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SEPARATION OF CHYMOPAPAIN FROM PAPAYA LATEX (*CARICA PAPAYA*) ON AMBERLITE IR-120 (Hg^{2+})

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SUMMARY

The quantitative separation of chymopapain from papaya latex has been carried out by chromatography on Amberlite IR-120 (Hg^{2+}). The product obtained was further studied to determine its homogeneity.

INTRODUCTION

Several methods exist for the purification of enzymes and proteins. Over the last 25 years chromatographic procedures involving the use of modified cellulosic ion exchangers[1,2], or weak cation exchangers such as Amberlite IRC-50 have been developed[3]. Use of Sephadex or Bio-Gels of assorted grades, which serve as molecular sieves, have further aided purification. Sophisticated electrophoretic techniques permit characterization and can be scaled up for preparative purposes. As a result of these improvements, a number of enzymes have been obtained in a highly purified, homogeneous crystalline state.

However, the resolution of a naturally occurring complex mixture of enzymes or proteins continues to be a problem. Enzymes and proteins being macromolecules, derived from twenty or so different amino acids, offer numerous possibilities in structural variation, even when their total charges or chain-lengths are identical. Thus several molecular species can be closely related, yet, although appearing to be homogeneous, may reveal heterogeneity if a separation is attempted by another means. To this end newer methods for the separation of enzymes are desirable.

For several years we have been attempting the separation of macromolecules such as enzymes[4,5], DNA[6] and RNA[7] on cation exchangers equilibrated with metal ions. The present paper reports studies on chymopapain, a proteolytic enzyme from papaya latex (*Carica papaya*), on Amberlite IR-120 equilibrated with Hg^{2+}.

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EXPERIMENTAL

Preparation of Amberlite IR-120 (Hg^{2+}) column

Ten grams of dry regenerated Amberlite IR-120 (Na^+) were allowed to swell in water for 2–3 h. The slurry was then packed in a clean Pyrex glass tube (35 × 1 cm) and equilibrated with mercury(II) ions by passing through it 50 ml of 0.2 *M* mercury(II) chloride solution at a rate of about 15 ml/h. The adsorbent was then washed with 100 ml of distilled water to remove excess of mercury(II) chloride. Finally, the pH of the column was adjusted to 7.0 by passing through it 100 ml of phosphate buffer (pH 7.0, 0.1 *M*). The column was then used for the subsequent chromatographic studies.

The amount of Hg^{2+} retained on the column was independently determined by the diphenylthiocarbazone (dithizone) method[8] after eluting it with 6 *N* sulphuric acid. The resin retained 3 mg of Hg^{2+} per gram of resin under the conditions used.

Enzyme solution

About 1 ml of fresh papaya latex (*Carica papaya*) was diluted with 50 ml of sodium phosphate buffer (pH 7.0, 0.1 *M*) and was centrifuged at 3000 *g* for 10 min to remove the insoluble residue. This solution was further clarified by filtration through Whatman No. 1 filter paper and was suitably diluted with sodium phosphate buffer (pH 7.0, 0.1 *M*) before chromatography.

METHOD OF ASSAY

Reagents

The following reagents were used: Casein solution (1%) was prepared by dissolving 1.0 g of casein (re-precipitated at pH 4.8 with glacial acetic acid and repeatedly washed with acetone and dried) in 100 ml of sodium phosphate buffer (pH 7.0, 0.1 *M*); enzyme activator solution (prepared freshly as needed) was a 0.08 *M* neutralized solution of sodium cyanide in sodium phosphate buffer (pH 7.0, 0.1 *M*) that was 0.02 *M* in EDTA; enzyme solution (0.005–0.05 mg/ml); and 5% trichloroacetic acid.

Procedure

The procedure used for the assay with casein is based on the method of Kunitz[9] that had been adapted for papain[10] with slight modifications. The casein solution was brought to a temperature of 37° prior to the assay. To several test tubes containing the enzyme solution, in 0.1–0.8-ml amounts, was added 0.2 ml of activator solution and the volume was adjusted to 1 ml with sodium phosphate buffer (pH 7.0, 0.1 *M*); 1-ml aliquots of casein solution were added to each tube at an interval of 30 sec. After 10 min of incubation at 37° the reaction was stopped by adding 3 ml of 5% trichloroacetic acid solution. The mixture was allowed to stand for 30 min and was then filtered through Whatman No. 1 filter paper. The concentration of reaction products in the filtrate was determined by the method of Lowry *et al.*[11]. The readings obtained were corrected for the values of the blanks, which were prepared by first mixing 1 ml of the mixture that had the maximum concentration of enzyme used, containing activating agent, with 3 ml of 5% trichloroacetic acid solution and adding

1 ml of casein solution, and then proceeding in a similar way with the lower concentrations.

Definition of unit

One unit of enzyme activity is defined as the activity that gives rise to an increase of one optical density unit (at 660 nm) of Lowry positive material per 1-minute digestion, under the conditions described.

RESULTS

Chromatographic studies

Fifteen millilitres (1 mg/ml) of the enzyme solution was applied to the Amberlite IR-120 (Hg^{2+}) column at pH 7.0 and maintained at 25°. The solution was allowed to pass through the column at a rate of about 15 ml/h. The effluent was collected and the column washed with eight bed volumes (80 ml) of sodium phosphate buffer (pH 7.0, 0.1 *M*) to remove any loosely retained enzyme/protein. The adsorbed enzyme/protein was then eluted with 0.2 *M* ammonium acetate solution at a rate of 20 ml/h. Fractions, each of 10 ml, were collected and assayed for protein content and proteolytic activity.

The adsorptions (at 25°) were carried out at pH 7.0, as at this pH the enzyme is stable and the adsorbent exhibits higher capacity, particularly in the presence of phosphate ions. At higher temperatures irreversible binding and concomitant loss of enzymic activity occurs. There is no significant difference in retention even if the temperature is varied from 10 to 30°. Minor variations of flow-rate have no effect on the profiles. Slower rates permit a slightly more efficient separation, but the enzymes are then exposed to an unfavourable environment for a longer time, which is undesirable. No appreciable elution was obtained with 0.1 *M* ammonium acetate solution, whereas a 0.2 *M* solution proved to be satisfactory.

The percentages of total enzyme and protein adsorbed and eluted are given in Tables I and II. Fig. 1 gives the chromatographic profiles.

TABLE I

PERCENTAGE RETENTION OF PAPAYA LATEX PROTEASES/PROTEINS ON AMBERLITE IR-120 (Hg^{2+})

Macromolecule	*Amount loaded*	*Amount retained*	*Retention (%)*
Enzyme	75 units	75 units	100
Protein	15 mg	14.1 mg	94.03

TABLE II

RECOVERY OF PAPAYA LATEX PROTEASES FROM AMBERLITE IR-120 (Hg^{2+})

Macromolecule	*Eluted with 0.2 M ammonium acetate (%)**		*Recovery (%)* (total in all the fractions)*
	Fraction FI (total)	*Fraction FII (total)*	
Enzyme	4.55	89.28	95.72
Protein	4.82	41.42	56.17

* Expressed as percentage of the total retained enzyme/protein.

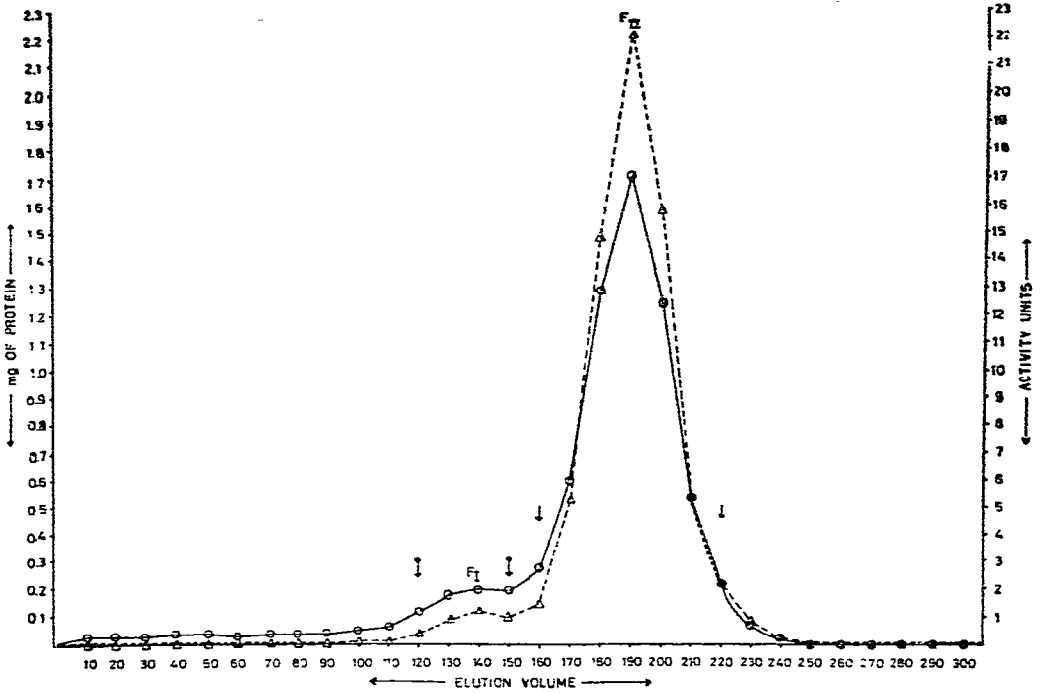

Fig. 1. Elution pattern of papaya latex proteases on Amberlite IR-120 (Hg^{2+}). △—△, activity; ○—○, protein.

These data show that two fractions were obtained. They were further characterized as follows. Fraction FI is somewhat spread and shallow in contrast to fraction FII. The precise shape of the fraction FI profile varies considerably and depends on the history and handling of the latex. Under controlled conditions fraction FI can appear as a sharp peak. In this work, however, attention was focused only on fraction FII, which is the major and more easily reproducible component. An early emerging protein peak is occasionally observed when stored enzyme solutions are used. This could result from autolysis of the enzyme due to storage.

Characterization of the fractions

(1) The stability in acid of proteases differs markedly. While papain loses its activity completely below pH 3, chymopapain is stable[12].

To identify the enzymic activity in fractions FI and FII, the respective peak fractions were pooled and the pH was adjusted to 2 by slowly adding dilute hydrochloric acid at 10°. After standing for 30 min the pH was re-adjusted to 7.0 by the slow addition of dilute sodium hydroxide solution. The proteolytic activities of both fractions were then tested as above. Fraction FI had lost its activity completely, but fraction FII had retained almost all of it.

(2) The success of any chromatographic fractionation is reflected in the homogeneity of the isolated fractions. Homogeneity was in this case studied by acrylamide gel electrophoresis.

The major active fraction (FII) was concentrated by dialysing it against solid sucrose before subjecting it to electrophoresis. Disc gel electrophoresis was carried out essentially according to the method of Reisfeld *et al.*[13], adapted for papain[14] (7.5% acrylamide at pH 4.5, β-alanine–acetic acid buffer). Pre-electrophoresis was carried out for 3 h using the gel buffer (potassium hydroxide–acetic acid, pH 4.3) and a current of 3 mA per tube.

After pre-electrophoresis, about 25–50 μg of the sample, in the presence of β-mercaptoethanol (0.05 *M*), was added to each tube and electrophoresis carried out for 1.5 h using β-alanine–acetic acid buffer (pH 4.5) with a current of 3 mA per tube. The gels were then removed, stained with Coomassie brilliant blue and de-stained as recommended by Weber *et al.*[15].

Fraction FII was electrophoretically homogeneous up to 50 μg of enzyme loaded. Electrophoretic characterization of fraction FI could not be carried out satisfactorily as the amount available was inadequate. Fig. 2 shows the disc gel electrophoretic patterns at pH 4.5.

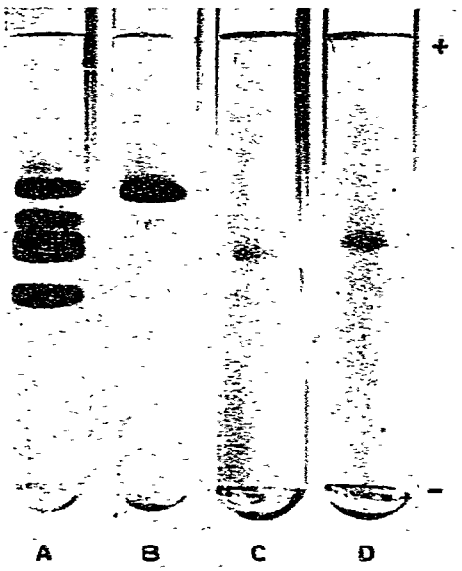

Fig. 2. Electrophoretic patterns at pH 4.5 on polyacrylamide disc gels. A, Crude latex; B, papain (V. P. Chest Institute, New Delhi, India); C, chymopapain (Sigma, St. Louis, Mo., U.S.A.); D, fraction FII.

DISCUSSION

Papain and similar enzymes have been obtained as stable active derivatives in the form of mercuri-papain[16]. Such heavy-metal derivatives are useful for crystallographic studies and full enzymatic potency is exhibited on detaching the mercury. Therefore, Hg^{2+} ions do not irreversibly affect these enzymes and this behaviour prompted us to attempt to fractionate such enzymes from papaya latex on IR-120 equilibrated with Hg^{2+} ions. Recently, similar efforts have been made to immobilize

chymopapain and papain[17,18] on agarose to which *p*-aminophenylmercury(II) acetate had been attached.

The failure of Amberlite IR-120 (Na^+) and IR-120 (NH_4^+) to retain sizeable amounts of enzyme and the significant increase in adsorption on IR-120 (Hg^{2+}) indicate that complex formation with Hg^{2+} is the basis of retention.

An enzyme solution that had previously been equilibrated with Hg^{2+} ions/ *p*-chloromercuri benzoate at 37° for 0.5 h (10^{-3} *M* effective concentration is adequate to block the -SH active site) behaved in a manner similar to the untreated sample as regards retention and elution characteristics. This, to some extent, shows that the -SH group does not have a direct role in the chromatographic process, though exchange is not discounted completely.

Ammonium acetate and ammonium dihydrogen orthophosphate solutions adjusted to pH 7.0 with ammonia are identical in their elution capabilities. Failure of sodium acetate to elute the enzyme, even at higher molarity, shows that the anion of the eluent does not have much influence on the elution. Elution may be due to the interaction of ammonium ions with Hg^{2+} ions, because, during elution, Hg^{2+} is associated with all the fractions, the amount of Hg^{2+} present in fractions FI and FII being 0.36 and 0.385 mg, respectively. The other fractions even if devoid of protein do contain Hg^{2+}.

Fraction FI, which appears to be papain, is comparatively small and scattered and is unstable at pH 2. The specific activity of this fraction is 6.0 compared with its initial specific activity of 5.0, giving a 1.2-fold purification.

Fraction FII constitutes the major fraction of the enzymatic activity. It appears to be chymopapain, as judged by its stability at pH 2 and an electrophoretic mobility identical with that of crystalline chymopapain. The specific activity of fraction FII is 13.07 compared with an initial specific activity of 5, thus a 2.6-fold purification was achieved. This degree of purification is of a comparable magnitude to that reported for highly purified preparations of chymopapain[12].

A simple separation of proteolytic activities together with almost quantitative recovery of the loaded enzyme has been achieved.

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