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STUDIES ON PROTEINASES FROM *CALOTROPIS GIGANTEA* LATEX

I. PURIFICATION AND SOME PROPERTIES OF TWO PROTEINASES CONTAINING CARBOHYDRATE

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Summary

Two proteinase containing carbohydrate, called calotropain-FI and calotropain-FII, were purified from *Calotropis gigantea* latex by CM-Sephadex C-50 chromatography.

Both calotropain-FI and FII were found to be homogeneous by rechromatography on CM-Sephadex C-50, gel filtration on Sephadex G-100, electrophoresis on polyacrylamide gel and by N-terminal amino acid analysis.

Some properties of these enzymes are reported.

Introduction

Several proteinases of plant origin have been characterised, including the latex proteinases [1—6]. Preliminary studies [7—9] have been made on the occurrence, isolation and properties of a proteinase from the latex of *Calotropis gigantea* and from *Calotropis procera*.

In the present study, we report the isolation, purification, characterisation and some properties of two proteinases which contain carbohydrate from *C. gigantea* latex.

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Material and Methods

CM-Sephadex C-50 and Sephadex G-100 (fine) were obtained from Pharmacia Fine Chemicals. Casein (vitamin free) and crystalline lysozyme (egg white) were products of Nutritional Biochemical Corporation. Casein was further purified as previously described [10]. Urea-denatured haemoglobin was from V.P. Chest Institute (New Delhi). Crystalline bovine serum albumin and ovalbumin were obtained from Sigma.

All the other chemicals and solvents were used of AnalaR grade. Water distilled in all-glass apparatus was used throughout.

Buffers

Buffers of sodium citrate (pH 3.0—5.5), sodium acetate (pH 5.0), sodium phosphate (pH 6.0—8.0) and glycine/NaOH (pH 8.6—10.5) of desired molarity were prepared as described [11].

Source of enzyme

Latex of *C. gigantea* plant, collected in the early morning hours in glass containers, was used as the enzyme source.

Assay of enzyme activity (Caseinolytic activity)

Caseinolytic activity was measured as described for papain [2] at 37°C, except the buffer used was 0.1 M sodium phosphate (pH 7.0).

One unit of enzyme activity is defined as the activity which produces an increase of 1 absorbance unit at 280 nm/min.

Estimation of protein

The protein content of the samples was estimated, at 660 nm on a Klett-Summerson colorimeter, according to Lowry et al. [12] using crystalline bovine serum albumin as standard.

Estimation of carbohydrate

The carbohydrate content of the samples was estimated at 500 nm on a Klett-Summerson colorimeter by the phenol/sulphuric acid method [13] with D-glucose as standard.

Purification of calotropain-FI and FII

Extraction of enzyme. Fresh *C. gigantea* latex was mixed with 0.01 M sodium phosphate buffer, pH 7.0 (resultant pH 6.1—6.2), kept at 4°C for 30 min and centrifuged at 12 000 × *g* for 10 min and the clear supernatant was collected. Extraction under these conditions was bound to yield maximum enzyme activity.

Precipitation with acetone and p-chloromercuribenzoate inhibition. Chilled acetone (—15°C) was added with stirring to the precooled extract (4°C) and the temperature of the mixture was kept at 4°C. After 15 min at 4°C, the precipitate was collected by centrifuging at 2800 × *g* and dissolved in 25 ml 0.01 M sodium phosphate buffer (pH 7.0). To this solution 1 mg neutral *p*-chloromercuribenzoate was added per 25 mg protein. After 30 min at 4°C, the solution was diluted to 125 ml with 0.02 M sodium acetate buffer (pH 5.0).

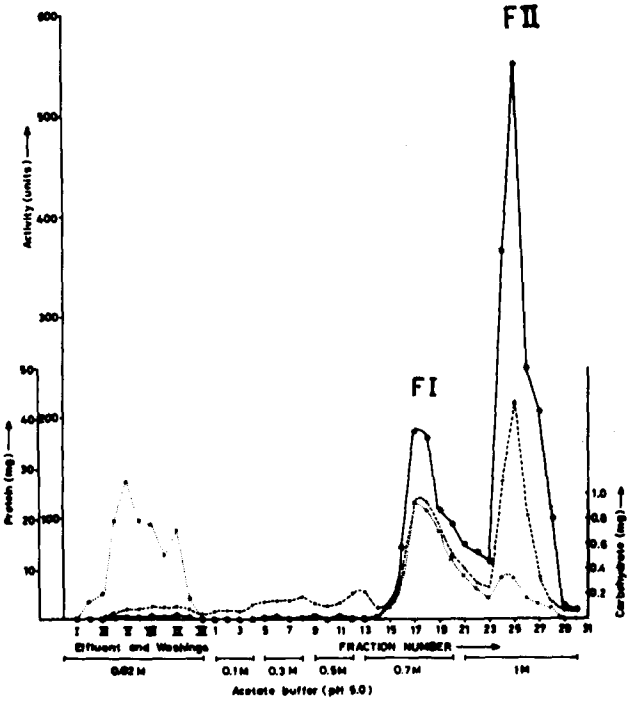

Fig. 1. Typical chromatographic elution profiles of *C. gigantea* latex proteases on CM-Sephadex C-50 column. ○———○, activity: ●- - - - -●, protein; ●· · · · ·●, carbohydrate. FI, calotropain-FI; FII, calotropain-FII.

The small precipitate formed was sedimented at 12 000 × *g* and discarded. The clear supernatant was used directly for the next step.

CM-Sephadex C-50 chromatography. CM-Sephadex C-50 (130 ml gel) was packed in a glass column (2.5 × 25 cm) and equilibrated in 0.02 M sodium

TABLE I

PURIFICATION OF *C. gigantea* LATEX PROTEINASES

Step of purification	Total activity (a) (units)	Total protein (b) (mg)	Total carbohydrate (mg/glucose equiv.)	Specific activity (a/b)	Purification (-fold)	Recovery of activity (%)
Crude extract	2535	455	62.5	5.57	1.0	100
Precipitation with acetone	2500	359.37	12.5	6.95	1.25	98.6
CM-Sephadex C-50 chromatography (total recovery)	2411.24	298.96	10.72	—	—	95.1
(a) Calotropain-FI (fraction Nos. 17 + 18 only)	187	23.52	0.95	7.97	1.43	—
(b) Calotropain-FII (fraction Nos. 24 + 25 only)	553.12	43.16	0.33	12.81	2.30	—

acetate buffer (pH 5.0). The enzyme, inhibited with *p*-chloromercuribenzoate, was applied to the column at 4°C (flow rate, 50 ml/h). After washing the column with 0.02 M sodium acetate buffer (pH 5.0), elution was carried out with a discontinuous gradient of 0.1—1 M sodium acetate buffer (pH 5.0). 25-ml fractions were collected and assayed for proteinase activity, protein and carbohydrate content (Fig. 1, Table I). The first peak is called calotropain-FI and the second peak, calotropain-FII.

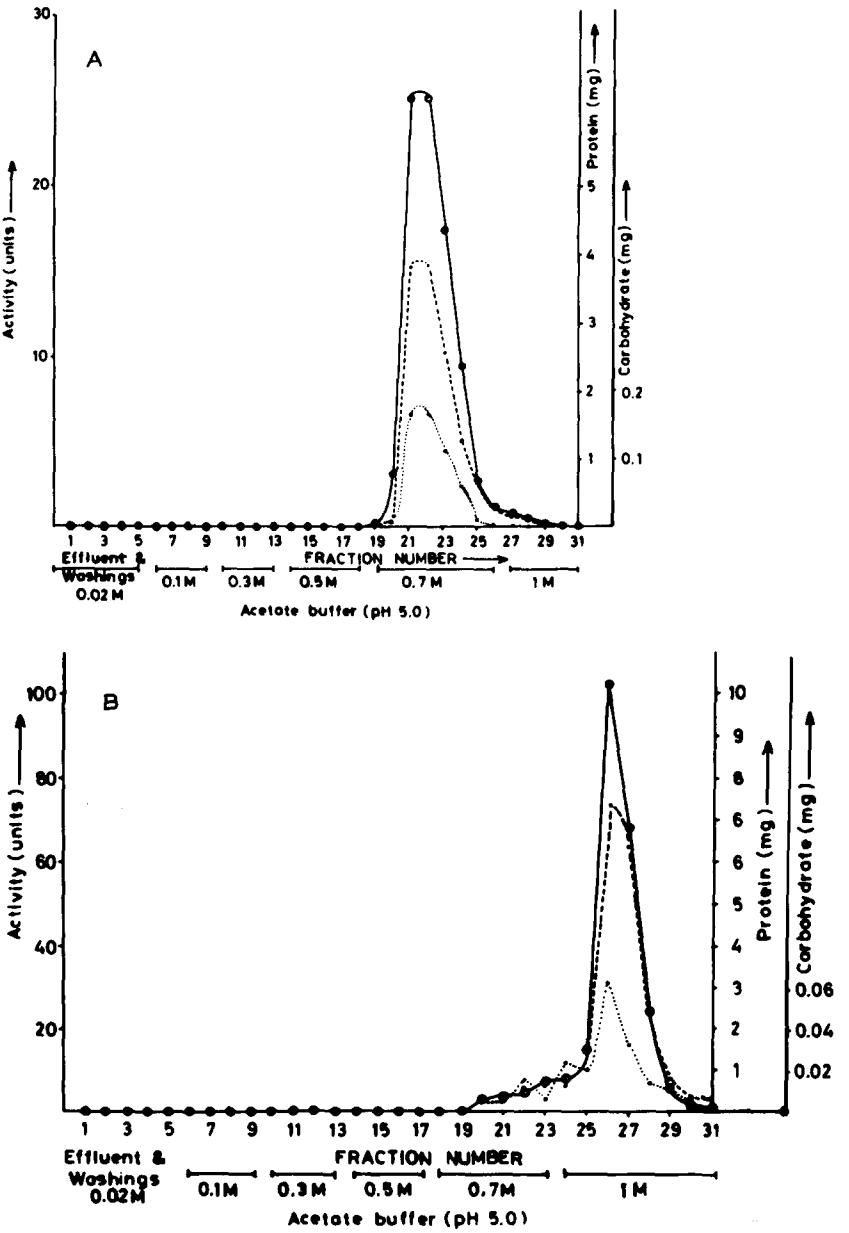

Fig. 2. Rechromatographic profile of (A) calotropain-FI and (B) calotropain-FII on CM-Sephadex C-50 column. ○———○, activity; ●- - - - -●, protein; ●· · · · ·●, carbohydrate.

Characterization of the fractions

Rechromatography on CM-Sephadex C-50. Fraction numbers 17 and 18 (calotropain-FI) and fraction numbers 24 and 25 (calotropain-FII) were separately dialysed against 0.02 M sodium acetate buffer (pH 5.0) at 4°C, and rechromatographed on two separate columns of CM-Sephadex C-50 (50 ml gel, 1.7 × 20 cm). 10-ml fractions were collected (Figs. 2A and B).

Gel filtration on Sephadex G-100. Fractions of calotropain-FI (fraction 17 and 18) and calotropain-FII (fraction 24 and 25), after extensive dialysis against 0.02 M sodium acetate buffer (pH 5.0) at 4°C, were precipitated by addition of 5 vols. chilled (−15°C) acetone. The precipitate formed was collected by sedimentation at 12 000 × *g* and dissolved in 0.1 M sodium phosphate buffer (pH 7.0) and chromatographed on two separate columns of Sephadex G-100 (100 ml gel, 2.3 × 24 cm) which was previously equilibrated with 0.1 M sodium phosphate buffer (pH 7.0). Elution was carried out with the same starting buffer (flow rate, 30 ml/h, 5-ml fractions, Figs. 3A and B).

Electrophoretic studies. The purity of calotropain-FI and FII were checked by polyacrylamide gel electrophoresis, at pH 4.3 as adapted for papain and chymopapain [10,14] (Fig. 4A) and at pH 7.5 as described by Gabriel [15] (Fig. 4B) and also in the presence of sodium dodecyl sulphate (SDS) [16] (Fig. 4C). All gels were stained with Coomassie brilliant blue and destained as described by Weber et al. [16].

N-terminal amino acid analysis. The N-terminal amino acids of calotropain-FI and FII were determined by the method of Porter [17]. The dinitrophenylated-amino acids were detected by the method of Levy [18].

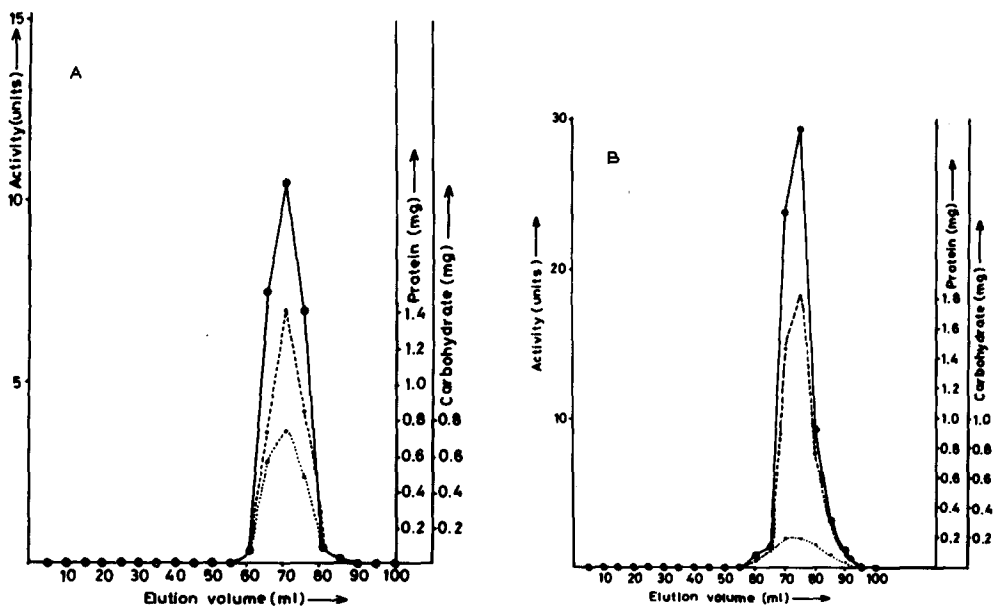

Fig. 3. Chromatographic elution profile of purified (A) calotropain-FI and (B) calotropain-FII on Sephadex G-100 column. ○———○, activity; ●- - - - -●, protein; ●· · · · ·●, carbohydrate.

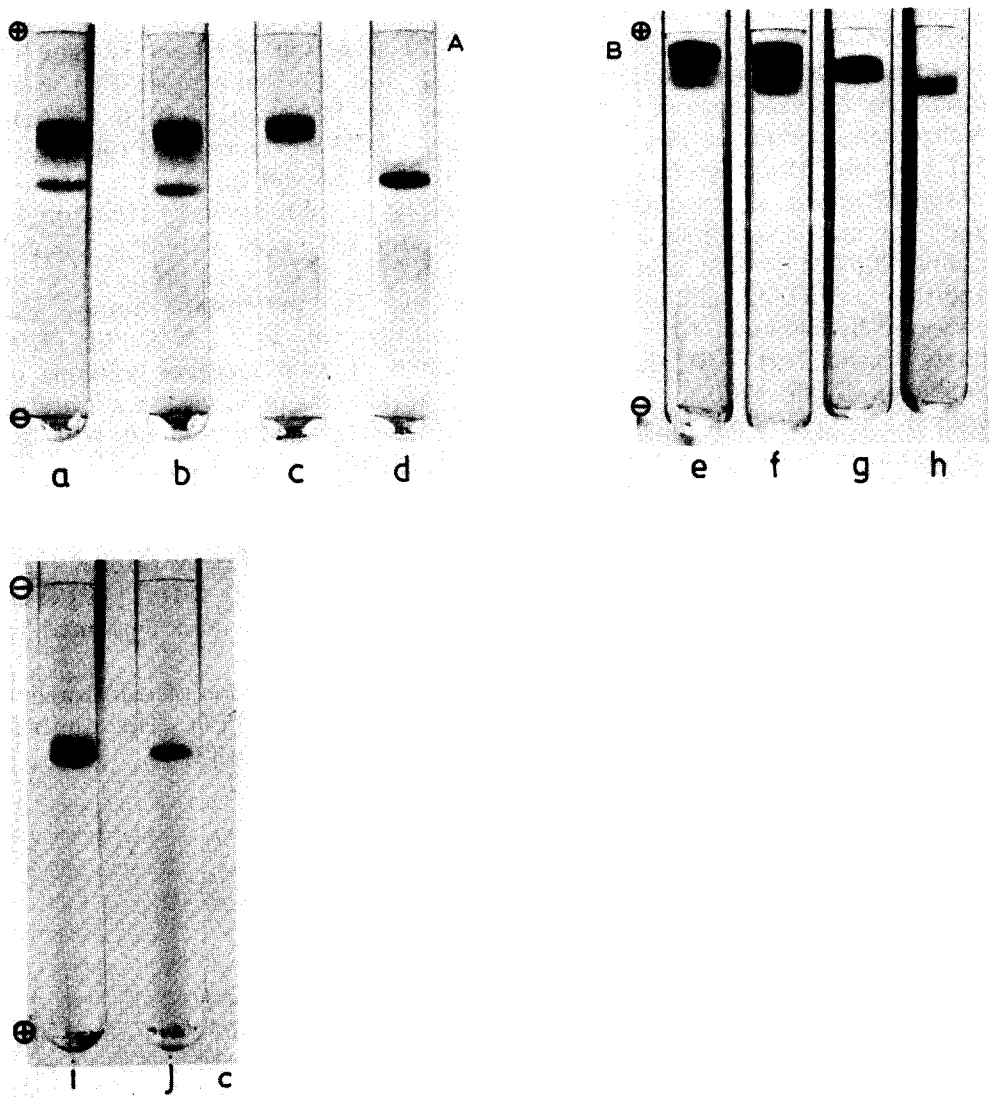

Fig. 4. Disc gel electrophoretic pattern of crude enzyme and purified calotropain-FI and FII. Electrophoresis at (A) pH 4.5, (B) pH (both at 7.5% acrylamide gel) and (C) at pH 7.0 in presence of SDS (10% acrylamide gel). a,e, crude latex extract; b,f, crude enzyme precipitated with acetone (*p*-chloromercuribenzoate inhibited); c,g,i, purified calotropain-FI; d, h, j, purified calotropain-FII.

Characteristics of calotropain-FI and FII reaction. The optimum pH, optimum temperature and heat stability of both the enzymes were studied using casein and haemoglobin as substrate.

The influence of urea concentration on the enzyme reaction was studied. Both enzymes, in 0.1 M sodium phosphate buffer (pH 7.0), were exposed to the desired urea concentration at 30°C for 1 h and assayed subsequently at 37°C. All solutions for the assay including casein, were prepared in corresponding urea solution (Table II).

TABLE II

PROPERTIES OF CALOTROPAIN-FI AND F-II

Property	Calotropain-FI	Calotropain-FII
Specific activity (units/mg) for casein substrate	7.97	12.81
Carbohydrate content (%)	4.04	0.76
N-Terminal amino acid	Glutamic acid	Glycine
Optimum pH		
Casein substrate	6.5	7.6
Haemoglobin substrate	4.35 and 8.1	4.25 and 8.1
Optimum temperature (°C) *	70	65
Temperature stability (°C) (15 min) *	65	60
Urea stability (8 M, 1 h at 30°C) *	Stable and fully active	Stable and fully active
Optimum ionic strength (*I*) (maximum velocity, pH 7.0 sodium phosphate buffer)	0.26	0.26

* The assay was carried out using casein substrate and the assay mixture contains an effective molarity of 0.1 M sodium phosphate buffer (pH 7.0).

Results and Discussion

Precipitation of the enzyme with acetone during purification removed 80% of the total carbohydrate present in the crude latex extract (Table I). Acetone-precipitated proteinase was inhibited by *p*-chloromercuribenzoate to avoid self-degradation, especially in the case of calotropain-FII, which is liable to auto-digestion. Precipitation with acetone resulted in 1.25 fold purification with 98.6% recovery of the enzyme. The *p*-chloromercuribenzoate-inhibited enzyme was 99% activated with the activating agent (an effective concentration of 0.005 M cysteine) and 0.002 M EDTA present in the assay mixture.

The adsorbed enzyme from CM-Sephadex C-50 was eluted as calotropain-FI and FII at a concentration of 0.7 and 1.0 M sodium acetate buffer, respectively. NaCl in the starting buffer could also be used for elution. In such cases calotropain-FI and FII eluted at 0.45 M and 0.65 M NaCl, respectively. Although complete adsorption of the enzyme over pH 5—7 was observed, the resolution of the two enzymes was satisfactory at pH 5.0.

The overall recovery of the enzyme activity after CM-Sephadex C-50 chromatography was 95.1%. The peak fractions of FI and FII had specific activity of 7.97 and 12.8, respectively, with a purification of 1.43 and 2.3 fold, respectively. The purity and the specific activity obtained in the present case are comparable with those reported for papain [2,19], chymopapain A and B [20,21], ficin [22—24] and stem bromelain [25].

Fractions FI and FII were found to have, respectively, 4.04% and 0.75% carbohydrate associated with the enzyme protein (Table I). The carbohydrate content did not significantly alter during rechromatography and gel filtration (Figs. 2A, B and 3A, B). Hence, both the enzymes seem to be glycoproteins. Precipitation of calotropain-FI and FII (*p*-chloromercuribenzoate inhibited) by concanavalin A and dissociation of the complex by addition of glucose in 0.1 M sodium phosphate buffer (pH 7.0) has also been observed, and is added evidence of the glycoprotein nature of both enzymes. Similar glycoprotein nature of plant proteinases were reported [22,26,27].

The peak fractions FI and FII were found to be reasonably homogeneous by rechromatography on CM-Sephadex C-50 (Fig. 2A and B) and by gel filtration on Sephadex G-100 (Fig. 3A and B). No sizable increase in specific activity was observed during these steps and the recovery of the enzyme activity units was 90—100%.

Fractions FI and FII showed a single band on polyacrylamide gel electrophoresis at pH 4.5, pH 7.5 and on SDS gel electrophoresis (Fig. 4A—C).

A single N-terminal residue, glutamic acid for calotropain-FI and glycine for calotropain-FII, was detected.

The broad pH optimum obtained (between pH 6.5—8.1 for calotropain-FI and pH 7.6 calotropain-FII) with casein as substrate, were in good agreement with the pH optima of other plant proteinases [20,22,28—30]. With haemoglobin as substrate, both enzymes showed double pH optima, one acidic (pH 4.35) and one alkaline (pH 8.10). Calotropin-FI showed higher activity at the acidic pH optimum compared to the alkaline one, whereas the reverse is the case with calotropain-FII. This double pH optimum for both the enzymes has also been observed with bovine serum albumin, ovalbumin and lysozyme substrates. Schwimmer [31] discussed the theory of the double pH optima and suggested three situations which can result in double pH optima. A fourth situation can arise as a result of an enzyme acting on a protein substrate attacking different loci at different pH values [29]. Double pH optima for other plant proteinases have also been reported [4,28,29].

The optimum temperature, of 70 and 65°C for calotropain-FI and FII, respectively, is in good agreement with those of other plant proteinases [22,32—34]. The heat stability of calotropain-FI (65°C) and calotropain-FII (60°C) (Table II) is comparable with the values reported for chymopapain A [20], ficin S [22] and stem bromelains [29,30].

Both enzymes were stable and fully active in 8 M urea. A rise in activity, (70% and 55%, respectively, for calotropain-FI and FII) in the presence of high concentrations of urea (4—8 M), was observed. The reasons for this increase in activity in urea solution are difficult to explain. Papain [35,36] was shown to retain its full activity in the presence of 8 M urea. Papain does not undergo any conformational change in urea solution as confirmed by optical rotation measurements [37].

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