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PURIFICATION OF A CELLULASE FROM KIDNEY BEAN ABSCISSION ZONES

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Abstract—The purification of a cellulase isoenzyme with a pI of 9.5 from kidney bean abscission zones is described. An important step in the purification involved the adsorption of the cellulase isoenzyme onto an affinity column of CF-11 cellulose and the subsequent elution with cellobiose. Native and SDS polyacrylamide gel electrophoresis established that there was only one component in the purified cellulase samples. Antibodies raised against the purified pI 9.5 cellulase precipitated this isoenzyme from crude or purified solutions but did not cross react with pI 4.5 cellulase from 2,4-D-treated abscission zones. The antibody was shown to be monospecific by immunoelectrophoresis and by the fact that it precipitated only a single ^{14}C-labeled protein from an abscission zone extract heavily labeled with ^{14}C amino acids.

INTRODUCTION

Abscission zones from kidney bean seedlings contain cellulase (EC 3.2.1.4) in at least three forms [1, 2]. An intracellular form with an isoelectric point (pI) of 4.5 seems to be present at all times and is increased by auxin treatment. An extracellular form with a pI of 9.5 is stimulated by ethylene during the abscission process [2] and correlates with the progress of abscission. A third form of cellulase is associated with the plasma membrane [3]. The relationship among these forms of cellulase, and their functions are presently unknown. Our goals have been (a) to purify the pI 9.5 cellulase to obtain a better understanding of its enzymology and then (b) to use this purified enzyme to produce antibodies which could be used to study the synthesis, degradation, transport and localization of the enzyme. In this paper, the purification of the pI 9.5 form of the enzyme is reported and the production and characterization of monospecific rabbit antibodies are described.

RESULTS AND DISCUSSION

Purification

The pI 9.5 cellulase isoenzyme was purified 580-fold to a final sp. act. of 105 000 units per mg of protein (Table 1). The major purification step was the use of a cellulose

Table 1. Purification of pI 9.5 cellulase

	Cellulase (Units)	Protein (mg)	Specific activity (U/mg protein)	% Recovery	Fold-purification
0.5 M NaCl extract	2 360 000	13 000	181		
40–65% $(NH_4)_2SO_4$ pellet	1 630 000	440	3700	69	20
1st cellulose column	815 000	13.5	60 400	35	330
Concentration	616 000	12.4	49 600	26	270
2nd cellulose column	536 000	5.1	105 000	23	580

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affinity column to adsorb the pI 9.5 cellulase, followed by elution of the enzyme activity with cellobiose. Passage of the enzyme through a second column not only increased the sp. act. but also eliminated many contaminating proteins (Fig. 1).

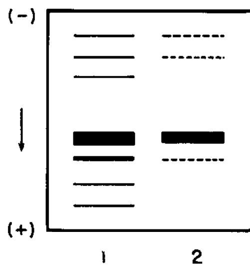

Fig. 1. A diagram of the SDS slab gel electrophoresis of purified pI 9.5 cellulase stained with Coomassie blue. Sample 1: 15 μg of cellulase after passage through one cellulose column. Sample 2: 10 μg of cellulase after passage through two cellulose columns.

The elution of the pI 9.5 cellulase from the cellulose column with cellobiose was successful, evidently because it acted as a substrate analog. In pea epicotyls [6], the auxin-induced cellulases needed a substrate of at least 3 hexose units in length for hydrolysis to occur. However, while cellobiose was not degraded, it did inhibit the activity towards carboxymethylcellulose, indicating an affinity of cellobiose for the enzyme. Here, no marked inhibition of pI 9.5 cellulase by cellobiose was observed. However, the cellobiose elution of the pI 9.5 cellulase clearly was specific for a particular protein in view of the purification achieved by this step.

It is likely that the purification procedure using the cellulose affinity column and cellobiose elution of the enzyme may be applicable to cellulases from other sources as well. In this laboratory, the technique has been successful for cellulase from ripening avocado. Often, the proper conditions of ionic strength and pH must be determined for adsorption of the enzyme to the column. We have also observed an affinity of bean pI 9.5 cellulase for a carboxymethylcellulose column matrix and, to some extent, for Sephadex gel.

The cellulose affinity column did not prove to be successful for the purification of the pI 4.5 cellulase. While a lower pH gave a selective adsorption of this isoenzyme to the column, the elution and recovery of adequate activity were not obtained, even though a variety of conditions were tried.

Purification of pI 9.5 cellulase

Purity of the pI 9.5 cellulase was demonstrated by polyacrylamide gel electrophoresis of the native enzyme in an acidic Reisfeld system [4] and in a SDS system [5]. The Reisfeld gel showed that the enzyme sample contained one protein-staining band which coincided with the extractable cellulase activity in the gel. The SDS gel (Fig. 1) showed one major protein band with a MW of *ca* 47 000 (three determinations) based on its position relative to ovalbumin (MW 43 000) and other MW standards. Isoelectric focusing showed that the purified cellulase contained only the pI 9.5 form of the enzyme, the form previously shown to increase after ethylene treatment [2].

Characterization of antibody

Antibodies raised in rabbits against this purified enzyme produced a single precipitin line on Ouchterlony plates and immunoelectrophoresis when both crude and purified cellulase samples were used. The ability of the antibody to precipitate the pI 9.5 cellulase was used to titer the serum; a value of 4900 cellulase units per ml of serum was obtained. To demonstrate the monospecificity of the antibody, a wide range of abscission zone proteins were labeled by inducing abscission in the presence of [^{14}C]-leucine. The antibody was allowed to react with a crude extract of these zones and the resultant precipitin complex was subjected to SDS gel electrophoresis. Only one radioactive band could be identified on these gels (Fig. 2), which indicated that the serum reacted with only one antigenic protein. The band corresponded to the 47 000 MW band of purified pI 9.5 cellulase. Therefore, the pI 9.5 cellulase preparation used in the production of the antibody contained no other antigenic protein in quantities sufficient to stimulate antibody production.

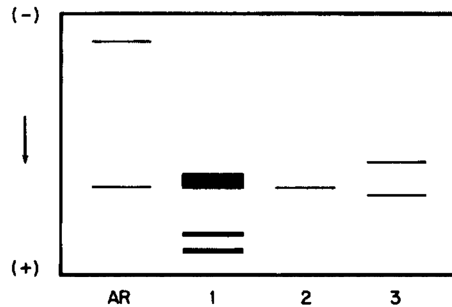

Fig. 2. SDS slab gel elctrophoresis of the immunoprecipitate from ^{14}C-labeled abscission zones. Sample 1: Coomassie blue staining of the immunoprecipitate obtained by reacting pI 9.5 cellulase antiserum with the supernatant II fraction from ^{14}C-labeled abscission zones. The antibody masked the position of the pI 9.5 cellulase. Sample 2: Purified pI 9.5 cellulase (2 μg). Sample 3: MW markers, ovalbumin (43 000) and bovine serum albumin (68 000). AR: An autoradiogram of sample 1. Labeled material appeared in a single band corresponding to the position of purified pI 9.5 cellulase (sample 2). An additional band of labeled material remained at the origin and did not migrate into the gel.

All attempts to demonstrate cross reactivity between the pI 9.5 cellulase antiserum and the pI 4.5 cellulase gave negative results. To make certain of this, we labeled the pI 4.5 form of the enzyme by inducing its formation with a 2,4-D-treatment of zones in the presence of [^{14}C]-amino acids. The serum was then added to the extracts but no precipitation was evident. To remove any traces of complex, goat anti-rabbit serum was added to precipitate all the rabbit IgGs. The absence of radioactivity in the resultant pellet and the absence of any detectable bands on autoradiograms of SDS gels confirmed that the pI 4.5 cellulase did not cross react with the antibody to pI 9.5 cellulase.

The lack of cross-reactivity between the pI 4.5 cellulase and pI 9.5 anticellulase is significant because it suggests no precursor–product relationship between the intracellular enzyme and the pI 9.5 extracellular enzyme. Therefore, the synthesis and secretion of the extracellular enzyme

evidently proceed via another pathway. It may be that the membrane-associated cellulase [3] plays a role in this regard.

With the antibody to the pI 9.5 cellulase now available, studies on the rate of enzyme synthesis, the secretion mechanism, the relationship to the membrane-associated cellulase, the localization of the pI 9.5 cellulase within the abscission zone itself, and the similarity to cellulase enzymes found throughout the kidney bean plant [7] can be carried out. In this way, we can attempt to directly assess the importance and involvement of this enzyme in the abscission process.

EXPERIMENTAL

Plant material. Seeds of *Phaseolus vulgaris* L. cv Red Kidney were germinated in a mixture of vermiculite and pea gravel (3:1) and grown for 10–12 days at 24° in the greenhouse. Plants were debladed by snipping off the leaf blade at the distal end of the petiole, leaving a very small amount of leaf tissue at the laminar abscission zone. Then plants were left in the flats and sprayed thoroughly with Ethrel 68–240 (Amchem Products Incorporated), at a conc equivalent to 7 mM (2-chloroethyl)phosphonic acid (CEPA) to increase the activity of the pI 9.5 cellulase. After 48 hr in the greenhouse, the laminar abscission zone including *ca* 2–3 mm of tissue above and below the zone was harvested.

Enzyme extraction. The abscission zone tissue was homogenized first in 0.02 M Tris buffer (pH 8.1) containing 3 mM EDTA to yield supernatant I, and then extracted with the same buffer containing 0.5 M NaCl giving supernatant II, the high salt supernatant. The extracts were filtered through a 50 μM mesh nylon cloth and centrifuged at 13 000 g for 20 min. All procedures were carried out at 0–4°.

Enzyme purification. Supernatant II containing the pI 9.5 cellulase was fractionated using $(NH_4)_2SO_4$ precipitation. The protein which precipitated between 40 and 65% satd with $(NH_4)_2SO_4$ was collected. Cellulase was quite stable as an $(NH_4)_2SO_4$ pellet and was stored at 0–4°. A cellulose affinity column (5 × 18 cm) was prepared from Whatman CF-11 cellulose powder and prewashed with column buffer (0.02 M Tris, pH 8.1, plus 0.1 M NaCl). The $(NH_4)_2SO_4$ pellet was applied to the column in column buffer, and washed with the same buffer until the A_{280} returned to the baseline. Cellulase was then eluted with 0.1 M cellobiose in column buffer. Fractions containing cellulase were precipitated with 85% $(NH_4)_2SO_4$ and chromatographed on a second CF-11 cellulose column.

Enzyme and protein assays. Cellulase activity was determined viscometrically by the method of ref. [1] and expressed as relative units. The substrate used was 1.25% carboxymethylcellulose, type 7H3SF (Hercules Corporation). Protein was determined by the method of ref. [8] using BSA as standard.

Antibody production. A young female white rabbit was used to produce antibodies. 250 μg of purified pI 9.5 cellulase was emulsified in Freund's complete adjuvant and injected directly into the lymph nodes of the hind legs. After 30 days the rabbit was boosted with 1 mg of pI 9.5 cellulase and subsequently with 50 μg injections as the titer fell off. Serum was collected starting 10 days after each boosting, checked on an Ouchterlony double diffusion plate [9] for antibody purity and titer, and pooled. The titer of the antibody was determined by running a series of dilutions of the enzymes with a constant amount of antiserum [10]. The cellulase conc at which the antibody no longer removed most of the activity from the supernatant was taken to be the titer of the serum.

Production of [^{14}C]*-cellulase.* The plant material was grown and explants were prepared as described in ref. [1]. The proximal ends of the explants were inserted into 1% (w/v) agar containing 1 mg/ml of chloramphenicol. To produce ^{14}C-labeled pI 9.5 cellulase, [U-^{14}C]-leucine was applied to the distal end of the explants in 10 μl droplets containing 1% (w/v) agar and 7 mM CEPA. The explants were incubated for 48 hr at 20–25° in a Plexiglass chamber which contained a 20% KOH trap to absorb evolved CO_2. The zones were harvested and the tissue homogenized as in the purification procedure. The supernatant II fraction containing the labeled pI 9.5 cellulase was used for immunoprecipitation.

To produce [^{14}C]-pI 4.5 cellulase, the same procedure was used, except that 23 μM 2,4-D was substituted for CEPA, a 90-hr incubation period was used, and intact plants were used instead of explants to reduce contamination. The labeled proteins were extracted as above except that the supernatant I fraction was used for the immunoprecipitation.

Immunoprecipitation of [^{14}C]*-cellulase.* The procedure essentially followed that of ref. [11]. The supernatant II fraction from ^{14}C-labeled plant material treated with CEPA was incubated overnight at 4° with pI 9.5 cellulase antiserum and unlabeled cellulase in 1% Triton X-100 and 1% deoxycholate. The immunoprecipitate was collected by centrifugation, washed 3 × in Pi-buffered saline, and further purified on a small 0.5/0.1 M discontinuous sucrose gradient. Unlabeled pI 9.5 cellulase was added to the immunoprecipitate and the mixture electrophoresed on an SDS polyacrylamide slab gel [5].

The same procedure was followed with pI 4.5 cellulase except that supernatant I was used. In case the pI 9.5 antibody was partially reactive with pI 4.5 cellulase, but did not pecipitate it, goat anti-rabbit serum was added to the mixture to precipitate the rabbit antibodies and any complexes that might have formed.

Autoradiography. The slab gel was stained with Coomassie blue, treated with PPO in DMSO according to the method of ref. [12], dried on Whatman 3MM filter paper, and exposed to Kodak XR-4 X-ray film for 2 weeks at 0°.

Electrophoresis. For immunoelectrophoresis, a 1.5% agar gel was prepared in Na barbitol buffer, pH 7.4 and ionic strength of 0.025. The electrophoresis buffer was the same but had an ionic strength of 0.05. The electrophoresis was run at 4 V/cm across the gel. After electrophoresis, the anti-cellulase rabbit serum was placed in the troughs and allowed to diffuse until precipitin lines were visible. The purity of the native enzyme was checked using cylinder gels and the acidic system of ref. [4]. Duplicate gels were either stained with Coomassie blue or sliced into 2-mm sections for cellulase extraction (using 0.1 M K Pi, pH 6.1) and assayed.

SDS polyacrylamide slab gel electrophoresis was conducted according to the method of ref. [5].

Isoelectric focussing. The isoelectric point determinations were made on an LKB 110 ml isoelectric focusing column using a pH 3.5–10 gradient, run at 300 V for 40 hr [13]. Fractions (3 ml) were collected, and the pH and cellulase activity determined in each.

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REFERENCES

1. Lewis, L. N. and Varner, J. E. (1970) *Plant Physiol.* **46**, 194.
2. Reid, P. D., Strong, H. G., Lew, F. and Lewis, L. N. (1974) *Plant Physiol.* **53**, 732.
3. Koehler, D. E., Leonard, R. T., VanDerWoude, W. J., Linkins, A. E. and Lewis, L. N. (1976) *Plant Physiol.* **58**, 324.
4. Reisfeld, R. A., Lewis, U. J. and Williams, D. E. (1962) *Nature* **195**, 281.

5. Conejero, V. and Semancik, J. S. (1977) *Phytopathology* **67**, 1424.
6. Wong, Y.-S., Fincher, G. B. and Maclachlan, G. A. (1977) *J. Biol. Chem.* **252**, 1402.
7. Lewis, L. N. and Koehler, D. E. (1979) *Planta* **146**, 1.
8. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
9. Ouchterlony, O. (1968) *Handbook of Immunodiffusion and Immunoelectrophoresis*, p. 21. Ann Arbor Science, Ann Arbor, Michigan.
10. Kabat, E. A. and Mayer, M. M. (1961) *Experimental Immunochemistry*, p. 69. Charles C. Thomas, Springfield, Illinois.
11. Taylor, J. M. and Schimke, R. T. (1973) *J. Biol. Chem.* **248**, 7661.
12. Bonner, W. and Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83.
13. Haglund, H. (1971) *Methods Biochem. Anal.* **19**, 1.