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ISOLATION OF HIGHLY ACTIVE PAPAYA PEPTIDASES A AND B FROM COMMERCIAL CHYMOPAPAIN

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Summary

Four enzyme fractions were isolated from commercial chymopapain (EC 3 4 22.6) by chromatography on carboxymethyl cellulose CM-32 and were further purified on an agarose-mercurial column Two fractions proved to be different forms of chymopapain B, the other two were papaya peptidase A and papaya peptidase B. The two latter enzymes were examined in detail. In contrast to previous findings, papaya peptidases exhibited high specific activity, similar to that of papain, (EC 3.4.22 2) and contained about 1 mol -SH group per mol enzyme. These results are not consistent with the idea that the essential -SH group of papaya peptidase A is 'masked' in the native state, but rather suggest that previous preparations contained a substantial amount of inactive enzyme.

Introduction

Papaya latex contains at least three thiol proteinases papain (EC 3.4 22.2), chymopapain (EC 3.4.22.6) and papaya peptidase A [1]. Papain has been extensively studied and some properties of chymopapain have also been described [1]. Papaya peptidase A was first isolated by Schack [2] from papaya latex and later by Robinson [3] from commercial chymopapain. Very recently Lynn [4] prepared papaya peptidase A and a similar enzyme, papaya peptidase B, which is presumably identical with the proteinase also noted by Schack

Papaya peptidases A and B as described by Lynn are rather peculiar thiol proteinases. As expected for a thiol enzyme, they are activated by thiol com-

Abbreviations DTNB, 5,5'-dithiobis(2-nitrobenzoic acid), PDS, 2,2'-dipyridyl disulfide

pounds and inactivated by iodoacetate. However, there was neither a free -SH group in the active papaya peptidase, nor an appreciable amount of carboxymethyl cysteine in the inhibited proteinase [4]. Therefore, it was suggested that the active site cysteine of these thiol enzymes must be significantly different from that of papain [4]. On the other hand, in the reaction of papaya peptidase A with iodoacetamide, Robinson found 0.6 mol cysteine labelled with carboxamidomethyl group per mol enzyme [3]

In the present paper it will be shown that papaya peptidase A isolated by the method of Robinson [3] can be further purified on an agarose-mercurial column In this way a few percent of highly active papaya peptidase A, not described so far, is separated, while the rest displays very low activity. This essentially inactive enzyme is presumably identical with that described by Lynn who isolated papaya peptidases from a protein fraction of papaya latex which did not bind to the agarose-mercurial column. The unusual behaviour of Lynn's papaya peptidase A preparation is readily interpreted in terms of a bulk inert protein being 'contaminated' with a small amount of highly active enzyme. Similar results were obtained with papaya peptidase B.

Materials and Methods

Reagents Chymopapain was obtained from Sigma Chemical Co, carboxymethyl-cellulose (CM-32) from Whatman Biochemicals Ltd. Pharmacia Ltd., supplied Sepharose 4B and the Sephadex gels; Serva Feinbiochemica the acrylamide, *N,N'*-methylene-bisacrylamide, sodium dodecyl sulfate (SDS), 5,5'-dithiobis(2-nitrobenzoic acid), Coomassie brilliant blue R-250 and Coomassie brilliant blue G-250. The *p*-aminophenyl mercuric acetate and 2,2'-dipyridyl disulfide were purchased from Aldrich Chemical Co. The *N*-carbobenzyloxyglycine *p*-nitrophenyl ester was obtained from Cyclo Chemical Company. The other reagents used were of analytical grade.

Enzymatic assays The activity of proteinases was measured with *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester as described for papain [5]. The reaction was followed at 340 nm on a Cary 118C spectrophotometer at 25°C in 0.1 M acetate buffer, pH 5.5/3.3% acetonitrile/1 mM EDTA. When the assay was carried out in the presence of cysteine (20 µl 0 2 M cysteine · HCl was added to 3 ml reaction mixture), the 'spontaneous hydrolysis' of the substrate augmented. Correction for the 'spontaneous hydrolysis' was always made, when its rate was higher than 3% of the enzymatic rate. Specific activity is expressed as µmol *p*-nitrophenol released/min per mg protein under the above conditions. The molar extinction coefficient of 6670 M^{-1} cm^{-1} was used for *p*-nitrophenol

The protein concentration was calculated by using $A_{280}^{1\%} = 18.3$ for all proteins Robinson found the values 17 9 for chymopapain in pool 1 and 18.3 for chymopapain in pool 2 and papaya peptidase A [3].

The second-order rate constants of acylation of papaya peptidase A with *N*-benzoyl-L-arginine ethyl ester were measured at 253 nm [6] and at 25.0°C as described previously for the acylation of papain [7].

Carboxymethyl-cellulose chromatography In a typical experiment 406 mg partially purified, commercial chymopapain, containing some salt, were dissolved in 7.0 ml standard buffer 0.15 M sodium acetate, pH 5.0/1 mM EDTA.

(The molarity of acetate buffer always refers to the Na^+ concentration.) This enzyme solution which contained 318 mg protein as measured at 280 nm, was activated by the addition of 0 7 ml 0.15 M cysteine adjusted to pH 5—6 before use. After incubation for about 30 min at room temperature, the enzyme was applied to a Sephadex G-25 column (approx 80 ml) equilibrated with standard buffer The pooled fractions (20 ml) contained 290 mg protein. This solution was immediately applied to a Whatman CM-32 cellulose column (1.5 × 20 cm) equilibrated with the standard buffer. Fractions of 10 ml were collected The chromatography was carried out at room temperature at a flow rate of 30 ml/h. The enzyme bound to the column was routinely washed with 20 ml standard buffer. Practically no protein was eluted from the column, even if washing was continued up to 50 ml.

The elution of the protein was effected with a linear gradient, the mixing chamber contained 300 ml standard buffer and the reservoir 300 ml 0 7 sodium acetate buffer, pH 5.0/1 mM EDTA. After the gradient elution, the column was washed with 1.5 M sodium acetate buffer, pH 5.0, but no more protein could be eluted.

Purification and concentration of the pools on agarose-mercurial column Agarose-mercurial was prepared according to Sluyterman and Wijdenes [8] The column was washed with 0 15 M acetate buffer, pH 5.0/1 mM EDTA. After binding the thiol enzymes were eluted with 0.05 M acetate buffer, pH 5.0/0 5 mM $HgCl_2$. For purification of pools 1, 2 and 3 about 2 ml, for pool 4 only 0.5—1 ml of agarose-mercurial was employed per 100 mg protein

Agarose-mercurial chromatography In a typical experiment 430 mg commercial chymopapain were dissolved in 12.3 ml activation mixture according to Sluyterman and Wijdenes [8] After 30 min incubation at room temperature, the solution was applied to an agarose-mercurial column (1 × 10 cm) at a flow rate of 18 ml/h. Fractions of 4 ml were collected The column was washed with 0.05 M sodium acetate, pH 5.0/10% dimethyl sulfoxide/0.5% *n*-butanol/0.1 M KCl until the enzymatic activity in the effluent decreased considerably. The remaining protein was eluted with a 'reverse' gradient the mixing chamber contained 40 ml 0.5 mM $HgCl_2$ in the washing buffer and the reservoir 40 ml 0.2 mM $HgCl_2$ in the same buffer. In this way the chymopapain forms were separated better than by a stepwise elution with 0 5 mM $HgCl_2$ It should be emphasized that the fractionation of chymopapain only occurs if the agarose-mercurial column is saturated with protein. When half of the affinity gel remains free, the protein elutes as single peak

Gel electrophoresis This was carried out on 7 5% polyacrylamide gels (pH 4.3) with the acidic β-alanine system [9] for 2 h, the first 20 min being run at 2 mA/tube then at 3 mA/tube. No tracking dye was used Preelectrophoresis was carried out for 2 h to remove residual peroxide. Staining was performed according to Blakesley and Boezi [10].

Molecular weight determination The molecular weight of proteinases was determined by SDS-polyacrylamide gel electrophoresis [11,12] and by gel-chromatography on a Sephadex G-75 column (1 × 54 cm) equilibrated with 0.1 M acetate buffer, pH 5.0/0 2 M sodium chloride.

Determination of thiol groups The procedures of Ellman with DTNB [13] and of Brocklehurst and Little with PDS [14] were employed. The molar

extinction coefficient 14 150 was used in the reaction of DTNB [15], in the PDS reaction the values 7480 at pH 8.2 and 7700 at pH 3.8 were used (B. Asbóth, unpublished data from this laboratory).

Results and Discussion

The chromatography of commercial chymopapain on carboxymethyl cellulose is seen in Fig. 1. The elution pattern of protein is similar to that found by Robinson [3] who has shown that the first protein peak comprising pools 1 and 2 is chymopapain and the second one comprising pool 4 is papaya peptidase A. However, there is some difference between the experimental conditions employed by Robinson and those pertinent to Fig. 1. Namely, he applied the enzyme solution directly to the column, whereas we activated and gel-filtered it before loading. This may explain that in the present experiment the total amount of protein binds to the ion-exchange column. Apparently, by gel filtration we eliminated the non-binding fraction, probably composed of degraded protein Activation with cysteine was important because the commercial preparation contained a significant amount of activatable thiol enzyme(s) which would have been lost in the following purification on the agarose-mercurial column. A further difference is that Robinson used 1.5 M sodium acetate buffer in the reservoir, whereas we could elute the protein with 0.7 M sodium acetate as illustrated in Fig. 1. With 0.5 M sodium acetate in the reservoir, the elution of papaya peptidase A (second peak) was less complete, but the bidentate character of the chymopapain peak become more expressed and the protein appeared in later fractions and in a larger volume. This variation did not affect

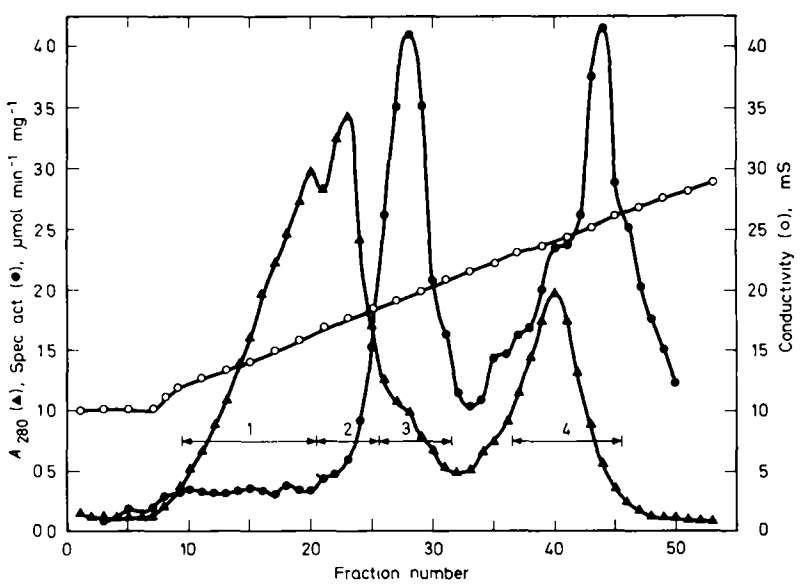

Fig 1 Chromatography of commercial chymopapain on CM-32 cellulose Chromatographic conditions are described in Materials and Methods Fractions were combined as indicated by the double arrows and are referred to in the text as pools 1—4

either the yield of protein or the specific activity of the different pools shown in Table I.

The curve of specific enzymatic activity in Fig. 1 reveals some important properties of the protein mixture. It is seen that there are two highly active enzymes in commercial chymopapain One elutes after chymopapain at the shoulder of the protein peak (pool 3) This is probably papaya peptidase B, which immediately followed chymopapain B in Lynn's experiment, too. The other is associated with pool 4, but the peak of specific activity is significantly shifted from the protein peak, which indicates that pool 4 cannot be homogeneous, at least in respect of activity Robinson has found pool 4 homogeneous by gel electrophoresis. Indeed, the protein peak of pool 4 in Fig 1 appears homogeneous as the small amount of active enzyme merges into the large quantity of inactive protein

The constant specific activity of the fractions in pool 1 indicates that the first part of chymopapain is homogeneous Chymopapain in pool 2 shows somewhat higher specific activity but this may be due to contamination with the highly active papaya peptidase B. Pool 3, which is composed of the shoulder fractions, seems to contain both chymopapain and papaya peptidase B.

All four pools were further purified on agarose-mercurial columns, which bind protein with free -SH group(s) [8] Besides purification, the great advantage of this covalent chromatography consists of the effective concentration of enzyme diluted during the preceding ion-exchange chromatography. The com-

TABLE I

COMPARISON OF CHROMATOGRAPHIC POOLS

Pools can be identified as follows 1 and 2, chymopapain B, 3, mixture of chymopapain B and papaya peptidase B, 4, crude papaya peptidase A, 3a, chymopapain B, 3b, papaya peptidase B, 4a, active papaya peptidase A (mercurial-bound fraction), 4b, 'inactive' papaya peptidase A (non-binding fraction)

Pool	1	2	3	4	
From CM-cellulose,					
mg	95	74	27	62	
ml	108	50	50	88	
Spec act *	0 40	0 67	3 07	1 93	
	(0 32)	(0 67)	(3 10)	(1 60)	
Pool				4a	4b
From agarose-mercurial,					
mg	35 5	20 6	11 6	3 5	52 4
ml	9 9	6 2	4 2	2 2	85
Spec act *	0 67	1 15	4 7	24	0 48
	(0 60)	(1 06)	(4 5)	(24)	(0 10)
-SH/mol enzyme (Nbs_2)			1 10	0 98	<0 01
-SH/mol enzyme (PDS, pH 8 2)			1 07		
-SH/mol enzyme (PDS, pH 3 8)			1 06	0 88	
Pool			3a	3b	
Re-chromatography of pool 3 on CM-cellulose, Spec act *			2 1	9 0	
-SH/mol enzyme (Nbs_2)				0 98	
-SH/mol enzyme (PDS, pH 3 8)				1 06	

* The values in the bracket were measured in the absence of cysteine

bined fractions from the CM-cellulose chromatography were directly applied to the agarose-mercurial column without the addition of organic solvent, a deviation from the original method [8]. Table I shows that in all cases, particularly with pool 4, only a minor part of the protein was bound. This indicates that the major part of the protein in the pools is inactive enzyme not containing free -SH group.

Table I shows that the specific activity of all four pools increased considerably after elution from the agarose-mercurial column. Papaya peptidase A (pool 4) is particularly interesting because only about 6% of its protein was obtained as active thiol enzyme (pool 4a) and about 94% was found in the non-binding fraction (pool 4b). The mercurial-bound papaya peptidase A exhibits the highest specific activity among the pools. This amounts to the specific activity of papain (19 5 μmol $min^{-1} \cdot mg^{-1}$), which has been thought to be the most active enzyme in papaya latex. The non-binding fraction shows a low specific activity even in the presence of cysteine.

The shoulder fraction (pool 3) displays the second highest specific activity. Since pool 3 appears to be a mixture of chymopapain and papaya peptidase B, the pure papaya peptidase B is expected to be more active. In fact, Fig 2 shows that rechromatography of pool 3 on CM-cellulose results in two peaks, the first one (pool 3a) having lower and the second one (pool 3b) higher specific activity than that of pool 3 (Table I). Both pools 3a and 3b seem to be homogeneous by activity.

The amount of free -SH groups in papaya peptidases was determined with DTNB and PDS. Measurements with DTNB and PDS at pH 8.2 yield the total free -SH groups, whereas PDS at pH 3.8 determines the active site -SH group in the case of papain and chymopapain [14,16] The -SH group content of both papaya peptidases A and B was calculated for the same molecular weight, 24 000, which was determined by two methods: SDS-polyacrylamide gel electrophoresis and gel chromatography on Sephadex G-75. This value agrees

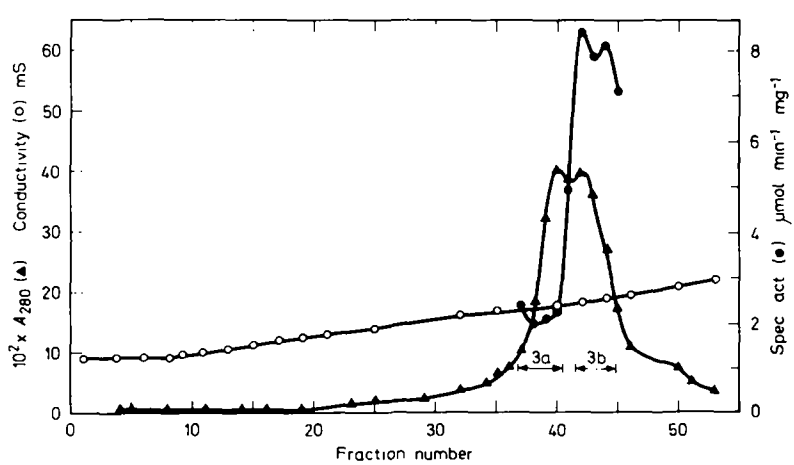

Fig 2 Re-chromatography of pool 3 on CM-32 cellulose The experimental conditions are the same as in Fig 1 , except that the reservoir of the gradient elution contained 0 5 M rather than 0 7 M acetate buffer The combined fractions designated by the double arrows represent pools 3a and 3b

well with that found by Robinson [3] and Lynn [4] for the less active papaya peptidases. Table I shows that the highly active papaya peptidase A (4a) and papaya peptidase B (3b) contain about 1 -SH group per enzyme molecule measured either at pH 8.2 or pH 3.8. This indicates that the thiol group is similar to the essential -SH group of papain. On the other hand, the -SH group content of pool 4b is negligible, which accounts for its very low specific activity.

The activity of papaya peptidase A (4a) was also measured with *N*-benzoyl-L-arginine ethyl ester. The pH-dependence of the second-order rate constant gave a bell-shaped curve as it was demonstrated by Robinson [3]. However, the value of rate constant at the pH optimum (1300 M^{-1} s^{-1}) was about 10-times as high as that observed by Robinson [3]. The high rate constant (1300 M^{-1} s^{-1}) is close to that found with papain (1660 $M^{-1} \cdot s^{-1}$ [7]).

Although the specific activities are similar, the highly active papaya peptidases can easily be distinguished from papain by electrophoresis on polyacrylamide gels. Fig. 3 shows that pools 3b and 4a run much farther than papain (see also Ref. 3). Furthermore, it was found (Dr. M. Sajgó in this institute, personal communication) that both papaya peptidases A and B have the same N-terminal amino acid, leucine as determined with the dansyl method, whereas the N-terminal of papain is isoleucine [1]. This is in agreement with Lynn's results [4] but in contrast to Robinson's finding [3], according to which papaya peptidase A possesses isoleucine at the NH_2-end.

A remarkable property of papaya peptidase A, and probably of papaya peptidase B, is that it binds to the agarose-mercurial column significantly weaker than chymopapain. This is illustrated in Fig. 4 which shows that the non-bind-

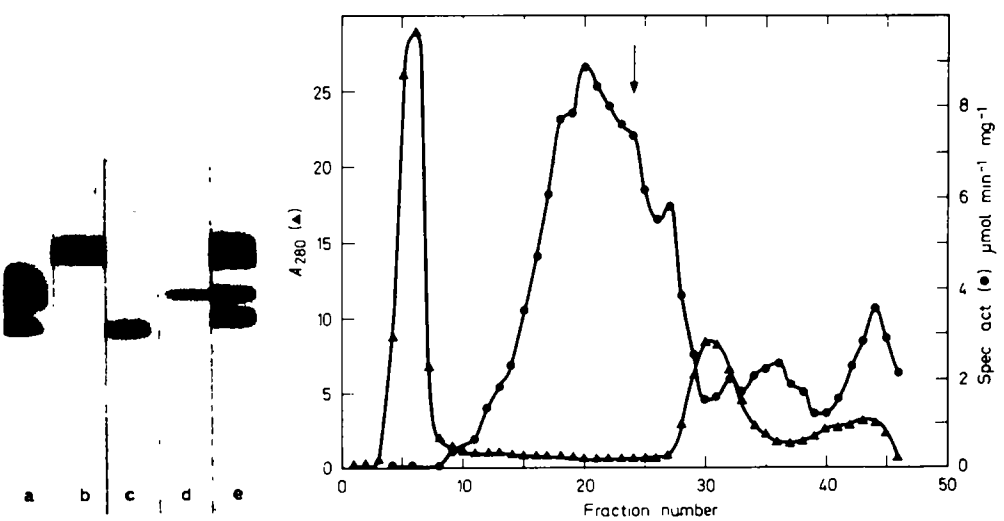

Fig 3 Comparison of papaya peptidases with papain Electrophoresis was carried out in the β-alanine system as described in Materials and Methods The protein bands migrated from the top towards the negative pole The gels are a, commercial chymopapain, b, papain, c, 4a, d, 3b, e, papain, 3b, 4a

Fig 4 Chromatography of commercial chymopapain on an agarose-mercurial column The arrow indicates the beginning of elution with the mercuric chloride gradient For details see Materials and Methods

ing, inactive protein fraction is immediately followed by a continuous release of protein at low concentration on washing the column with buffer not containing $HgCl_2$. On the basis of its high specific activity, this 'leaking' protein fraction should mainly contain papaya peptidases. This was confirmed by electrophoresis on polyacrylamide gels, which showed that in the 'leaking' fraction papaya peptidase A is the major component, and an additional minor band indicates the presence of some papaya peptidase B and/or chymopapain. Analysis of the non-binding fraction exhibited a similar pattern.

The above experiment clearly shows that depending on the extent of washing, different amounts of highly active papaya peptidase A can be present in the papaya peptidase A preparation of Lynn [4]. This can explain the occurrence of 0.2 mol -SH group per mol enzyme in the preparation and, more importantly the surprising behavior of the enzyme on alkylation. Obviously, if most of the activity arises from a few percent of highly active papaya peptidase A, then carboxymethylation of this enzyme abolishes practically the total activity without yielding an appreciable amount of carboxymethyl cysteine.

It can be concluded that the highly active form of papaya peptidases, first isolated in this work, does possess a single -SH group and exhibits an enzymatic activity which is comparable to that of papain and much higher than that of chymopapain.

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