PROTEIN DISULPHIDE-ISOMERASE IS LOCATED IN THE ENDOPLASMIC RETICULUM OF DEVELOPING WHEAT ENDOSPERM

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1. Introduction

The storage proteins of wheat and other cereals are synthesized during the mid and late phases of seed development and laid down as protein bodies within the endosperm. The nascent proteins are translated on membrane-bound ribosomes and translocated across the endoplasmic reticulum during translation [1]. Although the proteins are not secreted, but accumulate within the lumen of the endoplasmic reticulum, there appear to be many formal similarities between the post-translational processing of cereal storage proteins and that of secretory proteins in higher animals. The systems are homologous in many respects; in [2] mRNA for zein, the major storage protein of maize, was translated in *Xenopus laevis* oocytes and deposited within the lumen of the oocyte endoplasmic reticulum.

A proportion of the cereal storage proteins are present in the developing seed as disulphide-linked aggregates [3]. These aggregates are of particular importance in wheat, in that they have been correlated with breadmaking properties and are widely considered to be significant in conferring elasticity on wheat doughs [3,4]. Very little is known about the mechanism of formation of disulphide bonds during the biosynthesis of cereal storage proteins. Indeed the mechanism of formation of disulphide bonds during the biosynthesis of mammalian secretory proteins is not established for certain, but the weight of evidence suggests that under physiological conditions the rapid formation of the correct set of disulphide bonds requires enzymic catalysis [5]. The most likely candidate for this role in protein disulphide biosynthesis is protein disulphide-isomerase (EC 5.3.4.1), an enzyme which catalyses disulphide bond interchange within proteins. This enzyme has been found in a wide range of mammalian tissues active in the biosynthesis and secretion of disulphide-bonded proteins [6]. The tissue distribution, sub-cellular distribution, catalytic specificity and developmental properties are all consistent with its participation in the biosynthesis of disulphide-bonded secretory proteins [7,8].

Protein disulphide-isomerase has been detected in mature seeds of wheat, both in the germ and endosperm fractions [9], but its physiological role has not been established. We show that the enzyme is located in the endoplasmic reticulum of developing wheat endosperm, a location consistent with its playing a rôle in the formation of disulphide bonds during biosynthesis of wheat storage proteins.

2. Experimental

The growth, homogenization and sub-cellular fractionation procedures were based on [10]. Wheat (variety Highbury) was grown in a greenhouse with supplemental light in winter; it was harvested at the mid-phase of development when the rate of storage protein biosynthesis was close to maximal. Endosperms were isolated by hand and chopped with a razor blade until finely divided in an extraction medium containing 15% (w/w) sucrose (1.5 ml medium/g endosperm material). Two extraction media were used, one with and one without Mg^{2+}. The former ($+Mg^{2+}$ buffer) contained magnesium acetate (10 mM) and potassium acetate (100 mM) in 50 mM tricine buffer (pH 7.5). The latter ($-Mg^{2+}$ buffer) contained EDTA (5 mM) and potassium acetate (100 mM) in 50 mM tricine buffer (pH 7.5). After the material was chopped, a further 0.5 ml medium/g endosperm was added and the homogenate was filtered with squeezing

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through 4 layers of cheesecloth. About 5 ml of the homogenate were then loaded onto a linear sucrose density gradient (16–60% (w/w); 30 ml total vol.) made up either in $+Mg^{2+}$ buffer or in $-Mg^{2+}$ buffer but containing EDTA at only 1 mM. The density gradients were centrifuged for 2.5 h at 25 000 rev./min in a Beckman SW 27 rotor. The gradients were then fractionated by upward displacement (ISCO model 640) into 1.2 ml fractions which were then diluted 1:1 with a buffer containing 2 mM phenylmethyl sulphonyl fluoride (and 5 mM $MgCl_2$ in the case of the $-Mg^{2+}$ gradients) in 100 mM Tris–HCl (pH 7.5).

The isolated fractions were assayed for NADH:cytochrome *c* reductase activity in the presence of antimycin A [11] and for protein disulphide-isomerase activity [12,13]; the unit of isomerase activity is as defined in [12,13]. Sucrose content was determined by refractometry. Aliquots from the fractions with peak protein disulphide-isomerase activity were pelletted and the pellets treated with 2.5% (w/v) glutaraldehyde in 0.085 M sodium cacodylate. The pellets were washed and fixed using OsO_4. After dehydration, the material was embedded in Spurr's resin and sectioned on an LKB microtome. The sections were viewed on an AEI 801A electron microscope.

3. Results and discussion

Fig.1a shows the distribution of protein disulphide-isomerase activity in a sucrose density gradient run in $+Mg^{2+}$ buffer. A small fraction of the total activity is present at the top of the gradient, but most is found in a band from 38–52% (w/w) sucrose. Maximum activity is found in a fraction containing 44% sucrose, corresponding to 1.196 g/cm^3. In a repeat run in these conditions using a separate homogenate, the peak of activity was found at 41% sucrose (1.182 g/cm^3). The distribution in the gradient of protein disulphide-isomerase activity corresponds closely to that of NADH:cytochrome *c* reductase and the 2 activities reach a maximum in the same fraction. The antimycin-insensitive cytochrome *c* reductase in these preparations is established as a marker for the endoplasmic reticulum [10]. Fig.1b shows a corresponding sucrose density gradient run in $-Mg^{2+}$ buffer. It is noticeable that in these conditions a greater proportion of protein disulphide-isomerase activity is solubilised, but the major band of isomerase activity again cosediments with the NADH:cytochrome *c* reductase activity. In this case the maximal activities occur in a fraction containing 31% (w/w) sucrose, corresponding to 1.132 g/cm^3. In a repeat run the value was 1.146 g/cm^3. In $-Mg^{2+}$ conditions, ribosomes are lost from the endoplasmic reticulum and the median density of vesicles derived from this organelle shifts to a lower value. In both conditions, the peaks of protein disulphide-isomerase and NADH:cytochrome *c* reductase activity are well separated in the gradient

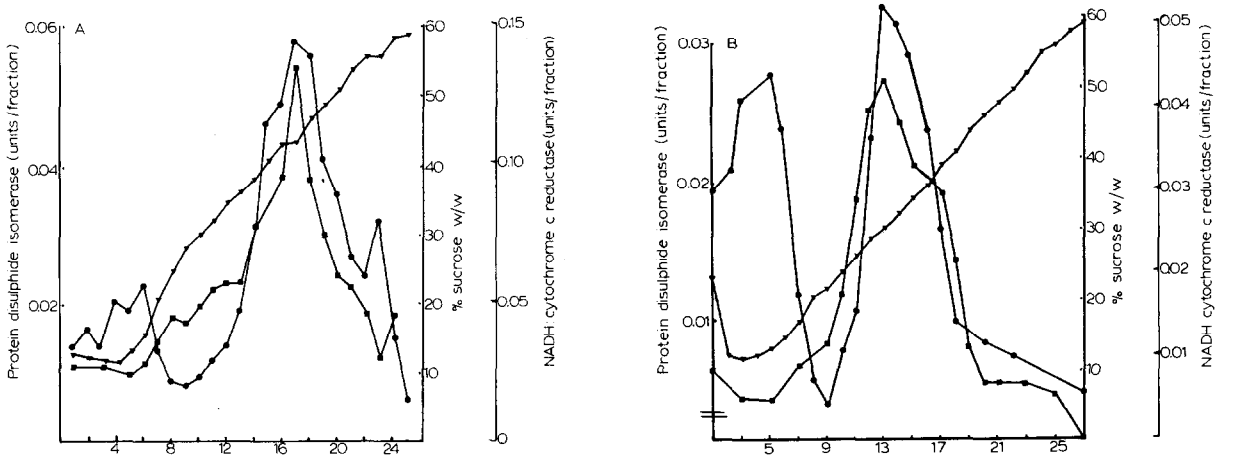

Fig.1. The distributions of protein disulphide-isomerase (●) and NADH:cytochrome *c* reductase (■) activities after centrifugation in a sucrose density gradient (▲). (a) Homogenate was prepared from 2.8 g endosperm material in $+Mg^{2+}$ buffer and Mg^{2+} was present in the density gradient. The endosperm was derived from caryopses of 44 mg av. (b) Homogenate was prepared from 1.9 g endosperm material in $-Mg^{2+}$ buffer and Mg^{2+} was absent from the density gradient. The endosperm was derived from caryopses of 47 mg av.

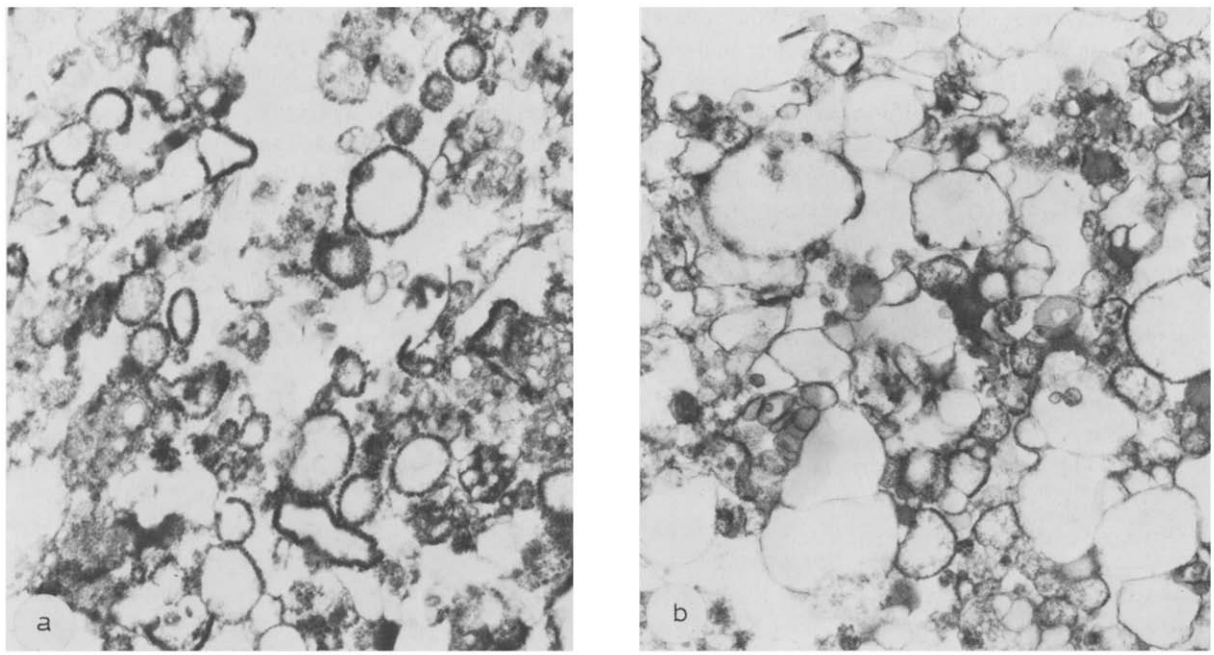

Fig.2. Electron micrographs of material obtained by centrifugation of the fractions from fig.1a and 1b, respectively, containing maximal protein disulphide-isomerase activity: (a) $+Mg^{2+}$ conditions, ×25 000; (b) $-Mg^{2+}$ conditions, ×28 000.

from protein bodies. These sediment as sharp bands at the bottom of the gradients of density 1.275 and 1.264 in $+Mg^{2+}$ and $-Mg^{2+}$ conditions, respectively, as indicated by the A_{280} of the density gradients (not shown).

The fractions containing peak protein disulphide-isomerase activity were pooled and pelletted, and fig.2a and 2b show electron micrographs of the pellets derived from the $+Mg^{2+}$ and $-Mg^{2+}$ gradients, respectively. The enzymically-active material, isolated in the presence of Mg^{2+}, contains characteristic rough vesicles derived from the rough endoplasmic reticulum, while that isolated in the absence of Mg^{2+} contains mainly smooth or degranulated vesicles.

Thus, several lines of evidence indicate that protein disulphide-isomerase is located in the endoplasmic reticulum (ER) of the developing wheat endosperm. The enzyme cosediments with antimycin-insensitive NADH:cytochrome *c* reductase, an ER marker, the enzyme is clearly resolved in the density gradient from protein bodies, and electron microscopy shows that the enzyme is associated with vesicular material. Furthermore, the absence of Mg^{2+} from the density gradient leads to a parallel shift to a lower density of the peaks of protein disulphide-isomerase and the ER marker enzyme, and to the loss of ribosomes from vesicles in the active fraction. This action of Mg^{2+} confirms that the vesicles with which isomerase is associated are derived from the endoplasmic reticulum. Furthermore, the position and behaviour of the other cellular organelles in this gradient system is known [10]; none of them correlate with the density distribution of protein disulphide-isomerase reported here.

It was noted that the evidence for the involvement of protein disulphide-isomerase in protein biosynthesis, derived from studies in animal systems, was based on 4 findings; its tissue distribution; its subcellular distribution; its catalytic specificity; and its developmental properties. We have here presented evidence for one such correlation in a plant system, namely that the enzyme in developing wheat endosperm is located in the endoplasmic reticulum, the site of storage protein biosynthesis and transmembrane translocation. In other experiments we have studied protein disulphide-isomerase at various stages of wheat development and have shown that the enzyme is not detectable during germination but is present in the developing seed over 10–50 days after anthesis, the period during which disulphide-bonded storage proteins are synthesized and deposited (L. T. R., R. B. F., unpublished).

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References

[1] Larkins, B. A. and Hurkman, W. J. (1978) Plant Physiol. 62, 256–263.
[2] Hurkman, W. J., Smith, L. D., Richter, J. and Larkins, B. A. (1981) J. Cell Biol 89, 292–299.
[3] Miflin, B. J., Field, J. M. and Shewry, P. F. (1982) in: Seed Proteins (Daussant, J. C. and Mosse, J. eds) Academic Press, London, in press.
[4] Ewart, J. A. D. (1977) J. Sci. Fd. Agric. 28, 191–199.
[5] Freedman, R. B. and Hillson, D. A. (1980) in: The enzymology of posttranslational modification of proteins (Freedman, R. B. and Hawkins, H. C. eds) vol. 1, pp. 157–212, Academic Press, New York.
[6] Freedman, R. B. (1979) FEBS Lett. 97, 201–210.
[7] Creighton, T. E., Hillson, D. A. and Freedman, R. B. (1980) J. Mol. Biol. 142, 43–62.
[8] Brockway, B. E., Forster, S. J. and Freedman, R. B. (1980) Biochem. J. 191, 873–876.
[9] Grynberg, A., Nicolas, J. and Drapron, R. (1978) Biochimie 60, p. 547–551.
[10] Miflin, B. J., Burgess, S. and Shewry, P. R. (1981) Exp. Botany 32/126, 199–219.
[11] Tolbert, N. E. (1974) Methods Enzymol. 31, 734–746.
[12] Ibbetson, A. L. and Freedman, R. B. (1976) Biochem. J. 159, 377–384.
[13] Hillson, D. A. and Freedman, R. B. (1980) Biochem. J. 191, 373–388.