Assembly of *Agrostemma githago* (corn-cockle) storage proteins and their precursor proteins into oligomers

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(Received 10 April 1985/10 June 1985; accepted 17 June 1985)

The major fraction of seed storage proteins of *Agrostemma githago* (corn-cockle), a non-leguminous dicot, occurs as material with $S_{20,w}$ values of $\sim 11S$ and $\sim 2S$, and a minor fraction as oligomers with $S_{20,w}$ values of $\sim 6.5S$. The $11S$ proteins are of the legumin type and consist of disulphide-linked α - and β -subunits of $M_r \sim 39000$ and ~ 23000 respectively. The oligomeric assembly of the precursor polypeptides of the $11S$ proteins was examined. The ~ 65000 - M_r precursor polypeptides of two $11S$ proteins, which consist of 38000 – 25000 - M_r subunits and 36000 – 22000 - M_r subunits respectively, were assembled into oligomers of $\sim 7S$ and subsequently cleaved. Thereafter the $11S$ oligomer was formed. The 88000 - M_r precursor of a third $11S$ protein, which consists of 41000 – 23000 - M_r subunits, was assembled into an $\sim 8S$ oligomer and then cleaved, yielding two disulphide-linked intermediates of $M_r 59000$ and 24000 . Thereafter, the $11S$ oligomer was formed. Processing of the 59000 - M_r to the 41000 - M_r polypeptide occurred both in the $8S$ and in the $11S$ form.

In dicotyledonous plants, seed storage proteins have been investigated most extensively in legumes. Separation by ultracentrifugation showed that leguminous storage proteins fall into two major types, namely legumins and vicilins, sedimenting at 11 – $12S$ and 7 – $8S$ respectively. In some species additional proteins of 2 – $4S$ occur. The legumin holoprotein consists of six α - ($M_r \sim 40000$) and six β - ($M_r \sim 20000$) polypeptides linked together in pairs by disulphide bridges. The vicilin holoprotein appears to consist of three ~ 50000 - M_r subunits, some of which may be cleaved post-translationally. Legumin-like proteins and the 2 – $4S$ proteins are the major storage proteins in other dicots, whereas vicilin is reported to be a minor constituent in some non-leguminous species [for reviews, see Derbyshire *et al.* (1976), Higgins (1984), Casey & Domoney (1984) and Boulter (1984)].

In crop seeds, many storage proteins are synthesized via high M_r polypeptides, which undergo post-translational cleavage (Higgins, 1984). In order to determine whether this is a general mode of storage-protein synthesis, we have initiated a study on the synthesis of storage

proteins in the non-leguminous dicot *Agrostemma githago* (corn-cockle), a member of the Caryophyllaceae. This study supplements our investigations on protein synthesis in relation to dormancy and germination of *Agrostemma githago* seeds (de Klerk, 1983; de Klerk & Smulders, 1984). Previously we reported that *Agrostemma* storage proteins are synthesized through post-translational cleavage of large precursor polypeptides (de Klerk, 1984a). Since the oligomeric assembly of precursor polypeptides has been examined thoroughly only in peas (*Pisum sativum*) (Spencer & Higgins, 1980; Chrispeels *et al.*, 1982), we report here on the oligomeric assembly of *Agrostemma* storage proteins and their precursor polypeptides.

Experimental

Materials

Developing seeds were derived from plants of *Agrostemma githago* L. cultivated in the Botanical Garden of the University of Nijmegen from May until August 1983. Pods were selected with seeds of the last maturation stage. This stage was recognized by the colour of the seed coat (black). During this stage there is a high level of synthesis of bands VIa–VIIIb and little synthesis of -VIb,o–VIIIa,c proteins (de Klerk, 1984b).

Methods

Incubation conditions. Samples consisting of five embryos were excised from the seeds and incubat-

Abbreviation used: SDS, sodium dodecyl sulphate.

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ed for 1 h in 150 μ l of 10 mM-KCl/1 mM-CaCl₂ and chloramphenicol (50 μ g $\cdot$ ml⁻¹) to which 570 kBq of a ¹⁴C-labelled amino-acid mixture (2.15 GBq $\cdot$ mg-atom of C⁻¹; The Radiochemical Centre, Amersham, Bucks., U.K.) was added. After the pulse, the embryos were rinsed in distilled water and incubated for various periods on filter paper moistened with the KCl/CaCl₂/chloramphenicol solution without addition of label.

Separation of oligomers on a sucrose density gradient and gel electrophoresis. After incubation, the samples of five embryos were homogenized in an all-glass homogenizer in 2 ml of 0.3 M-NaCl/0.1 M-Tris/HCl (pH 8.0)/1% Tween-20. The homogenates were centrifuged for 20 min at 31 000 $g_{av.}$. The supernatant was loaded on a linear 8–24% (w/v)-sucrose gradient containing 0.3 M-NaCl and 0.1 M-Tris/HCl, pH 8.0. The gradients were centrifuged in a Beckman SW41 rotor at 5°C at 196 000 $g_{av.}$ for 24 h. After centrifugation, 14 fractions were collected from each gradient, including the buffer loaded on the gradient. Proteins were precipitated by ethanol added to a final percentage of 80% (v/v), subsequently dissolved in a solution containing 2% (w/v) SDS/10% (v/v) glycerol/5% (v/v) 2-mercaptoethanol and 0.005% Bromophenol Blue, and heated for 3 min at 100°C. SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli (1970) with a 13%-polyacrylamide slab gel. The gels were stained with Coomassie Blue R-250, destained, photographed and finally prepared for fluorography (Bonner & Laskey, 1974) with Kodak X-Omat R film. The $s_{20,w}$ values were calculated. M_r values were obtained by using lysozyme (M_r 14 300), β -lactoglobulin (18 400), trypsinogen (24 000), carbonic anhydrase (29 000), pepsin (34 700), egg albumin (45 000), bovine serum albumin (66 000), and phosphorylase *b* (97 400) (all from Sigma) as standards.

Results

Separation of *Agrostemma githago* storage proteins by ultracentrifugation

After SDS/polyacrylamide-gel electrophoresis, *Agrostemma* seed proteins yield several major Coomassie Blue-stained bands, indicated by VIa (41 000), VIb (38 000), VIc (36 000), VIIa (25 000), VIIb (23 000), VIIc (22 000), IXa₁ (17 000), IXa₂ (17 000), IXb (16 000), X (12 000) and XI (8 000) (Fig. 1, lanes C and T; M_r values are given in parentheses and are from de Klerk, 1984a). Since these proteins are synthesized in large amounts in developing seeds and are broken down after germination (de Klerk, 1983, 1984b), they are classified as storage proteins.

Ultracentrifugation of *Agrostemma* seed pro-

Fig. 1. Fractionation of proteins extracted from developing *Agrostemma githago* embryos on a sucrose density gradient, followed by analysis of the gradient fractions by SDS/polyacrylamide-gel electrophoresis and staining with Coomassie Blue

Equal portions of each fraction were loaded on the gel. Roman numerals indicate the various storage proteins (cf. de Klerk, 1984a). The Arabic numerals refer to fractions of the gradient with fraction 14 at the bottom of the gradient. T and C are total extracts stored during centrifugation at -20°C and 5°C respectively. 'vic.' indicates a vicilin component.

teins on a sucrose gradient yielded two major components. The first (Fig. 1, lanes 1–3) sedimented at ~2S and consisted of bands IX and X. The second fraction (Fig. 1, lanes 7–10) was ~11S and consisted of bands VI and VII. Band VIa is disulphide-linked to VIIb, VIb to VIIa, and VIc to VIIc (de Klerk, 1984a). So the 11S proteins are clearly of the legumin-type. By analogy with legumin like proteins in other species (Derbyshire *et al.*, 1976) the 11S oligomers in *Agrostemma* seeds probably consist of six pairs of disulphide-linked VI and VII subunits, representing the α - and β -legumin subunits respectively. It should be noted that a considerable amount of the VI and VII subunits, especially of VIb,c and VIIa,c, precipitated in fractions 1–6. This indicates that the oligomers are labile during extraction.

A minor component sedimented at ~6.5S and by gel electrophoresis was shown to contain an M_r 65 000 polypeptide (Fig. 1, lanes 5–7). This polypeptide is synthesized in developing seeds (Fig. 2c, lanes 5–7), is a globulin and is degraded after germination (G. J. de Klerk, unpublished work). These data indicate that it is a storage protein of the vicilin type.

Assembly of the precursor polypeptides of VIb–VIIa and VIc–VIIc

As reported previously (de Klerk, 1984a), the VIb–VIIa and VIc–VIIc complexes are synthe-

Fig. 2. Fractionation of labelled proteins extracted from developing *Agrostemma githago* embryos after pulse or pulse-chase, on a sucrose density gradient followed by analysis of the gradient fractions by SDS/polyacrylamide-gel electrophoresis and fluorography

The embryos were pulse-labelled with a ^{14}C -labelled amino-acid mixture for 1 h (a), or pulse-labelled for 1 h followed by a 2 h chase (b), a 5 h chase (c), or a 23 h chase (d). Equal portions of each fraction were loaded on the gel. Roman numerals indicate the various storage proteins and their precursors. The arabic numerals refer to the fractions of the gradient with fraction 14 at the bottom of the gradient. T and C are total extracts stored during centrifugation at -20°C and 5°C respectively. The M_r markers were bovine serum albumin, pepsin, trypsinogen and lysozyme. Staining with Coomassie Blue showed that, in (a), (b) and (c), bands VI and VIII precipitated in fractions 7–10 and in (d), in fractions 7–14. In (c), 'vic.' indicates a vicilin component.

sized from precursor polypeptides in which the $\alpha(\text{VI})$ - and $\beta(\text{VIII})$ -subunits are peptide-bonded. The precursors form a triplet of polypeptides of M_r 69 000, 66 000 and 65 000, indicated by III (Fig. 2a). Legumin in legumes (Croy *et al.*, 1980; Spencer & Higgins, 1980; Barton *et al.*, 1982), and legumin-like storage proteins in oats (*Avena*) (Brinegar & Peterson, 1982) and rice (*Oryza*) (Yamagata *et al.*, 1982) are also synthesized from precursors in which the α - and β -subunits are peptide-bonded.

After centrifugation, the group-III polypeptides sedimented to the greatest extent at $\sim 7\text{S}$ (Fig. 2a, lanes 6–7). This indicates that they had assembled rapidly into a trimer. If the group-III polypeptides had not assembled rapidly into an oligomer, then

they would be expected to sediment in the top gradient fractions 1–5.

During the chase, the group-III polypeptides were cleaved and yielded the two pairs of disulphide-linked α - and β -subunits, VIb–VIIa and VIc–VIIc (de Klerk, 1984a). After a 2 h chase, fractions 6 and 7 had more label at the positions of VIb,c and VIIa,c than had fractions 8–10 (Fig. 2b). Coomassie Blue staining of the same gel showed, however, that the bulk of proteins VIb,c and VIIa,c was present in fractions 8–10 (see Fig. 1). This shows that the cleavage products remained initially in the 7S form. After a 23 h chase, the Coomassie Blue staining and the amount of label at the positions of VIb,c and VIIa,c showed identi-

cal distributions (Fig. 2d). The results may be summarized as follows:

In peas it was found that the assembly of the legumin precursor polypeptides and their cleavage products occurs in the same way. The assembly of the legumin precursor polypeptides into the intermediate 7S and the final 12S oligomers was, however, much slower (Spencer & Higgins, 1980; Chrispeels *et al.*, 1982). Similar data are reported for glycinin in soya beans (Barton *et al.*, 1982). It should be noted that, after a 23h chase, VIc—VIIIa sedimented to a large extent in fractions 3–6 (Fig. 2d). The same was observed after Coomassie Blue staining (see Fig. 1). This indicates that the 11S oligomers are unstable during the extraction.

Assembly of the precursor polypeptides of VIa–VIIIb

As shown previously (de Klerk, 1984a), the disulphide-linked VIa (α) and VIIIb (β) polypeptides are synthesized from a precursor of M_r 88000, which is indicated by 'I' (Fig. 2a). Precursor polypeptide I sedimented at $\sim 8\text{S}$ (Fig. 2a, fractions 6–8), indicating that it is present as a trimer. Since band I was present in only small amounts in fractions 1–5, precursor I appeared to have assembled rapidly into the 8S form.

After a 2h chase, band I had been partially processed to the polypeptides IV (M_r 59000) and VIIIId (M_r 24000). With longer chase times, IV and VIIIId were subsequently cleaved to yield VIa and VIIIb respectively (de Klerk, 1984a). Since IV and VIIIId are disulphide-linked and as they are intermediates in the synthesis of the α (VIa)- and β (VIIIb)-polypeptides respectively, cleavage of I into IV and VIIIId represents a legumin-type cleavage.

Band IV sedimented both at 8S and at $\sim 11\text{S}$; after a 2h chase, it sedimented predominantly at 8S and 11S (Fig. 2c, fractions 6–7 and 8–9 respectively). This shows that the IV–VIIIId complex was assembled from an 8S into an 11S oligomer and that apparently the 18kDa polypeptide, which is peptide-linked to VIa in intermediate IV, did not hinder this assembly.

The cleavage product of IV, band VIa, became visible only after a 5h chase (Fig. 2c; see also de Klerk, 1984a). Band VIa sedimented both at 8S and at 11S (Fig. 2c, fractions 6–7 and 8–9 respectively). The presence of intermediate IV in the 11S oligomeric form and of its cleavage

product, VIa, in the 8S oligomeric form indicates that the proteolytic cleavage of IV occurred both in the 8S and in the 11S oligomer. Apparently this cleavage was independent of the oligomeric form. This contrasts with legumin-type cleavage (both of I into IV–VIIIId, and of III into VIb–VIIIa and VIc–VIIIc), which occurred in the 7–8S form only.

After a 23h chase, band IV had almost completely disappeared. Furthermore, VIIIId had been processed to VIIIb, which probably represents a deglycosylation event (de Klerk, 1983; Vitale & Chrispeels, 1984). From Figs. 2(c) and 2(d) it cannot be concluded with certainty in which oligomeric form the processing of VIIIId to VIIIb occurred, but the data indicate that this was in the 11S form only. The processing and assembly of the VIa–VIIIb complex may be summarized as follows:

We thank Professor Dr. H. F. Linskens for his stimulating interest and Dr. T. J. V. Higgins for the critical reading of the manuscript.

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