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Soybean 11S globulin polypeptides have an antigenic homology with 11S globulins from various plants

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Summary. Rabbit serum antibodies (AB) against glycinin acidic polypeptides were separated by cross exhausting, and the antibody fractions for each of the two subfamilies of glycinin subunits (A_1 and A_3) were obtained. The antibodies were used in the immuno blot assay with seed protein of various plant classes. Polypeptides homologous to soybean glycinin were detected. Homology with A_1 polypeptide was revealed in more cases than with A_3. Total seed protein preparations were subjected to centrifugation in sucrose density gradient, and the polypeptides, imunochemically related to glycinin, occurred only in fractions with sedimentation constant about 11S. The nativity of conservative antigenic determinants of 11S globulins is discussed.

Key words: Antigenic determinants – Enzyme-linked immuno sorbent assay – Soybean – Storage protein

Introduction

Storage 11S globulins, which have initially been characterised in some legumes (Danielsson 1949), are known to be prevalent seed protein components in several orders of monocots and dicots (Derbyshire et al. 1976), and possess some common features of structural organization. They are hexamers assembled from homologous, but not identical, subunits, each consisting of an acidic (A) and basic (B) polypeptide bound together with disulphide bonds and synthesised as a single high molecular weight precursor (Barton et al. 1982; Croy et al. 1981). Nucleotide and amino acid sequence analysis of five soybean 11S globulin (glycinin) subunits $A_{1a}B_2$, $A_{1b}B_{1b}$, A_2B_{1a}, A_3B_4 and $A_5A_4B_3$ showed that they can be subdivided into two subfamilies (A_1–A_2 and A_3–A_4) according to their homology level (Nielsen 1984). This probably reflects the peculiarities of the glycinin gene family evolution. Comparative sequence study of 11S globulin genes from various species showed their significant homology, especially in the 3′-terminal region, coding the basic polypeptide. The current primary structure of 11S globulin is known for only nine plant species from five families (Borotto and Dure 1987). The immuno-chemical assay is an alternative method of evolutionary studies that has been used successfully in studies on this class of proteins (Danielsson 1950; Kloz and Turkova 1963). Earlier works in this field ignored the sturctural complexity of proteins coded by multigenic families and dealt with native globular proteins, thus, the data obtained were used for elucidating the phylogenetic relations on the level of genera and tribes (Dudman and Millerd 1975; Eggi and Gavriljuk 1985). Alternatively, the storage protein transport and deposition mechanism seems to be universal for flowering plants and functions on the stage of protein precursor rather than native 11S aggregate (Adeli and Altosaar 1984). This mechanism most probably determines the conservativity of the specific regions of the polypeptide chains of storage proteins.

The aim of the present work was to investigate the homology of storage proteins in a wide variety of plants for which the gene sequences are not yet identified. For this purpose, we used immuno-chemical methods of analysis of individual polypeptides – products of individual storage protein genes.

Materials and methods

Plant seeds were reproduced in Vavilov's Plant Industry Institute (Leningrad) and Selection and Genetics Institute (Odessa). The seed meal was suspended in 35 m*M* sodium phosphate buffer containing 0.4 *M* NaCl and 0.01 *M* 2-mercaptoethanol,

pH 7.4. After incubation at room temperature for 3 h the suspension was centrifuged to remove the insoluble material. Then the soybean seed globulin was subjected to the procedure of glycinin separation (Alexenko et al. 1985). Glycinin was reduced and carboxyamido-methylated, and the ion exchange chromatography of acidic subunits was carried out on DEAE-Sephadex A-50 (Kitamura and Shibasaki 1975). Acidic polypeptides A_1, A_2, A_3 and A_4 were used for immunisation of adult male rabbits. 50 mg of protein were injected intramuscularly and subcutaneously in 0.15 *M* NaCl solution with equal volume of Freund complete adjuvant, repeatedly 30 days later without adjuvant, and 10 days later blood was taken. The globulin fraction was precipitated from cell-free serum by adding the ammonium sulphate saturated solution to 40%; then the immunoglobulins were separated on columns with immobilized glycinin acidic polypeptides (Casey 1979). The preparation was previously exhausted on a column with heterological acidic glycinin polypeptide, then the unbounded fraction was loaded on the column with a homologous antigene and the specific antibodies (AB) were eluted with a buffer pH 2.8. The specificity of antibodies was detected by double immuno diffusion technique (Garvey et al. 1976). Donkey antibodies against rabbit immunoglobulins provided by V. Poltoranina (Oncological Scientific Center, Moscow) were conjugated with horseradish peroxidase according to Wilson and Nakane (1978) and used in a double immuno blot assay with antibodies against glycinin polypeptides and total globulin to plant seeds. Electrophoresis was carried out according to the procedure of Laemmli (1970) in 12.5% polyacrylamide gel. Electrophoretic transfer of proteins to nitrocellulose sheet BA 85 ("Schleicher and Schull") immuno sorbent assay and developing of replica were carried out as described by Towbin et al. (1979); the developing solution contained 0.6 mg/ml 4-chloro-1-naphtol and 0.1% H_2O_2. Protein centrifugation in sucrose density gradient 10% – 35% was carried out as described (Hill and Breidenbach 1974; Alexenko et al. 1985): 2 – 5 mg of protein were loaded on the centrifuge tube of 12 ml. The protein content was determined after centrifugation in each of 24 fractions with Coomassie blue G (Sedmac and Grossberg 1977); 1/5 of fraction volume was loaded on the gel for protein visualization, and 1/20 for transfer to nitrocellulose after precipitation in 6.6% trichloroacetic acid.

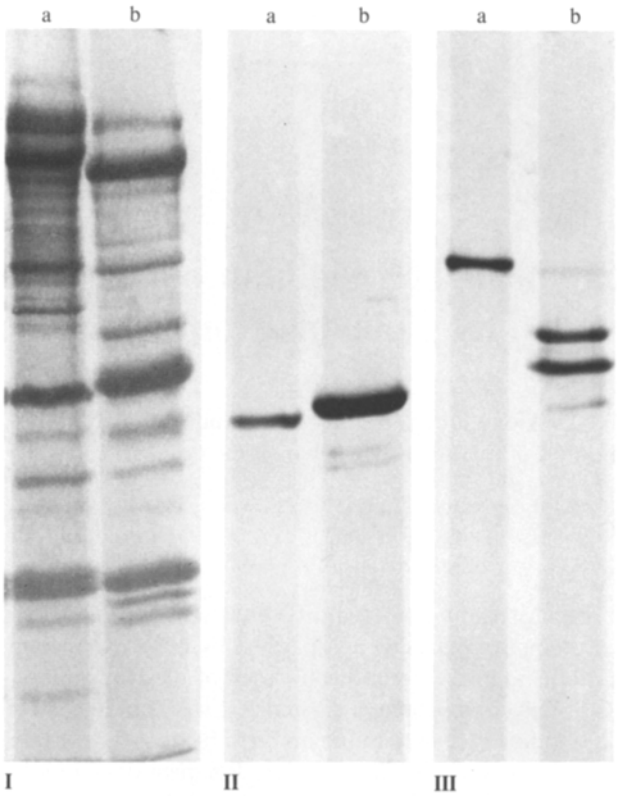

Fig. 1. Electrophoresis of soybean seed protein. **I.** Total protein vizualisation; **II.** Replics on nitrocellulose is tested with AB1; **III.** Replics is tested with AB3. Samples: a *Glycine tabacina*; b *G. max.* 35 μg, 0.1 μg and 0.1 μg of protein is loaded per gel track in I, II and III, respectively

Results

The polypeptides A_1, A_2, A_3 and A_4 used for immunization did not contain electrophoretically detected impurities. However, the sera obtained exhibited significant cross-reactivity, revealed both in the double immuno diffusion reaction and on antibody-tested replica. The characteristics of cross reactions are in agreement with Moreira et al. (1982), but the influence of impurities cannot be neglected. In order to obtain antibody fractions specific to each of the two subfamilies of polypeptides and possessing no minor activity, the antisera were exhausted on immobilized antigenes. Serum against A_1 was exhausted on A_3 or A_4, then loaded on the column with immobilized A_1 and the bounded fraction containing antibodies for determinants that are specific for the 1st (but not the 2nd) subfamily, was eluted. Antibodies against the 2nd subfamily were purified similarly. This procedure allowed to eliminate the influence of common antigene impurities; however, it did not allow to separate antibodies distinguishing polypeptides within each subfamily, because of their high immunochemical homology or identity. The purified antibodies for A_3 and A_4 exhausted on A_1 (AB3 and AB4) bound on replica with electrophoretic bands of A_3 and A_4 in equal ratio; the antibodies for A_1 and A_2 exhausted on A_3 (AB1 and AB2), with a band of about 38 kD, corresponding to polypeptides A_1 and A_2 (Fig. 1).

The results of the assay of seed total protein from various plant species with AB1 and AB3 (Fig. 2) how that seeds of most of the investigated species, including monocots and gymnospermous (*Larix sibirica*), contain polypeptides immunochemically related to the soybean 11S globulin. The mobility of these bands corresponds to mol. weight of 30 to 50 kD. The seed protein patterns of some species contain bands detected only with AB1 but not AB3. The antibodies used exhibited sorbtion on soybean glycinin electrophoretic bands at 0.02 μg of protein per gel track. The same density of colour bands in reactions with total protein of other species, both legumes (data not shown) and other families and orders required 300 – 800 times as much protein, without any correlation

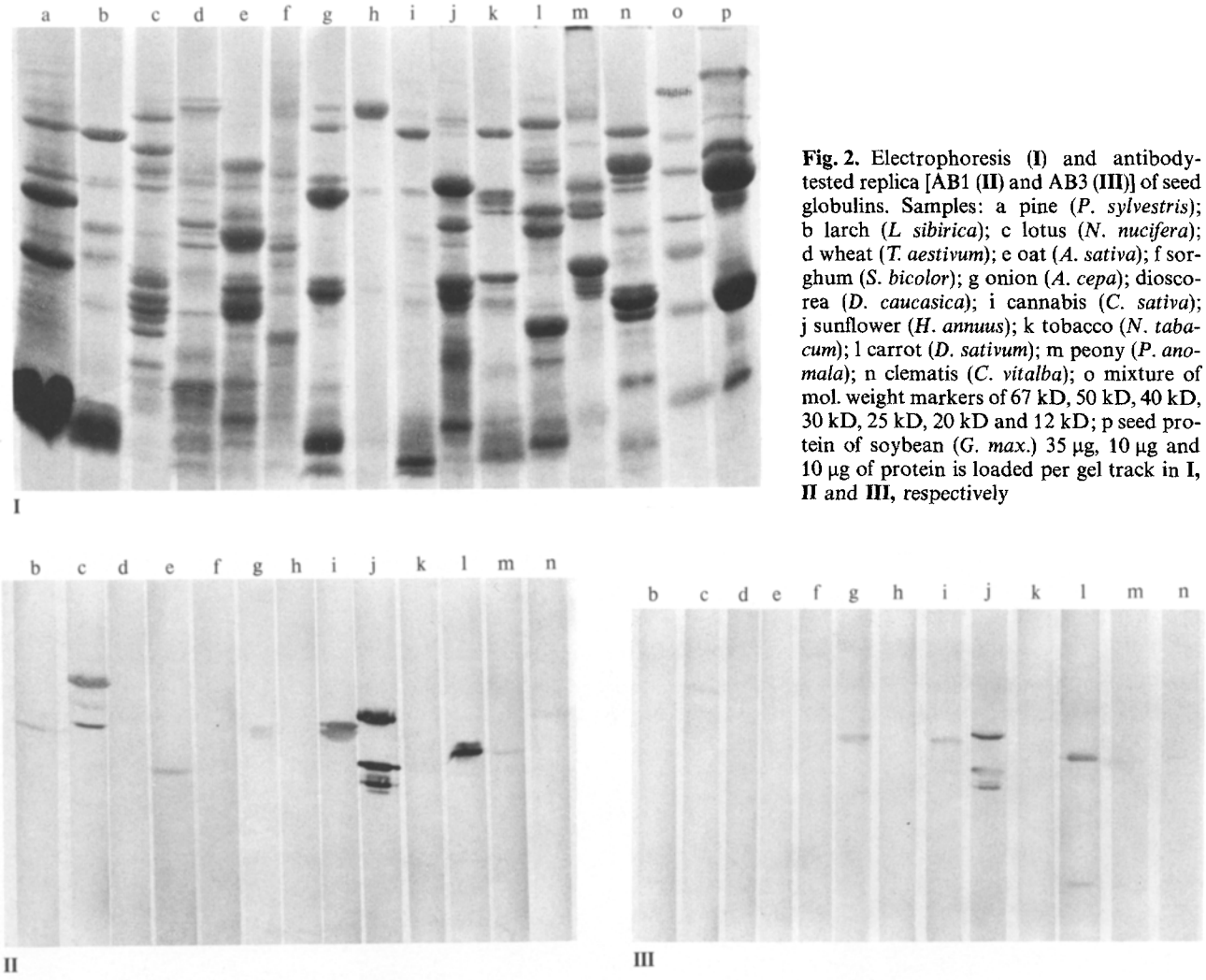

Fig. 2. Electrophoresis (**I**) and antibody-tested replica [AB1 (**II**) and AB3 (**III**)] of seed globulins. Samples: a pine (*P. sylvestris*); b larch (*L sibirica*); c lotus (*N. nucifera*); d wheat (*T. aestivum*); e oat (*A. sativa*); f sorghum (*S. bicolor*); g onion (*A. cepa*); dioscorea (*D. caucasica*); i cannabis (*C. sativa*); j sunflower (*H. annuus*); k tobacco (*N. tabacum*); l carrot (*D. sativum*); m peony (*P. anomala*); n clematis (*C. vitalba*); o mixture of mol. weight markers of 67 kD, 50 kD, 40 kD, 30 kD, 25 kD, 20 kD and 12 kD; p seed protein of soybean (*G. max.*) 35 μg, 10 μg and 10 μg of protein is loaded per gel track in **I**, **II** and **III**, respectively

with the taxonomical distance between species. It was necessary to check whether the homology detected proceeds from "random" antigenic determinants of independent origin in different species, or is due to homologous proteins. In order to make a reliable choice between these opportunities, total protein preparations were subjected to centrifugation in sucrose density gradient, and fractions were analysed via electrophoresis and immuno assay. The polypeptides homologous to acidic glycinin subunits occured in the 11S fraction and were absent or occured in smaller amounts in the remaining fractions (Fig. 3). Samples that did not provide peaks of 11–13S in their sedimentation patterns also did not contain polypeptides homologous to soybean glycinin. Comparison of antibody-tested replica with the electrophoretic patterns of seed protein (Fig. 4) shows that the homologous signal is concentrated in one of the predominant protein fractions, which probably belongs to storage proteins rather than enzymes. Thus, the data obtained confirm that the homologous polypeptides belong to 11S globulins of common origin.

Discussion

A principal problem arising from the data obtained is to consider what conservative protein structures are responsible for the antigenic homology of storage proteins of taxonomically distant plant species. Since the immunochemical specificity was not damaged by heating with the reducing agent and precipitation with trichloroacetic acid, it may be concluded that these antigenic determinants are determined by the primary, or at least secondary, rather than the tertiary structure. These sequences are specific for 11S globulins and are not found in other polypeptides from seeds of species analyzed; they are probably extensive enough to avoid a random coincidence. The decrease in the assay sensibility revealed when

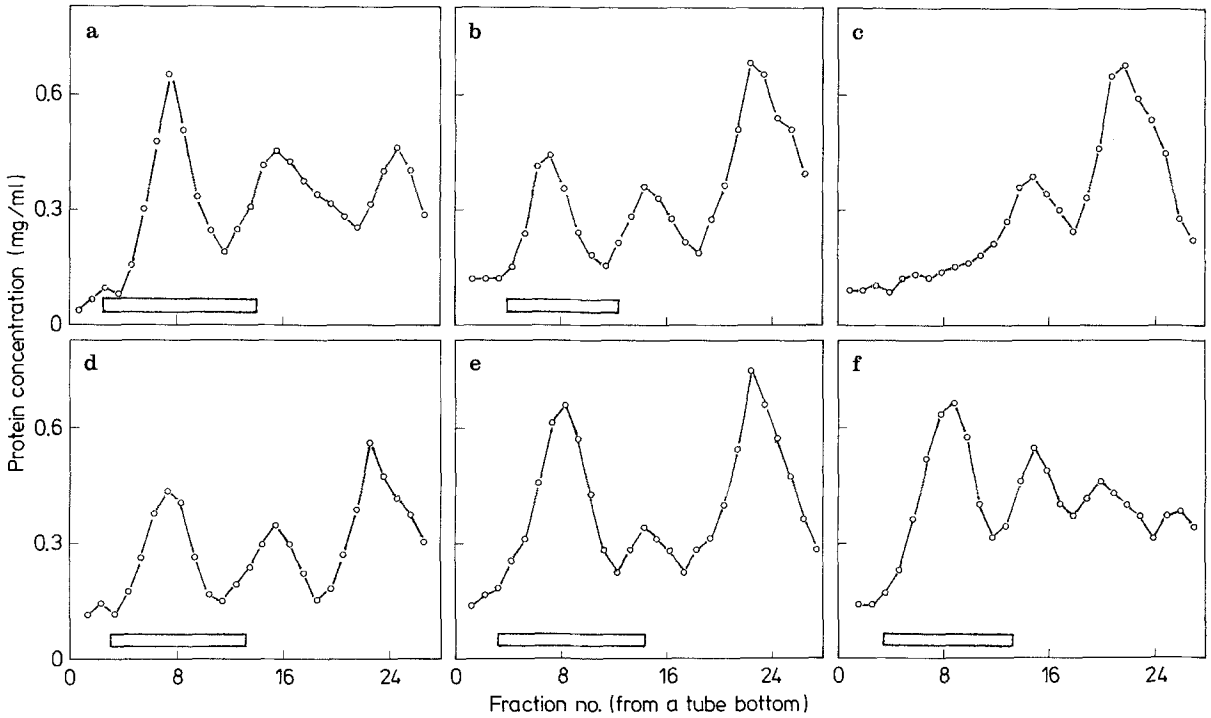

Fig. 3. Centrifugation patterns of seed proteins in sucrose density gradient: **a** soybean; **b** sunflower; **c** pine; **d** larch; **e** onion; **f** lotus. Fractions containing homologous polypeptides are marked under the profiles

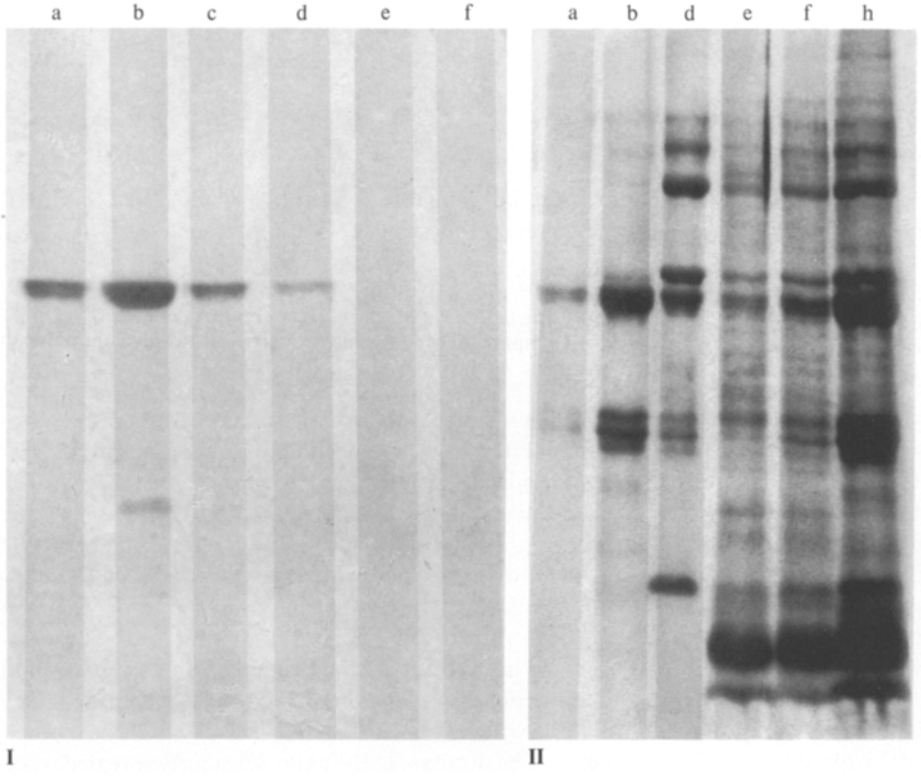

Fig. 4. Antibody-tested (AB1) replics (**I**) and electrophoresis (**II**) of the sucrose density gradient fractions of onion seed protein (see also Fig. 3e). Samples: a fraction 3; b fraction 6; c fraction 9; d fraction 12; e fraction 15; f fraction 18; h total seed protein

passing from soybean to plants of other genera and orders can be interpreted in terms of a small number of conservative determinants among all protein epitops, or alternatively as a decreased affinity of many determinants. However, the affinity stays constant for species of various taxonomical distance, which supports the former explanation. Provided the homology is due to one or few conservative determinants, they are the most probable candidates to be considered as "functional centers" of 11S globulins. The conceptions of storage protein functions are now intensively being developed (Adeli and Altosaar 1984). The principal function is participation in the intracellular system of protein transport, processing and deposition, which seems to be universal for at least flowering plants. The amino acid boxes, related to the detected conservative determinants, can occur in molecular regions providing these functions. Comparison of the results obtained with AB1 and AB3 shows that, in some species, these functions are distributed between the members of the multigenic family, so that the soybean glycinin polypeptide A_1 possesses more important and conservative functions than A_3.

The presence of 11S globulins homologous to soybean glycinin in seeds of flowering and gymnospermous plants proves an ancient evolutionary origin of this protein class, possibly simultaneous with the formation of the seed storage function. Thus, the evolutionary stability of 11S globulins appears to be comparable with that of enzymes.

The absence of detectable homology between glycinin and storage globulins of some species does not mean that the homology is absent. For example, the oat seed globulin exhibits antigenic homology with globulins of wheat and pea (Robert et al. 1985), and the polypeptides homologous to soybean glycinin were detected in pea (not shown) and oat, but not in wheat. It can be explained, presuming that even one or few amino acid substitutions in the conservative regions can damage the immuno specificity without loss of functional integrity. Besides that, some proteins can stay insoluble at the described extraction conditions.

Borroto and Dure (1987) reported the results of computer analysis of 11S globulin gene sequences of various plant species and deduced that 11S globulins were derived from a single ancestral gene. The analysis of comparative matrices indicates that the homology between 11S globulins is detected in extensive fragments of basic polypeptides adjacent to the cleavage site, with only a few short fragments in acidic polypeptides (especially in distant species). Some of these fragments could be responsible for the immunochemical homology detected.

The presence of a few highly conservative boxes as well as variable domains in 11S globulin molecules allows us to consider these proteins as a convenient universal model for studying the synthesis and transport of heterological proteins in plant cells.

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