In vitro synthesis of oat globulin

G.J. Matlashewski*, K. Adeli*, I. Altosaar*, P.R. Shewry+ and B.J. Miflin+

*Department of Biochemistry, School of Medicine, University of Ottawa, Ottawa, Canada and +Biochemistry Department, Rothamsted Experimental Station, Harpenden, Herts. AL5 2JQ, England

Received 30 June 1982

Avena sativa *Oat* *Seed* *Globulin* *In vitro translation*

1. INTRODUCTION

The majority of the seed storage proteins of oats (*Avena sativa* L.), unlike those of most other cereals, are globulins [1,2]. The major globulin component has a sedimentation coefficient ($s_{20,w}$) of 12.1 and a molecular weight (M_r) of 320 000 and is thought to consist of six subunits of M_r 21 700 and six of M_r 31 700 [3]. In many regards the major oat globulin resembles legumin, one of the two major seed storage proteins of legumes. Legumins from different species have $s_{20,w}$-values of 11 to 14, M_r-values of around 350 000 and consist of 6α and 6β subunits with M_r-values of 22 000 and 37 000 respectively [4]. The amino acid composition of oat globulin [3] falls within the range of values reported for legumins [4]. The α and β subunits of legumin exist as dimers stabilized by disulphide bonds [4] and are synthesized in vitro and in vivo as a single 60 000 M_r precursor which undergoes post-translational processing in vivo to produce the individual subunit polypeptide chains [5–7]. There is no evidence that the two types of oat globulin subunits associate as a disulphide stabilized dimer and it has been reported that the individual subunits are synthesized in vitro [8]. This paper reports that the oat globulin subunits are associated as dimers, of M_r about 58 000, which can be dissociated into subunits upon reduction. We also show that polysomes isolated from developing endosperms direct the synthesis of a protein of M_r about 58 000–60 000 which is specifically precipitated by oat globulin antiserum.

2. MATERIALS AND METHODS

Oats (*Avena sativa* L.) was harvested 2–3 weeks after anthesis and endosperms collected by squeezing them into liquid nitrogen. The tissue was stored either in liquid N_2 or at $-80°C$. Mature seed was harvested 6 to 8 weeks after anthesis and stored at $-20°C$.

Oat globulin was isolated from mature seed by a procedure essentially similar to that published previously [3,8]. Albumin, prolamin and glutelin fractions were isolated according to [9].

Membrane-bound polysomes were isolated as described previously for barley endosperms [10]. The A_{260}/A_{280} ratio of these polysomes was 1.7 indicating a high degree of purification.

In vitro protein synthesis was carried out using a wheat germ S30 cell free extract prepared according to the method of Roberts and Paterson [11] omitting the preincubation step. Translations were carried out in 20 μl volume containing 2.5 μl of S30 extract, 1 mM GTP, 8 mM creatine phosphate, 300 mM spermidine, 50 mM GTP, 2 mM DTT, 50 mg/ml creatine phosphokinase, 50 mM of each unlabelled amino acid, 4 μCi [^{3}H]leucine or 2 μCi [^{35}S]methionine, 5–25 μg polysomes. After incubation at 28°C for 60 min 2 μl samples were removed and spotted onto strips of Whatman No. 1 filter paper. These were placed in cold 10% CCl_3COOH, boiled in 5% CCl_3COOH followed by washing in cold 5% CCl_3COOH. They were then washed in ethanol and diethylether, placed in non-aqueous scintillation fluid and their radioactivity determined by scintillation spectrometry.

Published by Elsevier Biomedical Press

Radioactive polypeptides synthesized in vitro and authentic protein were analyzed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS–PAGE) [12]. Separating gels contained either 18% or 15% acrylamide/bisacrylamide, 60:1, and a 5% stacking gel. Radioactive bands were detected by fluorography [13] using Fuji RX X-ray film.

Globulin (0.5 mg) in 0.5 ml Tris/HCl (pH 7.5), 1 M NaCl was emulsified in an equal volume of complete Freund's Adjuvent and injected intramuscularly in New Zealand White rabbits at 7-day intervals for 4 weeks. Antisera containing anti-globulin IgG were chromatographed on protein A–Sepharose. IgG was eluted in 1.0 M acetic acid in PBS (0.15 M NaCl, 20 mM Na_2HPO_4). The eluted IgG was quickly neutralized and rechromatographed on a globulin–Sepharose column [14]. Anti-globulin IgG was eluted with 0.2 M glycine (pH 2.8) and neutralized with 0.2 M Na_2HPO_4. Fractions were stored at −20°C. Immunoprecipitation of in vitro synthesized products was done essentially as described by Roberts and Lord [15].

3. RESULTS

When oat globulin was separated by SDS–PAGE the major component was a broad band with an apparent M_r of about 58 000 (fig.1c). The same sample after reduction gave two major sets of bands with M_r-values around 22 000 and 36 000 (fig.1b). Each of these bands consists of a number of polypeptides of slightly different mobilities and they can be resolved further by isoelectric focusing which shows that the larger subunits have pI-values of 5–7 and the smaller pI-values of 8–9 (Burgess, Shewry, Matlashewski, Altosaar and Miflin, in preparation). These results suggest that the subunits exist as dimers stabilized by disulphide bonds in a manner directly analogous to that of legumin subunits [4].

Antisera against the major oat globulin were raised in rabbits and the IgG fraction purified by affinity chromatography (table 1). The amounts of anti-globulin IgG were estimated from the maximal serial dilution giving a detectable precipitation line in a double-diffusion test [16]. The purified anti-globulin IgG was tested for cross-reactivity against oat prolamin, albumin and glutelin (fig.2)

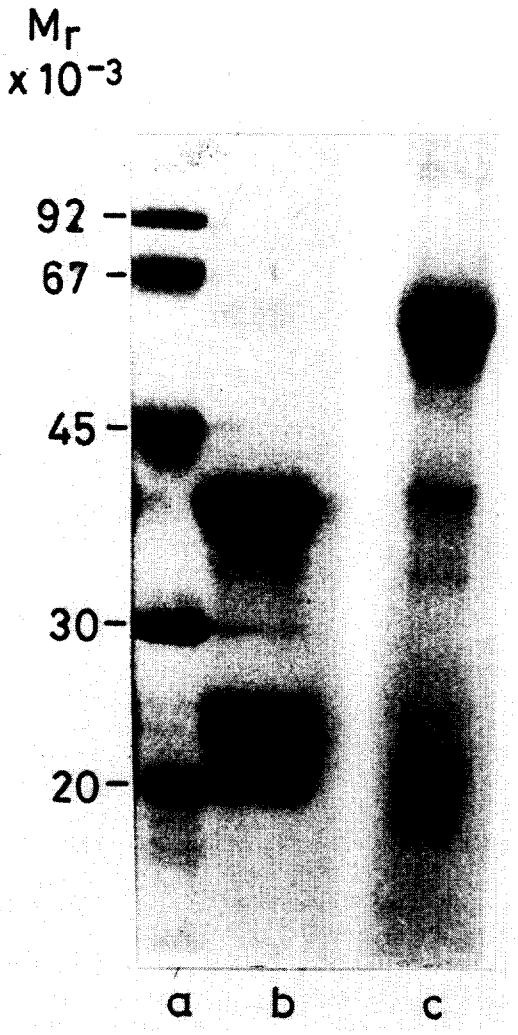

Fig.1. SDS–PAGE of reduced and unreduced globulin. (a) Molecular weight standards. (b) Globulin in the presence of 1% SDS, 0.5% 2-mercaptoethanol and 4 M urea. (c) Globulin in the presence of 1% SDS and 4 M urea.

but it only showed a specific reaction with the globulin fraction.

To see if the globulin subunits were synthesized as a precursor, polysomes were isolated from the membrane fraction of developing oat endosperms. These were fractionated by sucrose gradient centrifugation and the presence of 6-mers was demonstrated (not shown). These polysomes were able to direct the incorporation of [^{3}H]leucine into

Table 1
Purification of anti-globulin IgG

	Total protein (mg)	Specific activity[a] (Serial dilution/mg)	Purification
Serum (4 ml)[b]	500	0.128	–
IgG eluted from protein A–Sepharose with 1 M acetic acid (5 ml)	40	0.80	6.25
IgG eluted from globulin–Sepharose with 0.2 M glycine, pH 2.8 (5 ml)	1.25	25.6	200

[a] Specific activity was determined by dividing the maximal serial dilution factor, where a positive double immunodiffusion test could be obtained, by the total protein recovered

[b] Serum was obtained by letting the blood clot at 37°C for 15 min followed by centrifugation at 6000 × *g* for 15 min

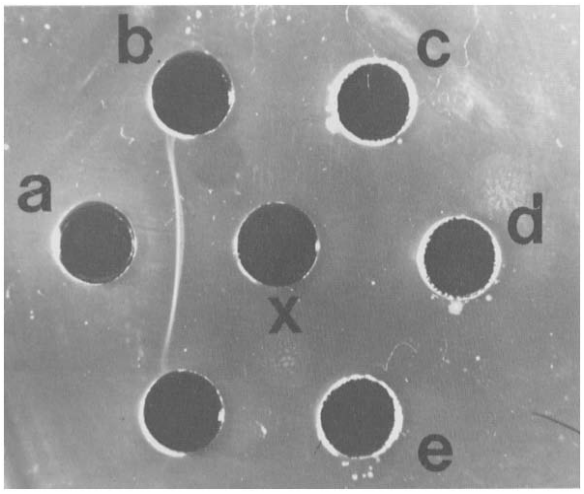

Fig.2. Immunodiffusion of anti-globulin IgG against oat seed protein fractions. Wells a, b, c, d contain respectively 50 μg of globulin, albumin, prolamin and glutelin in 10 μl Tris/HCl (pH 7.5), 1.0 M NaCl. Well X contains 0.5 μg of anti-globulin IgG in 10 μl phosphate buffered saline (pH 7.5).

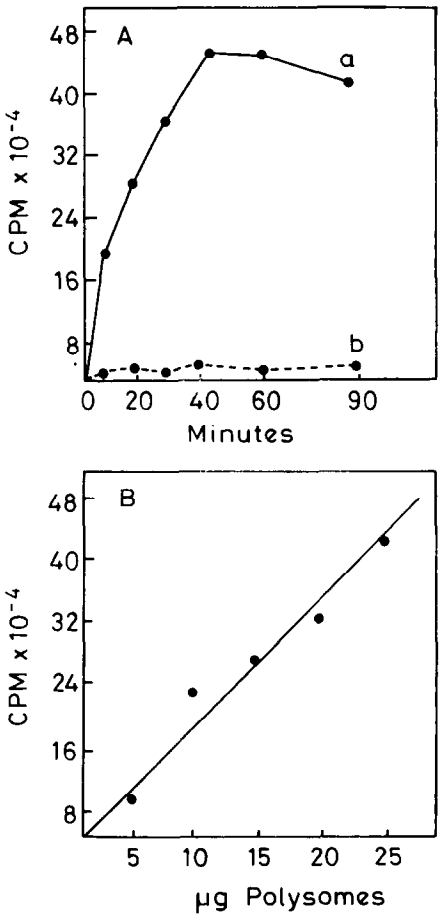

Fig.3. Conditions for translation in the wheat germ system: (A) shows a time course with (a) 25 μg of polysomes added to the translation mixture, (b) with no polysomes added; (B) shows the effect of the amount of polysomes on translation.

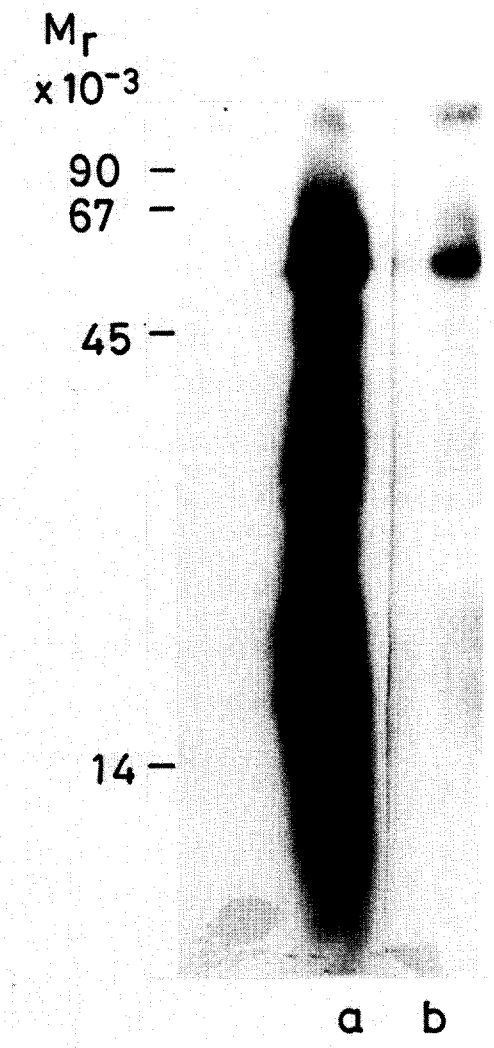

Fig.4. Fluorograph of SDS–PAGE polypeptides synthesized in vitro. Polysomes isolated from developing oat endosperms were translated in the presence of [^{35}S]methionine in a wheat germ system for 60 min. (a) Total polysomal translation products electrophoresed in the presence of 1% SDS, 0.5% 2-mercaptoethanol and 4 M urea. (b) Selective immunoprecipitation of globulin from the products of the reaction as in (a) separated under similar conditions.

CCl_3COOH-precipitable products over a period of 40 min (fig.3a). The incorporation was dependent on the amount of polysomes added over the range studied (fig.3b). The Mg^{2+} requirement of the translation system showed a broad optimum around 1.5 mM. When the products were analyzed by SDS–PAGE under reducing conditions a range of products was observed with M_r-values up to 70 000 (fig.4a), the most intense bands having M_r-values around 60 000. This differs from the observations of Luthe and Peterson [8] who found that the major products had M_r-values in the range of the globulin subunits.

To determine if any of the products were immunologically related to oat globulin they were precipitated with anti-globulin IgG and then analyzed by SDS–PAGE under reducing conditions. The results show (fig.4b) that one major band of M_r about 58 000 to 60 000 is specifically precipitated. We interpret these results as showing that oat globulin is synthesized in vitro as a precursor molecule approximately the same size as the unreduced dimer (fig.1c).

4. CONCLUSIONS

The results presented here provide further evidence for the proposed homology of 11 S storage proteins of seeds [4,17]. They show that the major oat globulin consists of dimers of a M_r around 58 000 and that, upon reduction, these dimers yield subunits of M_r-values 22 000 and 36 000 ([3], fig.1b). Like legumins the large and small subunits of oat globulin have basic and acidic pI-values respectively and show microheterogeneity (Burgess et al. in preparation). Further evidence for homology is that the major oat globulin synthesized in vitro by translation of isolated polysomes is a polypeptide of M_r about 58 000; analogous results have been reported for 11 S proteins of legumes [5–7]. It remains to be proved that this precursor is processed in vivo to the two types of subunit. Recent results with rice storage proteins [18], which are classified as glutenins, also show that they are synthesized in vivo as a relatively stable 57 000 precursor which is subsequently processed to give 22 000–23 000 and 37 000–39 000 M_r components. Taken together these results suggest that the legumin-like group of seed storage proteins have been widely conserved during the evolution of higher plants.

After this work was completed we became aware

that others have observed similar results which are reported in abstract form [19,20].

ACKNOWLEDGEMENTS

The authors are grateful to Janice Forde and Martin Kreis for advice and discussion. Part of this work was supported by an Agriculture Canada research contract (DSS 01SU.01531-1-0808).

REFERENCES

[1] Miflin, B.J. and Shewry, P.R. (1981) in: Nitrogen and Carbon Metabolism (Bewley, J.D. ed), pp. 195–258, Nijhoff/Dr. W. Junk, The Hague.
[2] Larkins, B.A. (1980) in: The Biochemistry of Plants – A Comprehensive Treatise, Proteins and Nucleic Acids (Marcus, A., Stumpf, P.K. and Conn, E.E. Series, eds) Vol. VI, pp. 449–489, Academic Press, New York.
[3] Peterson, D.M. (1978) Plant Physiol. 62, 506–509.
[4] Derbyshire, E., Wright, D.J. and Boulter, D. (1976) Phytochemistry 15, 3–24.
[5] Croy, R.R.D., Lycett, G.W., Gatehouse, J.A., Yarwood, J.N. and Boulter, D. (1982) Nature 295, 76–79.
[6] Tumer, N.E., Thanh, V.H. and Nielsen, N.C. (1981) J. Biol. Chem. 256, 8756–8760.
[7] Sengupta, C., Deluca, V., Bailey, D.S. and Verma, D.P.S. (1981) Plant Mol. Biol. 1, 19–34.
[8] Luthe, D.S. and Peterson, D.M. (1977) Plant Physiol. 59, 836–841.
[9] Peterson, D.M. and Smith, D. (1976) Crop Sci. 16, 67–71.
[10] Matthews, J.A. and Miflin, B.J. (1980) Planta 149, 262–268.
[11] Roberts, B.E. and Paterson, B.M. (1973) Proc. Natl. Acad. Sci. USA 70, 2330–2339.
[12] Laemmli, U.K. (1970) Nature 227, 681–685.
[13] Chamberlain, J.P. (1979) Anal. Biochem. 98, 132–135.
[14] Tan-Wilson, A.L., Reichlin, M. and Noble, R.W. (1976) Immunochemistry 13, 921–927.
[15] Roberts, L.M. and Lord, M.J. (1981) Eur. J. Biochem. 119, 31–41.
[16] Ouchterlony, O. (1968) Handbook of Immunodiffusion and Immunoelectrophoresis. Ann Arbor Science Publishers, Ann Arbor MI.
[17] Dieckert, J.W. and Dieckert, M.C. (1976) J. Fd. Sci. 41, 475–482.
[18] Yamagata, H., Tanaka, K. and Kasai, Z. (1982) Agric. Biol. Chem. in press.
[19] Walburg, G. and Larkins, B.A. (1982) Pl. Physiol. 69, Suppl. 123.
[20] Brinegar, A.C. and Peterson, D.M. (1982) Plant Physiol. 69, Suppl. 156.