Notes

CHROM. 3478

Column chromatography of papaya proteinases on hydroxylapatite

The first reported attempt at purification of the enzymes in the papaw (*Carica papaya*) was in 1879 by alcoholic precipitation of the protein components[1], but it was nearly sixty years later that a papaya enzyme was first crystallized[2]. This paper describes a method of preparing papain (EC 3.4.4.10), one of several proteinases present in the latex, by direct column chromatography without preliminary separation or concentration techniques. How temperature affects the resolution of the separation may be judged from chromatograms at 24° and 10°.

Experimental

Choice and preparation of the adsorbent. Comprehensive studies by TISELIUS *et al.*[3] on the use of various calcium phosphates as adsorption media in protein work, pointed to a possible approach by using hydroxylapatite for separating the papaya enzymes. The apatite was prepared as described[3], except that, when the sodium hydroxide is added to the brushite, the suspension is heated on a boiling water-bath without stirring. Apart from gentle mixing by rotating the flask used in the preparation, vigorous stirring was avoided at all stages to preserve the granular form of the apatite and ensure adequate flow rates. JENKINS[4] proposes the use of a tilting steam-jacketed kettle for heating, but this is costly equipment if only small batches of the adsorbent are required.

The partition coefficient of papaya extract in equilibrium with hydroxylapatite at various phosphate buffer concentrations was determined at pH 7.0 and 24°. The

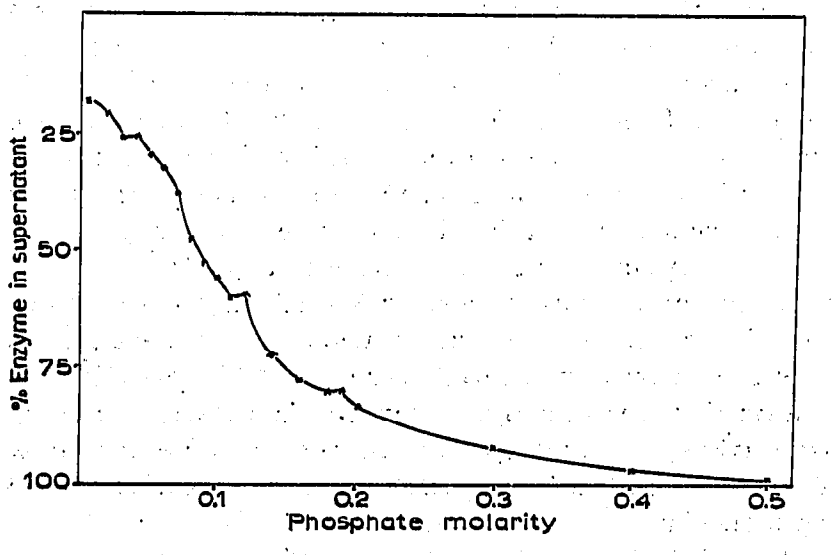

Fig. 1. Partition coefficient for the system: Papaya extract/phosphate buffer/hydroxylapatite.

curve in Fig. 1 shows three plateaux corresponding with critical buffer concentrations required for elution of the components.

In preparative work, columns 2 cm in diameter and 35–40 cm long were found suitable. To a water-jacketed column with a sintered glass plate, a small quantity of clean sand was added, the column was half-filled with 0.01 *M* buffer, and the apatite sludge was poured in. The adsorbent was allowed to pack itself by settling above the sand layer, the column being occasionally tapped and twisted to avoid furrowing.

Preparation of the sample. The use of freshly-bled latex facilitated the extraction procedure: a quantity of fresh latex was mixed with about five times its weight of 0.01 *M* phosphate buffer, pH 7.0, containing glutathion to a concentration of 0.005 *M*. The mixture was stirred for an hour at 20° and then filtered through a 1 cm layer of Hyflo Supercel on a Büchner funnel; the insoluble residue was re-extracted with half the volume of buffer used in the first extraction, and the combined filtrates were kept at 0°.

Eluting agents. Sodium phosphate buffers, pH 7.0, were used throughout. Stepwise elution was found preferable, using successively 0.01, 0.09, 0.15 and 0.40 *M* buffers. (Concentration gradients were avoided so as not to incur the risk of overlapping of the components during elution).

Column. The load applied to a 35 × 2 cm column was 400 mg protein, and adequate flow rates of 8–9 ml/h. were obtained when chromatography was carried out with a 50–60 cm hydrostatic head of pressure.

Fractions. The optical density (absorbance) of each fraction was determined at 280 mμ using a 0.5 cm quartz cuvette; the fractions were obtained using a fraction collector with timing unit. Micro-Kjeldahl analyses were carried out for protein nitrogen and extinction coefficients.

Proteolytic activity. The activities of papaya proteinases were determined with benzoylarginineamide as substrate, in the presence of glutathion, the specific activity, C_1, being defined as K_1 (first order reaction velocity constant) per mg protein N per ml reaction mixture.

Results

The chromatogram of a latex extract at 24° is shown in Fig. 2. Peak A consists of enzymically inactive nitrogenous substances. Peak B is papain; the crystalline enzyme may be prepared from these fractions. The composite nature of peak C is apparent, and may possibly be the result of mutual displacement effects; proteolytic activity, due to chymopapain, increases progressively as successive C2 fractions are eluted, while lysozyme activity in peak C1 decreases as further fractions emerge from the column. Peak D, which is a proteinase strongly adsorbed by the apatite, requires high phosphate concentrations for its desorption, and exhibits high activity.

However, the susceptibility of papaya proteinases, in solution, to autolysis, and also partial loss of activity if subjected to temperatures above 20° for extended periods, made the use of lower temperatures in preparative work imperative to maintain activity levels. The corresponding chromatogram at 10° is shown in Fig. 3.

As a result of the lower temperature, the multiple components in Peak C now emerge unseparated from the column, and "tailing" is marked but cannot be avoided without risk of eluting peak D prematurely.

Pure papain may be prepared by the above chromatographic method at 10°;

re-chromatography using the same adsorbent results in a single symmetrical peak, all fractions having identical specific activities ($C_1 = 1.2$). These fractions were pooled, dialysed against 0.01 *M* phosphate buffer, pH 7.0, and concentrated *in vacuo* in the

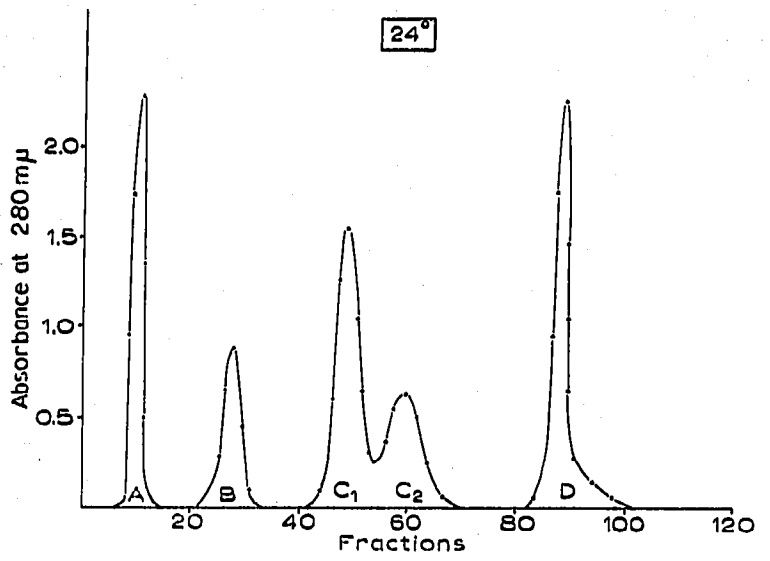

Fig. 2. Chromatogram of fresh crude papaya latex fractionated on hydroxylapatite column, 35 × 2 cm, at 24°; elution was effected with sodium phosphate buffers, 0.01 *M*, 0.09 *M*, 0.15 *M* and 0.40 *M* for peaks A, B, C and D, respectively.

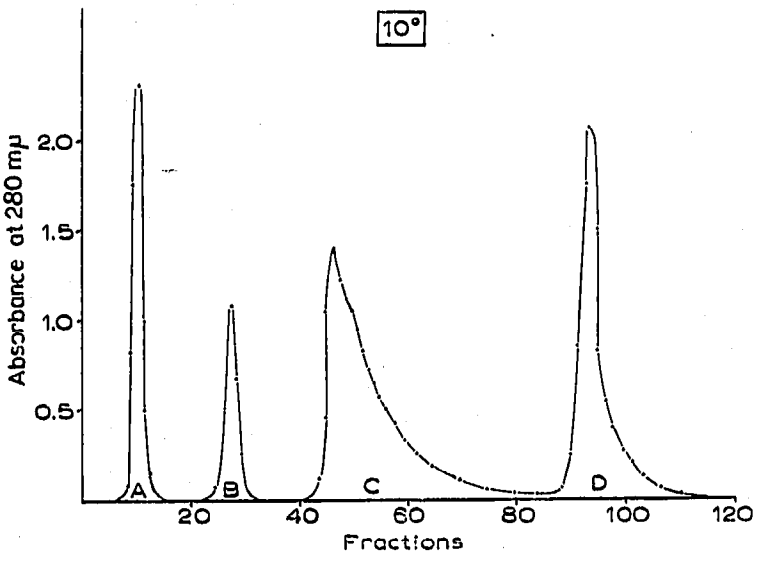

Fig. 3. Chromatogram of fresh crude papaya latex fractionated on hydroxylapatite column, 35 × 2 cm, at 10°; elution was effected with sodium phosphate buffers, 0.01 *M*, 0.09 *M*, 0.15 *M* and 0.40 *M* for peaks A, B, C and D respectively.

cold to one-quarter of the original volume; glutathion was then added to a concentration of 0.01 *M*, and sodium chloride was then added slowly with stirring to 40% saturation; the solution was left for 12 h at 4°, and after this time, the fine needles were centrifuged off in the cold. No increase in specific activity was obtained by crystallisation. Activity tests did not reveal any denaturation of the papain as a result of chromatography on hydroxylapatite.

The extinction coefficient, $E^{1\%}_{1cm}$ at 280 mμ was measured for papain prepared by re-chromatography on hydroxylapatite, and found to be 22.4, in good agreement with the value of 24 obtained by the checkers of the method of papain preparation described by KIMMEL AND SMITH[5].

Chymopapain may be prepared from the fractions in peak C by following the procedure of EBATA AND YASUNOBU[6]; studies on the proteinase in peak D are being carried out currently in these laboratories.

Remarks

The chromatographic method described is suitable for the isolation of pure papain, and this method may be preferred for preparing small quantities of the enzyme, being more direct and rapid than the salting-out procedure of KIMMEL AND SMITH[5]. The adsorbent is inexpensive to prepare, and even if purchased, is less costly than ion-exchange resins.

No artifacts were encountered with the buffer concentrations used; on re-chromatographing separately the proteinases in peaks B and C, the same buffer concentrations as used initially were required; the enzyme in peak D, when subjected to a second chromatography, could be eluted at a slightly lower buffer concentration, but "tailing" is avoided when the higher concentration is used.

Comparison of the chromatograms at 24° and 10° shows that the tendency for resolution of the two components in peak C is compromised by lowering the working temperature, necessary to protect the activities of the three proteinases. Fractions from the composite peak may, however, be used to prepare chymopapain by another method.

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