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Purification and some properties of sodom-apple latex proteinases

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Two thiol-activated proteinases with isoelectric points (pIs approximately 9–9·5) were purified from sodom-apple latex by chromatography on Q-Sepharose and Superdex 200. Proteinase I had an estimated molecular weight of approximately 25 000 and proteinase II one of about 30 000. The proteinases degraded casein and azocoll, proteinase I having a lower specific activity than proteinase II. Proteinase I was most active at pH 8–10, with the optimum at about pH 8. Proteinase II was most active at pH 6–8, with the optimum at about pH 7.

INTRODUCTION

Sodom apple (*Calotropis procera*), a shrub that is widely distributed in West and East Africa and other parts of the tropics (Irvine, 1961), is a member of the plant family Asclepiadaceae and is botanically unrelated to the cultivated apple (*Pyrus malus*). The green, puffy inedible fruits are obliquely ovoid in shape when mature and have a thick spongy pericarp (Irvine, 1961).

Apart from being used as a milk coagulant in traditional cheese-making (Ogundiwin & Oke, 1983; Aworh & Nakai, 1986; Aworh & Muller, 1987), extracts from parts of the sodom apple were reported to be used for the dehairing of hides and treatment of various diseases (Dalziel, 1937; Irvine, 1961) These applications are presumably linked to the presence of proteinases in sodom-apple extracts (Aworh & Nakai, 1988). A low-molecular-weight (27 000–30 000), thiol-activated proteinase, with pI approximately 9, was recently partially purified from sodom-apple leaves (Aworh & Kasche, 1992). The enzyme degraded casein, gelatin, and denatured type 1 collagen (azocoll).

This paper reports on the purification and some properties of two thiol-activated proteinases from sodom-apple latex.

MATERIALS AND METHODS

Materials

Sodom-apple latex was collected by detaching the leaves from the stem at the petioles (Fig. 1). Q-Sepharose fast-flow and isoelectric-focusing calibration standards were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Azocoll and dithioerythritol were products of Sigma Chemical Co. (St. Louis, MO, USA). Pure casein was obtained from Serva Feinbiochemica (Heidelberg, FRG). Sodium tetrathionate was a product of ICN Biomedicals Inc, (Plainview, NY, USA). Bovine-serum gamma globulin was obtained from Bio-Rad Laboratories (Richmond, CA, USA). All other chemicals were of analytical reagent grade.

Stabilisation of latex

Crude sodom-apple latex was centrifuged at 4°C (13 000 × *g*, 20 min). Enzyme activity in 10 ml of the resulting supernatant (pH 5·1) was reversibly inhibited by the addition of sodium tetrathionate to a final concentration of 10mM. After centrifugation, the precipitate was discarded, and the supernatant (9·6 ml) containing the tetrathionate-modified crude enzyme was collected.

Ion-exchange chromatography

The tetrathionate-stabilised latex was dialysed extensively at 4°C against 20mM ethanolamine buffer pH 9·4, containing lmM sodium tetrathionate. The dialysed solution (2 ml) was applied to a Q-Sepharose column (2·5 × 8·0 cm), equilibrated with the same buffer. Elution (0·46 ml/min at room temperature) was first with the pH-9·4 buffer (207 ml), followed by a linear gradient of 0–0·32M NaCl. Fractions were collected at 10-min intervals. Enzyme activity was eluted in two peaks (Fig. 2) and the active fractions making up each peak were pooled.

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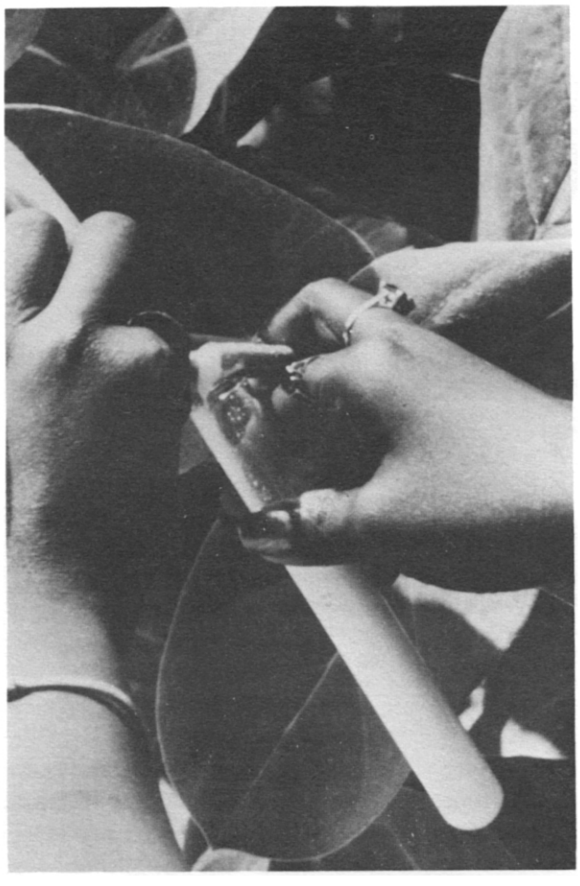

Fig. 1. Collection of sodom-apple latex.

Gel filtration

After concentration to 2 ml by ultrafiltration (10-kDd omegacell membrane from Filtron Technology Corporation, Northborough, MA, USA), the pooled fractions from ion-exchange chromatography were applied to an FPLC Superdex 200 (fractionation range 5000–600 000) Hiload column (1·6 × 60 cm) equilibrated with 0·05M sodium phosphate buffer, pH 7·1, containing 0·1M NaCl, 1mM sodium tetrathionate and 1mM EDTA. During elution (0·5 ml/min at room temperature fractions were collected at 5-min intervals. For molecular-weight estimation, the gel-filtration column was calibrated with the following markers: bovine-serum albumin (67 000), ovalbumin (43 000), chymotrypsinogen (25 000), myoglobin (17 800), and cytochrome c (12 500).

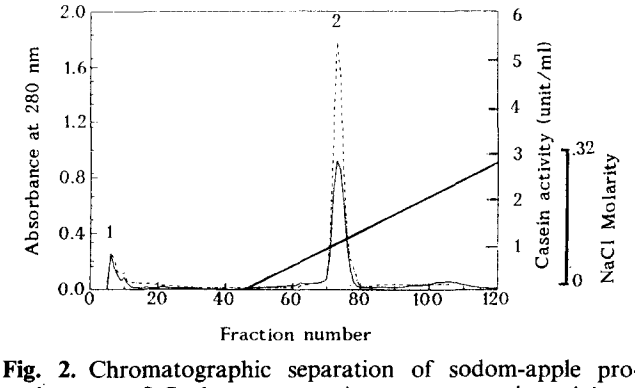

Fig. 2. Chromatographic separation of sodom-apple proteinases on Q-Sepharose. —— A_{280}; - - - - casein activity.

Isoelectric focusing

Purification was monitored by isoelectric focusing in 125 × 125-mm (150-μm thickness) Servalyt Precote, pH 3–10, polyacrylamide gels (Serva Feinbiochemica, Heidelberg, FRG). Electrofocusing was run for 3 h at 200 V (initial)–1000 V (final). The pH profile was determined in the gel with a Hamilton single-pore surface electrode and by cutting the gels into small pieces, macerating in distilled water, and determining the pH and proteinase activity. In addition, the Pharmacia IEF calibration kit was used for pI markers. Gels were stained with Coomassie brilliant blue R-250.

Electrophoresis

Purification was also monitored by sodium dodecyl sulphate-polyacrylamide–gel electrophoresis (SDS-PAGE) (Laemmli, 1970). A 5% stacking gel and a 10% running gel were used. The sample buffer included 5% (v/v) dithioerythritol, and samples were boiled for 5 min before electrophoresis to dissociate the proteins. Staining was with Coomassie brilliant blue R-250.

Assay of enzymatic activity

General proteinase activity was determined on casein and azocoll substrates. Casein-digestion activity was determined at 65°C by the method of Kunitz (1947) with some modification (Aworh & Nakai, 1988). The reaction medium consisted of a total volume of 2·0 ml of 0·1M sodium phosphate buffer (pH 8·0) containing 1–20μl of enzyme sample, 0·5% casein, 2mM EDTA, and 10mM cysteine as activator. After incubation at 65°C for up to 10 min, the reaction was terminated by the addition of 3 ml of 5% trichloroacetic acid (TCA), and the resultant precipitate was removed by centrifugation after standing for 1 h. Blanks were prepared for each sample in a similar manner, except for the addition of TCA to the enzyme solution before mixing it with the casein. A unit of enzyme activity was defined as that which caused an increase of 1 absorbance unit at 280 nm/min.

The azocollhydrolysis assay was based on the method of Chavira *et al.* (1984). Enzyme solutions (5–20 μl) were added to 2·5 ml prewashed azocoll suspension (10 mg/ml) in 0·1M acetate buffer (pH range 4–6) or 0·1M Tris-HCl buffer (pH 7–10) containing 2mM EDTA and 10mM dithioerythritol as activator. After incubation with agitation (330 r/min) at 50°C for 10 min, the reaction was terminated by rapidly drawing the suspension into disposable plastics syringes and filtering through Schleicher & Schuell (Dassel, FRG) disposable filter holders (0·2 μm). The absorbance at 520 nm of the clear filtrate, freed from insoluble azocoll, was read against buffer. Blanks, containing azocoll but no enzyme, were prepared in a similar manner.

Protein determination

Protein was determined by the dye-binding method (Bradford, 1976), with bovine-serum gamma globulin as a standard.

RESULTS AND DISCUSSION

Purification of sodom apple latex Proteinases

Crude sodom-apple latex showed marked proteinase activity on casein and azocoll substrates. The addition of 10mM sodium tetrathionate completely inhibited proteinase activity, which was fully restored by the addition of 10mM cysteine and 2mM EDTA to a solution containing the inactivated latex at a concentration of 20 μg protein/ml. This observation is consistent with the results of an earlier study (Aworh & Nakai, 1988) and supports the hypothesis that a free sulphydryl group is required for the proteinase activity.

Figure 2 shows a typical elution profile of enzyme activity on Q-Sepharose. Two proteolytic peaks were separated. Peak 1 did not bind to the column at pH 9·4 and eluted before the NaCl gradient began. Peak 2 was eluted at about 0·1M NaCl. On further purification of peak 1 on Superdex 200, enzyme activity was eluted in one peak (Fig. 3). Peak 2 also eluted as a single peak on Superdex 200 (Fig. 4). The active fractions of which each peak was composed were pooled and concentrated as sodom-apple latex proteinase I and II, respectively.

Some properties of sodom-apple latex Proteinases

Electrofocusing with pH 3–10 ampholytes showed that the enzymes have pIs of approximately 9–9·5, proteinase I having a higher pI than proteinase II (Fig. 5). The exact pIs would have to be determined with a narrower ampholyte range.

Proteinase I produced a single band on SDS-PAGE at an approximate relative molecular weight of 25 000 (Fig. 6). Proteinase II, which was apparently homogeneous on isoelectric focusing (Fig. 5) produced two protein bands on SDS-PAGE at an approximate relative molecular weight of 30 000 (Fig. 6), consistent with the value of 29 000 estimated from its elution volume

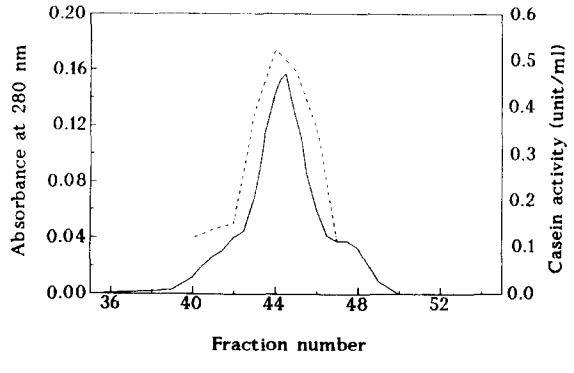

Fig. 3. Gel filtration of peak 1 from ion exchange on Superdex 200. —— A_{280}; - - - - casein activity.

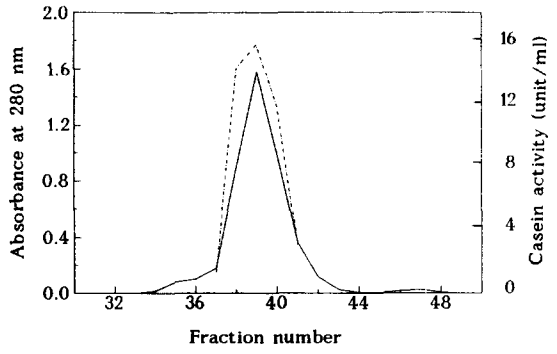

Fig. 4. Gel filtration of peak 2 from ion exchange on Superdex 200. —— A_{280}; - - - - casein activity.

on Superdex 200. The doublet nature of the main band of proteinase II could be indicative of non-identical subunits or different isoenzymes as is the case with papaya proteinases, where different isoenzymes, with relative molecular weight of 23 000–28 000, have been identified (Goodenough & Owen, 1986).

pH-activity profiles of sodom-apple proteinases on azocoll substrate (not presented) show that the enzymes had little activity at pH 4. Proteinase I was most active at pH 8–10, with the optimum at about pH 8. Proteinase II was most active at pH 6–8, with the optimum

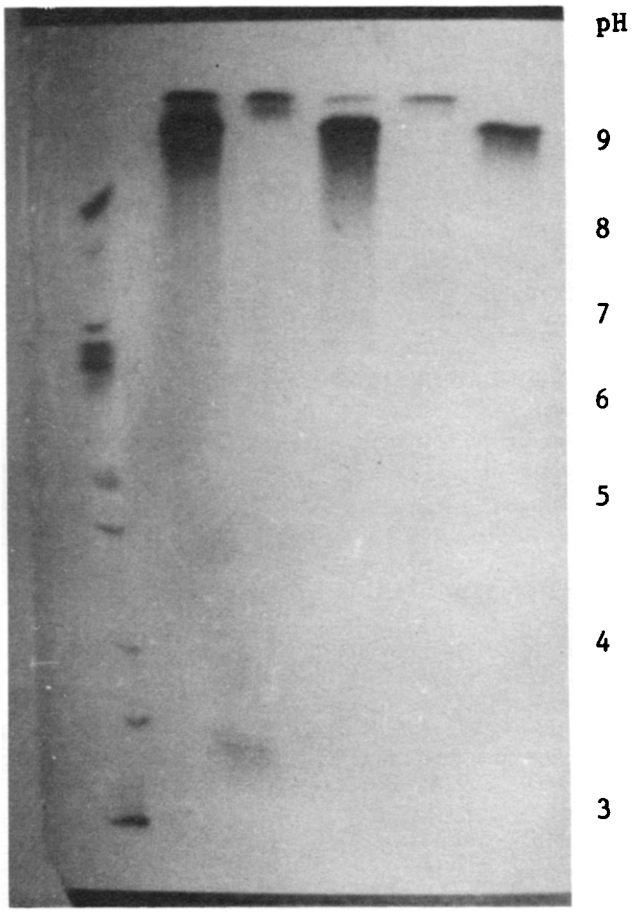

Fig. 5. Isoelectric focusing of pooled fractions from purification steps on Servalyt Precote, pH 3–10, polyacrylamide gels. From left to right: Lane 1, Pharmacia pI standards; lane 2, tetrathionate—stabilised crude latex; lane 3, peak 1 from ion exchange; lane 4, peak 2 from ion exchange; lanes 5 and 6, sodom-apple latex proteinases I & II.

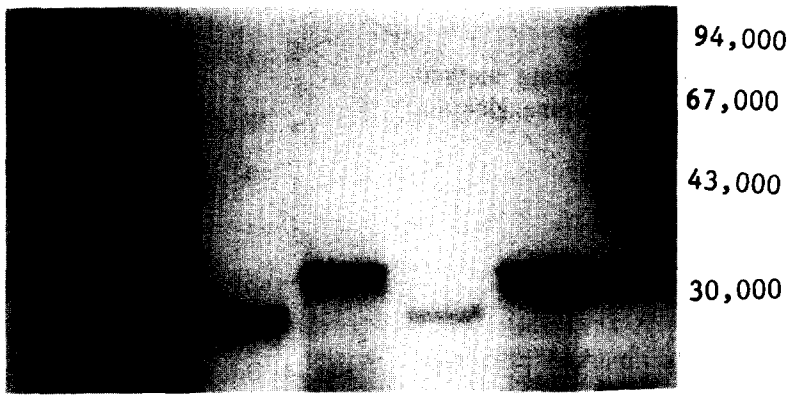

Fig. 6. SDS/polyacrylamide-gel electrophoresis of pooled fractions from purification steps. From left to right: Lane 1, MW standards; lane 2, tetrathionate-stabilised crude latex after dialysis; lane 3, peak 1 from ion exchange; lane 4, peak 2 from ion ex change; lanes 5 and 6 sodom-apple proteinases I and II; lane 7; MW standards.

at about pH 7. The enzymes are similar to papaya proteinases in optimum pH (Ebata & Yasunobu, 1962; Arnon, 1970).

The biochemical properties of sodom-apple proteinases determined in this study tend to support the hypothesis (Aworh & Nakai, 1988) that these enzymes belong to the group of cysteine proteinases (EC 3.4.22). These endoproteinases require a free sulphydryl group for activity and are characterised by an essential cysteine at the active site, low relative molecular weight (20 000–35 000), pI (8–11), and optimal pH of 6–8 (Ebata & Yasunobu, 1962; Murachi *et al.*, 1964; Englund *et al.*, 1968; Goodenough & Owen, 1986). Studies are in progress on further characterisation of sodom-apple latex proteinases, including their specificity on a number of synthetic peptide substrates. These would allow a more informed comparison to be made of the enzymes relative to other thiol proteinases from higher plants.

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