NUCLEAR CRYSTALLOIDS IN SIEVE ELEMENTS OF BORAGINACEAE: A PROTEIN DIGESTION STUDY

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SUMMARY

Nuclear crystalloids have been found in sieve elements of several Boraginaceae. Nuclei of differentiating sieve elements of Echium and other genera except Amsinckia contain one or more crystalloids composed of thin rods densely packed in parallel arrangement. After the nuclei disintegrate in the maturing sieve element the crystalloids are released into the cell lumen where they persist intact. In Amsinckia the crystalloid consists of two components: a dense component, similar to the crystalloid in the other genera and a loosely arranged paracrystalline component. The proteinaceous nature of the nuclear crystalloids and their possible similarity to P-protein was investigated by enzyme digestion techniques. Three proteolytic enzymes were employed in this study: protease, pepsin and trypsin. Successful digestion of the dense crystalloid in both Echium and Amsinckia was obtained with each enzyme tested. P-protein plugging the sieve plate pores was also digested. The loose component in Amsinckia and the aggregated and dispersed P-protein were not affected by the enzyme digestion procedures. These results seemed to indicate that the density or compactness of the proteinaceous inclusions may play a role in the differential response.

INTRODUCTION

Nuclear crystalloids have been identified in sieve elements of several members in the family Boraginaceae. In Echium, 16 species were found containing nuclear crystalloids (Esau & Thorsch, 1982). In additional surveys, the nuclear inclusions were identified in sieve elements of Amsinckia douglasiana (Esau & Magyarosy, 1979a,b), Amsinckia intermedia and in species of Ehertia, Myosotis and Trichodesma, and in one more species of Echium. Comparison of different genera revealed two forms of nuclear crystalloids. In Echium, Ehertia, Myosotis and Trichodesma the crystalloids are composed of extremely tightly packed tubules; in Amsinckia the crystalloids consist of two components, a dense component (dc), similar to the crystalloid in the other four genera, and a loosely arranged paracrystalline component (lc). During sieve element differentiation the nuclear envelope breaks open and the crystalloids are released into the cytoplasm, where the P-protein is also located. The possible similarity between the P-protein and the component of the crystalloids suggested an examination of the chemistry of the nuclear inclusions in Boraginaceae.

Crystalloids are common inclusions in plant cells and are generally assumed to be proteinaceous. To test this assumption with regard to the sieve element crystalloids in Boraginaceae we adapted a procedure (Behnke, 1975; D. L. Thomas, personal communication) for protein digestion of thin sections prepared for electron microscopy. The present paper reports on results obtained from protein digestion tests with
A. intermedia F. & M. and Echium judaeum Lacaita using three different proteolytic enzymes.

MATERIALS AND METHODS

Two species of Boraginaceae, Echium judaeum Lacaita and Amsinckia intermedia F. & M. were used. Echium plants were grown from seed in a greenhouse. Amsinckia material was collected from plants growing along the coastal cliffs at the University of California, Santa Barbara. Midveins of young leaves were excised into cold 4% glutaraldehyde buffered at pH 6.8 with 0.1M-sodium cacodylate. The material was fixed at room temperature under vacuum for 3 h and post-fixed in 0.5% osmium tetroxide in sodium cacodylate buffer overnight at 4°C. The tissue was dehydrated through a graded acetone series and embedded in Epon. Serial sections approximately 900 Å thick were mounted on nickel grids that had been coated with a thin film of Formvar. After drying overnight the grids were submerged in 10% hydrogen peroxide for 10 min at 38°C. The hydrogen peroxide is reported to de-osmicate the tissue (as cited by Bechtel & Pomeranz, 1981) and possibly increase the porosity of the tissue and embedding medium (Anderson & Andrè, 1968). After a brief wash in water the experimental sections were treated at 38°C with 0.2% aqueous protease, pH 7.6 (Protease Type VI, Sigma Chemical Company) for 1—6 h; 0.15—0.20% pepsin (Sigma Chemical Company, P-7000) in 0.1M-HCl for 4—9 h; or 0.3% aqueous trypsin, pH 8.0 (trypsin 1:250, GIBCO Laboratories) for 2—4 h. The grids were washed briefly in water and allowed to dry overnight. Sections were stained with uranyl acetate and lead citrate and viewed with a Siemens Elmiskop 101. Control grids containing serially cut sections of the same cells that were used for the digestion tests were run simultaneously with the substitution of water for the enzymes.

OBSERVATIONS

The digestibility of the nuclear crystalloids in Echium was tested in young sieve elements in which the inclusions were located in the nuclei (Figs 1, 3, 6, 7) and in older cells in which the crystalloids were free in the cytoplasm after nuclear breakdown (Fig. 4). In a young untreated sieve element the nucleus shows a tightly packed crystalloid and aggregates of dense chromatic material, and the cytoplasm contains a large mass of P-protein (Fig. 2). The section shown in Fig. 1, representing the same cell as Fig. 2 from a serially cut block, received a 2 h treatment with protease. The nuclear crystalloid was partially digested. The chromatic material in the nucleus and the mass of P-protein in the cytoplasm, as well as all other cellular components, were not affected by the enzyme treatment. Another section of the same cell from a serially cut block was treated for 4 h with protease and showed a completely digested nuclear...
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Figs 1-3
crystalloid (Fig. 3). No other cellular components were affected by the longer digestion procedure. The control section shown in Fig. 2 was subjected to the same digestion procedure as the section used for Fig. 1, but with omission of the enzyme (see Materials and Methods). Figs 1–3 strikingly suggest differential responses of proteinaceous cell components to the same digestion procedure: the crystalloid was digested, whereas all other proteinaceous entities remained intact.

Digestion of crystalloids with pepsin is illustrated for a cell in which the crystalloid was free in the cytoplasm after nuclear disintegration (Fig. 4) and a cell in which the nucleus was still intact (Fig. 6). After a 9 h digestion in pepsin the free nuclear crystalloid was almost completely digested except for a darkish fringe at its periphery (Fig. 4). The same cell in the control section showed an intact crystalloid (Fig. 5). In the mature sieve element illustrated in Figs 4 and 5, the P-protein was dispersed and formed a loose array of tubules in the cytoplasm. The P-protein showed no difference in appearance in the control and treated sections (Figs 4, 5). Thus, the P-protein was not digested in either the early form of massive protein body (Fig. 1, protease digestion) or in the late highly dispersed state (Fig. 4, pepsin digestion).

Treatment with pepsin of a sieve element in an early stage of differentiation with the crystalloid enclosed in an intact nucleus is shown in Fig. 6. The nuclear crystalloid was almost completely digested after a 6 h treatment with pepsin. A darkish fringe at the periphery of the crystal is apparent, although it is not as prominent as the fringe in Fig. 4. The increase in digestion time from 6 h (Fig. 6) to 9 h (Fig. 4) did not substantially alter the degree of digestibility of the crystalloid with pepsin.

Protein crystalloids free in the cytoplasm (mature cell, Fig. 4) and those contained within the nucleus (young cell, Fig. 6) were not expected to respond differently to the enzyme treatments. The tissue was thin-sectioned (900 A) to expose the crystalloids directly to the enzymes, thereby eliminating diffusion barriers such as cell walls, plasma membranes and nuclear envelopes.

Protease and pepsin are general proteolytic enzymes, while the third enzyme employed in our study, trypsin, is a more specific enzyme, catalysing the hydrolysis of peptide bonds whose carbonyl function is donated by either a lysine or an arginine residue (Lehninger, 1975, p. 106). The use of trypsin for 1 h in our digestion

**Figs 4, 6. Pepsin digestion. Fig. 5. Pepsin control.**

**Fig. 4. E. judaenum.** Part of a sieve element in a late stage of differentiation. The nuclear envelope has become disrupted and the crystalloid has been released into the cytoplasm. The tissue was treated for 9 h with 0.2% pepsin in 0.1M-HCl at 38°C. The nuclear crystalloid is almost completely digested except for a darkish fringe at its periphery. The P-protein (p) dispersed in the cytoplasm was not digested by the procedure. ×24000.

**Fig. 5. E. judaenum.** View of a section from a serially cut block containing the same sieve element as shown in Fig. 4. The control tissue was subjected to the same procedure as the section in Fig. 4 minus the enzyme. p, P-protein. ×24000.

**Fig. 6. E. judaenum.** Cross-section of a sieve element in an early stage of differentiation when the nuclear envelope is intact and the crystalloid is contained within its boundary. The tissue was treated for 6 h with 0.2% pepsin in 0.1M-HCl at 38°C. Almost complete digestion of the crystalloid has occurred and a darkish fringe at its periphery is apparent. ×15000.
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Fig. 7. Trypsin digestion. Fig. 8. Trypsin control.

Fig. 7. *E. judaeum*. Cross-section of a young sieve element treated with 0.3% trypsin (pH 8.0) at 38°C for 1 h. The nuclear crystalloid has been partially digested while the other cellular components are unaltered by the procedure. ×15,000.

Fig. 8. *E. judaeum*. View of a cell from the same block as shown in Fig. 7. The cell is at a slightly more advanced stage of differentiation. The control tissue was subjected to the same treatment as the section in Fig. 7 minus the enzyme. er, endoplasmic reticulum; m, mitochondrion. ×15,000.

The procedure resulted in a partial digestion of the crystalloid (Fig. 7). Figs 1, 4 and 7 reveal similar digestibility of the nuclear crystalloids with all three enzymes used. The control section for the digestion with trypsin taken from the same serially cut block

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Fig. 9, 11. Protease digestion. Fig. 10. Protease control.

Fig. 9. *A. intermedia*. Cross-section of a young sieve element treated with 0.2% protease (pH 7.6) at 38°C for 4 h. The dense crystalloid component has been partially digested while the loose component has not been altered. A small stack of endoplasmic reticulum (er) is present near the periphery of the cell. m, mitochondrion. ×28,000.

Fig. 10. *A. intermedia*. View of a section from a serially cut block containing the same sieve element as shown in Fig. 9. The control tissue was subjected to the same treatment as the section in Fig. 9 minus the enzyme. dc, dense component; lc, loose component. ×26,000.

Fig. 11. *A. intermedia*. Cross-section of a young sieve element treated with 0.2% protease (pH 7.6) at 38°C for 4 h. Several dense crystalloids are present in one nucleus and they have been digested by the enzyme treatment. The loose component remained intact. ×24,000.
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Figs 9-11
as the treated section was subjected to the same procedure but without the enzyme (Fig. 8).

In the genus *Amsinckia* the two forms of the nuclear crystalloid, lc and dc, responded differently to the enzyme treatments (Fig. 9). The dense crystalloid (dc) was partially digested after a 2 h protease treatment, while the loosely organized component (lc) was not affected by this procedure. Fig. 9 also shows that the stacked endoplasmic reticulum, a common feature of sieve elements in early stages of differentiation, was not affected by the digestion procedure. A section of the same cell from a serially cut block used for a control is shown in Fig. 10.

It is not uncommon to find more than one dense crystalloid per nucleus in *Amsinckia* (Fig. 11). This cell was treated with protease for 4 h. With this longer treatment (4 h instead of 2 h) the dense crystalloids were almost completely digested, whereas the loosely organized component persisted without alteration.

The two components of nuclear crystalloids in *Amsinckia*, although different in

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**Fig. 12.** *E. judaeanum.* Cross-section of a sieve element treated with 0.2% pepsin in 0.1 M HCl at 38 °C for 6 h. The sieve element was sectioned parallel with the surface of the sieve plate exposing the sieve plate pores. The P-protein plugging the pores was completely digested but the dispersed P-protein adjacent to the sieve plate was not digested. ×16,000.

**Fig. 13.** *E. plantagineum.* Cross-section of a sieve element to illustrate sieve plate pores plugged with P-protein. (We chose a section from a different species to illustrate plugging because our control for Fig. 12 was technically imperfect although it showed pores filled with undigested P-protein.) ×15,000.
form, could have been assumed to be of a similar proteinaceous composition. The digestion experiments, however, seemed to suggest a different composition as judged by the response to the enzymes. The protein digestion studies also failed to indicate a similarity between the dense crystalloids and P-protein, although both consist of tubular subunits (Esau & Magyarosy, 1979b), a characteristic macromolecular structure of proteins.

While studying the problem of the apparent differential digestion of obviously proteinaceous components, a rather unexpected observation on the behaviour of P-protein seemed to provide a solution. Some cross-sections of \textit{Echium} prepared for the protein digestion treatment contained a sieve element that was sectioned almost exactly parallel with the surface of the sieve plate and exposed the sieve plate pores. The pores were plugged with P-protein, a common feature of sieve elements in which the protoplast was disrupted during the fixation process (illustrated for a different species in Fig. 13). The P-protein plugging the pores was completely digested after treatment with protease for 4 h (Fig. 12) and with pepsin for 4 h (not illustrated). The dispersed P-protein adjacent to the sieve plate and free in the cytoplasm, however, was not digested (Fig. 12).

The digestion of P-protein plugging the sieve plate pores and the lack of digestion of the aggregated and dispersed P-protein (Figs 1, 4) seemed to indicate that the density or compactness of the proteinaceous inclusions may play a role in the differential response. If some proteinaceous cell components are so densely packed that the fixative (glutaraldehyde) and/or embedding medium (Epon) cannot penetrate them, they may be susceptible to digestion. Conversely, free penetration of fixative and/or embedding medium may make proteinaceous material inaccessible to enzyme digestion.

The dark fringe at the periphery of the crystalloid that was apparent in some instances after the digestion (Figs 4, 6) may represent a thin layer of proteinaceous material that was penetrated by the fixative and/or embedding medium and became inaccessible to the enzymic action.

**DISCUSSION**

Protein digestion techniques have been employed for many years for determining the composition of cellular structures that have been prepared for electron microscopy. The validity of these techniques, however, has been questioned by many workers (Anderson & André, 1968; Bechtel & Gaines, 1982; Behnke, 1975; Weintraub, Ragetli & Schroeder, 1971). Bechtel & Gaines (1982) listed several factors that may affect enzymic extraction of proteinaceous components, including: (1) exposing sections to the electron beam prior to extraction; (2) using osmium tetroxide as a fixative; and (3) using an embedding medium that penetrates the tissue so well that it prevents the enzyme from reaching the substrate. Behnke (1975) noted that the close packing of protein filaments into a crystal-like body probably inhibits the overall penetration of the embedding media and affords a larger surface area for enzymic digestion of sectioned material. Weintraub \textit{et al.} (1971) questioned the effect of...
aldehyde fixation on the digestibility of nuclear crystals in leaf cells of *Lychnis* and *Dianthus*. They assumed that the fixative was penetrating the crystalloids and as a result interfering with subsequent enzymic digestion procedures. Anderson & André (1968) suggested that routine fixation and embedding techniques prevent adequate penetration of enzymes and render the tissue components resistant to enzymic hydrolysis. This is not an irreversible effect, however, because the active sites can be enhanced by oxidizing the thin sections prior to enzyme treatment (Anderson & André, 1978; Marinozzi, 1961; Monneron & Bernhard, 1966). Complete reversal of the effects of fixation and embedding, however, do not seem probable in the light of the differential digestion of proteinaceous material reported in the present study.

In an attempt to determine the effect of the embedding medium on digestibility, Bechtel & Gaines (1982) removed the resin by floating sections mounted on Formvar-coated gold grids on 2-5% sodium ethoxide for 30 s. Removal of the embedding medium did not affect the digestibility of any components. Using another approach we attempted to determine the penetrability of the embedding medium in our tissue by subjecting 1 μm sections to Sudan Black B staining. Sudan Black B is taken up by structures embedded in plastic, while those that are not infiltrated by the plastic remain unstained. The nuclear crystalloids in both the sections treated with Sudan Black B and the control sections appeared dark when viewed with a light microscope under oil immersion. Because of this inherent density of the nuclear crystalloids, we were unable to determine whether penetration by the embedding medium had occurred.

Further studies are needed to determine the reliability of protein digestion techniques and to ascertain the effects of fixatives (glutaraldehyde) and/or embedding media (Epon) on the digestibility of proteinaceous components. In view of the important developments in the field of chemotaxonomy, the use of enzyme digestion techniques may become an important diagnostic tool.

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