Homology between legumin-like polypeptides from cereals and pea

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(Received 2 November 1984/Accepted 20 November 1984)

The presence of legumin-like constituents within the globulin fractions of wheat (*Triticum aestivum*), rye (*Secale cereale*) and corn (maize, *Zea mays*) was demonstrated. Two-dimensional analysis of wheat globulins in the presence and absence of a reducing agent revealed the existence of reducible ~60 kDa polypeptides. Western-blot analysis with ¹²⁵I-labelled antibodies raised against the oat (*Avena sativa*) 12S globulin holoprotein or its α -subunits demonstrated, firstly, the immunological homology between the α - and β -subunits of pea (*Pisum sativum*) legumin and oat 12S globulin, and secondly, the similar occurrence in wheat of antigenically homologous ~20 kDa and ~40 kDa polypeptides that associate via disulphide linkage to form ~60 kDa dimers. Western blotting also showed the presence of disulphide-linked ~20 kDa and ~40 kDa legumin-like subunits within the globulin fractions of rye and corn.

Pea (*Pisum sativum*) legumin characteristically displays a sedimentation coefficient ($s_{20,w}$) of approx. 12S and is composed of subunit pairs (~60 kDa) of disulphide-bridged acidic (~40 kDa) and basic (~20 kDa) subunits (Croy *et al.*, 1979). The individual legumin subunits are believed to arise from the post-translational cleavage of precursor polypeptides of approx. 60 kDa (Croy *et al.*, 1980).

Although legumin-like proteins are common to many dicotyledonous seeds, reports of their occurrence among cereals are limited (Derbyshire *et al.*, 1976). However, recent characterization of oat (*Avena sativa*) globulins revealed that the major component exhibited numerous similarities to legumin. It was shown to have an $s_{20,w}$ of approx. 12S (Peterson, 1978) and to consist of disulphide-linked pairs of acidic (~40 kDa) and basic (~20 kDa) subunits (Brinegar & Peterson, 1982a; Robert *et al.*, 1983a; Walburg & Larkins, 1983; Burgess *et al.*, 1983). The oat globulin subunits were also found to arise from precursor polypeptides (~60 kDa) that were post-translationally cleaved (Matlaszewski *et al.*, 1982; Brinegar &

Peterson, 1982b; Walburg & Larkins, 1983; Adeli & Altosaar, 1983, 1984a). The high level of similarity existing in the N-terminal amino acid sequences of their basic subunits further demonstrated the homology between oat 12S globulin and legumin from *Pisum sativum* and *Vicia faba* (broad bean), along with glycinin from *Glycine max* (soya bean) (Walburg & Larkins, 1983).

Together, these results raise the possibility of legumin-like globulins occurring in other cereals. However, few data are available to favour the presence of legumin-like globulins among cereals other than oat. Nonetheless, a 11–12S globulin component has been identified in barley (*Hordeum vulgare*) (Danielsson, 1949) and in wheat (*Triticum aestivum*) (Pence & Elder, 1953). These may involve proteins similar to legumin. Globulins resembling those of legumes have been reported to occur in corn (maize, *Zea mays*) (Khavkin *et al.*, 1977). In addition, the glutelin fraction of rice was recently shown to possess polypeptides homologous with pea legumin (Zhao *et al.*, 1983).

The present study dealt with the characterization of wheat globulins to investigate the presence of legumin-like constituents and their possible homology with those observed in oat and pea.

Experimental

Dry mature seeds of rye (*Secale cereale* L. cv. Puma), wheat (*Triticum aestivum* L. cv. Fredrick),

Abbreviations used: i.e.f., isoelectric focusing; SDS, sodium dodecyl sulphate; phosphate-buffered saline, 0.14 M-NaCl/2.7 mM-KCl/8.1 mM-Na₂HPO₄·7H₂O/1.5 mM-KH₂PO₄, pH 7.4; NRS, normal rabbit serum.

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rice (*Oryza sativa* L. cv. M101), corn (maize, *Zea mays* L. cv. F64-11-6-1) and oat (*Avena sativa* L. cv. Hinoat) were kindly provided by Dr. V. Burrows, Ottawa Research Station, Central Experimental Farm, Department of Agriculture; Ottawa, Ont., Canada. Dry pea seeds (*Pisum sativum* L. cv. Little Marvel) were generously supplied by Dr. K. Joy, Department of Biology, University of Carleton, Ottawa, Ont., Canada.

Peas and dehulled cereal seeds were ground to a flour in an electric coffee grinder. Globulins were extracted according to a modification of the method used for oat by Peterson (1978). Individual flour samples (3g) were stirred magnetically for 2 h at room temperature in 100 ml of 1 M-NaCl/0.05 M-Tris, pH 8.5. The slurry was centrifuged (15000g, 30 min, 24°C) and the supernatant dialysed for 48 h against several changes of distilled water at 4°C to precipitate the globulins. The dialysis residue was centrifuged (22000g, 45 min, 4°C) and the pellet (globulins) freeze-dried.

Oat, wheat and pea globulins were analysed by two-dimensional analysis as described previously (Robert *et al.*, 1983b) in the presence or absence of 2-mercaptoethanol. Globulins were resolved in the first dimension by i.e.f. in a pH 3.5–10 gradient (Ampholines; LKB), followed by SDS/polyacrylamide-gel electrophoresis in a 14% (w/v) polyacrylamide gel for the second dimension. Molecular-mass markers used were: phosphorylase *b* (94 kDa); bovine serum albumin (67 kDa); ovalbumin (43 kDa); carbonic anhydrase (30 kDa); soya-bean trypsin inhibitor (20.1 kDa); and α -lactalbumin (14.4 kDa).

Oat 12S holoproteins were purified by pI precipitation and three cycles of sucrose-gradient centrifugation (Burgess *et al.*, 1983; Adeli & Altosaar, 1984b). The acidic (α) subunits of oat 12S globulin were obtained by preparative SDS/polyacrylamide-gel electrophoresis (Matlashewski, 1983). Antibodies were raised separately against the 12S holoprotein and its α -subunits in New Zealand White rabbits and generously provided by Dr. K. Adeli and Dr. P. R. Shewry respectively. The immune serum was passed through a protein A-Sepharose column. The IgG fraction was eluted with 1.0 M-acetic acid in 0.15 M-NaCl/20 mM-Na₂HPO₄/NaH₂PO₄, pH 3.0. The IgG solution was then neutralized to pH 7.4 (Matlashewski *et al.*, 1982).

These two separate IgG fractions were labelled with ¹²⁵I (low pH, high concentration; New England Nuclear Corp.) by a modification of the method of Greenwood *et al.* (1963). A 1 mCi portion of Na¹²⁵I was added to 50 μ g of IgG in phosphate-buffered saline. A 10 μ l portion of 8.1 mM-chloramine-T in phosphate-buffered saline was added and the solution incubated for 10 min on

ice. After the addition of 10 μ l of 52 mM-sodium metabisulphite in phosphate-buffered saline, the solution was applied to a 10 ml Sephadex G-25 column. The fractions from the first peak (containing the labelled IgG) were pooled, dialysed against phosphate-buffered saline, and diluted with 30 ml of 5.0% (v/v) bovine serum albumin/3.0% (v/v) NRS in phosphate-buffered saline. The specific radioactivities were approx. 3 μ Ci/ μ g of IgG.

After SDS/polyacrylamide-gel electrophoresis in 12% gels, proteins were immediately transferred electrophoretically on to nitrocellulose sheets (0.45 μ m pore size; BA85; Schleicher and Schuell) for 24 h at 135 mA, as outlined by Towbin *et al.* (1979). The sheets were then incubated for 1 h at 40°C in 50 ml of 3.0% bovine serum albumin and 3.0% NRS in phosphate-buffered saline. After washing in phosphate-buffered saline (2 $\times$ 15 min), the sheets were incubated for 6 h with either the labelled oat 12S globulin holoprotein antibody or the oat globulin α -subunit antibody solutions. They were then washed in phosphate-buffered saline (6 $\times$ 10 min), dried and exposed at -70°C to Kodak X-Omat AR film in the presence of an intensifying screen.

Results and discussion

There are common features that generally distinguish components of 12S legumin-like globulins. Their subunits have molecular masses of approx. 40 kDa (α) and 20 kDa (β), have respectively acidic and basic pI values, and are disulphide-bridged to form dimers of approx. 60 kDa (Derbyshire *et al.*, 1976). All of these characteristics can usually be detected by two-dimensional analysis of these globulins in the presence and absence of a reducing agent. Typically, under non-reducing conditions, only the ~60 kDa dimers should be evident, whereas reducing conditions should dissociate the dimers and reveal their constituent subunits. The amounts of legumin-like proteins within the total globulin fraction will determine the facility of their detection.

When comparing the two-dimensional electrophoretic patterns obtained for the total globulin fractions from oat, pea and wheat, significant differences between them became apparent (Fig. 1). As previously reported, the oat globulins were found to consist primarily of legumin-like proteins (Brinegar & Peterson, 1982a; Robert *et al.*, 1983a; Walburg & Larkins, 1983; Burgess *et al.*, 1983). The dimers (~60 kDa) predominated in the absence of a reducing agent (Fig. 1, O⁻) and dissociated to their constituent α - and β -subunits in the presence of a reducing agent (Fig. 1, O⁺).

Although the pea globulin fraction contains a significant amount of vicilin (Derbyshire *et al.*,

Fig. 1. Two-dimensional analysis (i.e.f., pH 3.5–10 followed by SDS/polyacrylamide-gel electrophoresis) of the reduced oat (O^+), pea (P^+), wheat (W^+) and unreduced oat (O^-), pea (P^-) and wheat (W^-) total globulin fractions

1976), legumin was detected as both the unreduced dimers (Fig. 1, P^- , arrows) and the reduced α - and β -subunits (Fig. 1, P^+ , arrows). With the wheat globulins, a minor group of proteins was also found in the 60 kDa region under non-reducing conditions (Fig. 1, W^- , arrows), which was absent under reducing conditions (Fig. 1, W^+). Like the

~60 kDa polypeptides of oat and pea, these might have dissociated to subunits. However, the dissociation products of the wheat ~60 kDa group of polypeptides were not immediately evident. The subunits forming these reducible ~60 kDa polypeptides might be co-migrating with other wheat globulins.

When the SDS/polyacrylamide-gel-electrophoretic patterns of oat, pea and wheat globulins were challenged with the labelled oat globulin α -subunit antibody, various polypeptides from all three plants were selectively recognized (Fig. 2). As expected, the antibody hybridized strongly with the ~ 40 kDa oat globulin α -subunits under reducing conditions (Fig. 2, lane o^+). The antibody also cross-reacted with polypeptides of the pea α -subunit (Fig. 2, p^+). Hence the α -subunits from these two distantly related plants possess homologous antigenic determinants. The wheat globulin fraction also contained polypeptides of ~ 40 kDa that were selectively recognized by the oat globulin α -subunit antibody, and these cross-reacted strongly (Fig. 2, w^+). These polypeptides are therefore homologous with the oat and pea α -subunits.

Since the α -subunits of oat and pea are components of ~ 60 kDa dimers, the absence of a reducing agent should cause a shift of the radioactive signal from the ~ 40 kDa α -subunits to the disulphide-linked dimers. A similar phenomenon should be observed with the wheat globulins if the cross-reacting ~ 40 kDa polypeptides are involved in the formation of the ~ 60 kDa reducible polypeptides observed in Fig. 1 (W^-). Indeed, a relative increase in the radioactive signal found in the 60 kDa region, accompanied by a decrease in the ~ 40 kDa region, was observed, not only within the oat and pea globulin patterns, but with that of wheat also (Fig. 2, o^- , p^- , w^-). The persisting radioactivity in the ~ 40 kDa region under non-reducing conditions, probably resulted from disulphide bonds broken during sample boiling before electrophoresis (Robert *et al.*, 1983a). Hence, like those of oat

and pea, the wheat ~ 40 kDa polypeptides recognized by the oat globulin α -subunit antibody are part of reducible ~ 60 kDa polypeptides. The antibody also hybridized to polypeptides found between 50 and 70 kDa, as well as polypeptides smaller than ~ 40 kDa in the oat, pea and wheat globulin fractions (Fig. 2). The positions of these bands were apparently not affected by the presence or absence of a reducing agent.

Western-blot analysis with the antibody raised against the oat 12S globulin holoprotein showed that, along with their α -subunits, the β -subunits of both oat and pea were detected by the antibody under reducing conditions (Fig. 3, o^+ and p^+ respectively). This immunological cross-reactivity of the oat and pea β -subunits is in agreement with the homology implied from the significant resemblance of their *N*-terminal amino acid sequences (Walburg & Larkins, 1983). Similarly, along with the previously observed ~ 40 kDa subunits, wheat polypeptides of ~ 20 kDa were also recognized by the oat 12S globulin antibody under reducing conditions (Fig. 3, w^+). These ~ 20 kDa wheat polypeptides may be the necessary counterparts that associate with the ~ 40 kDa subunits to form the reducible ~ 60 kDa polypeptides observed in Figs. 1 (W^-) and 2 (w^-).

The cross-reaction patterns of the oat and wheat globulins observed under non-reducing conditions displayed a relatively stronger radioactive signal in the ~ 60 kDa region and much weaker signals in the ~ 20 kDa and ~ 40 kDa regions (Fig. 3, o^- , w^-) in comparison with the patterns obtained under reducing conditions (Fig. 3, o^+ , w^+). This indicates that the wheat globulin fraction includes legumin-

Fig. 2. Western blot analysis: 125 I-labelled antibodies raised against the purified oat globulin α -subunits hybridized to the SDS/polyacrylamide-gel-electrophoretic patterns of the reduced oat (o^+), pea (p^+), wheat (w^+) and unreduced oat (o^-), pea (p^-) and wheat (w^-) total globulin fractions

Lane (c) is a control lane with no protein.

Fig. 3. Western blot analysis: 125 I-labelled antibodies raised against the purified oat 12S globulin holoprotein hybridized to the SDS/polyacrylamide-gel-electrophoretic patterns of the reduced oat (o^+), pea (p^+), wheat (w^+), rye (r^+), corn (c^+) and unreduced oat (o^-), wheat (w^-), rye (r^-) and corn (c^-) total globulin fractions

Lane (c) is a control lane with no protein.

like subunits of ~40kDa and ~20kDa that are associated via disulphide linkage(s) to yield dimers of ~60kDa. These wheat subunits are antigenically homologous with the α - and β -subunits forming the oat and pea 12S globulins. However, when the wheat globulins (the total extract or only the reducible ~60kDa polypeptides) were analysed by two-dimensional analysis involving non-reducing conditions in the first dimension and reducing conditions in the second dimension, the only dissociation products detected were the ~40kDa polypeptides (results not shown). The reasons for this inconsistency are not known.

Repeated Western-blot analysis of the pea globulins under non-reducing conditions (using the 12S globulin holoprotein antibody) consistently yielded a very faint radioactive signal (results not shown). In comparison with the oat α -subunit antibody, the 12S holoprotein antibody always hybridized weakly to the ~60kDa dimers of all the plants studied. Since background staining was decreased to a minimum in these experiments, the variations in the intensity of the radioactive signals are believed to arise from differences in the availability of the antigenic sites between the three forms of the protein (i.e. subunits, dimer, holoprotein) and between the different plants studied.

It is interesting to note the variation in the relative proportions of the legumin-like polypeptides within the oat, pea, and wheat globulin fractions (see Fig. 1). Oat globulins are essentially all legumin-like, whereas the reverse holds true for those of wheat. Pea is intermediate in this respect. Thus the genes coding for these proteins seem to be differentially regulated within these three plants.

Some of the polypeptides (other than the ~40kDa subunits) that had cross-reacted with the oat globulin α -subunit antibody (Fig. 2) were also recognized by 12S holoprotein antibody (Fig. 3). This was particularly the case with the polypeptides in the 50–70kDa region. These polypeptides might represent a group of uncleaved globulin molecules. In oat and pea, precursors of the individual α - and β -subunits fall within this molecular-mass range (Matlashewski *et al.*, 1982; Brinegar & Peterson, 1982b; Walburg & Larkins, 1983; Adeli & Altosaar, 1983, 1984a; Croy *et al.*, 1980). Further study is required to establish the relationship existing between the typical legumin-like constituents and these other antigenically related polypeptides.

A Western-blot analysis was also performed on the globulin fractions from rye and corn by using the oat 12S holoprotein antibody. Both cereals, like wheat, possessed immunologically homologous legumin-like ~20kDa and ~40kDa subunits (Fig. 3, r^+ , c^+), which associated to form reducible ~60kDa polypeptides (Fig. 3, r^- , c^-).

The high-molecular-mass band (>100kDa) observed in corn under non-reducing conditions (Fig. 3, c^-) may result from the pairing of dimers as observed in oat globulins (Robert *et al.*, 1983a) and rice glutelin (L. S. Robert, C. Nozzolillo & I. Altosaar, unpublished work). The cross-reacting corn polypeptides may correspond to the legume-like globulins previously observed in this species (Khavkin *et al.*, 1977).

The proportions of the legumin-like proteins within the separate globulin fractions of wheat, rye and corn are much decreased compared with those of oat and pea. In fact, most of the globulin polypeptides from wheat, barley, rye, corn and rice were shown to be homologous with vicilin-like proteins found in legumes (L. S. Robert, K. Adeli & I. Altosaar, unpublished work). This may explain why only a minor 12S globulin component was found in wheat (Pence & Elder, 1953) and no such component was detected in rye and corn (Danielsson, 1949).

In summary, the globulin fractions from pea, wheat, rye and corn were all shown to possess polypeptides antigenically homologous with the legumin-like proteins present in oat. Similarly to the α - and β -subunits of pea legumin and oat 12S globulin, the wheat, rye and corn ~40kDa and ~20kDa subunits were found to associate via disulphide linkage to form ~60kDa dimers. It would thus appear that legumin-like genes are present and expressed in these cereals. Important variations in the relative amounts of the legumin-like proteins observed in oat, pea and wheat point to significant differences in the regulation of these genes.

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (A6711), the Medical Research Council (MA-8715), the Quaker Oats Company of Canada and Agriculture Canada (OSU-81-00411). We thank Dr. B. J. Mifflin and Dr. P. R. Shewry (Rothamsted Experimental Station, Harpenden, Herts., U.K.) for kindly providing the oat globulin α -subunit antibody and we also thank Dr. K. Adeli of the Department of Biochemistry of this University for kindly supplying the oat 12S globulin antibody. We also thank Dr. V. Burrows, Ottawa Research Station, and Dr. J. Erratt, Bio Logicals Inc., for their useful help and advice.

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