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Biochemical genetics of a 7S globulin-like protein from *Pinus pinaster* seed

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Abstract The megagametophytes of seeds of *Pinus pinaster* Ait. contain two types of oligomeric globulins of approximately 175 and 190 kDa that are comprised of 47-kDa and 27- and 22-kDa, monomers, respectively, joined by weak interactions. The 27- and 22-kDa components were purified and their N-terminal sequences determined. Both polypeptides were inherited as if they were coded by a single unit of recombination. The results obtained suggest that these two polypeptides originate from a single protein that undergoes proteolytic processing. The characteristics of this *P. pinaster* globulin indicate that it is a member of the 7S globulin family of seed storage proteins.

Key words 7S globulins · Seed storage proteins · *Pinus* · Megagametophyte proteins

Introduction

The 7S and 11S globulins are the two principal types of seed storage proteins in dicotyledonous plants. The relative amounts of both types vary with species, and in some cases only one of the types is present. The 7S globulins are trimeric proteins of 150–200 kDa consisting of monomers linked by weak interactions. Two essentially different 7S globulin classes have been described, and both types may be present in the same species. The first class is comprised of monomers of approximately 50 kDa, whereas the second class consists of 70-kDa subunits (Casey et al. 1986; Shotwell and Larkins 1989). Within each group there is usually some heterogeneity. In some 7S globulins, like, for example, in the vicilins of *Pisum sativum* and *Vicia faba*, the 50-kDa monomers may suffer proteolytic processing, thereby forming smaller polypeptides that remain associated within the native molecule (Gatehouse et al. 1983; Scholz et al. 1983; Spencer et al. 1983). Homology exists between all of the 7S globulins that have been well characterised, leading to the suggestion that their genes have evolved from a common ancestral gene that already existed at the start of angiosperm evolution (Borroto and Dure 1987; Casey et al. 1986; Gibbs et al. 1989).

Studies on the seed storage proteins of gymnosperms are still few in number. In several species, electrophoretic analyses have indicated the presence of proteins with characteristics similar to those of the angiosperm 11S and 7S globulins (Flinn et al. 1991; Gifford 1988; Hakman et al. 1990; Misra and Green 1990). This homology has been confirmed in the case of the 11S globulins by means of protein and cDNA sequence studies (Allona et al. 1992; Häger et al. 1992; Leal and Misra 1993). One 7S globulin cDNA has been also characterised from *Picea glauca* somatic embryos that corresponds to a 44-kDa mature polypeptide (Newton et al. 1992). In *Pinus pinaster* we have observed that while the major storage proteins consist of glutelins showing homology to the 11S proteins, there is also a considerable proportion of 150- to 200-kDa globulins formed by monomers of 22–23, 27 and 47 kDa that are not interlinked by disulphide bridges (Allona et al. 1992). In the present article, we report the characterisation of an oligomer formed by 22- to 23-kDa and 27-kDa components, the purification of these monomers and their inheritance, and we postulate that they are the result of a 7S globulin proteolytic process.

Materials and methods

Isolation of high-molecular-weight globulins

Mature seeds were used in all cases. The coats and the embryonic axes of *P. pinaster* seeds were removed before extraction. Megagametophytes were ground in diethyl ether (20:1 v/w; 1 h; 4 °C) to eliminate lipids. A second removal of lipids was carried out with

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acetone (20:1 v/w; 1 h; 4 °C). All traces of acetone were eliminated in vacuo, and the dried flour was stored at −20 °C. The salt-soluble proteins were extracted with 0.05 *M* Tris-HCl, pH 8.2, 0.5 *M* NaCl (10:1 v/w; 1 h; 4 °C), then dialysed against H_2O (48 h; 4 °C) and centrifuged (30,000 *g*; 30 min). The precipitate constituted the globulin fraction. This fraction was chromatographed on Ultrogel AcA 22 (2.5 × 80-cm column; flow rate of 9 ml/min) using 50 m*M* Tris-HCl, pH 8.2, as elution buffer.

Purification of high-molecular-weight globulin subunits

The globulin fraction was chromatographed on a reversed phase-high performance liquid chromatography (RP-HPLC) column (Nucleosil C-4; 8 × 250 mm; particle size, 5 µm) using a linear gradient of 28–34% acetonitrile in 0.1% trifluoroacetic acid. The peaks containing the 22- and 27-kDa polypeptides were re-chromatographed under the same conditions.

Electrophoretic methods

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Two-dimensional electrophoretic procedures, pore gradient-PAGE (▽PAGE) (non-dissociating conditions) × SDS-PAGE and isoelectrofocusing (IEF) (non-dissociating conditions) × SDS-PAGE were carried out as previously described (García-Olmedo et al. 1988). The pH gradient in the IEF gels was estimated according to Drysdale et al. (1971). All gels were stained with Coomassie Brilliant Blue G as described in Blakesley and Boezi (1977).

Preparation of the antibodies against the 22-kDa globulin component and immunoblotting were carried out as described previously (Allona et al. 1992).

Amino acid analysis and protein sequencing

Samples were hydrolysed with 5.7 *N* HCl, 0.5 m*M* phenol at 110° ± 1 °C. Cysteine was determined after performic acid oxidation. Amino acid analysis was carried out by RP-HPLC following the method of Bidlingmeyer et al. (1984). Protein sequencing was performed by standard methods in an Applied Biosystem 470 A gas phase sequenator.

Genetic analysis

Salt-soluble proteins from individual megagametophytes were extracted with 1.2 ml 0.05 *M* Tris-HCl, pH 8.2, 0.5 *M* NaCl. After centrifugation of the extraction mixtures at 9,000 *g* for 8 min, proteins were precipitated from the supernatants with trichloroacetic acid (12.5% final concentration). The precipitates were washed with 1 ml acetone, air dried and fractionated by SDS-PAGE. Heterozygous mother trees should segregate in a 1:1 ratio in haploid tissue (megagametophyte). Chi-square analysis for "goodness-of-fit" to the expected ratio was utilised for single-locus segregation tests. Linkage relationships may be postulated from the segregation data of pairs of protein loci. Linkage analysis was carried out according to Bailey (1961).

Results

Oligomeric structure of high-molecular-weight globulins

The isolation of the 150- to 200-kDa globulins present in the megagametophyte (haploid storage tissue) of *P. pinaster* seeds was carried out by molecular filtration of the saline extract on Ultrogel AcA22 under non-dissociating conditions (Allona et al. 1992). The results obtained from subjecting this protein preparation to two-dimensional electrophoresis (pore gradient PAGE, non-dissociating conditions × SDS-PAGE) indicate its oligomeric structure (Fig. 1A). The two-dimensional map shows the presence of two different oligomers, one formed by 47-kDa subunits with an apparent molecular mass of 175 kDa, and another formed by components of approximately 22 and 27 kDa, with a molecular mass close to 190 kDa. Analysis of this same preparation of globulins by IEF, non-dissociating conditions × SDS-PAGE, also revealed the presence of two oligomers and indicated that the one comprised of the 47-kDa subunits (pI = 6.5) is slightly more basic than the other (pI = 6.0) (Fig. 1B).

Genetic analysis of the 22- and 27-kDa components

The two-dimensional analysis mentioned above is for globulins obtained from a mixture of pine seeds from Coca, in Spain. The study of globulins from different

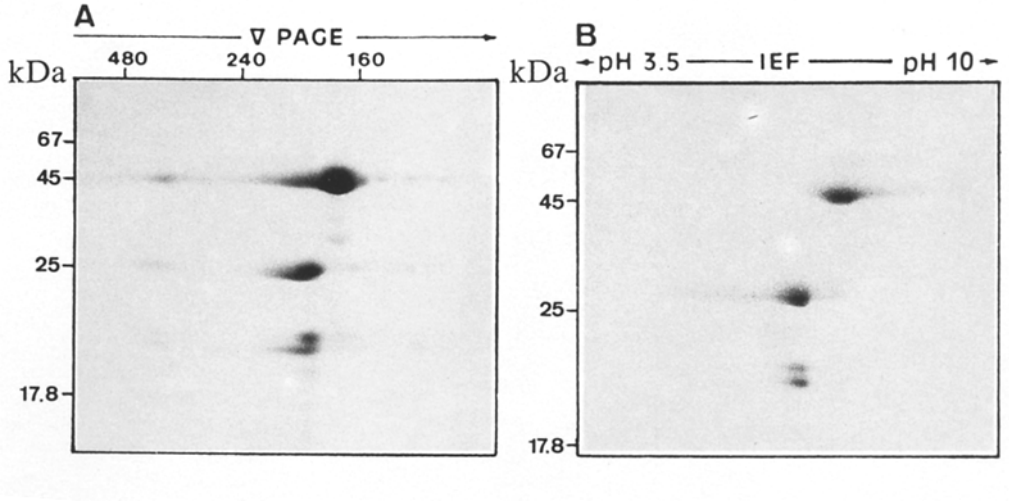

Fig. 1A, B Two-dimensional maps of *P. pinaster* high-molecular-weight globulins obtained by **A** pore-gradient PAGE, ▽PAGE × SDS-PAGE and **B** IEF × SDS-PAGE. Molecular mass markers are indicated on the *top* and *left* of figure

provenances gave similar results, and heterogeneity was seen in all of the 22- and 27-kDa electrophoretic zones. However, if the salt-soluble proteins (albumins and globulins) of individual gametophytes were analysed by SDS-PAGE only, a major polypeptide in each zone was visible that showed variants in electrophoretic mobility. In fact, three electrophoretic variants were identified for each zone, and these have been numbered 1–3 in descending order of mobility (Fig. 2).

The possibility that there was in fact only a single protein with allelic variants in each case was examined. Since megagametophytes are products of meiosis, allelic proteins from heterozygous mother trees are expected to segregate in a 1:1 ratio. Thus, by analysing the segregation patterns of megagametophytes from individual mother trees, we could determine the genetic control of proteins and linkage relationships without the need for making crosses and studying seedling progeny (see Adams 1983). Therefore, gametophytes of trees putatively heterozygous for these proteins were examined. The frequencies that were found indicate that the variants of the 22- and 27-kDa polypeptides behaved as alleles, except in the case of variants 2 and 3, which correspond to the 22-kDa band (Table 1). It must be noted that distortions in the segregation of certain allelic variants are relatively frequent phenomena in conifers (Perry and Knowles 1989; Geburek and von Wuehlisch 1989). Therefore, since the segregations in heterozygous trees for variants 1 and 2 and 1 and 3 did not deviate from Mendelian predictions, we infer that they are alleles at a locus. Megagametophytes that were heterozygous for both globulin components were analysed to test for linkage between the two loci. At this level of observation, the results indicate that both polypeptides were inherited as if they were coded for by a single unit of recombination (Table 2).

Table 1 Observed segregation of allelic variants of 22- and 27-kDa globulins from megagametophytes of heterozygous mother trees and goodness-of-fit to the 1:1 expected ratio

Protein	Electrophoretic variants				Deviation from expected to 1:1 ratio	
	1	*2*	*3*	*n*	$\chi^2(1)$	*P*
22 kDa	22	26	–	48	0.33	50–75%
22 kDa	–	14	34	48	8.33	< 0.5%
22 kDa	85	–	97	182	0.79	25–50%
27 kDa	56	40	–	96	2.66	10–25%
27 kDa	85	–	97	182	0.79	25–50%

Purification and characterisation of the 22- and 27- kDa components

Purification was achieved from globulins obtained from the seeds of tree p-4, from Coca, which is homozygous for the number 1 allele of the 22- and 27-kDa polypeptides. The globulin fraction was subjected to RP-HPLC using an H_2O-acetonitrile gradient (Fig. 3A). A control by SDS-PAGE of the peaks obtained in the elution showed that the 22- and 27-kDa components seemed homogeneous in fractions 2 and 3, respectively (Fig. 3B). However, the presence of 47-kDa polypeptides in fractions 4–9 indicated the possible heterogeneity of this subunit. The 22- and 27-kDa components were rechromatographed under the same conditions before being characterised.

Analysis of the amino acid composition of the two purified components was undertaken (Table 3). Both polypeptides showed a high proportion of glutamic acid (Glx) and had no cystine ($\frac{1}{2}$ Cys). The fundamental difference between them is that the 22-kDa component has a greater proportion of leucine (Leu) and tyrosine

Fig. 2 Different patterns obtained by SDS-PAGE of salt-soluble proteins from megagametophytes (haploids) of individual seeds. Electrophoretic variants for the 22- and 27-kDa globulin components, and their designations, are indicated on the *left* of figure

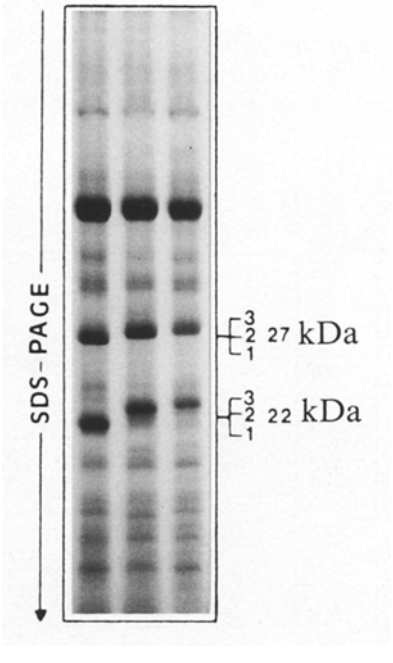

Table 2 Two-locus megagametophyte segregation data for the double heterozygous combinations of the 22- and 27-kDa globulins' loci and chi-square analysis for the detection of linkage

Combination	Observed numbers by allelic combinations					Deviation from expected to independent segregation	
22 kDa-27 kDa	*1-1*	*1-3*	*3-1*	*3-3*	*n*	$\chi^2(1)$	*P*
	85	0	0	97	182	182	< 0.5%
22 kDa-27 kDa	*1-1*	*1-2*	*2-1*	*2-2*	*n*		
	22	0	0	26	48	48	< 0.5%
22 kDa-27 kDa	*2-1*	*2-2*	*3-1*	*3-3*	*n*		
	0	14	34	0	48	48	< 0.5%

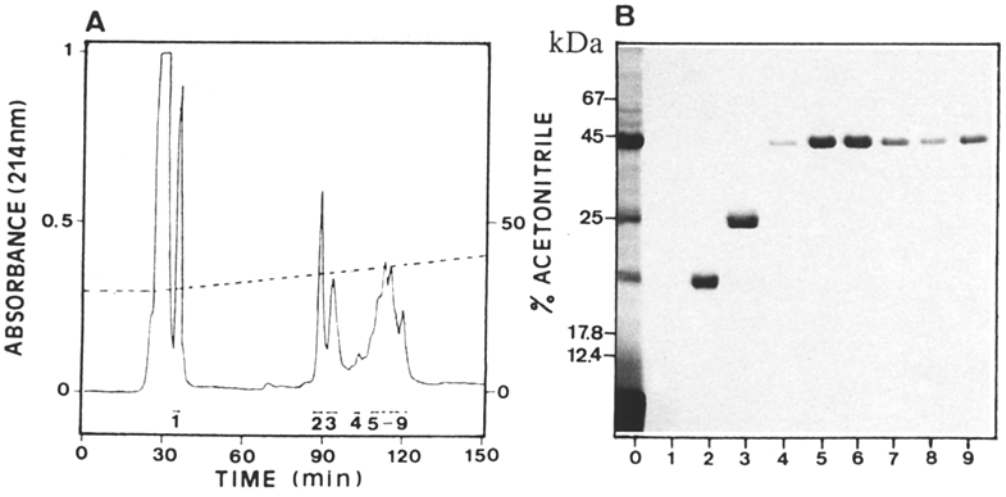

Fig. 3A, B **A** RP-HPLC fractionation of the total globulins obtained from the p-4 tree of *P. pinaster*. **B** SDS-PAGE of the following samples: *0* total globulins, *1–9* RP-HPLC peaks 1–9, respectively. *Numbers* on the *left* of figure refer to the molecular mass of the marker polypeptides

Table 3 Amino acid compositions and molecular weights of the purified 22- and 27-kDa globulin components and peak 6 of Fig. 3A containing polypeptides of the 47-kDa globulin

Amino acid[a]	moles/100 moles of amino acid			Residues/mol[b]	
	22 kDa	27 kDa	47 kDa	22 kDa	27 kDa
Lys	5.7	7.6	2.5	11	18
His	4.5	3.0	4.7	8	7
Arg	7.5	12.0	10.6	14	28
Asx	7.2	7.6	8.7	13	18
Thr	3.4	2.4	5.3	6	6
Ser	5.5	7.1	5.0	10	17
Glx	19.0	22.9	14.7	35	54
Pro	3.5	3.3	5.0	7	8
Gly	8.2	7.4	9.8	15	17
Ala	6.3	5.6	6.4	12	13
Val	3.9	4.2	6.5	7	10
1/2 Cys	0.2	0.2	0.7	0	0
Met	1.4	2.9	1.9	3	7
Ile	5.4	4.3	4.5	10	10
Leu	9.6	4.7	6.5	18	11
Tyr	4.2	1.4	3.3	8	3
Phe	4.5	3.4	3.9	8	8
Total residues/molecular weight				185	235
MW(1)[c]				22,000	27,000
MW(2)[c]				21,290	27,365

[a] Trp was not analysed
[b] Number of residues adjusted by the method of Delaage (1968) to the molecular weight determined by SDS-PAGE
[c] MW: (1) determined by SDS-PAGE, (2) calculated from the adjusted amino acid composition

(Tyr) and less arginine (Arg) and methionine (Met) than the 27-kDa component. The amino acid composition of one of the major fractions containing 47-kDa polypeptides (peak 6 in Fig. 3A) that was obtained in the fractionation of globulins by RP-HPLC is also shown in Table 3.

The N-terminal sequences of the 22- and 27-kDa polypeptides were determined. The latter showed homology to internal sequences of subunits of 7S globulins from *Picea glauca* and several angiosperms (Fig. 4). The sequence of the 22-kDa component (GSEEEEEE) was found to be characterised by the occurrence of eight glutamic residues together in positions 3–10.

Antibodies raised against the purified 22-kDa polypeptide were used in a Western blot analysis of the 27- and 47-kDa components. No significant cross reactivity was observed (results not shown).

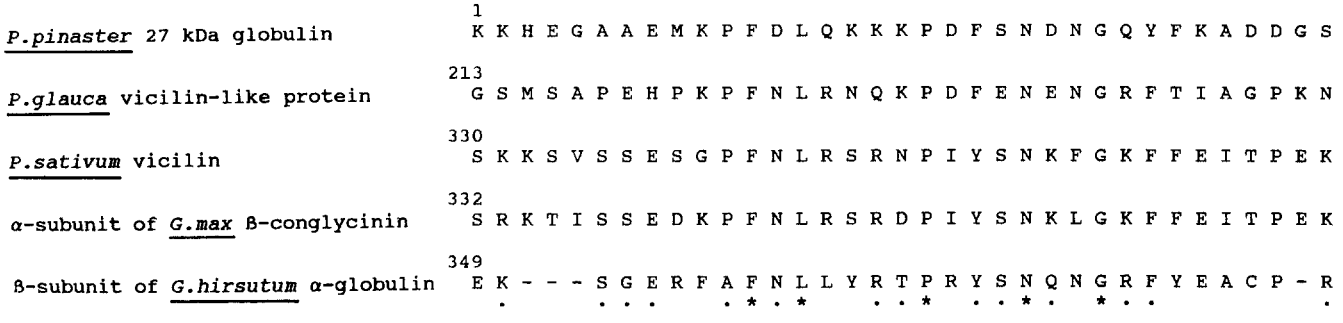

Fig. 4 Alignment of the N-terminal sequence of the 27-kDa component from *P. pinaster* globulins with sequences reported for the following 7S globulins: *P. glauca* vicilin-like protein (Newton et al. 1992), *P. sativum* vicilin (Lycett et al. 1983), α-subunit of *G. max* β-conglycinin (Sebastiani et al. 1990) and β-subunit of *G. hirsutum* α-globulin (Chlan et al. 1986). The *numbers* on the *left of sequences* indicate the position occupied in the mature protein by the amino acid where the sequence begins. The sequences are aligned to maximise homology resulting in the introduction of some gaps (Higgins and Sharp 1988). Identical residues are indicated by an *asterisk below the sequence*, and conserved residues by a *dot*

Discussion

Two oligomeric proteins with molecular masses of 175 and 190 kDa have been identified in the high-molecular-weight globulins of *P. pinaster*. These two proteins are comprised of 47-kDa and 27- and 22-kDa monomers, respectively, interlinked by weak interactions. If one assumes that the 22- and 27-kDa components could have arisen from the proteolytic cleavage of a single protein, which is what happens in pea and bean vicilins (Gatehouse et al. 1983; Scholz et al. 1983; Spencer et al. 1983), the characteristics mentioned above are compatible with those of a trimeric structure like the one typical of angiosperm 7S globulins (Plietz et al. 1983). However, unlike in those species, no subunits should be found in the native molecules that had not suffered proteolysis. Our assumption is supported by the following points. (1) The 22- and 27-kDa polypeptides were inherited as if they were coded by a single unit of recombination, which could indicate the presence of just one gene that carries information for a protein that is subsequently processed. (2) There was homology between the N-terminal sequence of the 27-kDa component and internal sequences of *Picea glauca* and angiosperm 7S globulins (Fig. 4). This could mean that the complete subunit was first formed by the 22-kDa component, then by the 27-kDa component. (3) The N-terminal sequence of the 22-kDa component having a cluster of Glu residues could constitute the hydrophilic zone contiguous to the processing site of the signal peptide. In some 7S globulins this zone is also rich in Glu (Newton et al. 1992; Slightom et al. 1983). (4) The amino acid composition of a protein formed by the 22- and 27-kDa polypeptides joined together should be similar to the one described for 7S globulins (Derbyshire et al. 1976).

Comparison of the N-terminal sequence of the 27-kDa component with internal sequences of several 7S globulins showed that the percentage of amino acid identities in this region varied from 28% for angiosperm proteins to 34% for *P. glauca*. The sequence similarity level increased if adjustments for conservative substitutions of amino acids are considered (Fig. 4). Even if the fact that the degrees of amino acid identity of the 7S globulins follow taxonomic grouping only between the most closely related taxa is taken into account (Newton et al. 1992), the low degree of identity between the *P. glauca* vicilin-like protein and the *P. pinaster* 27-kDa component is still surprising since both species belong to the Pinaceae family. In this context the non-existence of serological cross-reactivity between the 22- and the 47-kDa components of *P. pinaster* is also noticeable. These results suggest that in gymnosperms, as it has been demonstrated in angiosperms, there are different types of 7S globulins. We have confirmed the presence of proteins structurally homologous to that formed by 27- and 22-kDa components by showing cross-reactivity with antibodies raised against the purified 22-kDa component in different species of Pinaceae, such as *Cedrus atlantica*, *Abies alba* and *Picea abies* (unpublished results).

The inheritance analysis of the 27- and 22-kDa components and the biochemical results indicate the presence of one unique functional coding sequence for these polypeptides in the *P. pinaster* genome. However, the possibility of the presence of genes codifying homologous proteins that are expressed at lower levels cannot be excluded. This situation contrasts with the existence of a gene family encoding genetic variants of *P. glauca* 44-kDa vicilin-like protein (Newton et al. 1992) and with the heterogeneity found by us for the *P. pinaster* 47-kDa component. The number of genes encoding the different types of angiosperm 7S globulins is variable; for example, while pea convicilin is encoded by one gene (Domoney and Casey 1985), the β-subunit gene family of soybean β-conglycinin consists of 8–13 members (Tierney et al. 1987).

Our results confirm the presence of 7S globulins in gymnosperm species that were demonstrated by Newton et al. (1992) and suggest the existence, in this taxon, of different types inside this globulin class.

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