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PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST 11S STORAGE PROTEIN FROM PEA SEEDS

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Key Word Index—*Pisum sativum*; Leguminosae; pea; 11S storage proteins; monoclonal antibodies; structural probes.

Abstract—Antibodies can be used as probes to investigate the structure of 11S storage proteins, their subunit composition and structural modifications induced by technological treatments. Monoclonal antibodies have been raised against *Pisum sativum* legumin (11S storage protein). Their binding characteristics were examined by direct, sandwich and competitive ELISA and by immunoblotting against legumin and 11S type storage proteins from other species. One of the MAbs, reacting with all 11S proteins tested, recognizes a discontinuous epitope accessible on the surface of the native hexameric protein but destroyed in dissociated legumin. Two antibodies recognize sequential epitopes belonging to a region of the acidic polypeptides present on the surface of the native legumin. These two MAbs cross-react only with pea and bean 11S proteins. Two other MAbs are specific for sequential epitopes buried in the native protein, localized, respectively, on acidic and basic polypeptides. The MAb reacting with the acidic polypeptide exhibits very specific binding for pea legumin. By contrast, the MAb specific to the basic polypeptide cross reacts with 11S proteins studied. This work shows the potential of this approach for the characterization of the conformation of the 11S proteins and the investigation of structural modifications.

INTRODUCTION

In the last decade, interest in pea seeds (*Pisum sativum* L.) as a source of protein has increased. The pea crop is now commonly used as a major component in animal feeds [1] but it is also processed for the preparation of protein isolates, starch and fibres for use by the food industry. As in most legume seeds, pea proteins contain two main classes of oligomeric storage proteins, legumin and vicilin, belonging, respectively, to the 11S and 7S protein-type families. The structure model of legumin is based, as for other 11S type proteins, on the arrangement of six subunits ($\alpha\beta$) (M_r 60000) linked together by non-covalent bonds in a trigonal antiprism [2]. Each subunit consists of an acidic (α) and a basic (β) polypeptide (M_r 40000 and 20000, respectively) linked together by a disulphide bond [3, 4]. This pairing is a consequence of the synthesis of legumin as a covalently linked NH_2-acidic-basic-CO_2H precursor (M_r 60000). These subunits ($\alpha\beta$) although homologous are not identical, being derived from a multigene family [5].

The nutritional as well as the technological value of legume seeds proteins was shown to be dependent on their subunit composition as well as on their folded or unfolded states. From *in vitro* enzymatic assays, it was shown that the unfolding of these globular and oligomeric proteins induced by controlled denaturations increased markedly the rate of, and yield on hydrolysis [6–8]. In food applications, dissociation and conformational changes were also shown to modify the functionalities of these proteins, e.g. surface [9, 10] and gelling properties [11–13]. In addition to these modifications of the conformation, which are generally induced by technological treatments, the other main factor influencing the properties of these oligomeric proteins is their subunit compositions. For example, the level of trypsinolysis for various 11S type proteins depends on their subunit composition [14]. The antigenic activity of these proteins and the related allergenicity may also be related to the primary sequence of the subunits [15, 16].

These variations in the composition of the subunits as well as of the quaternary and tertiary structures of these oligomeric seed proteins is amenable to study by using antibodies of defined specificity as molecular probes. Recent work on the soybean 11S globulin illustrated the value of monoclonal antibodies for such purposes [17, 18]. In the case of pea proteins, nothing has been done despite their increasing use in feeds and food either as new ingredients or traditionally consumed vegetables. The work presented here reports on the production of monoclonal antibodies against pea 11S globulin (legumin) and their ability to probe the structure of this complex molecule in terms of subunit composition and conformation changes.

RESULTS

Monoclonal antibody (MAb) characterization: antigenic reactivity towards pea legumin

Eight stable hybridomas secreting antibodies against legumin have been obtained. Isotypic characterization shows that all the MAbs are of the immunoglobulin subclass IgG with the exception of MAb 9E5 and 6E5 which belong to the IgM subclass (Table 1). The binding characteristics of these antibodies were examined using both ELISA immunoassays and immunoblotting techniques.

Three ELISA methods were used to evaluate the reactivity of the MAbs towards legumin molecules under different conditions. In the indirect ELISA the protein is directly adsorbed to microtiter plates and it is well known that proteins undergo conformational changes by adsorption on the plastic surface of wells [19]. The native conformation is retained by using indirect sandwich or indirect competitive ELISAs. In the indirect sandwich ELISA, a polyclonal rabbit antiserum adsorbed to the microtiter plates immobilizes the antigen. In the indirect competitive ELISA, MAbs bind to legumin molecules in solution. Indirect and sandwich assays may reveal low affinity MAbs which are not detected by competitive ELISA. According to their reactivities in these three conditions (Table 1), the MAbs could be divided in four categories. Category I comprised MAbs 10B10 and 10C10 which showed a positive reaction in all three ELISAs. Category II comprised MAb 9E5 which was not detected by competitive ELISA but gave a positive reaction in indirect and sandwich ELISA. In category III, the MAbs (5E9–6E5) recognized only the legumin molecules when they were adsorbed on the microtiter plates surface in indirect ELISA. Category IV is represented by MAbs (2E2-6F7-9F10) which showed a poor specific reactivity, reacting not only with legumin but also with bovine serum albumin (BSA).

MAbs reactivity against legumin subunits and polypeptides in ELISA and immunoblotting assays

All MAbs were tested for their reactivity against legumin ($\alpha\beta$) subunits and separated α and β polypeptides using indirect ELISA (Table 2) and immunoblotting (Fig. 1). Amido Black staining of the nitrocellulose sheet revealed typical patterns of subunits (Fig. 1A track 1) and polypeptides (Fig. 1B track 1) of legumin. Three types of subunits were detected: the main subunit characterized by an apparent M_r of 54 000 and containing an α polypeptide of M_r 38 000, two minor 'big' subunits (M_r 58 000 and 55 000) with higher M_r α polypeptides (39 000–42 000) and a 'small' legumin subunit (M_r 35 000) with a lower α polypeptide (M_r 25 000). These different subunits correspond to those previously described by

Table 1. Specificities of MAbs for legumin in indirect or sandwich or competitive ELISA

Monoclonal antibodies	Classes Ig Isotypes	Indirect ELISA	Sandwich ELISA	Competitive ELISA	BSA coated
10B10	IgG3	+ +	+ +	+	—
10C10	IgG3	+ +	+ +	+	—
9E5	IgM	+ +	+	—	—
5E9	IgG1	+ +	—	—	—
6E5	IgM	+ +	—	—	—
2E2	Ig G2b	+	+	—	+
6F7	IgG2a	+ +	+ +	—	+
9F10	IgG2b	+	+	—	+

Table 2. Reactivity of monoclonal antibodies with legumin subunits and polypeptides

	Immunoblotting		Indirect ELISA		
Monoclonal antibodies	Non-reducing condition	Reducing condition	α coated	β coated	BSA coated
10B10	$\alpha\beta$ 60 000	α	+	—	—
10C10	$\alpha\beta$ 60 000	α	+	—	—
9E5	—	—	—	—	—
5E9	$\alpha\beta$ 35 000	β	—	+	—
6E5	$\alpha\beta$ 60 000	α	+	—	—
2E2	—	—	+	+	+
6F7	—	—	+	+	+
9F10	—	—	+	+	+

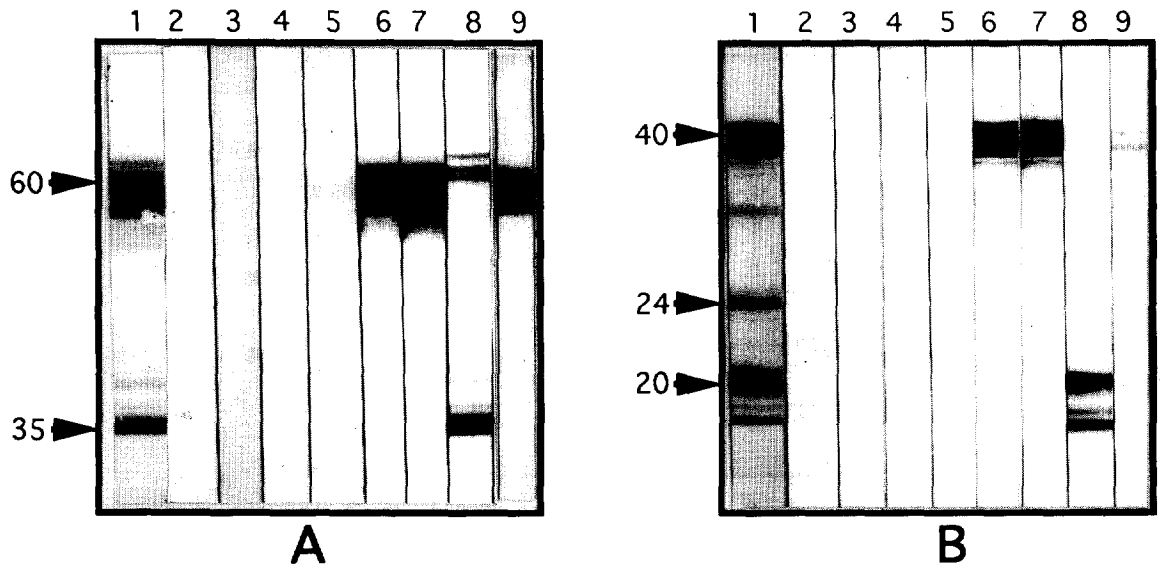

Fig. 1. Analysis of antibodies binding to legumin subunits by immunoblotting of SDS–PAGE gels under non-reducing (A) and reducing conditions (B). Efficiency control of electrotransfer by Amido Black staining (1); immunodetection with Mabs: 2E2 (2); 6F7 (3); 9E5 (4); 9F10 (5); 10B10 (6); 10C10 (7); 5E9 (8); 6E5 (9).

Matta *et al.* [20] and respectively called by these authors L4, L1 and L3 and L5. The MAbs reactivity against the three type of subunits and their constituent polypeptides were examined on the basis of Matta's data. MAbs from category I (10B10–10C10) exhibit very similar reactivity against polypeptides in immunoblotting. Both react very strongly with SDS dissociated ($\alpha\beta$) major subunits L4 (M_r 54 000) and in reducing conditions only with the major α polypeptides (M_r 38 000) without any reaction against the β-polypeptides (Fig. 1A). This specificity is also observed in the indirect ELISA (Table 2). MAb 9E5 from category II reacts neither in immunoblotting with legumin dissociated either into subunits or reduced polypeptides nor in ELISA against purified acidic or basic polypeptides. The epitope recognized by MAb 5E9 on the native hexameric protein should have been destroyed by the dissociating conditions used for separating the polypeptides in immunoblotting or in the purification procedure. MAb 6E5 from category III does not differ very much from those of category I except that the reaction towards α polypeptides is very weak in immunoblotting. Conversely, MAb 5E9 which is also classified in category III on the basis of the first set of data, shows very peculiar behaviours compared to all the other MAbs. It is the only one which mainly recognizes the minor 'small' subunit L5 (M_r 35 000) and shows a weak reaction towards the minor big subunits L1–L3 (M_r 55 000–58 000). Moreover, MAb 5E9 alone reacted specifically with the β polypeptides either in immunoblotting or in indirect ELISA.

However, after CNBr cleavage of the purified β polypeptides MAb 5E9 does not react with the resulting CNBr fragments (Fig. 2B). The polyclonal antibodies used as control faintly detect the intact β polypeptides and the large CNBr fragment but fail to detect the small fragment (Fig. 2C) although the control with Amido Black staining indicates that this fragment is efficiently transferred to nitrocellulose (Fig. 2A). MAbs from category IV (2E2-6F7-9F10) do not react in immunoblotting and show a high binding to BSA in ELISA. On using this binding as a baseline, it appeared that these antibodies do not particularly recognize any of the subunits examined. This result confirms their poor specificity as previously observed on the native oligomeric protein.

Cross-reaction of MAbs with 11S type storage proteins from other species

Cross-reactions with 11S type storage proteins from other species were assessed by indirect ELISA (Table 3) and immunoblotting (Figs 4–6). As shown in Fig. 3, these oligomeric proteins are characterized by very homologous subunits and polypeptide composition. All of them are composed of major subunits with M_r around 60 000 and minor ones around M_r 35 000–40 000 (Fig. 3A). In reducing conditions (Fig. 3B), they exhibit more or less complex patterns with bands around M_r 40 000 and 20 000 corresponding to the α-type and β-type polypeptides, respectively.

By indirect ELISA using 11S proteins adsorbed on the microtiter plates, the specificity of the polyclonal anti-pea legumin antibodies and of the various MAbs were compared. Polyclonal antibodies recognize 11S from pea, soyabean and fababean but do not react with 11S type proteins from non-leguminous seeds like sesame and pumpkin. MAb 9E5, belonging to category II, has broad specificity, reacting with soyabean, pumpkin, sesame and fababean 11S proteins to a markedly greater degree than with pea legumin. MAbs (10B10, 10C10, 5E9 and 6E5) which belong to categories I and III are characterized by

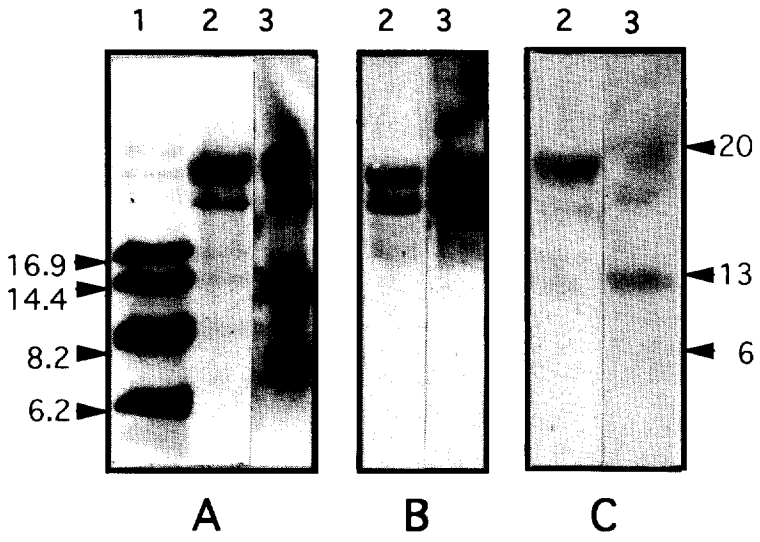

Fig. 2. Analysis of MAb 5E9 binding to β-polypeptides by immunoblotting. Efficiency control of electrotransfer by Amido Black staining (A). Immunodetection with MAb 5E9 (B); with polyclonal anti-legumin antibodies (C). Samples in the tracks are: protein M_r standards (1); β-polypeptides (2) and CNBr β-polypeptide fragments (3).

Table 3. Percentage of cross-reactions of monoclonal antibodies with 11S storage proteins from different species in indirect ELISA

	Cross-reaction (%)								
Storage protein	10B10	10C10	9E5	5E9	6E5	2E2	6F7	9F10	PolyAb
Fababean 11S	58	48	120	69	0	108	100	110	100
Sesame 11S	0	0	190	106	0	110	104	60	17
Soyabean11S	0	0	140	93	0	133	104	101	100
Pumpkin 11S	0	0	265	0	0	111	100	120	11
Pea 11S	100	100	100	100	100	100	100	100	100

Cross-reactions are expressed as a percentage of the binding to pea legumin at an appropriate dilution of culture supernatant.

a restricted reactivity towards 11S proteins. MAb 6E5 reacts very specifically with pea legumin. Both MAbs 10B10 and 10C10 exhibit similar recognition behaviours; they exhibit a strong binding to pea legumin and a weaker cross-reaction (50%) with fababean 11S protein. No reactivity with 11S globulins from other species is observed. Conversely, MAb 5E9 reacts with all 11S proteins tested, except the one from pumpkin.

According to this specificity, only MAbs from categories I and III were tested by immunoblotting of SDS–PAGE in non-reducing and reducing conditions. On the Western blot of unreduced 11S proteins revealed by using MAb 10B10, strong reactions are observed against pea and fababean proteins and a weaker one against pumpkin. The main difference between both sets of data, ELISA and immunoblotting, is the cross-reaction with pumpkin protein under dissociating conditions (Fig. 4). These MAbs recognized both subunits of high and low M_r for fababean but are specific for high M_r subunits in pea and pumpkin. Under reducing condition, only α polypeptides of high M_r from pea and pumpkin protein are reactive. On the other hand, both low and high M_r α polypeptides from fababean are detected (Fig. 4B). Similar results as for MAb 10B10 (Fig. 4) are obtained for the MAb 10C10.

When unreduced samples are analysed by immunoblotting using MAb 6E5, no cross-reaction with other 11S proteins is observed at all (results not shown) in agreement with the ELISA data. In contrast, under reducing conditions, MAb 6E5 exhibits an even stronger reaction with acidic polypeptides of sesame and pumpkin than with pea legumin (Fig. 5). Thus MAb 6E5 which appears to be extremely specific for pea legumin when non reduced 11S proteins are analysed, either in indirect ELISA or in immunoblotting, exhibits a cross-reactivity with acidic polypeptides of reduced 11S proteins of other non-leguminous species. A non-discontinous epitope, accessible on non-reduced acidic polypeptides of pea becomes accessible on sesame and pumpkin acidic polypeptides only after reduction. It is rather surprising to observe that this cross-reaction occurs with polypeptides from non-legume seeds whereas no cross-reaction is de-

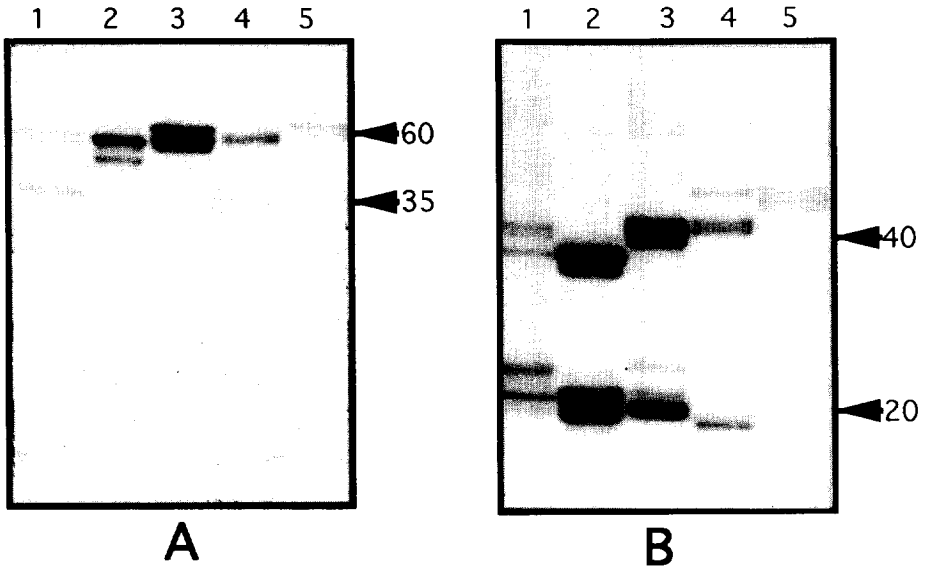

Fig. 3. Composition in subunits and polypeptides of 11S storage proteins from various species analysis by SDS–PAGE under non-reducing (A) and reducing conditions (B). Samples in the tracks are: (1) fababean; (2) sesame; (3) pumpkin; (4) soyabean; (5) pea.

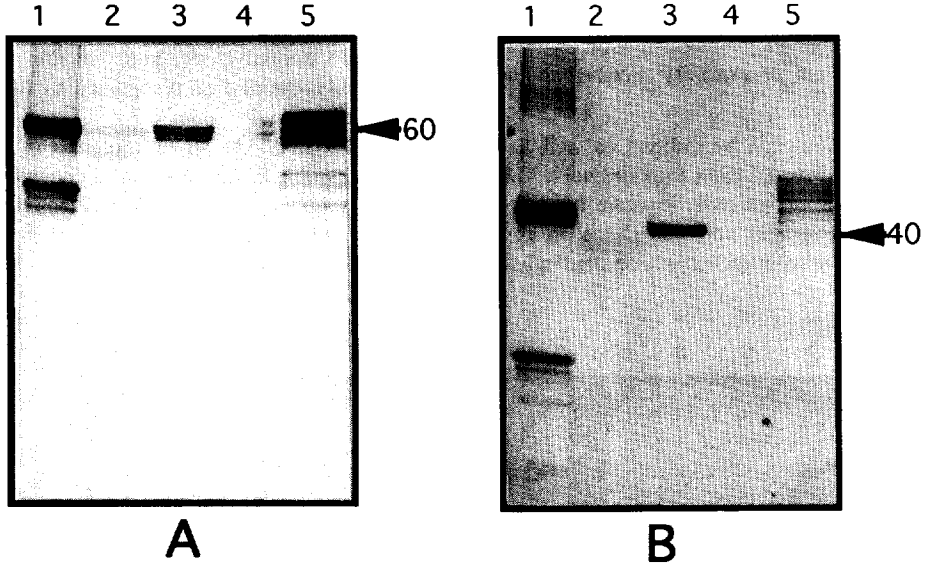

Fig. 4. Analysis of Mab 10B10 binding to 11S storage proteins from various species by immunoblotting of SDS–PAGE gels under non-reducing (A) and reducing conditions (B). Samples in the tracks are: (1) fababean; (2) sesame; (3) pumpkin; (4) soyabean; (5) pea.

tected with the corresponding polypeptides from 11S belonging to other leguminous seeds (soybean and fababean).

As in the ELISAs, MAb 5E9 recognizes subunits from fababean, soyabean, sesame and pea in immunoblotting of SDS-PAGE in non-reducing conditions (Fig. 6A). However, the type of reactive subunits varies depending on the species. In the case of fababean, only the lower M_r subunits are reactive. In soyabean, both minor small (A5B3) and minor 'big' subunits are detected whereas in sesame, only 'big' subunits are coloured. After reduction, a strong interaction is observed with basic β polypeptides for fababean, soyabean, sesame and pea. Even for pumpkin, which was unreactive in non-reducing conditions, a weak reaction with basic β polypeptides is observed under reducing conditions (Fig. 6B).

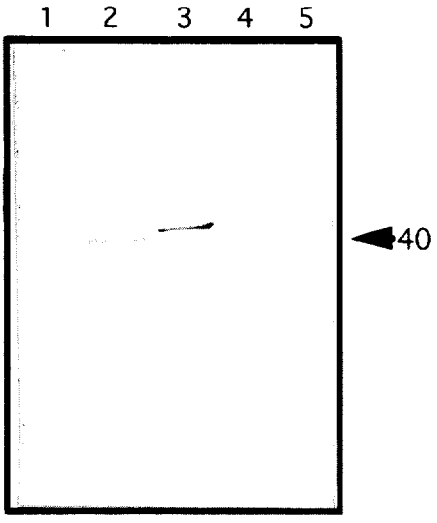

Fig. 5. Analysis of Mab 6E5 binding to 11S storage proteins from various species by immunoblotting of SDS–PAGE gels under reducing conditions. Samples in the tracks are: (1) fababean; (2) sesame; (3) pumpkin; (4) soyabean; (5) pea.

DISCUSSION

Epitope localization and accessibility: determination of structural probes

MAbs have been screened by indirect as well as by sandwich and competitive ELISAs to distinguish MAbs reacting against the native globular legumin from those specific for different oligomer unfolded states. In agreement with the observations of many investigators, the MAbs show substantial reactivity in indirect ELISA. Inversely, competition and sandwich ELISA methods allow us to distinguish two families in the MAbs which are reactive against the native (9E5, 10B10 and 10C10) and the denatured (5E9 and 6E5) antigen, respectively. This suggests that antigenic determinants recognized by MAbs 5E9 and 6E5 are exposed following coating on plastic, but are normally hidden in the native protein. When reduced legumin is analysed, the MAb 5E9 recognizes only β polypeptides. This fact explains its greater reactivity towards coated legumin in ELISA and the absence of recognition in sandwich ELISA since β polypeptides have most of their sequences buried in the native protein [21]. These results, obtained by different immunoassay procedures, clearly indicate that adsorption to plastic alters the native structure of legumin revealing additional epitopes. The epitope recognized by Mab 9E5 does not survive the dissociation conditions involved in polypeptide purification or in immunoblotting procedures. This epitope, accessible on the native protein, is probably discontinuous since it is dependent on the hexameric conformation of the molecule. However, it is not modified after coating to the plastic wells, indicating that the unfolding induced by plastic coating is partial. Conversely, sequential epitopes accessible on the native legumin and located on the α polypeptides are recognized by MAbs 10B10 and 10C10. Sequential, non-accessible epitopes on the native legumin that are located on the α and β polypeptides are recognized by MAbs 6E5 and 5E9, respectively.

This panel of MAbs, which recognize different types of epitopes, can be used as structural probes to investigate pea legumin conformational changes induced by technological treatments to improve the nutritional as well as the functional properties of this protein.

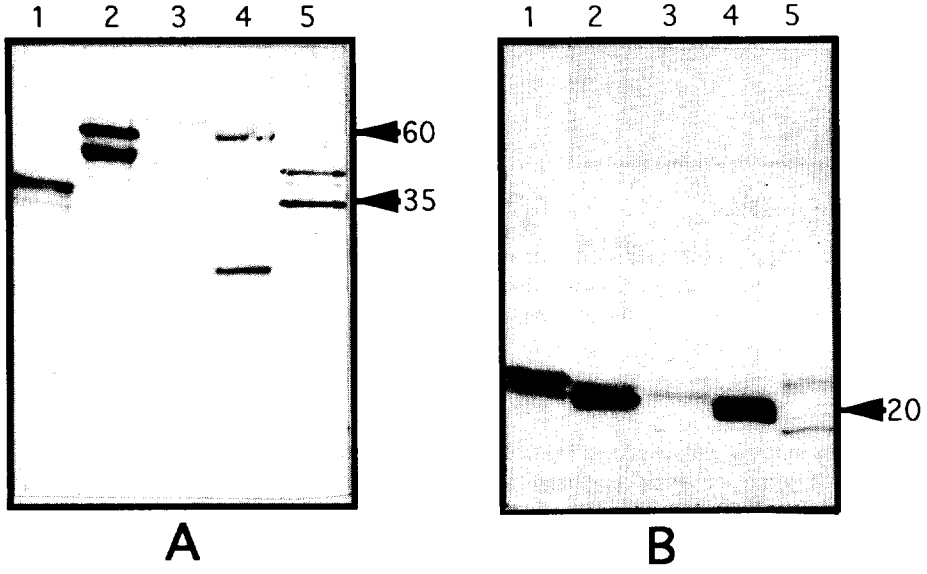

Fig. 6. Analysis of Mab 5E9 binding to 11S storage proteins from various species by immunoblotting of SDS–PAGE gels under non-reducing (A) and reducing conditions (B). Samples in the tracks are: (1) fababean; (2) sesame; (3) pumpkin; (4) soyabean; (5) pea.

Tools to analyse the polymorphism of legumin

As these MAbs also present different specificities towards the various constitutive subunits, they could also be considered as powerful tools to analyse the polymorphism of legumin subunits.

In the case of the pea legumin gene family, there are at least 10 legumin genes which can be further subdivided, on the basis of sequence homology, into three subfamilies [22]. The five genes of the first subfamily (genes legA–legE of which one, gene legD is a pseudogene) encode the major legumin subunits. MAbs 10B10 and 10C10 react with the major subunit L4 encoded by a gene of this family. The second subfamily (genes legJ–legL) encode the minor legumin subunits which include both large and small subunits. MAb 5E9 reacts with the minor small subunit L5 encoded by a gene of this family. The third subfamily corresponding to genes legM and legX was not distinguished by ours MAbs. The fact that the two categories of MAbs 10B10, 10C10 and 5E9 do not cross react with subunits encoded by the two subfamilies and allow us to distinguish subunits within each subfamily is not surprising according to the rather low sequence homology (38%) between the corresponding polypeptides [22].

Similar genes families are also mentioned for the 11S type protein from other species. Comparison of the cDNA sequence of legA and legJ genes with those of homologous genes from fababean (typeA and typeB) or soyabean [group I (A_2B_{1a}) and group II ($A_5A_4B_3$)] shows that they can be gathered into two main homology groups generally called type-A and type-B [23]. One of the main difference between the polypeptides encoded by these two families of genes, whatever the species, is the absence of methionyl residue in the type-B β polypeptides [24]. Consequently, the reaction of the MAb 5E9 only with the β polypeptides which have not been cleaved by CNBr, clearly shows that this MAb specifically reacts with the β polypeptides encoded by type-B genes. It allows the identification of the ($\alpha\,\beta$) subunits which react in immunoblotting with this MAb as type-B subunits. This is confirmed for pea, since the main ($\alpha\,\beta$) subunit (L4 M_r 54000) from type-A is not recognized, whereas for soybean since the low M_r subunit ($A_5A_4B_3$) encoded by a group II gene (type-B) [25] is revealed. The strong reaction with pea L5 subunit (M_r 35000) suggests that this polypeptide should be encoded by genes of type-B.

Distribution of the defined antigenic determinants among 11S proteins

A panel of broad or restricted specificity antibodies has been obtained. MAb 5E9 cross reacts with the basic polypeptides from the different species tested, in agreement with gene analysis [26] and comparison of protein sequences [27]. This indicates that the basic polypeptides contain the most conserved segments of the 11S globulin. MAbs 10B10, 10C10 and 6E5, reacting against the major acidic polypeptide, exhibit little cross-reaction, and thus detect more variable regions. These variable regions were shown to be hydrophilic regions, probably located at the surface of the oligomeric protein [27]. In ELISA, MAbs 10B10 and 10C10 only exhibited binding to pea and fababean 11S and did not recognize the other 11S proteins at all. This agrees with the fact that pea and fababean belong to the same taxonomic Viciae tribe. MAb 6E5 exhibited a strict reactivity against legumin. In immunoblotting, MAbs 10B10, 10C10 and 6E5 exhibit responses towards 11S protein from non-legume seeds that are much harder to explain. MAbs 10B10 and 10C10 detect 11S protein from pumpkin only in immunoblotting and MAb 6E5 reacts with 11S proteins from pumpkin and sesame only under reducing conditions, suggesting structural differences between these 11S globulins and pea legumin. Little information is available on 11S proteins from sesame and pumpkin, but the fact that the type-B subunits detected by MAb 5E9 are always minor components, except in sesame, confirmed the structural differences between 11S from sesame and other 11S globulins.

EXPERIMENTAL

11S protein purification. Legumin was purified from pea flour (*Pisum sativum* cv Amino) by a chromatographic procedure using successive ion-exchange and gel-filtration steps as described elsewhere [28]. 11S proteins from other species were the gift of G. Plumb, (AFRC Institute of Food Research, Norwich U.K.) and were purified as described elsewhere [29] from soya (*Glycine max*), fababean (*Vicia faba* cv Maris Bead), sesame (*Sesamum indicum*) and pumpkin (*Cucurbita pepo*).

α- and β-Polypeptide purification. The polypeptides were separated from reduced and alkylated legumin on a DEAE Sepharose CL6B column (phosphate buffer 0.05 M, pH 7, urea 6 M, β-mercaptoethanol 0.02 M; linear gradient of NaCl 0–0.75 M). The basic polypeptides were recovered as a single fraction in the non-retained peak whereas the acidic polypeptides were pooled from retained fractions. Methionine-containing β polypeptides were cleaved with cyanogen bromide [30].

Polyclonal antibodies. Rabbit polyclonal antibodies against legumin were obtained by immunizing rabbits subcutaneously with legumin (0.5 mg) first with complete Freund's adjuvant and then with incomplete adjuvant every 15 days. Bleeding was performed after the fourth injection. Sera were tested by indirect ELISA.

Monoclonal antibodies (MAbs). Female Balb/c mice were immunized three times with 10 μg of purified legumin. Three days after the last booster, their spleens were removed and splenocytes fused to the non-secreting myeloma Ag8 line as previously described [31]. Supernatants were tested by using indirect, sandwich and competitive ELISA tests. Hybridomas secreting anti-legumin antibodies were cloned by limiting dilution. Typing and subtyping were carried out using a mouse sub-typing panel (Biorad, France).

Indirect ELISA. 96-well microtiter plates were coated with 0.1 μg of 11S proteins (100 μl) in 0.05 M Na_2CO_3

Table 1. Effect of different Ti (IV) ascorbate treatments on Ti concentration of leaves, chloroplasts of the leaves, fruits and chromoplasts of the fruits of paprika plants grown in a crop chamber

Treatments	Leaves (mg g^{-1} fr. wt)	Chloroplasts (μg mg^{-1} chl*)	Fruits (mg g^{-1} fr. wt)	Chromoplasts (μg mg^{-1} cap†)
Reference	2.23 ± 0.45a	0.086 ± 0.008a	0.63 ± 0.10	0.071 ± 0.004
+ Ti $(IV)_R$	5.76 ± 0.85b	0.258 ± 0.045b	0.47 ± 0.08	0.081 ± 0.005
+ Ti $(IV)_L$	9.28 ± 0.53c	0.780 ± 0.089c	0.65 ± 0.07	0.069 ± 0.007
+ Ti $(IV)_{R\&L}$	10.13 ± 0.89c	0.831 ± 0.073c	0.51 ± 0.58	0.075 ± 0.005

Treatments followed by 'R' was applied via roots, by 'L' via leaves and 'R&L' via roots and leaves.
Mean ± SE within a column following by the same letter are not significantly different by LSD at $P = 0.05$ level.
*Chlorophyll.
†Capsanthene.

Table 2. Effect of different Ti (IV) ascorbate treatments on peroxidase and catalase activities of leaves and fruits of paprika plants grown in a crop chamber

	Leaves		Fruits	
Treatments	Peroxidase (UAP)	Catalase (UC mg^{-1})	Peroxidase (UAP)	Catalase (UC mg^{-1})
Reference	0.028 ± 0.004a	11.31 ± 0.45a	0.030 ± 0.004a	4.61 ± 0.36a
+ Ti $(IV)_R$	0.035 ± 0.004a	15.91 ± 0.45b	0.052 ± 0.005b	6.23 ± 0.45b
+ Ti $(IV)_L$	0.062 ± 0.005b	17.33 ± 0.60c	0.069 ± 0.007c	7.89 ± 0.46c
+ Ti $(IV)_{R\&L}$	0.060 ± 0.004b	18.56 ± 0.71c	0.071 ± 0.005c	7.02 ± 0.51bc

Treatments followed by 'R' was applied via roots, by 'L' via leaves and 'R&L' via roots and leaves.
Mean ± SE within a column following by the same letter are not significantly different by LSD at $P = 0.05$ level.

Table 3. Effect of different Ti (IV) ascorbate treatments on Fe^{2+} concentration of leaves, chloroplasts of the leaves, fruits and chromoplasts of the fruits of paprika plants grown in a crop chamber

Treatments	Leaves (mg g^{-1} fr. wt)	Chloroplasts (μg mg^{-} chl*)	Fruits (mg g^{-1} fr. wt)	Chromoplasts (μg mg^{-1} cap†)
Reference	0.50 ± 0.05a	6.73 ± 0.45a	1.11 ± 0.20a	1.54 ± 0.23a
+ Ti $(IV)_R$	0.63 ± 0.06b	7.91 ± 0.47a	2.02 ± 0.25b	2.97 ± 0.18b
+ Ti $(IV)_L$	0.79 ± 0.05c	9.06 ± 0.51bc	2.30 ± 0.34b	4.34 ± 0.34c
+ Ti $(IV)_{R\&L}$	0.95 ± 0.04d	10.18 ± 0.58c	2.26 ± 0.10b	4.62 ± 0.19c

Treatments followed by 'R' was applied via roots, by 'L' via leaves and 'R&L' via roots and leaves.
Mean ± SE within a column following by the same letter are not significantly different by LSD at $P = 0.05$ level.
*Chlorophyll.
†Capsanthene.

plant parts. These experiments and the fact that when Ti-treatments were applied, the concentration of this trace element did not change in fruits, enable us to affirm that the beneficial effect observed is due the Ti itself.

The Fe^{2+} concentrations in leaves, fruits, chloroplasts and chromoplasts are shown in Table 3. The Ti treatments enhanced the Fe^{2+} in every organ and organelle studied. The increase of this Fe fraction was greater when Ti was applied via leaves and soil together.

Some investigators suggested that the metabolically active form of Fe is Fe^{+2} [17–19], and also that this is the mobile fraction in the plants [20]. Iron is linked with a number of enzyme systems [21] and, furthermore, the change in activities of the enzymes peroxidase and catalase has been used as a measure to determine iron deficiency in plants [22]. In other work it has been demonstrated that the total content of iron in the chlorotic plants was higher than in the green plants [23, 24],