An Analysis of the Subunit Structure of the Crystalloid Protein Complex from Castor Bean Endosperm

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ABSTRACT

Chromatographic and electrophoretic studies have shown that the subunits of the crystalloid protein, isolated from mature castor bean (Ricinus communis L. cv Hale) seed endosperm protein bodies, are heterogeneous with molecular weights in the range 49 to 53.5 kilodaltons (kD), and are quantitatively in unequal amounts. Each subunit comprises an αβ polypeptide pair which are reduced by 2-mercaptoethanol in two subgroups with molecular weights in the 29 to 34 kD and 20.5 to 23.5 kD ranges. Subunits and corresponding polypeptide pairs are also seen to be heterogeneous in pI following isoelectric focusing. In general, large polypeptides are acidic (pI 4.8–6.2) and small polypeptides basic (pI 7.4–9.4), although overlap of some isoelectric isomers does occur, notably in polypeptides derived from subunits which are quantitatively present in smaller amounts.

The 11S globulins form the major storage reserve of dicotyledonous seeds (3). Within this group of proteins, two broad classes can be distinguished; those which are completely salt soluble, e.g. the legumin, and those which require the addition of SDS or urea to dissolve them, e.g. the crystalloids. Of these two classes, the former has received considerable attention with regards to protein structure and protein synthesis in developing seeds (14). In contrast, the crystalloids have been virtually ignored (6, 11, 15, 17). We have chosen to gain a better understanding of the crystalloid protein, therefore, using the castor bean endosperm where this protein makes up some 80% of the protein storage reserve in the mature seed (6, 15). In this paper, we examine the subunit structure of this protein, and show that it is a complex made up of at least six oligomeric proteins which exhibit considerable heterogeneity in both mol wt and pI.

MATERIALS AND METHODS

Isolation and Fractionation of Protein Bodies. Protein bodies were isolated from the endosperm of mature dry castor bean seeds (Ricinus communis L. cv Hale). This procedure, and those involved in the fractionation and extraction of the crystalloid protein were as described previously (6).

Ion Exchange Chromatography. The method of Tully and Beevers (15) was used with modifications. Crystalloid protein (5–7 mg/ml) was dissolved in 5 mM Na-acetate (pH 5) containing 6 M urea. This solution (5 ml) was loaded onto a CM-Sepharose (Sigma)

FIG. 1. SDS-PAGE profiles of crystalloid subunits (A) and polypeptides (B) extracted from protein bodies isolated from the endosperm of mature castor bean seeds. In each case, 10 μg of crystalloid protein in 10 μl of extraction buffer (± ME) were applied to the gel. Numerical values are mol wt in kD.

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column (27 x 1.5 cm) and the column washed with 50 ml acetyl-
urea buffer. The bound proteins were eluted with a 200-ml linear
NaCl gradient (0.1-0.5 M) in acetyl-urea buffer. The protein
eution profile was followed by monitoring the A at 280 nm. Prior
to SDS-PAGE\textsuperscript{2}, fractions (1 ml) from this profile were dialyzed
against 0.5 M Na-acetate (pH 5), and the precipitated crystalloid
was redissolved by boiling for 2 min in the extraction buffer: 65
mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, and 5% 
(v/v) ME. Protein content of the various fractions was determined
using the Lowry method (8).

Polycrylamide Gel Electrophoresis. Single-Dimension SDS-
PAGE. Electrophoresis was carried out in 0.75 mm 12% acrylam-
ide slab gels on a LKB-2001 vertical electrophoresis unit using the
method of Ref. 7. Subunit and polypeptide mol wt determinations
were as outlined by Weber and Osborne (16); mol wt markers
included phosphorylase B, 94 kD; BSA, 68 kD; ovalbumin, 43 kD;
carbonic anhydrase, 30 kD; soybean trypsin inhibitor, 21 kD; and
lysozyme, 14 kD (Bio-Rad).

Two-Dimensional SDS-PAGE. The method used was based
upon that of Matta et al. (9). In the first dimension, unreduced
crystalloid protein samples were separated on 0.75-mm 7.5% slab
gels as described above. Then, 1-cm vertical gel strips were incubated at 22°C for 30 min in the extraction buffer. After this
treatment, the subunits were fully reduced. The resulting polypep-
tides were separated in the second dimension by placing the gel
strip horizontally on the stacking gel of a 1.5-mm 12% acrylamide slab gel which was run as outlined for one-dimensional SDS-

\textsuperscript{2} Abbreviations: PAGE, polycrylamide gel electrophoresis; ME, 2-mer-
captoethanol; IEF, isoelectric focusing; NP-40, Nonidet P-40; TEMED,
$N,N,N',N'$-tetramethylenediamine.

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Fig. 2. Elution profile of crystalloid subunit separated by CM-Sepharose ion-exchange chromatography viewed by absorbance at 280 nm and
following SDS-PAGE under reducing conditions. A 10-µl aliquot of the protein present in each 3-ml fraction was applied to the gel after first being
dissociated and reduced as outlined in the "Materials and Methods." The standard was 10 µg of reduced crystalloid applied in 10 µl.

Two-Dimensional IEF/SDS-PAGE. IEF was carried out on
ultra-thin (0.5 mm) horizontal slab gels using a LKB-multiphore
system. The gel contained 7.2 g urea, 3.5 ml 29.1% (w/v) acryl-
amide, 3.5 ml 0.9% (w/v) bis-acrylamide, 0.5 ml NP-40, and 1.5
ml LKB-ampholines (pH 3.5-9.5) in a total volume of 20 ml. After
degassing, polymerization was achieved by the addition of 0.5 ml 1% (w/v) ammonium persulfate and 16 µl TEMED. Anodic and
cathodic solutions were 0.05 M glutamate and 0.1 M ethanol-
amine, respectively. Focusing was carried out for 90 min using a
LKB 2197 power supply. Under these conditions, complete sepa-
ration was only obtained with sample loading at the anode.

Following electrophoresis, 1-cm gel strips were cut and placed
in H$_2$O at 22°C for 60 min. The crystalloid protein immediately
precipitated \textit{in situ} as the urea and ampholines diffused from the
gel. Proteins were redissolved and dissociated by a further 15-min
equilibration in extraction buffer (± ME). Using this approach,
minimal intragel protein diffusion occurred. The gel was then
layered on top of the stacking gel of a 0.75-mm 12% SDS-acryl-
amide slab gel as outlined in the LKB-application manual. The
second dimension was then performed as outlined under single-
dimension SDS-PAGE.

Two-Dimensional SDS-PAGE/IEF. The SDS-PAGE first-di-

dension used either 7.5% acrylamide gels (in the case of unreduced
samples) or 12% gels (for reduced samples). Following electrophoresis, 1-cm vertical gel strips were cut and incubated at 22°C for
60 min in 9 M urea. The gel strips were then placed at the anode
end of an ultra-thin horizontal slab gel which had the same
composition as that described above. IEF was carried out as
indicated previously.

Gel staining was as outlined by Weber and Osborne (16) for

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Fig. 3. Two-dimensional SDS-PAGE analysis of crystalloid polypeptides. (1D), Electrophoresis under nonreducing conditions on a 7.5% gel. Band pattern of the subunit standard is presented as a track. (2D), Electrophoresis under reducing conditions on a 12% gel. Band pattern of polypeptide standard is given as a track. Aa through Ff represent subunit polypeptide pairs as defined in the text.

Table I. Specific Crystalloid Subunit-Polypeptide Pair Relationships

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Polypeptide Pairs</th>
</tr>
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<tbody>
<tr>
<td>53.5</td>
<td>34 (A) + 20.5 (a)</td>
</tr>
<tr>
<td>53</td>
<td>33 (B) + 21 (b)</td>
</tr>
<tr>
<td>52.5</td>
<td>32.5 (C) + 21 (c)</td>
</tr>
<tr>
<td>51</td>
<td>31 (D) + 23.5 (d)</td>
</tr>
<tr>
<td>50</td>
<td>30 (E) + 23 (e)</td>
</tr>
<tr>
<td>49</td>
<td>29 (F) + 22.5 (f)</td>
</tr>
</tbody>
</table>

SDS-PAGE gels and by the appropriate LKB application manual for IEF gels.

RESULTS AND DISCUSSION

The General Nature of Crystalloid Protein Subunits. The crystalloid protein was extracted from isolated protein bodies and dissociated into subunits using SDS, prior to electrophoresis. The profiles obtained for these subunits are shown in Figure 1A. Two groups can be discerned, a minor one of two bands in the 100 kD region of the gel and a major one of at least six bands in the 50 kD region. Under reducing conditions, two subgroups are obtained; one in the 30 kD region of the gel and the other in the 20 kD region (Fig. 1B). These data suggest that the basic crystalloid subunit has an approximate mol wt of 50 kD and comprises two polypeptides of approximate mol wt 30 and 20 kD, respectively.

The need for reducing conditions in order to generate the polypeptidases suggests interpolypeptide disulfide bridging which is consistent with the observed high cysteine content of the crystalloid protein (18). While this αβ subunit configuration is in keeping with that proposed for crystalloid proteins from other species (11), it does differ from the αβ2 configuration originally proposed for castor bean crystalloid subunits (15). However, inherent difficulties associated with mol wt determinations using tube gels, in contrast to the more accurate slab gel method used in the present study, could account for this discrepancy.

The castor bean crystalloid holoprotein has an approximate mol wt of 330 kD (3). A hexameric, αβ6, structure can therefore be envisaged for this protein. The different staining intensity of the bands, which is particularly obvious on the nonreducing gels (Fig. 1A), suggests that the crystalloid is not a single protein, however, but is heterogeneous. That is, it is made up of at least six proteins each with an αβ6 configuration, but which differ in overall size and quantity. Further evidence for quantitative differences can be seen in Figure 2 where the first peak of the elution profile (fractions 29–34), which accounts for some 50% of the total crystalloid protein (data not shown), is comprised mainly of the 53 kD subunit. In contrast, the other subunits are distributed throughout the remainder of the elution profile. Although the subunit mol wt heterogeneity discussed here is not uncommon in seed storage proteins of the globulin type (3), the degree of this heterogeneity is far less (49–53.5 kD) than that observed in some other seeds, e.g., the legumin of Vicia faba (37–79 kD; Ref. 9) and Lupinus albus (18–69 kD; Ref. 13).

If the proteins in the 50 kD region of the gel are considered to be crystalloid subunits, then those in the 100 kD region can be viewed as αβ2 dimers linked by disulfide bridges which are not fully accessible to SDS under nonreducing conditions. Evidence for this is 2-fold: first, no new bands outside the 30 and 20 kD regions are generated on reducing gels (Fig. 1A); and second,
following two-dimensional SDS-PAGE (Fig. 3), both high mol wt bands (I and II) are reduced to two polypeptide subgroups with mol wt in the same ranges as those obtained from the 50 kD subunits run on the same gel. While these observations are of considerable interest, they should be viewed with caution, inasmuch as we do not know the significance of the dimer in vivo, particularly in relation to subunit synthesis, nor have we ruled out the possibility that the 100 kD bands may be an artifact of the extraction procedure.

Specific Subunit Polypeptide Relationships within the Crystalloid Protein Complex. Although it is apparent from the above that each subunit comprises two polypeptides, the exact relationships of specific subunits and corresponding polypeptide pairs is not clear from the gels (Fig. 1). To examine these relationships, the protein complex was first separated as subunits on a CM-Sepharose column. Then, the subunits in each fraction throughout the elution profile were reduced to polypeptide pairs and run on single-dimensional SDS-acrylamide gels. Under these conditions, pairs of polypeptides within the 30 and 20 kD subgroups which are seen to coelute would be derived from the same single subunit. The results are shown in Figure 2. Three polypeptide pairings can be discerned: Aa (34 kD, 20.5 kD), Bb (33 kD, 21 kD), and Ff (29 kD, 22.5 kD). Additional evidence for these assignments is obtained following two-dimensional SDS-PAGE using nonreducing and reducing gels (Fig. 3). These data also clearly establish the remaining three polypeptide pairings: Cc (32.5 kD, 21 kD), Dd (31 kD, 23.5 kD), and Ee (30 kD, 23 kD). Also from Figure 3, a comparison of the relative position of individual subunits with polypeptide pairs, generated following the reduction of these subunits, indicates the probable subunit-polypeptide pair relationships within the crystalloid protein complex (Table I). We note that in each case there is a discrepancy between values for the apparent mol wt of a subunit obtained from the unreduced protein compared with that from the mol wt sum of the reduced polypeptide pairs. This has also been observed for other 11S seed storage proteins, notably legumin by Matta et al. (9). These workers have suggested that incomplete unwinding of unreduced subunit polypeptide chains due to the presence of inter- and intra-chain disulfide bridges results in a lowering of the apparent mol wt determined by SDS-PAGE. Because of this, the subunit mol wt determined from the polypeptide pairs are deemed to be the more accurate.

Subunit and Polypeptide Microheterogeneity following Isoelectric Focusing. It is well known that the subunits of many major seed storage proteins, including the 11S globulins, exhibit considerable electrophoretic microheterogeneity when viewed on gels.

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**Fig. 4.** A, Single-dimensional electrophoretic analysis of crystalloid subunits. B and C, Two-dimensional electrophoretic analysis of crystalloid subunits. Analysis by IEF under nonreducing conditions in the first dimension. Analysis in the second dimension by SDS-PAGE under nonreducing (B) and reducing (C) conditions. Standards are crystalloid subunits in either nonreduced (B) or reduced (C) form. Alphabetic and numerical assignments are those discussed in the text.
following IEF (2, 4, 9, 13). The crystalloid subunit complex was examined for similar sets of isoelectric isomers. In initial experiments, the subunits were separated on conventional single-dimensional IEF and two-dimensional IEF/SDS-PAGE (Fig. 4). It is apparent from the IEF gel (Fig. 4A) that each subunit exists in several isoelectric isomeric forms and that collectively these forms span a broad range of pI values. Two-dimensional electrophoresis (Fig. 4, B and C) shows that each subunit is made up of a set of at least seven isoelectric isomers. Also, significant variation in the pI ranges for individual isomeric sets can be seen; for example, the 53.5 kD (Aa) and 53 kD (Bb) subunits show approximate pI ranges of 6.5 to 8 and 5.5 to 7, respectively.

To examine the pI microheterogeneity of individual subunit polypeptides, the subunits were first reduced and separated using SDS-PAGE before being subjected to IEF in the second dimension. To demonstrate the validity of this approach, the subunit complex itself was first separated under nonreducing conditions and the pI profile compared with that obtained following conventional IEF. These data, presented in Figures 4A and 5A, show that similar profiles are obtained. When individual polypeptides are separated in this way, considerable differences in pI are seen (Fig. 5B). In general, polypeptides from the 30 kD subgroup are acidic (pI 5–6), whereas those from the 20 kD subgroup have a more basic character (pI 7.5–9.5). These kinds of differences have been noted for other globulin storage proteins (1, 5, 9). However, in castor bean there is also considerable pI overlap between subgroups. This is particularly true for polypeptides derived from the more minor subunits, e.g., Aa (34 and 20.5 kD) and Ff (29 and 22.5 kD). In this regard, polypeptide sets from the major subunit (53 kD) show the least heterogeneity in pI values: 4.8 to 5.8 for the 33 kD (B) polypeptide set and 8.4 to 9.2 for the 21 kD (b) set.

In summary, we have shown that the crystalloid is a complex made up of at least six oligomeric proteins present in different amounts. We have suggested that each holoprotein is a hexamer, and have shown that the subunits comprise two polypeptides with different mol wt and pI values. In addition, many different isoelectric isomeric forms for each subunit and corresponding polypeptide pairs are observed. The heterogeneity observed here occurs in a single seed. Thus, it is unlikely to be due to variations within a population of this seed cultivar. More likely, it probably reflects differences in primary structure since we have shown previously that the crystalloid proteins are not glycosylated (6). Whether these differences have arisen as a result of postranslational modification of initial single gene products or are due to the generation of a mixture of structurally related polypeptides coded for by a multigenic family, as has been recently proposed for the hordeins (12), is not clear. We do know, however, that the expression of the crystalloid genes occurs early during endosperm development (6) and that, in contrast to what has been observed for barley and soybean (10, 12), the synthesis of the entire castor bean crystalloid complex is probably initiated at the same time. Problems associated with the control of this initiation, and with the mode of crystalloid protein synthesis and deposition within developing protein bodies are currently under investigation.
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