Isolation and Characterization of Papaya Peptidase A from Commercial Chymopapain†

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ABSTRACT: Chromatography on a column of SP-Sephadex shows that commercial chymopapain contains three components with proteolytic activity. Each behaves as a single protein upon rechromatography and electrophoresis on polyacrylamide gels. The major component, which represents 31% of the activity applied to the column and is the most basic protein, was identified as papaya peptidase A. This enzyme has no methionine and isoleucine on its N-terminus. Its molecular weight is about 24,000 as determined by sodium dodecyl sulfate polyacrylamide electrophoresis and sedimentation equilibrium centrifugation. Its fluorescence emission as a function of pH resembles that for unactivated papain. Reduction is required for full activity, and in general it is less active than papain against substrates such as casein, *N*-benzoyl-L-arginine ethyl ester, *N*-tosyl-L-arginine methyl ester, *N*-benzoyl-L-arginineamide, and *N*-benzoyl-DL-arginine *p*-nitroanilide. Of the other components isolated from crude chymopapain, the more acidic enzyme contains 20% of the activity applied to the column, has a molecular weight of about 25,000, and N-terminal residues of tyrosine and glutamic acid. The other enzyme represents 26% of the initial activity, has a molecular weight of about 28,000, and tyrosine on its N-terminus. Both proteins have a single residue of methionine per molecule. The more acidic component resembles chymopapain A, and the other enzyme is similar to chymopapain B.

Papaya latex contains four components with proteolytic activity, three of which have been identified as papain, chymopapain, and papaya peptidase A. Each is a unique protein, and all require a sulfhydryl group for activity. Papaya peptidase A is the least known and least studied of these enzymes, although it represents 14% of the latex protein compared to 5% for papain and 27% for chymopapain (Shack, 1967).

Specificities of these papaya enzymes and of streptococcal proteinase, another thiol enzyme, are quite similar when the peptide bonds each enzyme hydrolyzes in the phenylalanyl chain of insulin are compared (Johansen and Ottesen, 1967; Gerwin et al., 1966). Amino acid sequences around the essential sulfhydryl groups of papain, chymopapain, and streptococcal proteinase (Light et al., 1964; Tsunoda and Yasunobu, 1966; Liu et al., 1965), and in key tryptophan-containing peptides in papain and the streptococcal enzyme, are also homologous (Robinson, 1970).

In the course of work to obtain additional structural information about the active sites of these enzymes, commercial preparations of chymopapain were found heterogeneous. A major component of these preparations has been identified as papaya peptidase A. This paper reports the isolation of papaya peptidase A from commercially available chymopapain and further characterization of its physical and chemical properties.

Experimental Procedure

Chymopapain (Lot 9DA) and papain were purchased from Worthington Biochemical Corp., Freehold, N.J. Commercial chymopapain is isolated from papaya latex essentially by the method of Jansen and Balls (1941).[1] Crude papaya latex (Type I) was bought from Sigma Chemical Company, St. Louis, Mo. A 10-g sample of crude latex was ground in a mortar (in the hood) and suspended in 100 ml of water. This suspension was dialyzed overnight at 5° against water, and undissolved material was removed by centrifugation. The solution was concentrated to half its original volume in an Amicon ultrafiltration device with a PM-10 membrane, and the concentrate was stored frozen.

Casein for assays and proteins for calibration of sodium dodecyl sulfate polyacrylamide gels were obtained from Schwarz/Mann, Orangeburg, N.Y. SP-Sephadex was purchased from Pharmacia, Piscataway, N.J. All chemicals were reagent grade or the best quality available.

Disc electrophoresis was carried out on 7.5 and 15% polyacrylamide gels with acidic β-alanine systems (Gabriel, 1971a).

Molecular weights were determined on dodecyl sulfate polyacrylamide gels (Weber and Osborn, 1969). Linear calibration curves on 10% gels were obtained with cytochrome *c*, lysozyme, myoglobin, trypsin, ovalbumin, and serum albumin.

Molecular weights were also determined by sedimentation equilibrium on a Beckman Model E ultracentrifuge equipped with interference optics and a temperature control unit. The meniscus depletion method (Yphantis, 1964) was used. Rotor speed was 33,450 rpm for the analyses of the components in pools 1 and 2; and 39,460 rpm, for the study of the protein in pool 3. An AN-H rotor with a 12-mm double-sector cell was used. A solvent blank was run and used to correct for window distortion.

Sedimentation coefficients were determined on the ultracentrifuge at 59,780 rpm, 25°, by analysis of Schlieren patterns. In all centrifugation studies, the protein solutions were dialyzed overnight at room temperature against 0.1 *M* sodium acetate buffer (pH 5.0) containing 1 m*M* EDTA.[2]

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[1] Worthington Biochemical Corporation, personal communication.

[2] Abbreviations used are: EDTA, ethylenediaminetetraacetate; Bz-Arg-OEt, *N*-benzoyl-L-arginine ethyl ester; Tos-Arg-OMe, *N*-tosyl-L-arginine methyl ester; Bz-Arg-NH_2, *N*-benzoyl-L-arginineamide; Bz-Arg-pNA, *N*-benzoyl-DL-arginine *p*-nitroanilide; Cm, carboxymethyl; Z, benzyloxycarbonyl.

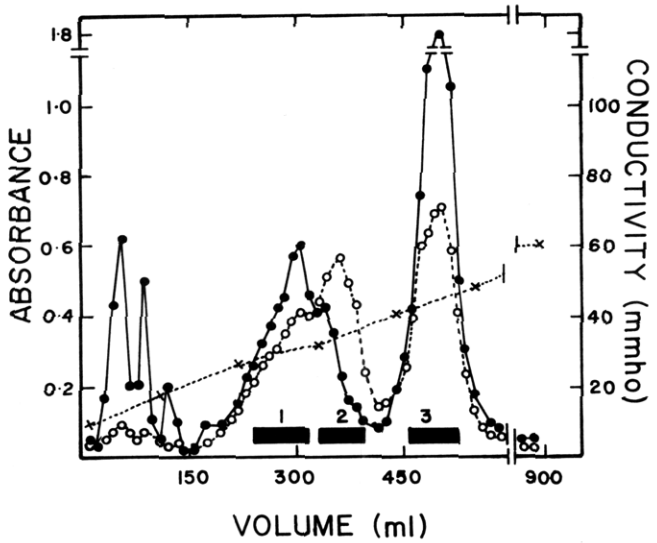

FIGURE 1: Chromatography of commercial chymopapain on a column (1.5 × 30 cm) of SP-Sephadex (C-50). The resin was equilibrated with 0.1 *M* sodium acetate buffer (pH 5.0) at room temperature, and a 300-mg sample of commercial chymopapain dissolved in 10 ml of this buffer was applied to the column. Elution was effected with a gradient formed in a nine-chamber mixer with 100 ml in each chamber. Chamber 1 contained 0.1 *M* sodium acetate buffer; chambers 2–4, 0.35 *M*; chambers 5–7, 0.7 *M*; and chambers 8–9, 1.5 *M*. The molarity represents the sodium ion content. All buffers contained 1 m*M* EDTA and were pH 5.0. 10-ml fractions were collected. Protein content (●) was indicated by the absorbance at 280 nm of the fractions, and proteolytic activity (○) was measured on 0.2-ml aliquots (read as absorbance at 280 nm) as described in the text. Conductivity (x--x) was measured with a Radiometer conductivity meter (Type CDM 2e) with a micro cell (Type CDCS 67021). Fractions were combined as indicated by the bars and are referred to in the text as pool 1, pool 2, and pool 3.

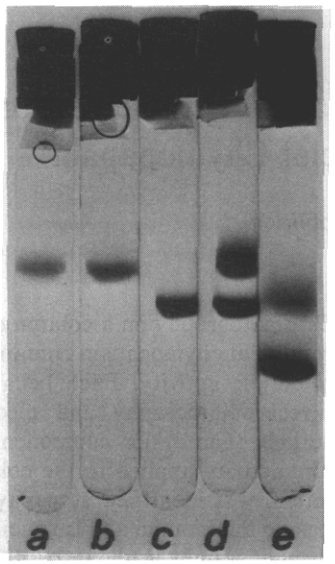

FIGURE 2: Disc electrophoresis of pools from SP-Sephadex chromatography. Samples from the three pools obtained as described in Figure 1 were applied to 7.5% polyacrylamide gels (5 × 60 mm) for electrophoresis using the β-alanine buffer system (Gabriel, 1971a). Electrophoresis was carried out at 5° and 2.5 mA/tube until the dye marker had traveled 45–55 mm. Gels were stained for 1 hr with Amido Black and destained by diffusion (Gabriel, 1971b). The gels are: a, pool 1; b, pool 2; c, pool 3; d, mixture of all three pools; e, commercial chymopapain. The sample of commercial chymopapain was run longer than the others.

Extinction coefficients were measured by a method developed by Pace.[3] Solutions of protein, 2–5 mg/ml, were dialyzed overnight against 0.1 *M* sodium acetate buffer (pH 5.0) containing 1 m*M* EDTA. Absorbance at 280 nm was measured in 1-cm cells, and the solutions were added to tared vials with a calibrated 2-ml pipet. The vials were heated at 110° for 12, 18, and 24 hr and weighed after cooling in a desiccator. Blanks, determined with the dialysis buffer, were subtracted from the solution weights before the extinction coefficients were calculated. All samples were run in triplicate.

Fluorescence measurements were made on a Perkin-Elmer fluorescence spectrophotometer (Model MPF-2A) equipped with a jacketed cell compartment that was kept at 25°. Excitation was at 292 nm, the wavelength for the maximum of the excitation spectrum of tryptophan as determined on this instrument. Emission spectra were scanned from 320 to 360 nm and maximal values were determined. Protein solutions (0.1–0.2 mg/ml) were dialyzed for 4 hr at room temperature against a wide-range buffer that was 0.02 *M* in $NaC_2H_3O_2$, NaH_2PO_4, and H_3BO_3 and 0.1 *M* KCl. The pH of this buffer was adjusted to 4.0 with HCl. Small amounts of 2.5 *N* NaOH or 2 *N* HCl were added with a microburet to 3-ml samples of protein, and pH was measured directly in the cuvet.

Amino acid analyses were performed on a Technicon Model NC-1 analyzer with a modified buffer gradient, ninhydrin reagent, and manifold.[4] Precision of the modified system was ±5%. Hydrolysis of proteins was done in 6 *N* hydrochloric acid at 110° under reduced pressure for up to 96 hr. Cysteic acid was measured on samples treated with performic acid (Moore, 1963), and tryptophan was determined after hydrolysis with *p*-toluenesulfonic acid (Liu and Chang, 1971).

Amino-terminal residues were determined by dansylation and analysis of the dansyl derivatives on thin-layer plates (Weiner et al., 1972).

Assays for proteolytic activity were carried out by the casein assay similar to one previously described (Kunimitsu and Yasunobu, 1970). The sulfhydryl enzymes were activated by adding 0.01–0.3 ml of enzyme sample to tubes containing 0.7 ml of 0.1 *M* sodium phosphate buffer (pH 7.2) that was 0.1 *M* in cysteine and 1 m*M* in EDTA. After the tubes were heated at 35° for 5 min, 1 ml of 1% casein solution in 0.1 *M* phosphate buffer (pH 7.2) was added, and the tubes were again heated at 35° for exactly 5 or 10 min. Addition of 3 ml of 5% Cl_3CCO_2H terminated the reaction. After the tubes had stood at least 30 min, the reaction mixture was filtered through Whatman No. 3 paper. Absorbance at 280 nm was measured on filtrates. Zero-time controls were run by adding Cl_3CCO_2H prior to the addition of casein; values for these controls were subtracted from the digestion samples. One unit of activity corresponded to a change of 1.0 unit of absorbance per min under the conditions described.

Esterase activity was measured at pH 7.2 with Bz-Arg-OEt as substrate by a titrimetric procedure for a papain assay (Arnon, 1970) and with Tos-Arg-OMe as substrate by a spectrophotometric method adapted from a trypsin assay (Walsh, 1970). In the Tos-Arg-OMe assay, the thiol enzymes were first activated by treatment with 0.014 *M* 2-mercaptoethanol at 35° for 5 min. Blanks to correct for spontaneous hydrolysis were run.

Amidase activity was measured at pH 7.5 with Bz-Arg-NH_2 and Bz-Arg-pNA as substrates by procedures used for

[3] C. Nick Pace, personal communication.

[4] G. W. Robinson, unpublished modifications.

Table I: Comparison of Amino Acid Compositions of SP-Sephadex Pools.[a]

Amino Acid	Pool 1	Pool 2	Pool 3
Asp	11.2	9.9	7.0
Thr	5.0	5.6	3.4
Ser	6.2	5.9	4.8
Glu	11.6	9.7	10.5
Pro	3.9	4.1	5.6
Gly	6.0	6.5	7.2
Ala	4.0	4.4	4.1
Val	6.0	6.5	7.2
½-Cys	3.1	2.6	2.4
Met	0.4	0.4	0
Ile	2.8	3.2	3.9
Leu	5.4	5.5	5.1
Tyr	8.6	9.3	7.7
Phe	3.3	4.0	2.0
Lys	10.6	11.2	11.6
His	1.4	1.7	2.1
Arg	3.3	3.3	6.8
Trp[b]	3.7	4.0	3.9
Recovery[c]	96.5	97.8	95.3

[a] Duplicate samples of pools 1, 2, and 3 from an SP-Sephadex column, described in the text, were hydrolyzed for 24 hr and analyzed by ion-exchange chromatography. Results are expressed as g of amino acid residue per 100 g of protein and are uncorrected for any losses or for incomplete release that may occur under these hydrolytic conditions. [b] Determined after hydrolysis with *p*-toluenesulfonic acid (Liu and Chang, 1971). [c] The ranges of recovery for the samples were ±5% for pools 1 and 3 and ±10% for pool 2. Amounts of protein hydrolyzed were calculated from absorbances at 280 nm.

Table II: Amino Acid Composition of Papaya Peptidase A.[a]

Amino Acid	Residues/Molecule: Time of Hydrolysis 24 hr	48 hr	64 hr	96 hr	Calcd
Asp	13.0	12.7	13.2	13.3	13
Thr[b]	10.6	8.8	8.7	8.4	12
Ser[b]	14.2	12.3	13.1	10.6	16
Glu	20.4	18.5	19.6	19.1	20
Pro	17.0		15.4	14.8	16
Gly	31.7	33.3	29.8	31.6	32
Ala	14.9	15.0	14.4	16.4	15
Val[c]	18.5	20.4	20.8	20.4	21
½-Cys[d]	6.81				7
Met[d]	0	0	0	0	0
Ile[c]	8.2	9.8	9.3	10.1	10
Leu[c]	10.1	10.9	11.2	11.0	11
Tyr[b]	12.8	11.6	12.4	10.4	14
Phe	3.3	3.0	3.4	2.9	3
Lys	22.8	22.6	21.2	22.8	23
His	3.9	4.0	4.3	4.1	4
Arg	10.8	10.9	10.0	10.8	11
Trp[e]	4.40	4.36	4.26		5

[a] Duplicate samples, hydrolyzed for 24, 64, and 96 hr, and a single sample, hydrolyzed for 48 hr, were analyzed by ion-exchange chromatography. Conditions for hydrolysis and analyses are given in the text. Results are expressed as residues per molecule of 24,100 molecular weight. Samples were taken from pool 3 of an SP-Sephadex column as discussed in the text. [b] Extrapolated to zero-time of hydrolysis. [c] Determined from time-course analysis. [d] Determined by presence of cysteic acid and absence of methionine sulfone after performic acid oxidation and hydrolysis (Moore, 1963). [e] Determined after hydrolysis with *p*-toluenesulfonic acid (Liu and Chang, 1971).

papain (Arnon, 1970), except that a continuous spectrophotometric assay was used with Bz-Arg-pNA.

All spectral assays were carried out with a Zeiss PMQ II spectrophotometer equipped with a transmission-emission converter and recorder. A water circulator kept the cell compartment at 25°. Rates of hydrolysis were proportional to enzyme concentration in each assay.

Results

Chromatography of commercial chymopapain on a column of SP-Sephadex reveals three components with proteolytic activity, as seen in Figure 1 for a typical separation. Pools 1 and 2 represented 20 and 26% of the activity applied to the column; pool 3 contained 31%. Chromatography on a column of Cm-cellulose with the same gradient system gave a similar pattern. Papain eluted from an identical column of SP-Sephadex at 150 ml.

Each pool eluted as a single component upon rechromatography on small columns of SP-Sephadex with evidence of some overlap between pools 1 and 2. Disc electrophoresis on polyacrylamide gels also indicated nearly pure components in pools 1 and 2 and a single protein in pool 3, as seen in Figure 2.

Solid ammonium sulfate, added to each pool, precipitated the protein in pools 1 and 2 at 70–75% saturation and in pool 3 at 85–90% saturation. A crystalline sheen appeared, and these slurries were stable for at least 6 months at 5°.

Because of the ease of isolation, the component in pool 3 was characterized more fully than the proteins in pools 1 and 2. The data, presented below, suggest that the proteins in pools 1 and 2 are chymopapain components and that the protein in pool 3 is papaya peptidase A.

Amino acid analyses, presented in Tables I and II, indicate that the components in pools 1 and 2 contain a small amount of methionine, whereas none is found in pool 3. All fractions have large amounts of lysine and glutamic acid. Isoleucine was found as the N-terminal residue of the protein in pool 3; tyrosine, in pool 2. Two residues, tyrosine and glutamic acid, were determined as N-terminals of the component in pool 1. No carbohydrate was detected in pool 3 by the phenol–sulfuric acid or anthrone methods (Ashwell, 1966; Spiro, 1966).

Table III: Molecular Parameters of Components from SP-Sephadex Column.

Property	Pool 1	Pool 2	Pool 3
Molecular Weight			
Dodecyl sulfate gel electrophoresis	25,900	27,300	24,100
Sedimentation equilibrium	24,000	28,900	23,800
$s_{20,w}^0$			2.55
f/f_{min}[a]			1.14
$\bar{v}$[b]	0.715	0.724	0.727

[a] Calculated from $s_{20,w}^0$, $\bar{v}$, and molecular weight (Tanford, 1961). [b] Calculated from amino acid composition (Cohn and Edsall, 1943).

Extinction coefficients at 280 nm of a 10-mg/ml solution in 0.1 *M* sodium acetate buffer (pH 5.0) were 17.9 for the component in pool 1 and 18.3 for the proteins in pools 2 and 3.

Table III lists molecular weight values for the components determined by dodecyl sulfate electrophoresis and sedimentation equilibrium. In the centrifugation studies of the three components, fringe displacements of 100–1000 μ resulted in linear plots when the natural logarithm of the displacement ($\ln f$) was plotted against the square of the ra-

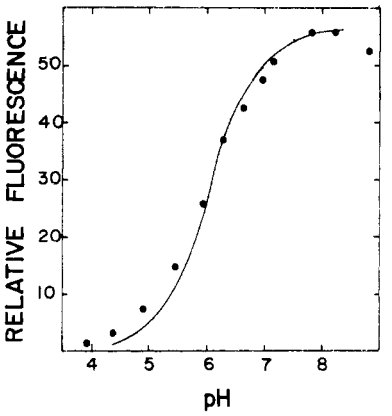

FIGURE 3: Fluorescence titration of papaya peptidase A. Fluorescence emission of the sample isolated in pool 3 (described in Figure 1) was measured as a function of pH. Details of the titration are given in the text, the intensity of fluorescence emission is given in machine units. The solid line represents the theoretical curve for a group with a pK of 6.05, and the closed symbols are the experimental values.

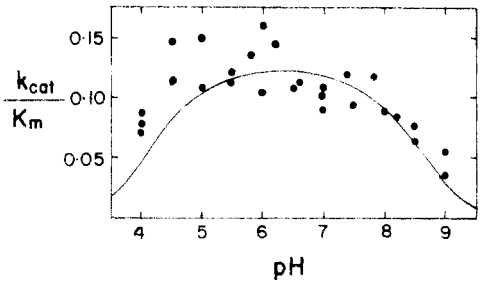

FIGURE 4: Plot of k_{cat}/K_m for papaya peptidase A with Bz-Arg-OEt. Kinetic constants for the hydrolysis of Bz-Arg-OEt catalyzed by papaya peptidase A were calculated by assuming a mechanism identical with one proposed for papain (Whitaker and Bender, 1965). The solid line represents a theoretical curve based on two groups with pK's of 4.3 and 8.5 that affect the hydrolysis. At each pH, data were obtained on six concentrations (8–80 mM) of substrate with 1–3 μM enzyme. Kinetic constants were calculated on a Wang 2200 computer from least-squares analyses of rate curves by a program for Michaelis–Menten kinetics (P. Method, personal communication). Units for k_{cat} are sec^{-1}; and for K_m, mM.

Table IV: Activity of Papaya Peptidase A in Catalyzing Hydrolysis of Ester and Amide Substrates.[a]

Substrate	pH	Substrate Concn (mM)	Enzyme Range[b] (μg/ml)	Specific Activity[c]
Bz-Arg-OEt	7.2	80	20–75	2.55
Tos-Arg-OMe	7.2	1	30–320	0.011
Bz-Arg-NH_2	7.5	80	50–150	0.076
Bz-Arg-pNA	7.5	1	10–170	0.002

[a] Hydrolyses catalyzed by the enzyme in pool 3 (Figure 1) were followed as described in the text. All assays were done at 25°. [b] Concentration range of the enzyme in the assay solution. [c] Specific activity is expressed as μmoles of substrate hydrolyzed per min per mg of protein.

dial distance (r^2). For the component in pool 3, an $s_{20,w}{}^0$ value of 2.52 S was extrapolated from a least-squares analysis of sedimentation velocity data of six concentrations between 1.7 and 4.6 mg/ml. Single determinations of $s_{20,w}$ for solutions (4 mg/ml) of the proteins in pools 1 and 2 gave values of 2.86 and 2.78. The schlieren patterns for all three components were symmetrical. Table III also lists other molecular parameters for the proteins in the three pools.

Intensity of fluorescence emission was determined as a function of pH; Figure 3 shows the results for the protein in pool 3. An apparent pK of 6.05 was calculated for an ionizing group affecting fluorescence. The components in pools 1 and 2 gave similar curves with calculated pK values of 6.45 and 6.35.

Enzymatic properties of the component in pool 3 were examined with a protein substrate after various treatments. Unreduced enzyme had only 4.8% the activity against casein of the reduced enzyme. A 10-min exposure at 35° to a 0.1 M solution of iodoacetamide at pH 7.2 completely inactivated the reduced enzyme. Amino acid analysis after this treatment and acid hydrolysis showed 0.6 residue of S-Cm-cysteine per molecule. After unreduced enzyme was similarly treated with iodoacetamide, no free sulfhydryl groups were evidenced by the absence of S-Cm-cysteine in an acid hydrolysate. When a solution of unreduced enzyme at room temperature was acidified to pH 2, then neutralized and made 0.1 M in cysteine to reduce the enzyme, more than 95% of the hydrolytic activity against casein was lost.

In the proteolytic enzyme assay against casein, the component in pool 3 had 0.74 unit/mg compared to 0.9–1.07 for chymopapain A (Ebata and Yasunobu, 1962), 3.4 for chymopapain B (Kunimitsu and Yasunobu, 1967), and 2.94 for papain (determined in this study). The components in pools 1 and 2 had 0.18 and 0.19 unit/mg.

Table IV summarizes the catalytic properties of the enzyme in pool 3 against amide and ester substrates. In Figure 4, a plot of k_{cat}/K_m against pH is shown for the hydrolysis of Bz-Arg-OEt, where k_{cat} is the maximal velocity divided by the enzyme concentration ($V_{max}/[E_0]$) and K_m the Michaelis constant. A mechanism identical with one applied to papain (Whitaker and Bender, 1965) is assumed. The curve is calculated by arbitrarily assigning pK values for two prototropic groups on the free enzyme of 4.3 and 8.5 in the expression $f = 1/(1 + [H^+]/K_1 + K_2/[H^+])$ where f is the fraction of the observed maximal value of k_{cat}/K_m. The enzyme in pool 3 did not catalyze the hydrolysis of the dipeptide substrates Z-Phe-Leu, Z-Phe-Tyr, Z-Phe-Gly, Z-Gly-Phe, and Ac-Phe-Tyr. No hydrolysis was detected by the ninhydrin reaction or amino acid analysis (Gerwin et al., 1966) when 8.3 mM solutions of peptide in 0.1 M sodium phosphate buffer (pH 7.2) were treated with 135 μg of activated enzyme for 24 hr at 35°.

Discussion

Three types of evidence indicate that the component in pool 3 is the same as the protein in papaya latex that has been named papaya peptidase A. First, both proteins lack methionine and have similar amino acid compositions as shown in Table V. The ratio of lysine to arginine is about 2 for these proteins and differs from that for papain and chymopapain. Secondly, the extrapolated value of 2.52 for $s_{20,w}{}^0$ found in this study agrees well with the value of 2.45 previously observed (Shack, 1967). Lastly, a component of papaya latex elutes from a column of SP-Sephadex at essentially the same salt concentration as that found for papaya peptidase A (Shack, 1967), as seen in Figure 5. This component has the same electrophoretic mobility as the fraction isolated from commercial chymopapain as compared in Figure 6.

Similar specificity requirements of the papaya enzymes

Table V: Comparison of Amino Acid Compositions of Papaya Proteinases.[e]

Amino Acid	Proteinase					
	Peptidase A[a]	Peptidase A[b]	Papain[c]	Chymopapain B[d]	Pool 1[a]	Pool 2[a]
Asp	13	13	19	29	25	25
Thr	12	9	8	17	13	16
Ser	16	14	13	24	18	19
Glu	20	19	20	30	23	22
Pro	16	13	10	14	10	12
Gly	32	32	28	41	27	33
Ala	15	15	14	20	15	18
Val	21	21	18	27	17	19
½-Cys	7		7	11	8	7
Met	0	0	0	1	1	1
Ile	10	10	12	13	6	8
Leu	11	11	11	16	12	14
Tyr	14	11	19	20	14	16
Phe	3	3	4	7	6	8
Lys	23	22	10	27	21	25
His	4	4	2	5	3	4
Arg	11	11	12	10	5	6
Trp	5		5	6	5	6
Total	233		212	318	229	247
Molecular weight calculated	25,165		23,406	34,452	24,950	28,119
N-Terminal residue	Ile		Ile	Tyr	Tyr,Glu	Tyr

[a] This study. [b] Calculated using a molecular weight of about 25,000 (Shack, 1967). [c] Glazer and Smith, 1971. [d] Kunimitsu and Yasunobu, 1970. [e] Results are expressed as residues/molecule.

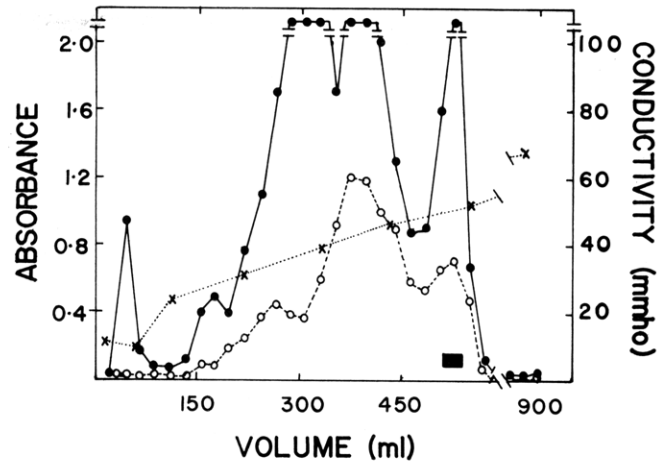

FIGURE 5: Chromatography of crude papaya latex on SP-Sephadex. A 10-ml sample of papaya latex, which was prepared as described in the text, was applied to a column (1.5 × 30 cm) of SP-Sephadex (C-50). Conditions of elution and analysis are given in Figure 1. Protein content (●) and proteolytic activity (○) are indicated as functions of absorbance at 280 nm. Conductivity (x--x) of the fractions was also determined. A pool, indicated by the bar, was taken for electrophoresis.

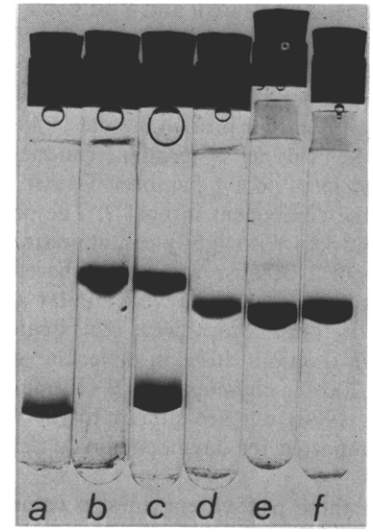

FIGURE 6: Comparison of papaya peptidase A with papain and crude latex fraction. Electrophoresis was carried out at 5° and 2.5 mA/tube with the β-alanine buffer system (Gabriel, 1971a), and gels were stained with Amido Black (Gabriel, 1971b). Gels a–c were 7.5% polyacrylamide; gels d–f, 15% polyacrylamide. Samples were: a, papaya peptidase A; b, papain; c, mixture of papaya peptidase A and papain; d, papaya peptidase A; e, fraction from papaya latex (Figure 5); f, mixture of papaya peptidase A and crude latex fraction. The dye marker had run off the gels a–c and had moved 55 mm on gels d–f.

and streptococcal proteinase (Johansen and Ottesen, 1967; Gerwin et al., 1966) and the limited comparison of amino acid sequences (Light et al., 1964; Tsunoda and Yasunobu, 1966; Liu et al., 1965; Robinson, 1970) suggest that these enzymes have homologous structures at their active sites even though they differ in many physical properties. Properties of papaya peptidase A reported here point out additional similarities among these thiol enzymes. Because the three-dimensional structure and extensive data about papain are available, papain serves as a model for comparison with other thiol proteinases (Drenth et al., 1971; Glazer and Smith, 1971).

Papain and papaya peptidase A lack methionine and have a residue of isoleucine on their N-termini. Both are inactivated at acidic pH in contrast to chymopapain which is stable at pH 2 (Kunimitsu and Yasunobu, 1967; Jansen and Balls, 1941). Their overall amino acid composition and electrophoretic behavior differ considerably, however, as shown in Table V and Figure 6.

Fluorescence titration curves for papaya peptidase A resemble those for unactivated papain (Shinitzky and Goldman, 1967; Barel and Glazer, 1969; Weinryb and Steiner, 1970; Sluyterman and DeGraaf, 1970). The sigmoid curve results from the fluorescence of a tryptophan residue(s) that is quenched at low pH by a protonated group near it and which then becomes unquenched upon ionization of this group. The apparent pK for such a group calculated for papain is 6.6 (Shinitzky and Goldman, 1967; Barel and Glazer, 1969) compared to 6.05 for papaya peptidase A.

Tryptophan–histidine complexes have been implicated in this fluorescence behavior by a study of model compounds (Shinitzky and Goldman, 1967), and such an interaction may exist between tryptophan-177 and histidine-159 at the active site of papain (Drenth et al., 1971). Fluorescence titrations with activated forms of papain, however, reflect ionization of groups with pK's of 4.6 and 8.5 (Barel and Glazer, 1969; Sluyterman and DeGraaf, 1970). These values suggest that a carboxyl or possibly a perturbed histidyl group is responsible for the lower pK and a sulfhydryl group for the higher one. Although no definite assignment can be made to groups in unactivated papaya peptidase A that affect its fluorescence, the similarity of its fluorescence titration curve to that of unactivated papain should be noted.

The catalytic activity of papaya peptidase A is generally lower than that observed for other proteolytic enzymes. It

hydrolyzes casein less than papain or chymopapain under identical conditions. Values of k_{cat}/K_m for Bz-Arg-OEt and Bz-Arg-NH_2 are also lower than values found for papain (Whitaker and Bender, 1965) at comparable pH values. The specific activity against the ester substrate Tos-Arg-OMe is 0.01 unit/mg compared to 247 units/mg for trypsin (Walsh, 1970) where a unit is the hydrolysis of 1 μmol/min. The enzyme is active, however, against several substrates which are hydrolyzed by other thiol proteinases except that its fails to split the model dipeptides which are readily hydrolyzed by streptococcal proteinase (Gerwin et al., 1966). Papain was also found inactive against these model substrates.

The chymopapain components isolated in this study resemble but are not identical with chymopapain fractions A and B that have been described (Ebata and Yasunobu, 1962; Kunimitsu and Yasunobu, 1967). Glutamic acid is the N-terminal residue on chymopapain A; tyrosine, on chymopapain B. The presence of both amino acids as N-terminal residues in pool 1 and of tyrosine in pool 2 suggests that pool 1 contains chymopapain A; and pool 2, chymopapain B.

No relationship between the A and B derivatives has been established (Kunimitsu and Yasunobu, 1967), and one may be the hydrolysis product of the other. The amino acid compositions and molecular weights found here suggest that the smaller component in pool 1 could have been derived from the slightly larger protein in pool 2. Also the presence of two amino acids as N-terminal residues may indicate that the protein in pool 1 has been formed by limited hydrolysis of the component in pool 2. The possibility exists, however, that pool 1 is slightly contaminated with the component in pool 2 because the electrophoretic behaviors of the proteins in the two pools were similar and because the N-terminal determinations were not quantitative. These chymopapain fractions differ in molecular weight from the value of 35,200 for chymopapain B (Kunimitsu and Yasunobu, 1967, 1970) but are similar to the value of 27,000 tentatively reported for chymopapain A (Ebata and Yasunobu, 1962).

Although these papaya enzymes in commercial chymopapain have similar size and catalytic properties, their differences presented here suggest that previous work using commercial chymopapain (Arnon and Shapira, 1967, 1968; Shapira and Arnon, 1967) may need reinterpretation. The large amounts of papaya peptidase A found in commercial chymopapain make assignment of particular properties to chymopapain difficult. The ease of isolation of papaya peptidase A, however, makes it suitable for further analysis in studying structure-function relationships in thiol proteinases.

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