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Note

Isolation of calotropain FI

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The occurrence of a protease in the latex of the madar plant (*Calotropis gigantea*) and certain preliminary studies on its isolation and activation have been reported[1,2]. Similar work by Atal and Sethi[3], on the isolation of a protease (calotropain I) from *C. procera* latex, has shown it to be heterogeneous. Recently, we have reported the purification and properties of two proteases (calotropains FI and FII) from *C. gigantea* latex[4,5]. This paper describes the use of chromatography on IR-120 (Hg^{2+}) for the isolation and purification of calotropain FI.

EXPERIMENTAL

Enzyme preparation

Latex (1 ml) from *C. gigantea* (collected in glass containers during the early morning hours) was mixed with 0.01 *M* sodium phosphate buffer of pH 7.0 (9 ml), and after 20 min at 4°, the mixture was centrifuged for 10 min at 12 000 *g*. To the supernatant liquid (9.5 ml) 47.5 ml of chilled (—20°) acetone were added and, after 20 min at 4°, the precipitate formed was collected by centrifuging at 3000 *g*. This precipitate was treated with 9 ml of the 0.1 *M* sodium phosphate buffer, and any insoluble material was removed by centrifugation at 12 000 *g*.

Method of assay

Protease activity was assayed, with casein as substrate, according to a method used for papain[6], except that the buffer solution was 0.1 *M* sodium phosphate of pH 7.0. Protein was determined by the method of Lowry *et al.*[7], with bovine serum albumin as standard.

Chromatography on IR-120 (Hg^{2+})

All operations were carried out at 25°. A packed column (21 × 1.7 cm) of 25 g of Amberlite IR-120 (Na^+) was converted into the Hg^{2+} form and equilibrated with the sodium phosphate buffer as described previously[8]. Enzyme solution (9.0 ml) was allowed to percolate through the resin at 25 ml/h, and the column was then washed with the same buffer. Elution of the adsorbed enzyme/protein was carried out with 0.2 *M* ammonium acetate, followed by 0.1 *M* potassium iodide, or with a mixture of 0.002 *M* EDTA and 0.01 *M* sodium cyanide at a flow-rate of 50 ml/h; 25-ml fractions were collected.

Characterisation of the fraction

The purity of the fraction obtained as described above was independently checked by chromatography on CM-Sephadex CG-50, electrophoresis in polyacrylamide gel and N-terminal amino acid analysis.

Fractions having maximum enzyme activity were pooled, and the combined solution was made 2 m*M* in EDTA and 5 m*M* in cysteine, then dialysed at 4° against 0.02 *M* sodium acetate buffer of pH 5.0. The non-diffusible material was further characterised as follows.

(1) The activated enzyme solution was applied to a column (15 × 1.3 cm) of CM-sephadex CG-50 that had been equilibrated with 0.02 *M* sodium acetate buffer of pH 5.0, and the adsorbed enzyme was eluted, at 25°, with a stepwise-increasing concentration of sodium acetate buffer at pH 5.0; 10-ml fractions were collected at a flow-rate of 50 ml/h. A single peak for protein, coinciding with enzyme activity, was obtained, thus indicating the essentially homogeneous character of the material. The recovery, with respect to enzyme activity, was 82.5%.

(2) The purity of the fractions at each step was checked by electrophoresis (7.5% polyacrylamide gel) at pH 4.5 according to the method of Reisfeld *et al.*[9] (as adapted for papain[10] and chymopapain[8]) and at pH 7.5 as described by Gabriel[11]. Up to 30 μg of protein in a solution 2 m*M* in EDTA and 5 m*M* in cysteine was applied to each gel. After electrophoresis as previously described[8], the gels were stained with Coomassie brilliant blue and de-stained as recommended by Weber *et al.*[12]. At both pH values, the purified enzyme moved as a single band. At highly alkaline pH, the enzyme protein does not enter the gel, even by reversing the polarity.

(3) The activated enzyme was precipitated by adding 5 volumes of chilled (−20°) acetone, the precipitate was collected by centrifugation at 12 000 *g* and dissolved in 0.01 *M* sodium phosphate buffer of pH 7.2, and the enzyme in this solution was inhibited by addition of iodoacetic acid to give an effective concentration of 1 m*M*. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis of the inhibited enzyme was carried out by the method of Weber *et al.*[12]; electrophoresis was carried out for 3.5 h at 8 mA per tube, and a 10% gel was used.

(4) The N-terminal amino acid of the purified Calotropain FI was detected by the method of Sanger[13] as modified by Porter[14]; detection of the dinitrophenyl derivative of the amino acid obtained was as described by Levy[15].

RESULTS AND DISCUSSION

Precipitation of the crude enzyme by acetone resulted in an increase in specific activity from 5.5 to 6.5, with a 1.2-fold increase in purity; 90% of the proteolytic activity was recovered in this step (see Table III).

The enzyme obtained after acetone precipitation was completely retained on the IR-120 (Hg^{2+}) column (see Table I). One of the proteases, calotropain FI, could be eluted from the column with 0.2 *M* ammonium acetate (see Fig. 1 and Table II); ammonium salts, irrespective of the anion, were identical in their ability to elute this protease. Higher concentrations of ammonium salts failed to elute protease FII (calotropain FII), and attempts to elute this species with a mixture of 0.002 *M* EDTA and 0.01 *M* sodium cyanide or with 0.1 *M* potassium iodide resulted in complete loss of enzyme activity. However, quantitative recovery of the inactive protein was

TABLE I

RETENTION OF *CALOTROPIS GIGANTEA* LATEX PROTEASES AND PROTEINS ON AMBERLITE IR-120 (Hg^{2+})

Macromolecule	*Amount loaded*	*Amount retained*	*Percentage of total activity or protein retained*
Enzyme	162.5 units	162.5 units	100.00
Protein	25 mg	24.2 mg	96.80

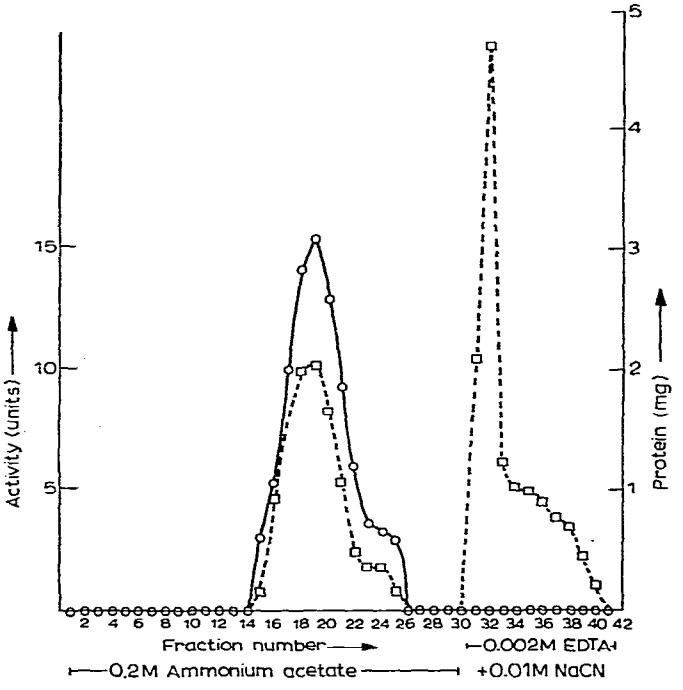

Fig. 1. Typical chromatographic profiles of *Calotropis gigantea* latex proteases on Amberlite IR-120 (Hg^{2+}) column. ○———○, Activity; □ - - - □ protein.

TABLE II

RECOVERY OF CALOTROPAIN FI FROM AMBERILTE IR-120 (Hg^{2+})

Macromolecule	*Elution with 0.2 M ammonium acetate*	*Elution with 0.002 M EDTA containing 0.01 M NaCN*	*Percentage recovery of retained activity or protein*
Enzyme	85.75 units	—	52.78
Protein	10.47 mg	13.32 mg	98.30

possible. All the eluted fractions contained Hg^{2+} ions (as estimated by the diphenylthiocarbazone method[16]). The ratio of protein content (mg) to Hg^{2+} content (mg) in the active fraction (fraction numbers 12, 14 and 15) was 8.8:1.

A 1.5-fold increase in purity is achieved for calotropain FI (see Table III).

TABLE III

PURIFICATION OF CALOTROPAIN FI BY CHROMATOGRAPHY ON AMBERLITE IR-120 (Hg^{2+})

Step No.	*Fraction*	*Enzyme activity (units)*	*Active enzyme protein (mg)*	*Specific activity**	*Purity***	*Percentage recovery of activity*
1	Crude extract	180	33	5.45	1	100
2	Acetone precipitate	162.5	25	6.50	1.2	90.25
3	IR-120 (Hg^{2+}) chromatography	85.75	10.47	8.20	1.5	47.60

* Units/mg of enzyme/min.
** Relative to crude extract.

The increase in purity attained for chymopapain B[17], ficin[18–20], stem bromelain[21] and papain[22] by earlier workers was also of this order. With a small amount of contaminating protein in the crude extract, the degree of purification obtained may not be great, yet the enzyme can be reasonably pure. In this connection, we must point out that the specific activity of purified calotropain FI (8.2 units/mg of enzyme/min at pH 7.0) is in good agreement with the specific activities reported for such similar enzymes as crystalline chymopapain A and papain[23] (5 and 10.11 units/mg of enzyme/min, respectively, at pH 7.2).

Calotropain FI obtained from the IR-120 (Hg^{2+}) column showed a single peak on chromatography on CM-sephadex CG-50 at pH 5.0 (see Fig. 2 and Table IV).

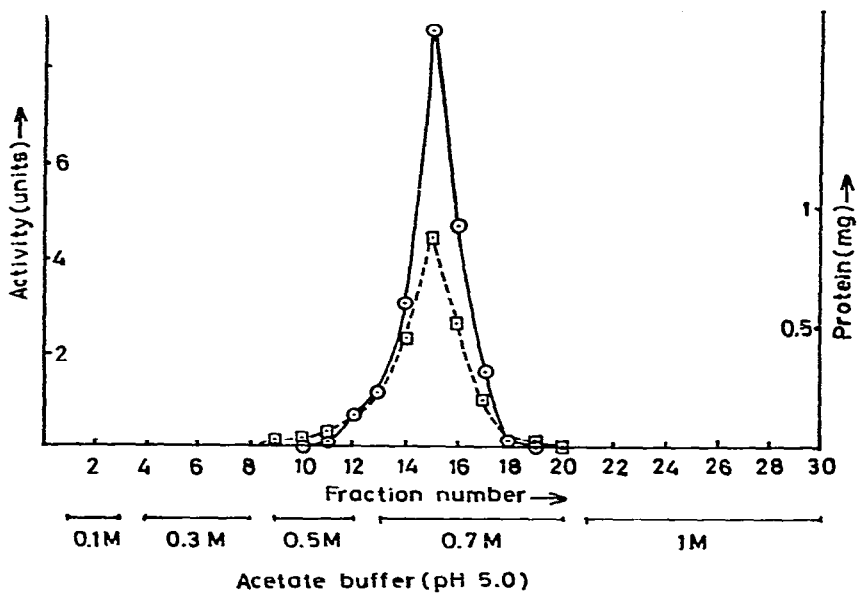

Fig. 2. Profile of calotropain FI from IR-120 (Hg^{2+}) column during further chromatography on CM-Sephadex CG-50. ⊙———⊙, Activity; ⊡- - -⊡ protein.

TABLE IV

RE-CHROMATOGRAPHY OF AMBERLITE IR-120 (Hg^{2+}) FRACTION (ACTIVE FRACTION; CALOTROPAIN FI) ON CM-SEPHADEX CG-50

Macromolecule	*Enzyme processed*	*Enzyme recovered*	*Percentage recovery of activity or protein*
Enzyme	24 units	19.8 units	82.5
Protein	3.63 mg	2.68 mg	73.7

It also yielded a single band on polyacrylamide gel electrophoresis at pH 4.5 and at pH 7.5, and on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (Fig. 3). A single N-terminal amino acid (glutamic acid) was detected for calotropain FI. Thus ,according to these criteria, the protease was homogeneous.

Amberlite IR-120 (Hg^{2+}) is thus suitable for the isolation of calotropain FI from *C. gigantea* latex.

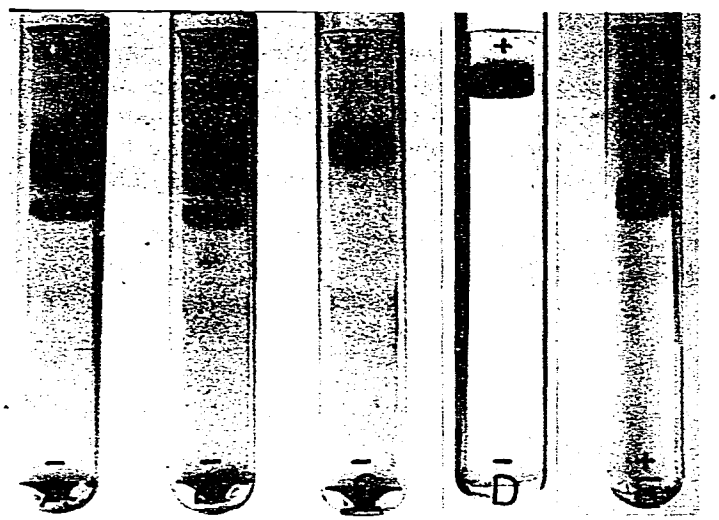

Fig. 3. Polyacrylamide gel electrophoretic patterns of purified calotropain FI from IR-120 (Hg^{2+}) column (unless otherwise indicated, electrophoresis was at pH 4.5 on 7.5% gel). A = Crude extract; B = acetone-precipitated enzyme; C = purified calotropain FI [IR-120 (Hg^{2+}) fraction]; D = purified calotropain FI at pH 7.5; E = purified calotropain FI (electrophoretic system 10% polyacrylamide gel–sodium dodecyl sulphate).

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