was 37 ml h^{-1} with a 32.5/2.6 mM K_2HPO_4/KH_2PO_4 buffer (pH = 7.5) containing 0.4 M NaCl (2.5 ml containing 35 mg). (A) Caraway and cumin, (B) flax and pine nut, and (C) fennel, dill, alfalfa and celery.

previously purified globulins from soybean, wheat, amaranth, and those crystallized globulins for hemp and pumpkin seeds. It was noted that derived molecular weights of globulins fall well within expected values (Fig. 3) and similarities exist with regard to molecular weights of all globulins isolated from dicotyledonous and monocotyledonous plant sources, i.e. typically in the very narrow range of 300 000–370 000 Da.

Closer examination of the gel filtration chromatograms revealed that in some cases a well-resolved protein peak preceded the characteristic 11S globulin peak. The K_{av}'s for these respective protein peaks were: 0.182 for wheat (Marcone and Yada, 1995); 0.270 for soybean (Marcone *et al.*, 1994); 0.295 for dill, oat, and mustard; 0.318 for alfalfa, 0.341 for flax and fennel; 0.409 for pea and rye; and 0.430 for amaranth. These corresponded to 567 000, 506 000, 490 000, 474 000, 459 000, 412 200, and 398 000 Da, respectively. These fractions were thought to be composed of aggregates of polymerized protein which had similar subunit profiles to their non-polymerized 11S globulin counterpart (data not shown). In fact, the presence of such a fraction has been demonstrated to occur in other globulin preparations, e.g. amaranth (Marcone and Yada, 1991); soybean (Marcone and Yada, 1994) and in wheat (Marcone and Yada, 1994) and is believed to be caused by a sulfhydryl/disulfide interchange reactions.

Table 4. Molecular masses of purified seed storage globulins from various sources obtained by gel filtration chromatography

Globulin source	Molecular mass (Da)	Literature value (Da)
Pea (*Pisum sativum* L.)	304 000	> (330 000–410 000)
Peanut (*Arachis hypogaea* L.)	335 000	(330 000–396 000)
Soybean (*Glycine max* Merr.)	(366 000)	(366 000) (Marcone *et al.*, 1994)
Corn (*Zea mays*)	304 000	166 000
Barley (*Hordeum vulgare*)	304 000	(300 000) (Shewry and Miflin, 1983)
Rye (*Secale cereale*)	304 000	(300 000) (Shewry and Miflin, 1983)
Oat (*Avena sativa*)	397 000	(322 000) (Walburg and Larkins, 1983), (Peterson, 1978)
Wheat (*Triticum aestivum*)	(474 000)	(474 000) (Marcone and Yada, 1994)
Amaranth (*Amaranthus hypochondriacus*)	(337 000)	(337 000) (Marcone and Yada, 1991)
Buckwheat (*Fagopyrum esculentum*)	335 000	(270 000) (Belozerskii *et al.*, 1968)
Hemp (*Cannabis sativa*)	ND	(300 000) (Derbyshire *et al.*, 1976)
		(334 000) (Brand *et al.*, 1955)
Pumpkin (*Cucurbita pepo*)	ND	(340 000) (Rao *et al.*, 1978)
Alfalfa (*Medicago sativa*)	350 000	(480 000) (Krochko and Bewley, 1994)
Caraway (*Carum carvi*)	350 000	
Celery (*Apium graveolens*)	335 000	
Cumin (*Cuminum cyminum*)	335 000	
Dill (*Anethum graveolens*)	397 000	
Fennel (*Foeniculum vulgar*)	320 000	
Flax (*Linum usitatissimum*)	320 000	
Pine Nut (*Pinus edulis*)	350 000	
Mustard (*Brassica alba*)	304 000	(263,000) (Rao *et al.*, 1978)

ND, not determined experimentally.

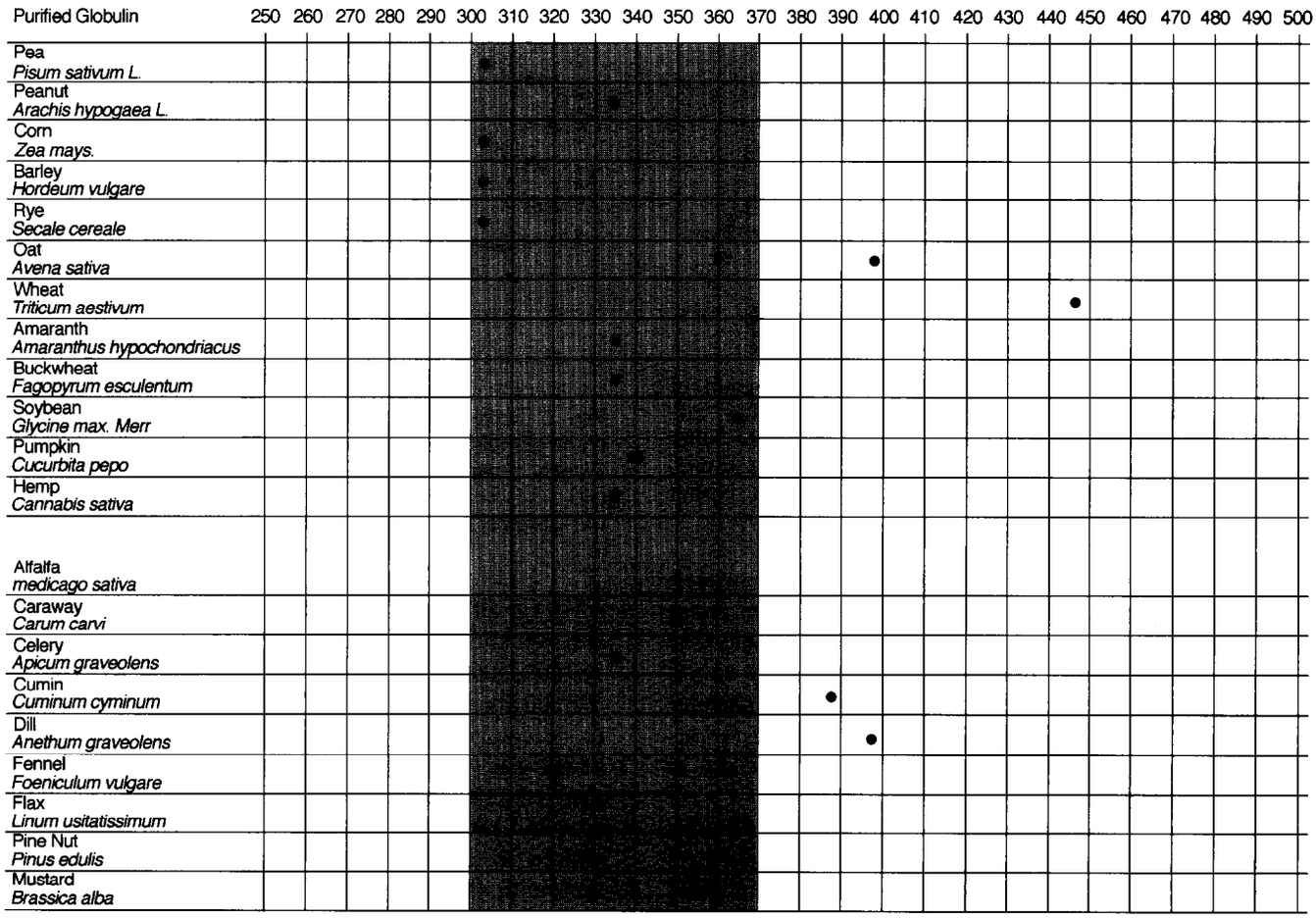

Fig. 3. Graphic representation of molecular weight distribution of purified seed storage globulins from various sources obtained by gel filtration chromatography. ■, Literature value (Values reported in kilodaltons).

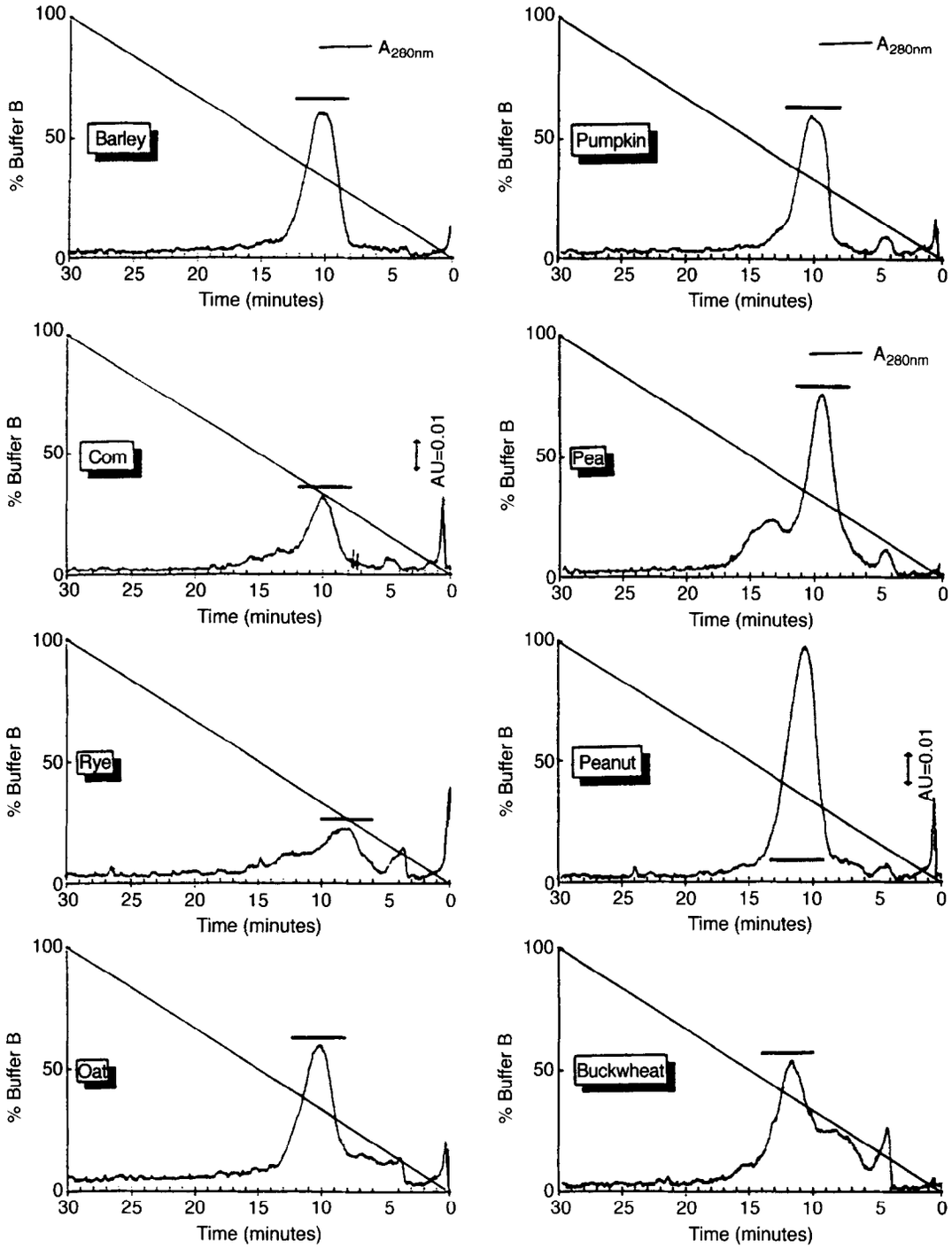

Fig. 4. Fast protein liquid chromatograms (FPLC) of various purified dicotyledonous and monocotyledonous seed globulins from a Sephacryl™ S-300 gel filtration chromatography run on a Mono-Q anion-exchange column.

Examination of the last gel filtration peak, i.e. labelled either peak 3 or 4, in select globulin preparations, revealed a variety of proteinaceous components. In the case of amaranth, this last peak was shown to be composed of water-soluble albumins (Marcone and Yada, 1991), whereas in the wheat globulin preparation it was composed of a purothionin and a smaller globulin component, possibly a 7S globulin (Marcone and Yada, 1995). In the case of soybean, flax, fennel and alfalfa preparations, this peak (molecular weight range 102 000–165 000 Da and glycosylated to 2.3–4.1%) was identified as the 7S vicilin globulin (Marcone *et al.*, 1994). When the 11S globulin (peak 2) from the above sources was tested for the presence of carbohydrate, no carbohydrate was detected indicating that they were not contaminated with the 7S glycosylated globulin. The 7S globulin can undergo a reversible dimerization reaction and elute in a position with a molecular weight similar to that of the 11S globulin (Lillford and Wright, 1981). Although reversible dimerization of the 7S globulin can theoretically occur, it would be highly improbable under the conditions of high ionic strength used in these experiments.

Anion-exchange chromatography

Following gel filtration chromatography, all isolated non-polymerized globulins (from peak 2) were further purified by anion exchange chromatography (representative sample of globulins shown; Fig. 4). In the majority of cases, only one major peak was observed with similar retention times (i.e. eluted at 0.15 M NaCl). The only exception to the above was the amaranth globulin which had a longer retention on the column, i.e. higher affinity, therefore indicating that it was more negatively charged than the other globulins studied. The major peaks of all globulins eluting from the anion-exchange column were collected for further analysis. Table 5 shows the protein yield for these globulins after chromatography.

Characterization of select crude globulin preparations, i.e. amaranth, soybean and wheat (prior to gel filtration) by anion-exchange chromatography revealed that they were composed of multiple components. More specifically, in the case of the amaranth globulin preparation, six distinct charge components were identified, whereas in the wheat and soybean preparations, four and two distinct charged species were noted, respectively (Marcone and Yada, 1991; Marcone *et al.*, 1994; Marcone and Yada, 1995). Since anion-exchange chromatography generally revealed that only one peak was present, it could be concluded that gel filtration chromatography was an excellent first step in eliminating many undesirable proteins.

Information obtained from gel filtration and anion-exchange chromatography indicates similarities with regard to molecular weight and similarity in elution profiles between the 11S proteins isolated from monocotyledonous and dicotyledonous sources. However, this information was still insufficient to decide if most seeds had a homologous 11S globulin counterpart. It was, therefore, necessary to examine their subunit structures.

Electrophoretic protein characterization

SDS–PAGE

In the past, the large molecular weight of globulins has been used to show the existence of a multi-subunit structure for 11S seed globulins (Derbyshire *et al.*, 1976). SDS–PAGE of the studied dicotyledonous and monocotyledonous globulins demonstrated that the globulins from both plant classes were composed of a variety of subunits differing in both molecular weight and concentration, i.e. existed as hetero-oligomers and were composed of a non-equimolar ratio of various subunits (Fig. 5). The various molecular weights calculated for the subunits composing these purified globulins are reported in Table 6.

In order to determine the nature of the bond forces holding these above-mentioned subunits together in their respective hetero-oligomeric type proteins, the purified globulins were subjected to SDS–PAGE in the absence of β-mercaptoethanol. Results indicated that intermediary disulfide-linked subunits were found in all globulins obtained from dicotyledonous plant seeds, i.e. peanut, buckwheat, hemp, pumpkin, dill, celery, fennel, flax, cumin, caraway, amaranth (Marcone and Yada, 1992) and soybean, with the exception of pea and alfalfa globulins (Fig. 6). In contrast, of the five monocotyledonous seed globulins examined only oat was found to possess intermediary subunits.

Table 5. Purification of globulins from various dicotyledonous and monocotyledonous seed sources

Globulin source	Crude globulin extract injected onto gel filtration (mg)	Yield from gel filtration in mg and (%)	Yield from anion exchange (coming from gel filtration) in mg and (%)
Pea (*Pisum sativum* L.)	30	14.0 (46.7)	7.9 (56.4)
Peanut (*Arachis hypogaea* L.)	30	17.7 (59.0)	12.7 (71.6)
Corn (*Zea mays*)	30	17.6 (58.5)	9.0 (51.1)
Barley (*Hordeum vulgare*)	30	15.3 (51.0)	11.5 (75.1)
Rye (*Secale cereale*)	30	16.6 (55.3)	6.7 (40.6)
Oat (*Avena sativa*)	30	13.7 (45.7)	7.9 (57.5)
Wheat (*Triticum aestivum*)	30	15.9 (53.0)	6.9 (43.5)
Amaranth (*Amaranthus hypochondriacus*)	25	13.5 (53.9)	10.9 (80.5)
Buckwheat (*Fagopyrum esculentum*)	30	16.1 (53.6)	8.7 (53.8)
Soybean (*Glycine max* Merr)	30	9.8 (32.5)	8.8 (90.0)
Pumpkin (*Cucurbita pepo*)	N/P	—	(81.8)
Hemp (*Cannabis sativa*)	N/P	—	(72.4)
Alfalfa (*Medicago sativa*)	30	16.1 (53.6)	16.1 (100)
Caraway (*Carum carvi*)	30	19.1 (63.8)	16.5 (86.4)
Celery (*Apium graveolens*)	30	20.5 (68.3)	14.8 (72.3)
Cumin (*Cuminum cyminum*)	30	18.8 (62.8)	12.3 (65.4)
Dill (*Anethum graveolens*)	30	15.5 (51.8)	7.9 (50.8)
Fennel (*Foeniculum vulgare*)	30	17.2 (57.3)	8.5 (49.4)
Flax (*Linum usitatissimum*)	30	17.1 (57.0)	14.1 (82.3)
Mustard (*Brassica alba*)	50	22.5 (45)	12.5 (55.7)
Pine Nut (*Pinus edulis*)	30	16.7 (55.8)	12.5 (74.6)

N/P, not performed (twice crystallized). Protein content determined by Kjeldahl.

Table 6. SDS–PAGE molecular mass distribution of subunits from various purified seed storage globulins

Globulin source	Number of subunits		Molecular mass of subunits ($\times 10^{-3}$)	
	Experimental	Literature	Experimental	Literature
Pea (*Pisum sativum* L.)	5	(Domoney *et al.*, 1980)	76.5, 47.3, 35, 21.1, 15.5	50, 33, 17, 14, 12 (Domoney *et al.*, 1980)
Peanut (*Arachis hypogaea* L.)	7	(Bhushan and Reddy, 1989)	63.6, 49.9, 40.5, 39.9, 36.2, 30.6, 25.8	75, 58, 50, 40, 33, 25, 23 (Bhushan and Reddy, 1989)
Soybean (*Glycine max* Merr.)		(Marcone *et al.*, 1994)	47, 34, 18	47, 34, 18 (Marcone *et al.*, 1994)
Corn (*Zea mays*)	3		69.7, 36.8, 33.7	62, 41, 25, 12 (Paulis *et al.*, 1975)
Barley (*Hordeum vulgare*)	4		60.1, 37.1, 25.8, 21.1	51.5, 54.5, 20.0, 19.0; 68.0, 36.0, 18.5 (Rhodes and Gill, 1980)
Rye (*Secale cereale*)	4		60.1, 37.1, 25.8, 21.1	
Oat (*Avena sativa*)	2	(Paulis *et al.*, 1975); (Rhodes and Gill, 1980)	37.1, 25.8	21.7, 31.7; 20–25 and 35–40 (Peterson, 1978; Walburge and Larkins, 1983)
Wheat (*Triticum aestivum*)	2	(Peterson, 1978)	35, 49	35, 49 (Marcone and Yada, 1994)
Amaranth (*Amaranthus hypochondriacus*)	5	(Walburg and Larkins, 1983)	37.5, 31.5, 26.5, 20.5, 14.5	37.5, 31.5, 26.5, 20.5, 14.5 (Marcone and Yada, 1991)
Buckwheat (*Fagopyrum esculentum*)	1(2)	(Marcone and Yada, 1994)	35.3, 25.8	40 (Belozershii *et al.*, 1968)
Hemp (*Cannabis sativa*)	3	(Marcone and Yada, 1991)	33.7, 22.7, 12.3	27, 23 (Derbyshire *et al.*, 1976)
Pumpkin (*Cucurbita pepo*)	2	(Belozershii *et al.*, 1968)	33.1, 24.3	36, 34, 22 (Ohmiya *et al.*, 1980)
Alfalfa (*Medicago sativa*)	7		37.4, 35.6, 33.3, 30.0, 22.8, 17.4, 5.4	49.0–39.0, 24, 23, 20 (Krochko and Bewley, 1994)
Caraway (*Carum carvi*)	3		31.9, 26.6, 21.0	
Celery (*Apium graveolens*)	5		37.6, 35.6, 31.9, 26.6, 22.8	
Cumin (*Cuminum cyminum*)	5		53.9, 31.9, 26.0, 21.0, 16.5	
Dill (*Anethum graveolens*)	6		53.9, 37.8, 35.2, 31.9, 22.8, 16.5	
Fennel (*Foeniculum vulgare*)	4		58.3, 35.6, 33.3, 22.8	
Flax (*Linum usitatissimum*)	5		50.9, 35.2, 30.0, 24.6, 14.4	
Pine Nut (*Pinus edulis*)	8		48.5, 37.8, 33.7, 26.6, 22.8, 18.0, 16.5, 14.9	47.0, 27.0, 22.0–23; 51–55, 31–34.5, 21.5–2 (Allona *et al.*, 1994)
Mustard (*Brassica alba*)	7	8	38.7, 32.6, 31.4, 25.2, 21.8, 13.5, 11.6	70.0, 50.0, 37.0, 34.0, 27.0, 20.0, 14.0, 11.0 (Gifford *et al.*, 1988; Rao *et al.*, 1978)

Values in parentheses are those reported in the literature.

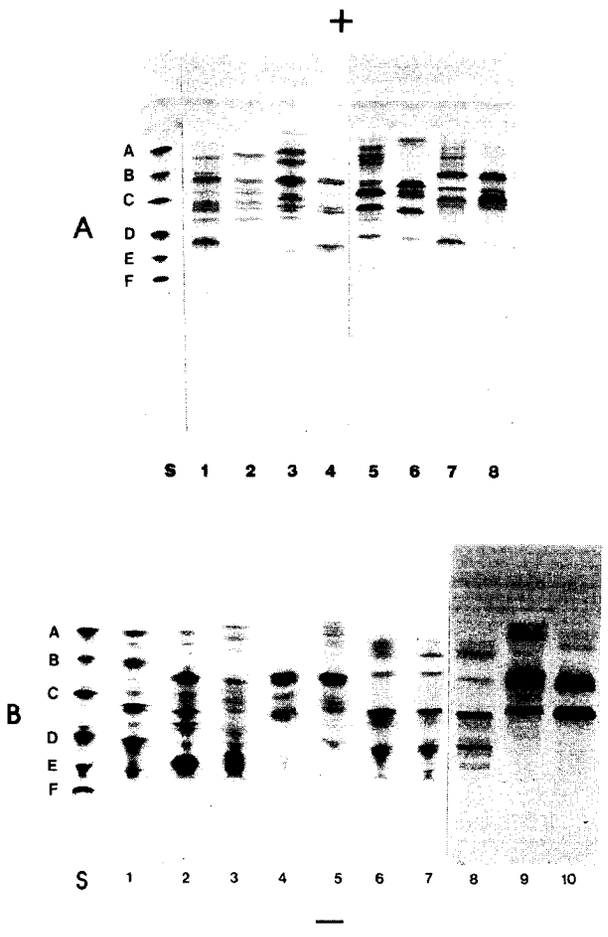

Fig. 5. (A) SDS–PAGE (reducing) (1 μl of 5 mg ml^{-1} protein solutions) applied to gradient 8/25 PhastGels (Pharmacia, LKB) Lane S, standards (A) α-lactalbumin, 14 400 Da, (B) soybean trypsin inhibitor, 20 100 Da, (C) carbonic anhydrase, 30 000 Da, (D) ovalbumin, 43 000 Da, (E) bovine serum albumin, 67 000 Da, (F) phosphorylase b, 94 000 Da. Lane: (1) pea, (2) peanut, (3) corn, (4) oat, (5) buckwheat, (6) rye, (7) barley, purified globulins, (8) wheat, (9) hemp, and (10) pumpkin. (B) SDS–PAGE (1 μl of 5 mg ml^{-1} protein solutions) applied to gradient 8/25 PhastGels (Pharmacia, LKB) Lane S, standards (A) α-lactalbumin, 14 400 Da, (B) soybean trypsin inhibitor, 20 100 Da, (C) carbonic anhydrase, 30 000 Da, (D) ovalbumin 43 000 Da, (E) bovine serum albumin, 67 000 Da, (F) phosphorylase b, 94 000 Da. Lane: (1) dill, (2) celery, (3) alfalfa, (4) fennel, (5) pine nut, (6) flax, (7) cumin, and (8) caraway.

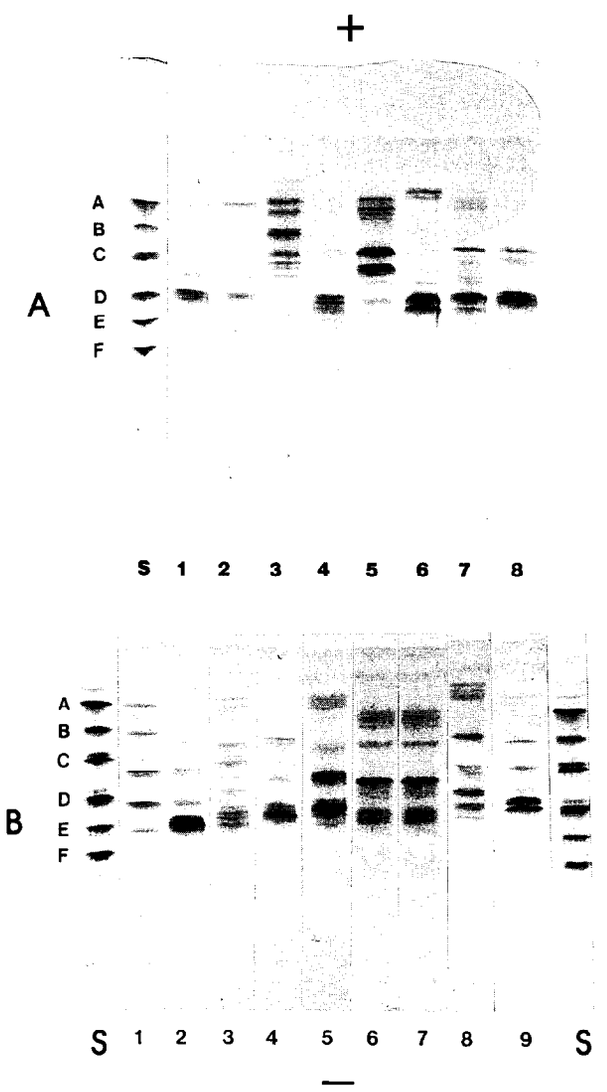

Fig. 6. (A) SDS–PAGE (non-reducing) (1 μl of 5 mg ml^{-1} protein solutions) applied to gradient 8/25 PhastGels (Pharmacia, LKB). Lane S, standards (A) α-lactalbumin, 14 400 Da, (B) soybean trypsin inhibitor, 20 100 Da, (C) carbonic anhydrase, 30 000 Da, (D) ovalbumin, 43 000 Da, (E) bovine serum albumin, 67 000 Da, (F) phosphorylase b, 94 000 Da. Lane: (1) pea, (2) peanut, (3) corn, (4) oat, (5) buckwheat, (6) rye, (7) barley, (8) hemp, and (9) pumpkin, purified globulins. (B) SDS–PAGE (non-reducing) (1 μl of 5 mg ml^{-1} protein solutions) applied to gradient 8/25 PhastGels (Pharmacia, LKB) Lane S, standards (A) α-lactalbumin, 14 400 Da, (B) soybean trypsin inhibitor, 20 100 Da, (C) carbonic anhydrase, 30 000 Da, (D) ovalbumin, 43 000 Da, (E) bovine serum albumin, 67 000 Da, (F) phosphorylase b, 94 000 Da. Lane: (1) dill, (2) celery, (3) alfalfa, (4) fennel, (5) pine nut, (6) flax, (7) cumin, and (8) caraway.

Although, it was generally found that only the dicotyledonous seed globulins had intermediary subunits (disulfide-linked subunits), these intermediary subunits constituted a very small percentage of the total subunits present ($< 25\%$). The relative ease of disruption of the molecular structures of monocotyledonous and dicotyledonous globulins (observed in the absence of β-mercaptoethanol) would indicate that most, if not all, of the interchain bonding between subunits was non-covalent in nature, and constituted a major similarity between these two classes of globulin.

A closer examination of the derived molecular weight data for these subunits (i.e. listed in Table 6), shown in Fig. 7, demonstrated that a striking similarity existed in the size distribution of subunits forming the 11S globulins from monocotyledonous and dicotyledonous origins; falling within two very narrow molecular weight ranges, i.e. 20 000–27 000 and 30 000–39 000 Da. In the past, researchers working with select globulins found that these two regions corresponded to basic and acidic subunits, respectively (Derbyshire *et al.*, 1976). The above observation was the first indication of the extent

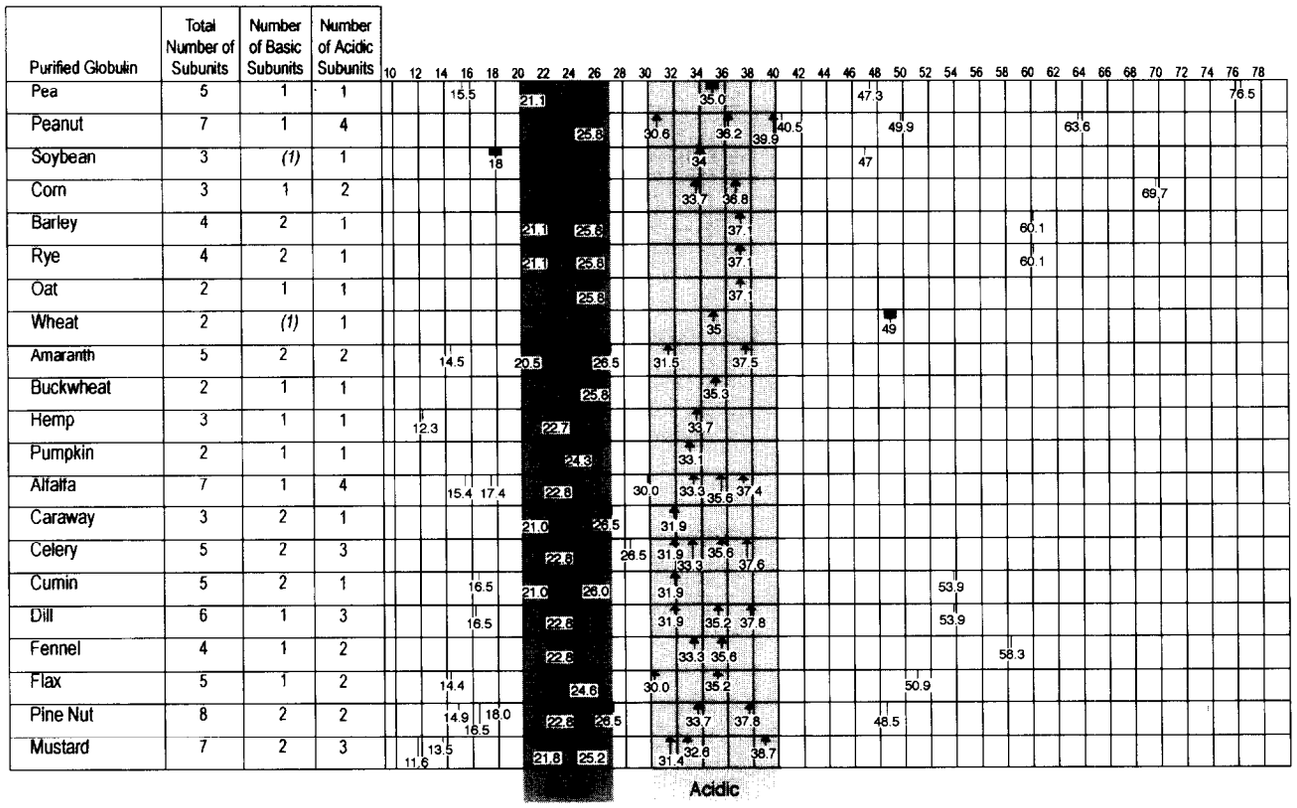

Fig. 7. Tabular representation of molecular weight distributions (i.e. Kilodaltons) of subunits from various purified seed storage globulins from various sources determined by SDS–PAGE (reducing). ■, Literature value; ▲, Acidic subunit.

Table 7. Molecular masses of purified globulins from various sources determined by Native–PAGE

Globulin source	Molecular mass (Da)
Pea (*Pisum sativum* L.)	328 600
Peanut (*Arachis hypogaea* L.)	354 600 dissociation products 265 400 and 140 000
Soybean (*Glycine max* Merr.)	ND (dissociation product found) (Marcone *et al.*, 1994)
Corn (*Zea mays*)	200 400
Barley (*Hordeum vulgare*)	317 400
Rye (*Secale cereale*)	298 900
Oat (*Avena sativa*)	200 400
Wheat (*Triticum aestivum*)	ND
Amaranth (*Amaranthus hypochondriacus*)	ND (dissociation product found) (Marcone and Yada, 1991)
Buckwheat (*Fagopyrum esculentum*)	324 900 dissociation product 183 000
Hemp (*Cannabis sativa*)	ND
Pumpkin (*Cucurbita pepo*)	ND
Alfalfa (*Medicago sativa*)	278 000 dissociation product 126 000
Caraway (*Carum carvi*)	278 000 dissociation product 160 000
Celery (*Apium graveolens*)	255 000
Cumin (*Cuminum cyminum*)	278 000 dissociation product 133 000
Dill (*Anethum graveolens*)	278 000
Fennel (*Foeniculum vulgare*)	255 000
Flax (*Linum usitatissimum*)	535 000
Pine Nut (*Pinus edulis*)	186 000 dissociation 336 000, 126 000
Mustard (*Brassica alba*)	240 000

ND, not determined.

of similarity between subunits of these two classes of globulins and may represent a general universal characteristic of globulins. A universal storage globulin-like protein could, therefore, be envisaged as having a subunit profile compatible with the above characteristics. Although subunits with molecular weights approximating those of the acidic and basic subunits already studied are listed in Table 6, further investigations are required.

Table 8. A comparison of the amino acid composition of various purified seed storage globulins from both dicotyledonous and monocotyledonous plants

Amino acid	Globulin source										
	Pea (*Pisum sativum* L.)	Peanut (*Arachis hypogaea* L.)	Soybean (*Glycine max* Merr.)	Corn (*Zea mays*)	Barley (*Hordeum vulgare*)	Rye (*Secale cereale*)	Oat (*Avena sativa*)	Wheat (*Triticum aestivum*)	Amaranth (*Amaranthus hypochondriacus*)	Buckwheat (*Fagopyrum esculentum*)	Hemp (*Cannabis sativa*)
Asx[a]	12.4 (±0.2)	11.8 (±0.6)	11.5 (±0.0)	6.8 (±0.0)	9.3 (±0.2)	8.1 (±0.1)	9.3 (±0.2)	8.7 (±3.0)	8.8 (±0.3)	10.3 (±0.4)	10.7 (±0.3)
Thr	3.4 (±1.2)	3.6 (±0.2)	4.5 (±0.1)	2.9 (±0.1)	3.6 (±0.1)	3.0 (±0.1)	3.4 (±0.1)	3.3 (±0.4)	4.1 (±0.2)	2.9 (±0.2)	3.0 (±0.1)
Ser	9.4 (±1.1)	9.4 (±2.6)	7.7 (±0.1)	10.3 (±0.5)	8.2 (±1.7)	9.0 (±0.8)	7.7 (±0.2)	8.9 (±3.0)	6.9 (±0.2)	8.1 (±0.7)	7.7 (±2.2)
Glx[b]	19.3 (±0.2)	19.9 (±0.9)	16.4 (±0.0)	16.5 (±0.1)	15.5 (±0.4)	16.2 (±0.2)	19.1 (±0.4)	14.9 (±0.5)	15.3 (±0.3)	17.9 (±0.7)	17.4 (±0.7)
Pro	6.2 (±0.3)	8.5 (±0.8)	6.3 (±0.1)	9.5 (±0.3)	9.0 (±0.4)	9.2 (±0.5)	9.2 (±0.4)	7.6 (±0.7)	6.1 (±0.4)	8.5 (±0.1)	10.0 (±1.3)
Gly	5.1 (±0.1)	7.0 (±0.4)	6.6 (±0.0)	8.8 (±0.1)	9.9 (±0.6)	9.4 (±0.1)	7.8 (±0.1)	9.3 (±0.5)	8.0 (±0.1)	7.6 (±0.3)	6.6 (±0.1)
Ala	4.6 (±0.2)	5.9 (±0.2)	5.9 (±0.1)	7.4 (±0.2)	8.3 (±0.8)	7.9 (±0.3)	6.6 (±0.3)	7.9 (±0.5)	5.7 (±0.1)	5.4 (±0.1)	6.0 (±0.5)
Cys[c]	0.2	0.8	1.1 (±0.0)	1.1	1.9	1.7	0.6	1.4 (±0.0)	0.9 (±0.0)	1.1	1.4
Val	4.3 (±0.1)	4.7 (±0.2)	5.6 (±0.4)	5.4 (±0.1)	5.5 (±0.4)	5.3 (±0.2)	4.8 (±0.1)	5.2 (±0.3)	6.3 (±0.1)	5.3 (±0.2)	4.8 (±0.3)
Met	0.3 (±0.1)	1.5 (±0.2)	1.2 (±0.2)	1.2 (±0.1)	1.4 (±0.7)	1.8 (±0.1)	1.4 (±0.3)	1.2 (±0.3)	1.8 (±0.1)	1.1 (±0.2)	1.3 (±0.9)
Ile	4.3 (±0.1)	3.7 (±0.2)	4.3 (±0.1)	2.7 (±0.1)	2.9 (±0.5)	3.3 (±0.1)	3.9 (±0.0)	2.8 (±0.2)	5.0 (±0.0)	3.7 (±0.1)	3.6 (±0.2)
Leu	8.6 (±0.0)	6.9 (±0.3)	8.6 (±0.2)	5.2 (±0.0)	5.0 (±1.6)	6.2 (±0.0)	6.6 (±0.1)	5.0 (±0.0)	6.8 (±0.1)	6.0 (±0.2)	5.9 (±0.2)
Tyr	2.3 (±0.0)	2.3 (±0.1)	2.3 (±0.1)	2.4 (±0.1)	1.9 (±0.1)	2.1 (±0.1)	3.0 (±0.1)	1.7 (±0.1)	2.1 (±0.1)	2.1 (±0.6)	2.3 (±0.1)
Phe	4.9 (±0.1)	4.6 (±0.2)	5.7 (±0.0)	4.7 (±0.1)	3.9 (±0.2)	3.8 (±0.1)	5.5 (±0.3)	2.8 (±0.1)	6.8 (±0.3)	5.5 (±0.7)	4.0 (±0.1)
Lys	6.8 (±0.3)	3.9 (±0.2)	4.5 (±0.0)	3.9 (±0.1)	4.3 (±0.7)	3.8 (±0.8)	2.7 (±0.8)	3.9 (±0.2)	4.3 (±0.2)	4.1 (±0.6)	2.1 (±0.4)
His	1.5 (±0.1)	2.1 (±0.2)	2.8 (±0.1)	2.9 (±0.1)	2.0 (±0.1)	2.6 (±0.1)	2.2 (±0.1)	1.9 (±0.2)	2.6 (±0.2)	1.7 (±0.1)	2.1 (±0.1)
Arg	6.2 (±0.1)	9.4 (±0.5)	5.4 (±0.1)	9.4 (±0.0)	9.1 (±1.6)	8.6 (±0.2)	6.3 (±0.1)	9.9 (±0.0)	10.1 (±0.7)	9.7 (±0.9)	12.4 (±3.0)
Trp	ND	ND	ND	ND	ND	ND	ND	3.6 (±0.1)	ND	ND	ND

—continued

Table 8—*contd*

Amino acid	Globulin source										Mean values
	Pumpkin (*Cucurbita pepo*)	Alfalfa (*Medicago sativa*)	Caraway (*Carum carvi*)	Celery (*Apium graveolens*)	Cumin (*Cuminum cyminum*)	Dill (*Anethum graveolens*)	Fennel (*Foeniculum vulgare*)	Flax (*Linum usitatissimum*)	Mustard (*Brassica alba*)	Pine Nut (*Pinus edulis*)	
Asx[a]	9.1 (±0.1)	13.4 (±0.3)	12.1 (±0.0)	11.7 (±0.4)	12.2 (±0.0)	11.0 (±0.0)	10.7 (±0.1)	12.0 (±0.0)	7.9 (±0.2)	10.6 (±0.0)	10.4
Thr	2.7 (±0.1)	2.5 (±0.1)	3.7 (±0.1)	3.5 (±0.2)	4.0 (±0.1)	4.0 (±0.2)	3.9 (±0.1)	3.8 (±0.1)	4.0 (±0.0)	3.4 (±0.1)	3.5
Ser	8.5 (±0.8)	8.2 (±0.1)	6.0 (±0.0)	7.3 (±0.3)	5.7 (±0.1)	6.7 (±0.2)	6.5 (±0.1)	6.9 (±0.1)	5.6 (±0.2)	6.8 (±0.1)	7.7
Glx[b]	17.3 (±0.5)	20.7 (±0.2)	19.4 (±0.1)	20.1 (±0.7)	20.5 (±0.0)	19.9 (±0.2)	19.7 (±0.1)	21.2 (±0.1)	22.5 (±0.7)	19.9 (±0.1)	18.6
Pro	10.4 (±1.1)	—	—	—	—	—	—	—	7.3 (±2.6)	—	8.3
Gly	7.1 (±0.1)	5.7 (±0.1)	8.3 (±0.1)	9.1 (±0.3)	9.1 (±0.1)	9.0 (±0.1)	9.2 (±0.0)	9.2 (±0.1)	10.4 (±0.3)	8.0 (±0.1)	8.2
Ala	6.8 (±0.3)	5.5 (±0.2)	7.2 (±0.0)	6.5 (±0.2)	6.5 (±0.0)	6.2 (±0.1)	6.4 (±0.1)	7.9 (±0.1)	5.9 (±0.1)	6.5 (±0.0)	6.5
Cys[c]	1.5	2.3	0.8	0.8	0.9	0.8	1.1	1.0	2.70	0.5	1.2
Val	4.6 (±0.2)	4.7 (±0.0)	6.1 (±0.0)	5.7 (±0.2)	5.2 (±0.0)	6.0 (±0.1)	5.8 (±0.0)	5.6 (±0.0)	4.5 (±0.1)	4.3 (±0.0)	5.2
Met	1.7 (±0.1)	0.4 (±0.1)	1.9 (±0.3)	1.3 (±0.1)	1.1 (±0.1)	1.9 (±0.3)	1.6 (±0.3)	1.1 (±0.7)	2.3 (±0.3)	1.5 (±0.3)	1.4
Ile	3.2 (±0.1)	4.5 (±0.2)	5.3 (±0.3)	4.3 (±2.5)	6.1 (±0.0)	5.0 (±0.3)	5.3 (±0.2)	5.5 (±0.1)	3.4 (±0.2)	4.4 (±0.3)	4.2
Leu	6.6 (±0.1)	9.2 (±0.1)	8.7 (±0.0)	8.4 (±0.3)	7.6 (±0.1)	8.2 (±0.1)	8.0 (±0.0)	5.8 (±0.0)	8.3 (±0.3)	8.0 (±0.1)	7.1
Tyr	2.6 (±0.1)	2.1 (±0.0)	1.1 (±0.0)	1.6 (±0.1)	1.2 (±0.0)	1.5 (±0.0)	1.6 (±0.0)	1.7 (±0.0)	1.7 (±0.0)	2.8 (±0.0)	2.0
Phe	4.7 (±0.1)	5.6 (±0.1)	5.9 (±0.0)	6.0 (±0.2)	6.2 (±0.0)	5.9 (±0.0)	5.9 (±0.0)	4.9 (±0.0)	3.3 (±0.1)	4.1 (±0.0)	5.0
Lys	2.7 (±0.2)	4.5 (±0.3)	5.1 (±0.1)	5.8 (±0.3)	3.7 (±0.1)	5.5 (±0.3)	5.8 (±0.1)	2.7 (±0.1)	3.6 (±0.1)	5.8 (±0.1)	4.3
His	1.9 (±0.1)	2.4 (±0.0)	2.4 (±0.0)	2.0 (±0.1)	2.3 (±0.0)	2.4 (±0.0)	2.5 (±0.0)	2.0 (±0.0)	2.1 (±0.1)	3.2 (±0.0)	2.3
Arg	10.1 (±1.8)	8.2 (±0.1)	6.1 (±0.0)	6.3 (±0.2)	7.7 (±0.7)	6.0 (±0.0)	5.8 (±0.0)	9.3 (±0.1)	4.6 (±0.1)	10.2 (±0.1)	8.1
Trp	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	

Reported in molar per cent. ND, not determined. Results are the mean value (±SD) of three replications.
[a]Asx = Asp + Asn.
[b]Glx = Glu + Gln.
[c]Cystine and cysteine measured as cysteic acid by performic acid treatment.

Table 9. A comparison of the amino acid composition of the various purified globulin fractions from *Amaranthus hypochondriacus*, *Glycine max* L. and *Triticum aestivum*

Amino acid	Fraction[ag]							
	Amaranth			Soybean			Wheat	
	2a	2b	Konishi *et al.* (1985)	2	3	Kitamura and Shibasaki (1975)	2	3
Asx[b]	9.40 (±0.25)	8.76 (±0.25)	10.1	12.0 (±0.5)	11.5 (±0.0)	11.8	8.73 (±3.00)	7.64 (±0.05)
Thr[d]	5.02	4.02	4.1	5.0 (±0.2)	4.5 (±0.1)	4.2	3.28 (±0.41)	2.54 (±0.00)
Ser[d]	7.28	6.90	8.6	8.0 (±0.1)	7.7 (±0.1)	6.6	8.93 (±3.41)	7.21 (±0.14)
Glx[c]	14.76 (±0.35)	15.29 (±0.31)	16.6	15.9 (±0.1)	16.4 (±0.0)	18.8	14.89 (±0.48)	16.96 (±0.28)
Pro	5.57 (±0.67)	6.14 (±0.39)	5.4	5.8 (±0.1)	6.3 (±0.1)	6.3	7.61 (±0.71)	6.15 (±0.41)
Gly	9.03 (±0.28)	8.00 (±0.14)	9.2	7.6 (±0.1)	6.6 (±0.0)	7.8	9.25 (±0.46)	9.15 (±0.18)
Ala	6.11 (±0.07)	5.71 (±0.10)	6.2	6.2 (±0.2)	5.9 (±0.1)	6.7	7.85 (±0.49)	7.27 (±0.12)
Cys[e]	1.39 (±0.01)	0.93 (±0.01)	0.9	1.8 (±0.0)	1.1 (±0.0)	1.1	1.42 (±0.01)	1.31 (±0.01)
Val[d]	5.73 (±0.01)	6.29 (±0.09)	6.1	5.0 (±0.2)	5.6 (±0.4)	5.6	5.16 (±0.33)	5.92 (±0.06)
Met	1.18 (±0.07)	1.77 (±0.05)	1.2	1.0 (±0.1)	1.2 (±0.2)	1.0	1.23 (±0.27)	0.85 (±0.00)
Ile[d]	5.29 (±0.01)	5.01 (±0.04)	5.2	4.7 (±0.0)	4.3 (±0.1)	4.6	2.75 (±0.20)	2.51 (±0.04)
Leu	7.53 (±0.07)	6.82 (±0.06)	7.5	9.0 (±0.1)	8.6 (±0.2)	7.2	5.02 (±0.03)	4.80 (±0.10)
Tyr[d]	2.34	2.13	2.7	2.3 (±0.2)	2.3 (±0.1)	2.5	1.73 (±0.05)	1.46 (±0.05)
Phe	4.80 (±0.12)	6.78 (±0.26)	5.3	4.2 (±0.1)	5.7 (±0.0)	3.9	2.79 (±0.09)	3.04 (±0.17)
Lys	4.14 (±0.08)	4.25 (±0.16)	3.7	4.2 (±0.0)	4.5 (±0.0)	4.1	3.91 (±0.21)	2.55 (±0.16)
His	2.03 (±0.09)	2.62 (±0.15)	2.8	1.9 (±0.1)	2.8 (±0.1)	1.8	1.93 (±0.15)	2.32 (±0.23)
Arg	10.96 (±0.28)	10.12 (±0.72)	7.9	6.4 (±0.2)	5.4 (±0.1)	5.9	9.91 (±0.01)	14.36 (±0.07)
Trp[c]	ND	ND		ND	ND		3.57 (±0.12)	3.95 (±0.23)

Fractions 2a, 2, 2 refer to the polymerized amaranth, soybean and wheat globulins, respectively, whereas 2b, 3, 3 refer to their non-polymerized forms.
[a]Reported in molar percent.
[b]Asx = Asp + Asn.
[c]Glx = Glu + Gln.
[d]Extrapolation from 24, 48 and 72 h hydrolysis times.
[e]Cystine and cysteine determined as cysteic acid by performic acid treatment. Results are the mean values (± SD) of three replications.
[f]Tryptophan determined by methane sulfonic acid.
[g]Fractions taken from the gel filtration column after further purification on the anion exchange column.

Native–PAGE

When purified dicotyledonous and monocotyledonous seed globulins were subjected to Native–PAGE, the molecular weights determined by this electrophoretic method did not correspond with those values previously determined by gel filtration chromatography (Tables 4, 7) The present finding was not surprising since it is known that the net charge of proteins is an important determining factor in overall electrophoretic mobility and, in select cases, can override any sieving-out effect (i.e. size effect), especially at the gel concentrations commonly used for large oligomeric plant storage proteins (Hedrick and Smith, 1968).

Further examination of the purified dicotyledonous globulins under Native–PAGE conditions (pH 8.3), revealed the presence of a minor protein band of slightly higher relative electrophoretic mobility in most of the globulins tested, i.e. peanut, buckwheat, alfalfa, caraway, cumin, amaranth (Marcone and Yada, 1991), and soybean (Marcone *et al.*, 1994). Other researchers have also reported this phenomenon in some plant storage proteins, i.e. soybean 11S globulin (Kitamura *et al.*, 1974; Wolf *et al.*, 1981), peanut globulin (Cater *et al.*, 1957), as well as sesame seed α-globulin (Prakash and Nandi, 1977). In cases where this dissociation phenomenon was found to occur, its occurrence was postulated as being the result of electrostatic repulsion between subunits of non-covalently linked protein subunits. Amino acid analyses of the studied globulins revealed high levels of glutamic/glutamine and aspartic/asparagine (to be discussed later); therefore, repulsive forces between individual subunits could result from an increase in the number of negatively charged moieties coupled with the deprotonation of the α-amino acid groups with increasing pH.

The observed dissociation under Native–PAGE conditions is consistent with SDS–PAGE data which showed that the majority of attractive forces between subunits of the dicotyledonous and monocotyledonous globulins involved weak secondary forces (i.e. hydrogen, hydrophobic and electrostatic). The dissociation mediated by disruption of covalent bonds, namely the alkaline hydrolysis of disulfide linkages, would be highly unlikely in the present case (i.e. pH 8.3), since the rate of such hydrolysis has been shown to decrease 10-fold for every pH unit below pH 11 (Donovan, 1973; Prakash and Nandi, 1977).

In the case of the monocotyledonous seed globulins (i.e. from corn, barley, oat, rye) and wheat (Marcone and Yada, 1995), alkaline dissociation was not

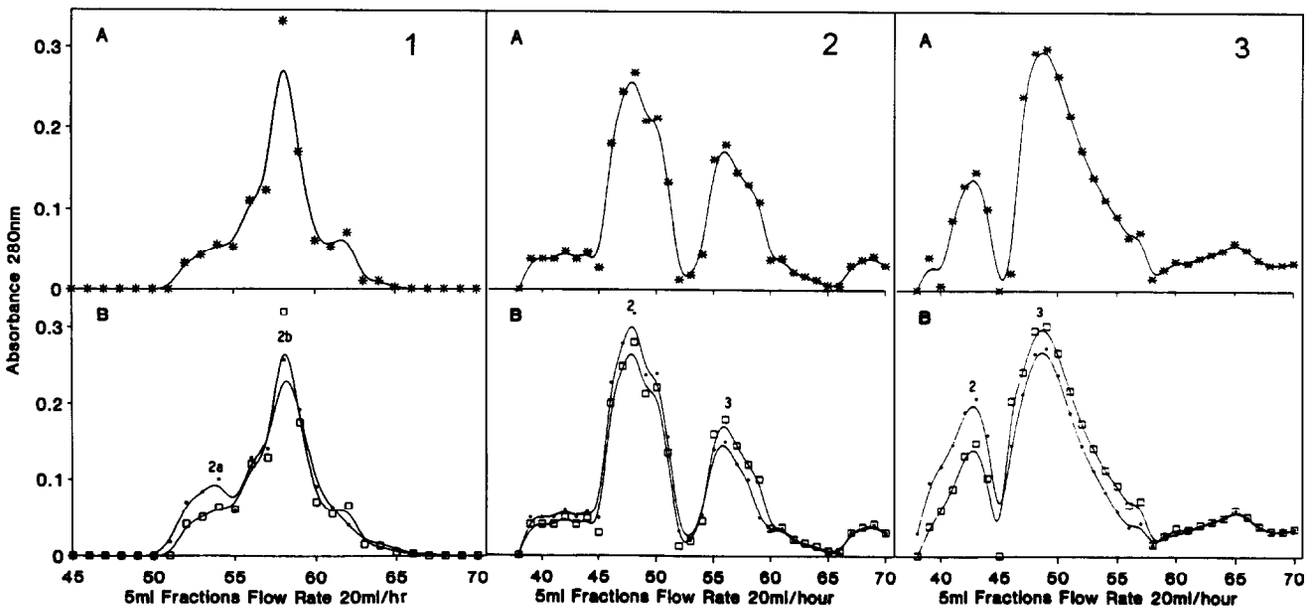

Fig. 8. (A) Sephacryl™ S-300 gel filtration chromatogram of crude globulin extract of (1) *A. hypochondriacus* (reprinted with the kind permission of Agric. Bio. Chem.), (2) *Glycine max* L., (3) *Triticum aestivum* (reduced/extracted and NEM treated) (2.5 ml containing 35 mg). (B) Sephacryl™ S-300 gel filtration chromatography of crude globulin from each of the respective materials (-●-, native; -□-, reversed native, i.e. reduced and NEM treated).

observed. It is possible that monocotyledonous, unlike dicotyledonous, seed globulins have stronger internal bond forces holding subunits in the molecule together, or alternatively do not have a large quantity of aspartic or glutamic acids (between subunits) which ionize at alkaline pH causing electrostatic repulsion.

To date, no biologically important function for the dicotyledonous plant globulins which undergo pH-induced dissociation has been reported. Control of hydrolysis rates of storage globulins (carbon and nitrogen reserves) during germination would seem to be a possibility (Sun *et al.*, 1974). The pH at which this dissociation occurs and the relative activity of proteolytic enzymes such as naphtylamidase and alkaline peptidase at pH 6–10 suggests that it can occur *in vivo* (Mikola, 1983). It is also conceivable that small, but physiologically important, amounts of free peptides (subunits) exist in equilibrium with the oligomer within the seeds (Sun *et al.*, 1974). According to Daussant, 1975, a progressive deamidiation of storage proteins takes place as a first step in degradation which is then quickly followed by the cleavage of disulfide bonds (when these are present). Disulfide bonds are found between intermediary subunits in dicotyledonous but not monocotyledonous seed globulins (as previously discussed).

A further decrease in size of proteins by the protease systems may occur, regulated by pH changes in the seed, which is brought about by the accumulation of amides thus tending to increase the overall pH. Therefore, it is possible that an increase in pH would favour dissociation of the globulins which in turn would allow for small amounts of free polypeptides to be more easily hydrolyzed by proteases.

Since globulins are not the major storage protein found in monocotyledonous plant seeds (as previously shown in Tables 1 and 3), but rather the prolamins/glutelins proteins, it would be reasonable to suggest that they do not serve as the major carbon and nitrogen reserve for seed germination. For this particular reason it is probable that dissociation is not observed since their regulatory role in protein hydrolysis during germination is not as important as compared to dicotyledonous seeds.

Amino acid analysis

Examination of the amino acid composition of both dicotyledonous and monocotyledonous seed globulins indicated a similar high content of amides (glutamic acids—glutamine, aspartic acid—asparagine and arginine; Table 8) indicating that they both have a storage role and may be equivalent proteins (i.e. have similar function, structure, etc.). A comparable level of other amino acids was also found.

Polymerization of seed globulins

In most of the crude dicotyledonous and monocotyledonous globulin preparations, the 11S globulin of molecular weight 300 000–370 000 Da was found to be the major protein (although a larger globulin was also found to exist in some of the species under investigation as evidenced by the gel filtration studies described earlier). It is not yet known if these polymers pre-exist in the intact seed; however, this possibility cannot be totally ruled out (Nash *et al.*, 1974). It is generally believed that these polymers occur after extraction

Table 10. Circular dichroism secondary structure fractions for the various purified amaranth, soybean and wheat 11 S globulins

Fraction[a,b]	(%)					
	Amaranth		Soybean		Wheat	
	2a	2b	2	3	2	3
α-Helix	18.4 (±1.1)	7.8 (±1.3)	17.9 (±1.0)	15.8 (±0.4)	26.4 (±0.3)	8.0 (±0.0)
β-Sheet	46.3 (±1.9)	57.6 (±1.1)	42.2 (±2.0)	56.0 (±2.0)	19.0 (±0.3)	53.3 (±0.4)
β-Turn	15.5 (±1.0)	17.6 (±0.5)	24.1 (±0.2)	21.2 (±0.3)	30.6 (±0.1)	13.8 (±0.2)
Random coil	19.9 (±0.3)	16.9 (±0.3)	15.8 (±0.5)	7.0 (±1.1)	24.0 (±0.1)	24.8 (±0.2)

Fractions 2a, 2,2 refer to the polymerized amaranth, soybean and wheat globulins, respectively, whereas 2b, 3, 3 refer to their non-polymerized forms.
[a] Expressed as a percentage of the total.
[b] Results are the mean values (±SD) of six scans per three replications.

(Derbyshire *et al.*, 1976). One of the major and important characteristics of these polymerized globulins is their contribution to the insolubility of protein isolates (Nash and Wolf, 1967; Wolf, 1970; Lillford and Wright, 1981; Hoshi *et al.*, 1982). These polymers have also been attributed to turbidity (Briggs and Wolf, 1957) and increased viscosity (Circle *et al.*, 1964) of globulin solutions. In the past, researchers were not able to effectively study these polymerized forms since they could not adequately isolate the proteins to homogeneity (Wolf *et al.*, 1962). In order to gain a clearer understanding as to the nature and cause(s) leading up to polymerization, selected dicotyledonous seeds (amaranth and soybean) and monocotyledonous seed (wheat) globulins were isolated to homogeneity and further studied.

Since a comparison of the amino acid composition of both amaranth globulin fractions, i.e. native and polymerized form, revealed the presence of 33% more cysteic acid in the polymerized form (Table 9) and 39 and 84% in the polymerized soybean and wheat globulins, respectively, it was postulated that some sort of interchange reaction between disulfide groups may have taken place and produced a polymeric species.

In order to verify if such a sulfhydryl–disulfide interchange reaction was occurring and was responsible for the appearance of the polymerized globulins in amaranth, soybean and wheat, their respective flours were treated with a sulfhydryl blocking alkylating agent, *N*-ethylmaleimide (NEM) prior to chromatography (Fig. 8). Similarly, when the unreduced extracted crude globulins were subsequently reduced and alkylated with NEM, Fraction 2a (amaranth), Fraction 2 for wheat and Fraction 2 for soybean decreased slightly, indicating that the polymerization reactions were partially reversible (Fig. 8).

The observed inability to completely eliminate the amaranth, soybean and wheat polymerized globulin fractions under restricted disulfide exchange may be attributed to the fact that sulfhydryl and disulfide residues have propensities to be buried in hydrophobic regions of the protein (Yamagishi *et al.*, 1981). Far-UV circular dichroism (CD) measurements of both amaranth globulin and its polymerized form revealed fairly high levels (i.e. 46.3 and 57.6%, respectively) of the β-sheet secondary structure fraction (Table 10) β-Sheet has a tendency to be buried in the interior of proteins (Cid *et al.*, 1982). The wheat globulin fraction, i.e. polymerized and native forms, also showed very high levels (i.e. 19.0, 53.4) and soybean (i.e. 42.2, 56.0) of β-sheet secondary structure fractions, respectively (Table 10).

The relative frequency of occurrence of cysteic and acid residues in the β-sheet fraction has been reported to be high (Burgess *et al.*, 1974; Chou and Fasman, 1974). It is, therefore, reasonable to suggest that such buried residues in all three globulins may be resistant to NEM attack (Yamagashi *et al.*, 1981). The other possibility is that NEM may be restricted from reacting with the sulfhydryl residue before the disulfide linkage is formed due to steric hindrance near the neighbourhood of the cysteine residue (Cecil and Thomas, 1965; Yamagishi *et al.*, 1981). The partial involvement of secondary forces such as hydrophobic interaction, and hydrogen and ionic bonding in polymerization, has also been reported (Yamagishi *et al.*,1981; Hoshi *et al.*, 1982) and may account for the partial reversal of the polymer to its native globulin form. Of these forces, hydrophobic interactions would appear most likely in view of the high surface hydrophobicity reported for the amaranth globulin (Konishi and Yoshimoto, 1989) while hydrogen and/or ionic bonding involvement would not be favoured in view of the high ionic strength buffers used in this study (Yamagishi *et al.*, 1981).

In a study which examined the effects of the ionic strengths of extraction solutions on the physicochemical properties of the mustard globulin, it was found that a polymerized fraction increased with increase in ionic strength (Marcone *et al.*, 1997). Since electrostatic forces are not favoured while hydrophobic forces are strengthened with increasing ionic strength, the observed increase in polymerization with increase in ionic strength would, therefore, likely be due, in part, to the increase in hydrophobic type forces.

When the polymerized amaranth and wheat globulins were subjected to Native–PAGE only one band was observed which had higher electrophoretic mobilities (data not shown) than their smaller native globulin counterparts (Marcone and Yada, 1991, 1995). This

observation would indicate that these polymers were more highly negatively charged above pH 8 and would explain this apparent anomaly in electrophoretic mobility. Examination of the secondary structure conformations of the polymerized versus native globulins revealed that the polymerized globulins had higher levels of α-helix, but lower levels of β-sheet, while β-turn and random coil fractions remained virtually unchanged. It is interesting to note that the structural conformational changes required for polymerization to occur through disulfide bond linkages are occurring in the buried secondary structure fractions which have the greatest propensity to possess these amino acid residues.

It appears that polymerization of globulins is occurring through a sulfhydryl–disulfide interchange mechanism although other physical forces such as hydrophobic interaction have been previously shown to play a role in the polymerization mechanism. Since the amounts of these polymers have been shown to be affected by prior treatment of the flours or crude globulin preparations with various chemical agents, it would appear that they do not pre-exist in the intact seed but occur during/after extraction. The polymerized globulins appear to possess similar internal conformation as evidenced by their secondary structure analyses, and to possess surface properties which are different from those of their native counterparts.

Summary of physicochemical comparison of 11S globulins

Detailed characterization of 21 seed globulins derived from both monocotyledonous and dicotyledonous plants indicated that globulins from both class types (as well as within the same class type) lay within a narrow molecular weight range of between 300 000–370 000 Da and were composed of multiple subunits. In all cases, globulins could be classified as hetero-oligomers being composed of a non-equimolar ratio of various subunits. The vast majority of subunits forming these globulins were shown to be held together by a non-covalent bond forces although a small percentage of linkages between subunits were also shown to be disulfide linked, in the case of dicotyledonous seed globulins. It was also found that the majority of subunits composing the dicotyledonous and monocotyledonous seed globulins fell within two very narrow molecular weight ranges, i.e. 20 000–27 000 and 30 000–39 000 Da, and were believed to correspond to basic and acidic subunits, respectively.

The amino acid composition of both dicotyledonous and monocotyledonous seed globulins suggests that they have a storage role and may be equivalent proteins since they were found to have similar and high contents of amides (glutamic acids—glutamine and aspartic acid—asparagine and arginine).

On the basis of the structural and chemical data obtained in this study, it is concluded that the 11S storage globulin, having several similar properties, exists in many leguminous and non-leguminous dicotyledonous plants as well as monocotyledonous plants. This similarity among 11-S storage globulins could be due either to convergent evolution in response to a common functional need, or to common ancestry. It is, therefore, important to conduct further studies to more closely examine the physicochemical relationships shared among globulins, especially those that pertain to protein conformation.

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