Biochimica et Biophysica Acta, 569 (1979) 193—201

BBA 68788

A PURIFICATION AND SOME PROPERTIES OF TWO PROTEASES FROM PAPAYA LATEX *

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(Received January 30th, 1979)

Key words: Protease; Latex; (Papaya)

Summary

Two proteases, one of which is papaya peptidase A and the other a previously unknown enzyme in papaya latex have been purified to homogeneity in a simple two stage process. Both are markedly less reactive than papain or chymopapain. Each has a molecular weight of 24 000, N-terminal sequences commencing Leu-Pro-Glu, and contains no carbohydrate. Their amino acid compositions differ for several residues. The essential -SH groups of the enzymes examined appear to be 'masked' in the native state.

Introduction

Three of the sulphydryl proteases from papaya latex which have been isolated and studied are papain (EC 3.4.22.2) chymopapain (EC 3.4.22.6) and papaya peptidase A [1]. Papain has been extensively examined and work on chymopapain has been lately reviewed [1]. Papaya peptidase A was first isolated by Schack [2] from papaya latex, in a partly purified form: more recently it was produced as a homogeneous enzyme from commercial chymopapain by Robinson [3]. A fourth protease from the latex was reported by Schack [2], but neither named nor further characterized.

In this report a simple two-stage isolation of papaya peptidase A from the latex is described, which uses affinity and ion-exchange chromatography. The fourth protease component of papaya latex [2] was simultaneously produced. These two enzymes were obtained in forms homogeneous in disc gel electrophoresis. Amino acid compositions and molecular weights are reported, those for the peptidase A agreeing with values found by Robinson for the enzyme isolated from commercial chymopapain [3]. N-terminal sequences of four

* This is paper NRCC No. 17537.

residues were determined for the two proteases described here, and compared with those for papain and chymopapain. Similarly, a comparison of pH profiles and residue specificities was made. The nature of the cysteine residue of papaya peptidase A was examined in some detail because of its apparent similarity to that in the second protease studied here, and its difference from the analogous residues of papain and chymopapain.

Experimental

Reagents. Sepharose 4B and the Sephadex gels were obtained from Pharmacia Ltd.; cyanogen bromide, *N,N'*-methylene bis-acrylamide, 2-mercaptoethanol and iodoacetic acid from the Eastman Co. The Aldrich Chemical Company supplied the *p*-aminophenyl mercuric acetate and 2,2'-dipyrydyl disulphide; ICN Pharmaceuticals Ltd. the 4,4'-bis-dimethylamino diphenyl carbinol, 5,5'-dithio-bis-(2-nitrobenzoic acid) and 1,3-dibromoacetone. The papaya latex (Crude Type 1), *N*-carbobenzoxyglycine-, -tyrosine- and -lysine-*p*-nitrophenyl esters, *N*-benzoyl-arginine-*p*-nitroanilide and dithiothreitol were obtained from the Sigma Chemical Co. Oxidized insulin B chain and pepsin were obtained from Schwartz-Mann, and $NaC^{14}N$ from New England Nuclear. Carboxymethyl-cellulose (CM-52) was purchased from Whatman Biochemicals Ltd.; acrylamide, sodium dodecyl sulphate and Coomassie Brilliant Blue R250 from the BioRad Laboratories. Other reagents used were of analytical grade.

Isolation of enzymes. Columns of agarose-mecurial (3.5 × 40 cm) were prepared as described by Sluyterman and Wijdenes [4]. These had capacities ranging from 1.1—1.7 μmol 5,5'-dithio-bis-(2-nitrobenzoic acid)/ml [4].

Papaya latex was ground with sand in 100 ml activation buffer [4] and applied to the agarose-mercurial after centrifugation at 3000 × *g* for 15 min. On washing with the same buffer and assaying the eluant for esterolytic activity as described below, an active peak was defined coincident with a brownish colour. Enzymes bound to the agarose-mercurial were eluted with buffer containing 0.05 mM $HgCl_2$ [4,5]; they were identified as papain and chymopapain [5] and were further purified, for use in this work, by ion exchange chromatography on a CM-52 carboxymethyl cellulose column (3.5 × 40 cm) in 10^{-2} M acetate buffer (pH 6.4) using a linear gradient of 0—0.7 M NaCl.

The non-binding esterolytic fraction isolated was clarified by centrifugation after dialysis versus 10^{-2} M sodium acetate (pH 6.0) and applied to a CM-52 column (3.5 × 45 cm) equilibrated with that buffer. Elution was with a succession of linear gradients comprising 400 ml each of 0—0.2, 0.2—0.25, 0.25—0.35, 0.35—0.5 and 0.5—0.7 M NaCl in acetate buffer. This series of gradients separated all components of the non-binding fraction described above (Fig. 1). Each component of the separation was recycled on the CM-cellulose column to ensure homogeneity before amino acid analyses were performed. Gel-electrophoresis [6] with 7.5% gels (pH 4.3) confirmed the homogeneity of the samples so obtained. A more direct isolation of papaya peptidase A was effected on the CM-cellulose column with a linear gradient consisting of 1 l each of 0—0.7 M NaCl in acetate buffer (pH 6.0). That enzyme was clearly separated from the other proteins as the final component eluted. Recycling on the ion-exchange column ensured that the peptidase A so obtained was homo-

geneous, which was confirmed by gel-electrophoresis using the procedure of Grabriel [6].

Enzymatic assays. To 2.9 ml acetate buffer (pH 7.0), $1 \cdot 10^{-4}$ M each EDTA, dithiothreitol, was added 0.1 ml ($3 \cdot 10^{-3}$ M) *N*-carbobenzoxy-glycine- or -lysine-*p*-nitrophenyl ester in acetonitrile or ethanol, respectively. Sufficient enzyme was added to give a convenient rate of hydrolysis, which was measured on a Beckman DB spectrophotometer at 410 nm for the first part of the reaction.

Assays with casein were conducted by the method of Arnon [7].

Amino acid analysis. Hydrolysates were prepared under vacuum, at 110°C for 22 h in 6 N HCl or 3 M *p*-toluenesulphonic acid [8]. Total cysteine content was determined by the procedure of Hirs [9]. The tryptophan content was assayed after the sulphonic acid hydrolysis [8] and by the method of Spies and Chambers [10].

N-terminal sequence. The procedure of Seligy et al. [11] was employed using a Beckman model 890-G sequenator and Edman degradation.

Molecular weight determination. Gels containing sodium dodecyl sulphate were prepared and used following Weber et al. [12]. Bovine plasma albumin, ovalbumin, pepsin and chymotrypsinogen were used as standards.

Sulphydryl assays. The procedures of Ellman with 5,5'-dithio-bis-(2-nitrobenzoic acid) [13], of Rohback et al. [14] with 4,4'-bis-dimethylamino diphenyl carbinol and of Brocklehurst with 2,2'-dipyrydyl disulphide [15] were used.

Carbohydrate content. The method of Dubois et al. was used with mannose as standard [16].

Pepsin digests. These were made in 5% formic acid at 35°C for 17 h. The ratio of substrate protein to pepsin was 25 : 1. Peptide maps were prepared on thin-layer cellulose sheets (Macherey-Nagel Polygram Cel 400). Electrophoresis in the first dimension was run at 400 V for 2 h at 10°C in water/acetic acid/pyridine/acetone (40 : 2 : 1 : 8, v/v), pH 4.4, using a Desaga chamber. Chromatography in the second dimension was in *n*-butanol/pyridine/acetic acid/water (15 : 10 : 3 : 12, v/v). The plates were developed with 0.2% ninhydrin.

Digestions of insulin B chain. Reactions were performed in 0.1 M NH_4HCO_3 at 35°C, using 1 ml solvent, 10 μg 2-mercaptoethanol, 2 mg oxidized B chain and 20 μg enzyme. After suitable times, samples were freeze-dried and peptide maps prepared as described above. To determine the sites of peptidase activity, peptides from several maps were pooled, after elution with 6 N HCl, hydrolysed at 110°C for 22 h and subjected to amino acid analysis.

Alkylation experiments. Reactions with iodoacetic acid were carried out in acetate buffer, pH 5.5 [17].

The procedure used in reactions with dibromoacetone was that of Husain and Lowe [18]: equimolar amounts of enzyme in $5 \cdot 10^{-2}$ M acetate buffer, pH 5.6 and dibromoacetone (in acetone) were mixed and assays for residual activity made. Samples were prepared for amino acid analysis, from reaction mixtures, by dialysis versus 5% acetic acid and freeze drying.

Cyanide activation. To 7 μmol papaya peptidase A in acetate buffer (pH 6.0) were added 21 μmol $Na^{14}CN$ (4.3 μCi) in the same buffer. Esterolytic activity was immediately assayed except that no reducing agent was added. Esterolytic

activity was also monitored after addition of a second amount of cyanide (21 μmol, $Na^{14}CN$). The reaction mixture from this experiment was filtered on Sephadex G25 (2.5 × 20 cm) in the acetate buffer used: 75% of the original amount of protein was recovered, and this, in the presence of 2-mercaptoethanol, had the same specific activity as the enzyme originally measured. The ^{14}C activity of the gel-filtered enzyme was measured on a liquid scintillation counter under conventional conditions.

Results and Discussion

The less active peaks of Fig. 1 (I-IV) were isolated and purified by recycling on CM-cellulose. Peptide maps from peptic digests, and amino acid analyses showed that these fractions differed only slightly from each other and from chymopapain. They were, apparently, the several forms of that enzyme which have been reported previously [19].

Maps of pepsin digests (Fig. 2) show that Fraction V of Fig. 1 is not chymopapain or papaya peptidase A. Amino acid analyses presented in Table I confirm this and demonstrate the compositional similarity of the papaya peptidase A, Fraction V and papain. That similarity of composition is most notable in the acidic and neutral residues of Table I. The position of elution of the Fraction V relative to that for papaya peptidase A on a CM-cellulose ion exchanger suggests that this is the fourth protease from papaya latex, the presence of which

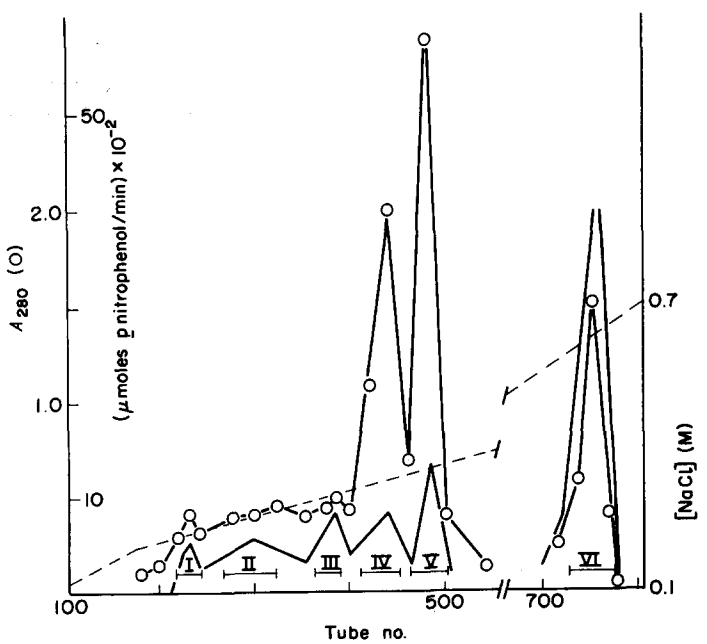

Fig. 1. Fractionation on CM-52 cellulose (2.5 × 45 cm) of the non-bound esterolytic peak from an agarose-mercurial column. Linear gradients of NaCl in acetate, pH 6.4, of 0—0.2, 0.2—0.25, 0.25—0.35, 0.35—0.5, 0.5—0.7 M.

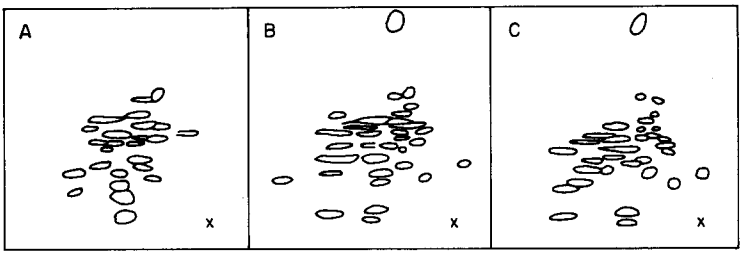

Fig. 2. Peptide maps of pepsin digests of A-chymopapain; B-papaya peptidase A; C-papaya peptidase B.

was noted by Schack [2]. The use of differing sources of latex may explain the comparative inactivity of the sample in Fig. 1. Other separations of this enzyme from latex yielded protein of specific activity comparable with that for papaya peptidase A, (in conformity with Schack [2] we shall refer to that fourth component as papaya peptidase B). Using casein as substrate [7], specific activities of 0.66 units/mg were obtained with both peptidase A and B. With the *N*-carbobenzoxy derivative of glycine-*p*-nitrophenyl ester the specific activities were 13 and 12 μmol *p*-nitrophenol/min/mg. The analogous lysine substrate yielded, in the same order 53 and 45 μmol *p*-nitrophenol/min/mg. Neither enzyme was active towards *N*-carbobenzoxy-tyrosine-*p*-nitrophenyl ester, and although the leucine analogue was unaffected by peptidase A; peptidase B produced 2 μmol *p*-nitrophenol/min/mg. Neither enzyme catalysed the hydrolysis of benzoyl-arginine-*p*-nitroanilide [3].

TABLE I

AMINO ACID COMPOSITIONS OF FRACTION V (Fig. 1), PAPAYA PEPTIDASE A, PAPAIN AND CHYMOPAPAIN: RESIDUES/MOLECULE

Peptidase B = Fraction V.

Residue	Papain *	Chymopapain	Peptidase A **	Peptidase B ***
Asp	19	24	14	18
Thr	8	16	9	8
Ser	13	18	13	14
Glu	20	27	20	19
Pro	10	15	13	8
Cys	7	11	7	7
Gly	28	37	34	27
Ala	14	19	15	13
Val	18	21	20	18
Met	—	1	—	—
Ile	12	10	10	8
Leu	11	15	12	9
Tyr	19	19	12	12
Phe	4	7	3	3
His	2	4	4	4
Lys	10	29	23	14
Arg	12	6	11	8
Trp	5	6	5	2

* Ref. 1.
** Residue, M_r = 24 297.
*** Residue M_r = 20 732.

The peptidase A isolated in this work had the amino acid composition presented in Table I which is in agreement with that reported by Robinson [3]. Determinations on dodecyl sulphate-gels showed the molecular weight of the enzyme to be 24 000, a figure confirmed by gel-filtration on Sephadex G-75, and which is in agreement with the earlier report [3]. By similar procedures the peptidase B protease was also shown to be of molecular weight 24 000.

N-terminal residue determination with the Edman procedure yielded the sequences: Papaya peptidase A, Leu-Pro-Glu-Asn; Papaya peptidase B, Leu-Pro-Glx-Ser. The N-terminal residue of the peptidase A is Leu, in contrast to the determination previously reported [3] of that residue being Ile. As the Edman method, in conjunction with amino acid analysis permits a clear distinction between Leu and Ile, while dansylation employed with thin layer techniques [3] is not so unambiguous, that leucine is the N-terminal residue of the protease may reasonably be stated.

A comparison of the data for N-terminal residues of the peptidases A and B with those from papain (Ile-Pro-Glu-Tyr) and chymopapain (Tyr-Pro-Gln-Ser) purified in this laboratory by procedures outlined above, may be made. Similarities of structure are apparent in the regular appearance of Pro and Glu residues at positions 2 and 3 respectively. For bromelain a comparable sequence has been found [20], namely Val-Pro-Glx. The N-terminal tyrosine of chymopapain is that reported also by Kunimitsu and Yasunobu [19]. The sequence for papain is the same as that found in the literature [1].

As has been noted above, unlike papain and chymopapain, neither of the papaya peptidases were bound to the agarose-mercurial column used in this work. Apparently, their sulphydryl groups were not available for attachment to the Hg moiety of the affinity packing. In the absence of conventional reducing agents such as dithiothreitol, 2-mercaptoethanol and cysteine, both the proteases discussed here displayed only approximately 30% of their maximal activities, which were developed equally by those three reducing agents. Those maximal activities, however, immediately decreased to the approximate 30% level when the enzyme and reducing agent were separated by gel-filtration on Sephadex G-25. Apparently both are very readily oxidized in the air.

With 5,5′-dithio-bis-(2-nitrobenzoic acid) used under the conditions recommended by Ellman [13] reaction of peptidase A indicated only 0.2-SH/molecule of enzyme. A comparable failure to react either with 2,2′-dipyrydyl disulphide [15] or with 4,4′-bis-dimethylamino diphenyl carbinol [14] was found. Papaya peptidase B was similarly unreactive in the Ellman procedure, 0.2 SH/mol enzyme were obtained under conventional conditions [13]. As the two enzymes discussed here were apparently similar in composition and reactivity, attention was focussed on the peptidase A to investigate further the nature of its sulphydryl groups.

Attempts to assay the sulphydryl content of papaya peptidase A [13] in the presence of such denaturing agents as dodecyl sulphate (2%), urea (4 M) and guanidine hydrochloride (3 M) yielded a maximum value of 0.5 SH/molecule.

Iodoacetic acid [17] completely inhibited the caseinolytic activity of the peptidase A both in the presence and absence of reducing agents. However, no evidence of -S-carboxymethylation was obtained by amino acid analysis; apparently non-specific reactivity of the iodoacetic acid accounts for the loss of of enzymatic activity observed.

Dibromoacetone is a bifunctional reagent which has been used in examining the active sites of papain and ficin [18]. Under conditions identical with those of Husain and Lowe [18] in the absence of reducing agent no effect on the activity of the papaya peptidase A was observed; effectively total inhibition by dibromoacetone occurred only in the presence of 2-mercaptoethanol or dithiothreitol. Again, however, no evidence of -S-carboxymethylation of cysteine was obtained on amino acid analysis, nor did that assay reveal the occurrence of carboxymethylated histidine [18]; no change in the histidine content of the enzyme was observed. Apparently the inhibitory action of dibromoacetone was non-specific.

The presence of such reducing agents as 2-mercaptoethanol are necessary for the full development of activity by papaya peptidase A, and so the enzyme is categorized as an -SH enzyme [1]. However, the active cysteine of this protease must be significantly different from those of papain and ficin, as the data reported above show. It was thus of interest to examine the nature of the sulphydryl group of the peptidase further. To that end, we examined the possibility of the essential sulphydryl residue existing, at least partly, in the form of a sulphenic acid. Evidence of the existence of such sulphenic acids in enzymes has recently been summarized by Allison [21]. Among the tests suggested for establishing the presence of these notably elusive acids is their reduction by arsenite [21]; it was observed that papaya peptidase A is fully activated (compared with 2-mercaptoethanol) by arsenite. If, however, a sulphenic acid residue is present in the peptidase A, reaction with a small molar excess of cyanide should not activate the enzyme, and binding of the cyanide to the protein through formation of a thiocyanate should occur, by analogy with results reported for oxidized papain [22,23] and glyceraldehyde-3-phosphate dehydrogenase [23]. Using a threefold molar excess of $C^{14}N^-$, papaya peptidase A was activated to 48% of its maximal activity; a sixfold excess of the cyanide activated the enzyme to 62% of the maximum measured (in the presence of 2-mercaptoethanol). An excess of one hundredfold of the cyanide activated the peptidase to 80% of the maximal value. No binding (<1%) of the $-C^{14}N$ to the enzyme was found, after gel-filtration of the reaction mixtures from the experiments described above. Cyanide ions apparently act on the peptidase A only as reducing agents, even in low molar ratios with the enzyme, and not as nucleophiles reacting as

$$RSOH + CN^- \rightarrow RSCN + OH^- \quad (1)$$

The failure of the papaya peptidase A to bind cyanide seems to eliminate the possibility of the enzyme containing a sulphenic acid moiety in its native state. The activation by arsenite must be re-examined. The observation that arsenite is an agent incapable of reducing -S-S- bonds (and so diagnostic for sulphenic acids) derives from its failure to reduce the disulphide bond of 5,5′-dithio-bis-(2-nitrobenzoic acid) [23]. However, if we assume that the peptidase A exists in solution largely in the inactive state because of intramolecular -S-S-bond formation binding the cysteine residue of the active site in a manner similar to that proposed for Pro-papain [24] the nature of the bond will be very different from that of the model disulphide. The intramolecular -S-S bond may allow reduction even by a reagent as weak as arsenite.

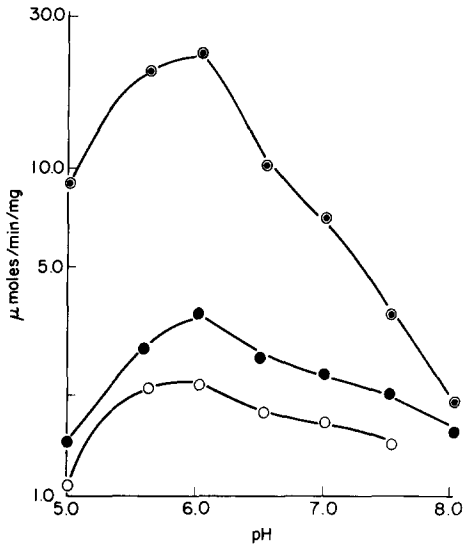

Fig. 3. Rate (in μmol of *p*-nitrophenol/min/mg) versus pH for catalysis of *N*-carbobenzoxy-glycine-*p*-nitrophenyl ester in (pH 5.0—5.6) 0.1 M acetate or (pH 6.0—8.0) 0.1 M PO_4^{3-}. Papain (⊙); chymopapain (●); papaya peptidases A and B (○).

Comparison between papain, chymopapain and papaya peptidases A and B was made in an examination of the pH/rate profiles of the enzymes with *N*-carbobenzoxy-glycine-*p*-nitrophenyl ester. The results are summarized in Fig. 3, and are comparable with those obtained using the lysine analogue as substrate. It is apparent that over the range of pH studied (5.0—8.0) the order of reactivities is papain >> chymopapain > papaya peptidases A and B. The similarities of modes of reactions are also apparent in the way the three curves of Fig. 4 have inflexions at similar pH values (6.6). A comparable ordering of reactivities was obtained when casein was employed as substrate (see, also, Ref. 3).

After digestion of the B chain of insulin, papain, chymopapain and the peptidases A and B produced comparable bond scissions as has been previously reported for the first three of these enzymes [25].

Papaya peptidase A, after recycling through the CM-cellulose ion exchanger and passage through Sephadex G-75 contained 0.36 mannose equivalents/mol of enzyme as assayed by the method of Dubois et al. [16]. This suggests that the enzyme is essentially free of carbohydrate, and is thus similar to papain (and unlike ficin and bromelain [1]). The peptidase B, contained 0.45 mannose equivalents/mole and so again resembles papaya peptidase A.

Acknowledgements

The aid of Dr. M. Yaguchi in performing the N-terminal sequences, Mr. W.J. Brockbank with the gel-electrophoresis and Mr. A. Castagne with the amino acid analysis is gratefully acknowledged.

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