BBA 65597

CHYMOPAPAIN

IV. THE CHROMATOGRAPHIC FRACTIONATION OF PARTIALLY PURIFIED CHYMOPAPAIN AND THE CHARACTERIZATION OF CRYSTALLINE CHYMOPAPAIN B

DONALD K. KUNIMITSU* AND KERRY T. YASUNOBU**
Department of Biochemistry and Biophysics, University of Hawaii, Honolulu (Hawaii)

(Received November 15th, 1966)

SUMMARY

1. The partially purified chymopapain fraction from dried papaya latex has been fractionated further by chromatography on Amberlite XE-64 resin and the distribution of proteolytic activity has been studied in greater detail. "Procedure A" resolves the chymopapain fraction into 8 major fractions.

2. In order to compare the properties of the various components, "Procedure B" was developed, which separates the chymopapain fraction into 4 major components. Amino acid content, N-terminal residues, chromatographic behavior, sulfhydryl content and specific activity were determined for each of the 4 components. Although all the fractions show the properties described previously for chymopapain, distinct differences were noted in the chemical and enzymatic properties.

3. The protease designated chymopapain B can be obtained in crystalline form from Fraction VIII by Procedure A and from Fraction IV by Procedure B. The enzyme differs from chymopapain A with respect to N-terminal residue, specific activity, sulfhydryl content and amino acid composition. The crystalline enzyme was shown to be monodisperse electrophoretically, by ultracentrifugation, N-terminal amino acid analysis and rechromatography.

INTRODUCTION

Studies of the protein constituents of papaya latex indicate the presence of at least four discrete enzymes. Papain[1] and papaya lysozyme[2] have been studied in detail

Abbreviations: BAA, *N*-benzoyl-L-arginine amide; PCMB, *p*-chloromercuribenzoate; DDPS-, *N*-(4-dimethylamino-3,5-dinitrophenyl) succinimido-; CM-, carboxymethyl-.

* NDEA Fellow 1960-1963, NIH Predoctoral Fellow 1964. Present address, Department of Chemistry, Cornell University, Ithaca, New York.

** To whom requests for reprints should be addressed.

but studies with chymopapain and glutamine cyclotransferase[3] are much less definitive.

SMITH AND KIMMEL[4] have reported that the crystalline chymopapain isolated by the procedure of JANSEN AND BALLS[5] was electrophoretically heterogeneous. Most recently, chymopapain A from dried papaya latex has been purified and crystallized in our laboratory by utilization of a modified JANSEN AND BALLS procedure which incorporates the use of cation exchangers[6].

During the course of isolating chymopapain from crude papaya latex, as opposed to the partially purified latex (Paul Lewis Laboratories, Wisconsin), we have been able to further fractionate the "chymopapain-rich" fraction on Amberlite IRC-50 (XE-64). Some of the properties of the various chromatographic species were studied and a new component, chymopapain B, has been obtained in homogeneous form. This protease differs in several respects from the chymopapain A previously purified in our laboratory[6].

In the present report, various methods for the preparation of chymopapain B are evaluated and the homogeneity of the crystalline enzyme is demonstrated. In addition, some of the enzymic properties of chymopapain B have been studied and compared with those of chymopapain A and papain.

MATERIALS AND METHODS

Materials

Dried papaya latex (Lot No. 20211010) was purchased from Paul Lewis Laboratories and was marked under the designation "Crude standardized papain". Papain was prepared from this latex in the absence of cysteine by a procedure previously described[6]. All preparations employed in this study were re-crystallized three times.

p-Chloromercuribenzoate (PCMB) and iodoacetate, as well as all of the synthetic substrates used, were products of Mann Research Laboratories, New York. Other chemicals and materials were obtained from the same sources as described in a previous report from this laboratory[6].

Determination of protein concentration

Protein concentrations were determined refractometrically in a Brice–Phoenix differential refractometer by the use of a refractive index increment of 0.00186 for a 1% solution, at 546 mμ of a deionized sample of enzyme. A similar determination of the refractive index increment for papain gave the same value of 0.00186 at 546 mμ.

Extinction coefficients ($E^{1\%}_{1\,cm}$ at 280 mμ) for chymopapain B, as well as for the other fractions, are summarized in Table II. Papain gave the value 24.0 in 0.10 M acetate buffer (pH 5), which is in contrast to that obtained previously in our laboratory[6], but is in agreement with the result of GLAZER AND SMITH[7].

Determination of enzymic activities

Proteolytic activity was measured with casein in essentially the same manner as described previously[6]. Amidase activity was assayed by the ninhydrin method of MOORE AND STEIN[8] with BAL as activator. The procedure was calibrated in a manner described by STOCKELL AND SMITH[9]. Peptidase activity was measured by the formal titration technique[10].

N-Terminal amino acid analysis

The procedure of SANGER[11] was used to determine the N-terminal amino acid. The DNP-protein was hydrolyzed 8, 12, and 24 h with constant-boiling HCl at 105°. Hydrolysis of the DNP-protein for 4 h in concentrated HCl indicated that no DNP-glycine or DNP-proline was present; the aqueous phase in all hydrolyzates was examined for DNP-arginine with negative results. The solvents, the corrections for manipulative losses, and the destruction factors during acid hydrolysis of pertinent DNP-amino acids were obtained as described by FRAENKEL-CONRAT, HARRIS AND LEVY[12].

In order to distinguish between isoleucine and leucine, and aspartic acid and glutamic acid, the DNP-amino acids, after elution from paper, were rechromatographed in solvent systems 2 and 4 (*cf.* ref. 13) on thin-layer plates.

Amino acid analysis

24-h hydrolysates of various fractions (6 mg) were analyzed in the Spinco Model 120 automatic amino acid analyzer. Separate analyses for methionine sulfone and cysteic acid were carried out after performic acid oxidation by the procedure of MOORE[14]. The total number of amide groups was determined essentially by the method of STEGEMANN[15]. The ammonia liberated was measured by the indophenol method catalyzed by sodium nitroprusside[16], and the values obtained were extrapolated to zero time.

Electrophoretic studies

Free-boundary electrophoresis was conducted at 1° in a Perkin–Elmer electrophoresis apparatus, Model 38, equipped with schlieren optics. The buffers were prepared by the method of MILLER AND GOLDER[17].

Ultracentrifugal studies

Ultracentrifugal studies were performed in the Spinco Model E analytical ultracentrifuge. The conventional 12-mm cell with the 4° centerpiece was used. The runs were made at 59 780 rev./min at 24°.

Determination of free –SH groups

The spectrophotometric method of BOYER[18] was routinely employed for these determinations. The concentration of mercaptide was determined at 234 mμ at pH 4.6 ($E_m = 1.74 \cdot 10^4$). The molar ratios of PCMB to protein varied from 0.10 to 3.0. Periodic readings at 255 mμ were followed for 30 min in each experiment, but the reaction was essentially complete in 10 min. At the higher concentrations of PCMB, the enzyme activity was completely abolished.

Free –SH groups were also determined as CM-cysteine after alkylating the protein (10 mg in 4 ml of 0.1 M sodium phosphate buffer, pH 7.0) in the absence and presence of activator (10^{-2} M cysteine, $5 \cdot 10^{-3}$ M EDTA) with four times recrystallized iodoacetic acid. The samples (including the cysteine when present) were incubated with a 20-fold molar excess of iodoacetic acid. After 30 min (100% inhibition), the carboxylmethylated proteins were exhaustively dialyzed. 24-h hydrolyzates of the protein were then examined for CM-cysteine in a Spinco Model 120 amino acid analyzer. The molar ratio (moles CM-cysteine per mole protein) was calculated by assuming that the molecular weight of each protein was 34 500 (ref. 19). From the known

amino acid composition of the various components (see Table IV), the amount of CM-cysteine was expressed in relation to the content of proline, glutamic acid, aspartic acid and alanine which were completely liberated after a 24-h hydrolysis.

RESULTS

Purification of chymopapain B

Evaluation of the procedure of Jansen and Balls. A previous report from this laboratory noted the failure of the procedure of JANSEN AND BALLS[5] to yield homogeneous crystalline chymopapain and similar results were obtained with the starting material used in the present study. The non-crystalline enzyme was electrophoretically heterogeneous (figure not shown) and the crystalline enzyme[4] has previously been shown to be impure. Chromatography of this fraction on Amberlite IRC-50 (XE-64) resulted in the appearance of at least eight chromatographically distinguishable components[19].

Development of "Procedure A" for preparing chymopapain B. This procedure incorporated all of the steps previously described for the isolation of chymopapain A (ref. 6) except for a slight modification in the buffers used to elute the enzyme from XE-64 resin. Fig. 1 illustrates a typical chromatogram; chymopapain B was obtained

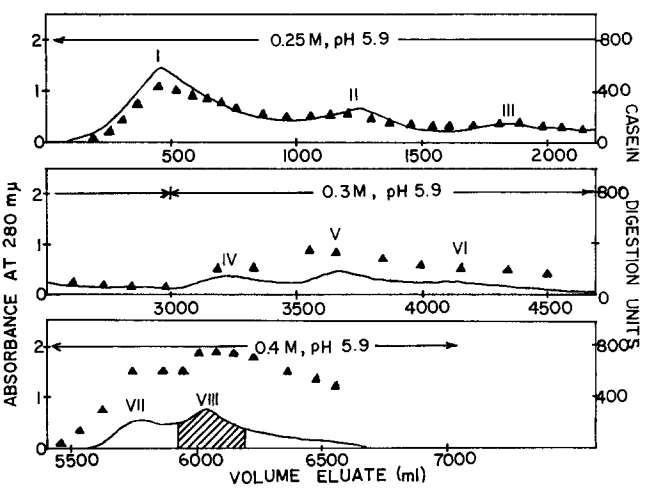

Fig. 1. The chromatography on XE-64 of the fully saturated NaCl precipitate. 1.7 g of protein were chromatographed on a 2.0 cm × 25.0 cm column eluted with buffers shown in the figure. (———) and (▲—▲) indicate absorbance and specific activity, respectively. Cross hatching indicates area where enzyme was collected. Procedure A was used.

by crystallizing the protein fraction present in Fraction VIII by the procedure described previously for chymopapain A (ref. 6). The results of the entire purification procedure are summarized in Table I. Fig. 2 shows a photomicrograph of crystalline chymopapain B.

Development of "Procedure B" for isolating chymopapain B. In this procedure, the NaCl fractionation step was eliminated to increase the yield of chymopapain B. Thus, the 0.7 M acetate buffer eluate obtained from the CM-cellulose chromatography

TABLE I

PURIFICATION OF CHYMOPAPAIN B

Step	*Procedure A*			*Procedure B*		
	Total protein (g)	*Specific activity* (casein digestion)*	*Activity recovered (%)*	*Total protein (g)*	*Specific activity* (casein digestion)*	*Activity recovered (%)*
1 Crude extract	21.22	2.77	100.0	59.82	2.80	100.0
2 0.45 saturated $(NH_4)_2SO_4$ supernatant	17.10	2.60	75.6	50.51	2.50	75.4
3 0.65 saturated $(NH_4)_2SO_4$ precipitate	8.67	3.08	45.4	31.62	3.08	58.1
4 CM-cellulose chromatography	5.44	2.70	25.0	15.61	2.70	25.2
5 Fully saturated NaCl precipitate	1.74	2.42	7.2	—	—	—
6 XE-64 chromatography	0.13	3.93	0.9	2.27	3.85	5.2
7 First crystals	0.10	3.50	0.6	1.59	3.40	3.2

* Assayed in the presence of 0.01 M cysteine + 5 mM EDTA.

step was directly chromatographed on XE-64 resin after dialysis against 0.25 M phosphate buffer (pH 5.90). The total number of enzyme units of chymopapain B was increased by a factor of 5 as shown in Table I. The chromatographic pattern on XE-64 resulting from Procedure B is shown in Fig. 3. What appeared to be crystalline chymopapain B could be isolated by Procedure B as well as by Procedure A, as shown in the subsequent sections. To reduce the number of analyses required, when the various fractions were compared, Procedure B was used.

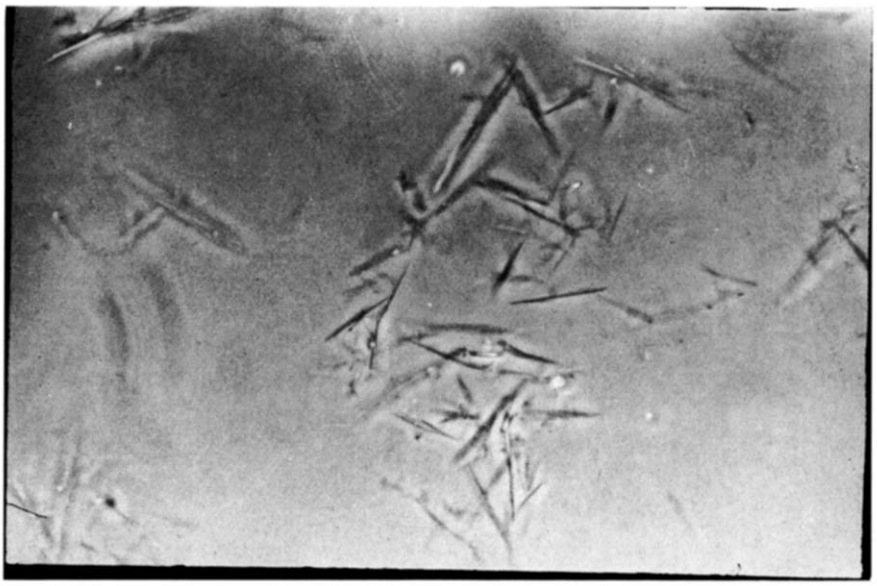

Fig. 2. Photomicrograph of once-crystallized chymopapain B (× 400).

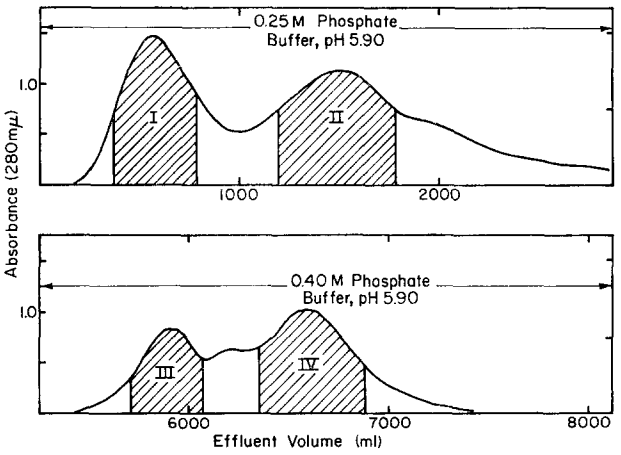

Fig. 3. The chromatography of the fully saturated NaCl precipitate on XE-64. 1.74 g of protein were chromatographed on a 2.0 cm × 25 cm column in a step-wise elution procedure with the buffers indicated. All operations were carried out at 4°. The solid line represents absorbance at 280 mμ. A few minor components eluted with 2 360 ml of 0.30 M phosphate buffer (pH 5.90) are not shown. Cross hatched areas demonstrate areas where enzyme was collected. Procedure B was used.

Properties of Fractions I–IV isolated by Procedure B

Specific activities. Table II presents the results of the specific activity measurements of Fractions I–IV as assayed with casein in the absence and presence of cysteine. It is evident that Fraction IV has the highest activity toward casein even in the absence of activator, in contrast to the other fractions which show a higher requirement for cysteine. The specific activities of Fractions I and II are similar to those reported previously for chymopapain A (ref. 6) and N-terminal studies (Table II) indicate that chymopapain A is probably present in these fractions.

TABLE II

SOME PROPERTIES OF FRACTIONS I–IV AND PAPAIN

Specific activity (casein digestion)			*—SH titer (moles —SH/mole protein)***			$E^{1\,cm*}_{1\%}$ 280 mμ	*N-terminal residues (% of total)*		
Fraction	*Non-activated*	*Activated*†	*Non-activated*		*Activated****		*Di-DNP-tyrosine*	*DNP-glutamic acid*	*Others*††
			PCMB	*Iodoacetic acid*	*Iodoacetic acid*				
I	0.39	1.13	0.88	0.83	1.32	18.40	40	52	8
II	0.42	1.18	0.86	0.80	1.30	18.45	65	26	9
III	1.58	2.80	0.75	0.60	1.26	18.60	90	10	Trace
IV	2.40	4.10	1.01	0.98	1.57	18.40	95	5	Trace
Papain	2.20	7.10	(*cf.* ref. 27)	(*cf.* ref. 27)		24.00	(*cf.* ref. 22)	(*cf.* ref. 22)	

* In 0.10 M acetate buffer, pH 5.0.

** Molecular weight of protein is assumed to be 33 500.

*** See MATERIALS AND METHODS for further details of the preparation of the carboxymethylcysteine-derivative and the analysis of carboxymethylcysteine.

† 0.01 M cysteine + 5 mM EDTA (final concentrations).

†† DNP-threonine, DNP-aspartic acid, DNP-isoleucine and DNP-alanine.

N-Terminal analyses. Examination of the N-terminal residues of the 4 major chromatographic components indicates that only glutamic acid and tyrosine can account for these residues, although much lower levels of threonine and aspartic acid can be found (Table II). These observations, coupled with the earlier observation that glutamic acid occupies the N-terminal position in purified chymopapain A (ref. 6), strongly indicate that the protease with the N-terminus of tyrosine constitutes a new proteolytic species. Moreover, the predominance of glutamic acid and tyrosine as N terminal residues in the proteins present in the crude extract of the latex (Table III)

TABLE III

SUMMARY OF SOME α-NH_2-TERMINAL AMINO ACID ANALYSES

Fraction	*α-DNP-amino acids found (% of total)*		
	DNP-di-tyrosine	*DNP-glutamic acid*	*Others**
Crude extract (treated with $HgCl_2$)**	60	30	10
0.65 saturated $(NH_4)_2SO_4$ precipitate	56	37	7
CM-cellulose fraction	59	34	7
Fully saturated NaCl precipitate	59	33	8
XE-64 Fraction IV (chymopapain B)	95	5	Trace
XE-64 Fraction IV (rechromatographed on XE-64)	95	5	Trace
Chymopapain B (recrystallized once)	95	5	Trace
Chymopapain B***	89	4	7

* DNP-threonine, DNP-aspartic acid, DNP-isoleucine, DNP-alanine.

** 0.01 M (final concentration): Some precipitation of protein occurred and therefore, the solution was clarified by centrifugation.

*** This fraction was left standing for 72 h at 25° in 0.1 M phosphate buffer (pH 7.0).

is evidence to support the contention that these two proteins exist in the starting material employed.

Free –SH content. The presence of free –SH groups in the chymopapain molecule had been first pointed out by JANSEN AND BALLS on the basis of the strong reaction of the enzyme with sodium nitroprusside[5]. CAYLE AND LOPEZ-RAMOS have reported that one cysteine residue per molecular weight of 35 000 combines readily with PCMB and iodoacetic acid and have stated that this sulfhydryl group is in the active site of chymopapain[20].

Determination of the free –SH content of Fractions I–IV also indicates that in the non-activated form of each of these four fractions approximately one cysteine residue reacts with PCMB and iodoacetic acid, but that in the presence of activator (cysteine and EDTA), there are small but significant increases in the free –SH titer (Table II).

Amino acid composition. In an attempt to ascertain specific physical differences among the 4 major fractions, amino acid analyses were carried out on 24-h hydrolysates of these fractions. Table IV summarizes these results.

It can be seen that all 4 fractions have very similar compositions except for slight differences in the content of lysine, glutamic acid, cysteine, glycine, methionine, tyrosine and phenylalanine. The abundance of lysine residues and the presence of

TABLE IV

AMINO ACID COMPOSITION OF FRACTIONS I–IV

The values are expressed as number of residues per molecular weight of 33 500 and are the results of duplicate determinations of 24-h hydrolysates.

Residue	*Fraction I*	*Fraction II*	*Fraction III*	*Fraction IV*
Lysine	26.1	25.7	24.5	25.1
Histidine	4.2	4.5	4.5	4.5
Arginine	9.3	9.6	10.0	9.7
Aspartic acid	27.0	27.2	27.0	27.1
Threonine	15.8	15.6	15.6	15.5
Serine	20.8	20.4	21.6	20.8
Glutamic acid	27.7	27.4	29.1	28.6
Proline	14.9	14.3	13.7	13.7
Glycine	42.2	39.0	38.1	38.6
Alanine	19.3	20.5	20.2	19.2
Half cysteine*	12.5	11.7	11.1	10.7
Valine	22.2	23.1	24.9	24.7
Methionine*	1.5	1.4	1.1	1.1
Isoleucine	11.2	11.6	12.0	12.2
Leucine	15.5	15.1	14.5	14.8
Tyrosine	18.3	18.5	18.7	19.5
Phenylalanine	8.0	7.4	6.3	6.5
Tryptophan**	5.6	5.9	5.8	5.7
Amide	29.1	29.1	30.1	30.1
Total residues	303	300	301	302
Calculated net charge***	+13	+16	+14	+14

* Performic acid oxidized sample.
** Spectrophotometric method of GOODWIN AND MORTON[29].
*** (Lysine + arginine + histidine) − (glutamic acid + aspartic acid − amide).

methionine in all 4 fractions are of particular interest since they immediately distinguish these proteins from papain.

The calculated net charges indicate very basic isoionic points and, in fact, determination of the isoelectric points of chymopapain A and chymopapain B has yielded values of 10.0 and 10.4, respectively[6].

Properties of crystalline chymopapain B

Homogeneity of the enzyme. The homogeneity of chymopapain B was ascertained on the basis of ultracentrifugation, free-boundary electrophoresis, N-terminal analyses and rechromatography.

Examination of the sedimentation patterns obtained during ultracentrifugation indicates that there is no gross heterogeneity evident at pH 4.75.

As had been observed during the purification of papain, the extent of purification cannot be judged solely by the increase in specific activity, since most of the proteins present in the papaya latex possess similar proteolytic activities. In fact, examination of Table I reveals that there is only a 1.3-fold purification. However, the multiple components present in the crude extract are reduced to a single component as can be seen in Fig. 4. Crystalline chymopapain exhibited only one electrophoretic band in the pH range of 4–11 (ref. 19). Therefore, the extent of purification of chymopapain B as judged by electrophoresis is much greater than can be assessed by the increase in specific activity alone.

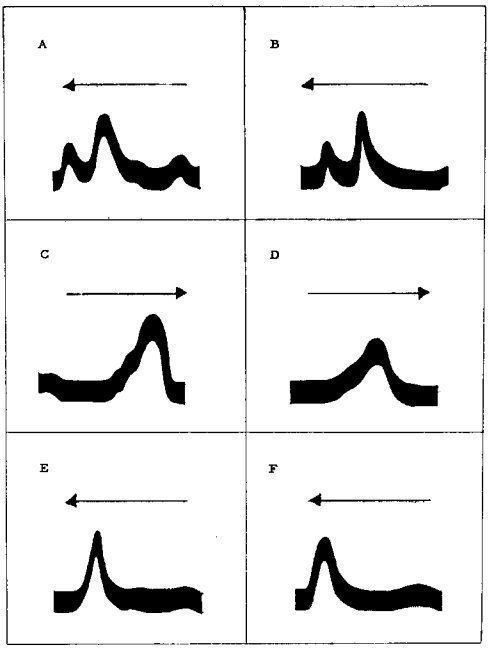

Fig. 4. The extent of purification of chymopapain A and chymopapain B was determined by electrophoresis. (A), crude extracts; (B), 0.45 saturated $(NH_4)_2SO_4$ supernatant fraction; (C), CM-cellulose fraction; (D), fully saturated NaCl precipitate; (E), chymopapain A; and F, chymopapain B. (A), (B) and (C) were run in 0.02 M acetate buffer (pH 4.0) at an ionic strength of 0.10. (D), (E) and (F) were run in 0.02 M phosphate buffer (pH 7.45) at an ionic strength of 0.17.

Table III summarizes the results of N-terminal analyses on different fractions obtained during the purification of chymopapain B, as well as on different samples of the enzyme. In general, di-DNP-tyrosine accounts for approximately 95% of the α-DNP-amino acids found in most recrystallized preparations.

Chymopapain B could be rechromatographed on XE-64 as a single, relatively symmetrical peak with essentially total recovery of protein and enzymic activity (Fig. 5). This chromatographic behavior is again suggestive of the homogeneity of this protease.

Stability. A property of chymopapain that was noted originally by JANSEN AND BALLS[5] was the relative stability of this protease at pH 2 when kept at temperatures below 10°. In fact, these workers utilized this stability as a basis for purifying chymopapain since, at pH 2, a considerable amount of inert protein (including denatured papain) could be removed by salt fractionation. It might be pointed out that the preparation procedures employed in the present study also incorporate this "acid step".

Chymopapain B is also stable at pH 2 but this stability depends markedly upon the manner in which the enzyme is stored. If the enzyme solution is frozen (about −10°), the protease loses approximately 50% of its activity within 24 h and then is further inactivated at a much slower rate. However, if the sample is kept at 4°, it will retain 85% of its activity after a period of two weeks. Papain, under comparable conditions, is rapidly and irreversibly inactivated.

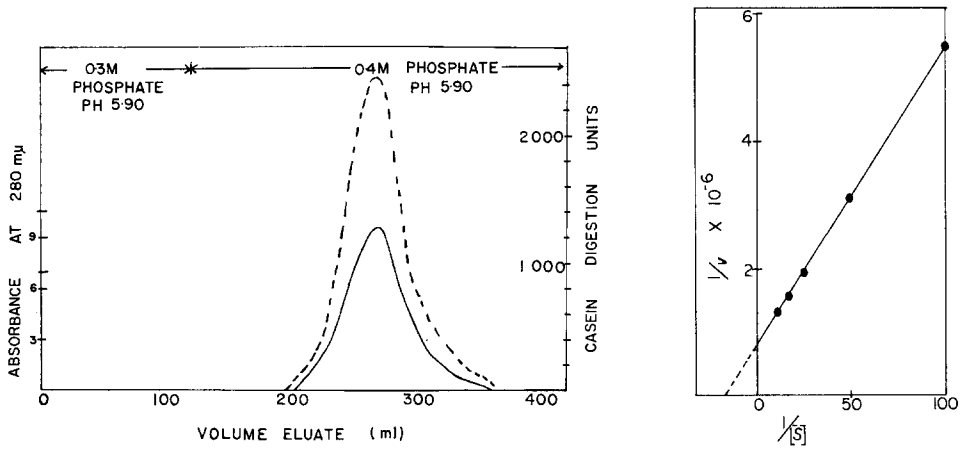

Fig. 5. Rechromatography of chymopapain B on XE-64. 425 mg of enzyme obtained by Procedure B was applied to a 1.0 cm × 20.0 cm column of XE-64 and elution was carried out with the buffers indicated. All operations were carried out at 4°. The solid line represents absorbance at 280 mμ and the dotted line represents casein digestion units.

Fig. 6. Plot of the reciprocal of the velocity versus the reciprocal of the substrate concentration for the hydrolysis of BAA. The reaction mixture contained in 1.5 ml: Citrate buffer, pH 5.50, 60 μM; KCl, 48 μM; enzyme, 0.45 mμM; BAL, 15 μM; and EDTA, 4 μM. The reaction was run at 39°. $v_{max.}$ and K_m were $13.1 \cdot 10^{-7}$ moles/l per sec and 0.061 M respectively.

In order to establish whether autolysis of chymopapain B was a significant factor in determining the general level of stability, a sample of the enzyme was allowed to stand at 25° for 72 h at pH 5. Activity measurements indicated that the protease lost approximately one third of its activity during this period, but a determination of the α-NH_2 groups by dinitrophenylation failed to reflect the occurrence of significant autolysis.

Influence of substrate concentration on enzymic activity. A Lineweaver–Burk plot for the chymopapain B-catalyzed hydrolysis of BAA at pH 5.5 is shown in Fig. 6. The linear relationship between 1/V and 1/S implies that the kinetics of BAA hydrolysis are amenable to treatment by the classical two-step Michaelis–Menten mechanism. The calculated value of k_3 (*i.e.*, k_3 in $E + S \rightleftharpoons ES \xrightarrow{k_3} P + 3$) for papain under comparable conditions is 10 sec^{-1} (see ref. 9). Compared on this basis, papain acts approximately twice as fast as does chymopapain B. CAYLE AND LOPEZ-RAMOS[20] and ourselves[6] have previously reported that papain acts ten times faster on BAA than does chymopapain, while KIMMEL AND SMITH[21] have reported that their preparations of chymopapain are about one-fifth as active as papain on BAA.

DISCUSSION

The isolation of crystalline chymopapain A has been previously reported[6]. The starting material was a partially purified papaya latex which is no longer commercially available. Therefore, it was necessary to use the crude latex as the source of the enzyme. Procedures A and B were developed which resolved the chymopapain fraction into 8 and 4 major fractions, respectively. Chymopapain B was obtained in crystal-

line form from Fraction VIII by Procedure A and from Fraction IV by Procedure B.

For the purpose of determining the physicochemical differences of the multiple chymopapain fractions, in the present study Procedure B was used in order to reduce the number of analyses. Another objective of the present investigation was to obtain at least one fraction in a homogeneous form so that its properties could be compared with those already determined for chymopapain A (ref. 6).

Concerning the first point, components I and II isolated by Procedure B appear to contain significant amounts of chymopapain A, while components III and IV appear to be a new proteolytic species (as judged by N-terminal analyses).

Although the amino acid compositions of the various fractions are quite similar, they can be distinguished on the basis of their N-terminal residues specific activities, and by their chromatographic behavior on XE-64. Determinations, by the Archibald method, of the molecular weights of chymopapain A and B indicate that their molecular weights are similar (approx. 35 000 g/mole) and therefore cannot account for the observed chromatographic behavior[19].

In this regard, it is significant that the presence of several proteolytically active and chromatographically distinguishable components has been observed in stem bromelain[23] and in various species of Ficus[24]. Moreover, WHITAKER and co-workers have clearly shown that species differences exist, and that the yield of the various chromatographic components depends upon the source of the starting material[25]. OTA, MOORE AND STEIN, on the other hand, report that reversibly inhibiting stem and fruit bromelain with phenylmercuric acetate yields a single component on chromatography[26], but in the absence of this inhibitor during purification, autolysis occurs yielding multiple components. FINKLE AND SMITH have in fact suggested that the wide distribution of proteolytic activity observed during the purification of the enzymes from dried papaya latex may be due to autolysis[27].

The question arises as to whether the multiple fractions we have observed are due to autolysis of the chymopapain molecule. This does not apparently seem to be the case since it has been shown that chymopapain A does not undergo autolysis[6] and chymopapain B did not autolyze significantly when left standing at room temperature for several days. Furthermore, when the crude extract of the papaya latex is dinitrophenylated, DNP-glutamic acid and di-DNP-tyrosine are found in significant amounts (Table III), indicating that chymopapain A and B are already present in the starting material employed in this study. However, we cannot unequivocally state that both of these species are true proteins in the sense that they will be found to exist in fresh papaya latex and, therefore, the possibility of conversion of native chymopapain to some degradation product during the preparation of the dried papaya latex cannot be ruled out.

The yields of chymopapain A and chymopapain B are 3% and 5%, respectively, as compared to a yield of 15% for papain (calculated on the basis of activity recovered). However, it is evident from this study that the chymopapain A and B isolated represent only a small portion of the total chymopapain fraction present in the starting material.

Although the reason for the wide spread of chymopapain activity has not been fully established, it would appear that the major reason for the low yield is the existence of chymopapain in a variety of forms which cannot be separated readily by conventional methods of protein fractionation. Among the possible forms which can be

separated from the native form by chromatography and salt fractionation is the partially denatured form, and inter-molecular disulfide bonded forms, and mixed disulfide forms with such compounds as glutathione whose existence in papaya latex has been demonstrated[28]. The extent to which these reactions alter the chromatographic behavior as well as the yields of the chymopapain components has not been fully established and should be subjected to more detailed studies.

We have attempted to minimize some of the complications attending the purification of an –SH enzyme by isolating chymopapain B as the inactive mercury derivative, but multiple chromatographically separable components[19] were still detected. Thus the fractionation effected by cation exchangers yields an essentially homogeneous enzyme preparation but, as a consequence of this fractionation, the yield is drastically lowered.

The properties of chymopapain B have been shown to resemble closely those reported for chymopapain A. Both of the enzymes are characterized by being stable at pH 2, possessing 1–2 essential sulfhydryl groups, having from one-ninth to one-half of the specific activity of papain on a weight basis, appearing to be more thermostable than papain, having greater milk clotting activity and by containing methionine. However, their substrate specificity[6] and the obligatory nature of at least one –SH group are properties which appear to be very similar to those of papain.

ACKNOWLEDGEMENTS

We should like to thank Dr. M. EBATA for helpful discussions and suggestions during the course of this work, and Miss PINKIE BEE and Miss ANN BENSON for their competent technical assistance. In addition, Dr. J. TSUNODA ran the fingerprint patterns of the peptic digest. This work was supported in part by research grants from the National Science Foundation and the National Institutes of Health.

REFERENCES

1 J. R. KIMMEL AND E. L. SMITH, *Advan. in Enzymol.*, 19 (1957) 267.
2 E. L. SMITH, J. R. KIMMEL, D. BROWN AND E. O. P. THOMPSON, *J. Biol. Chem.*, 215 (1955) 67.
3 M. MESSER AND M. OTTESEN, *Biochim. Biophys. Acta*, 92 (1964) 409.
4 E. SMITH AND J. R. KIMMEL, in P. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 4, Part A, Academic Press, New York, 1960, p. 133.
5 E. F. JANSEN AND A. K. BALLS, *J. Biol. Chem.*, 137 (1941) 459.
6 M. EBATA AND K. T. YASUNOBU, *J. Biol. Chem.*, 237 (1962) 1086.
7 A. N. GLAZER AND E. L. SMITH, *J. Biol. Chem.*, 236 (1961) 2948.
8 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 176 (1948) 367.
9 A. STOCKELL AND E. L. SMITH, *J. Biol. Chem.*, 227 (1957) 1.
10 N. C. DAVIS AND E. L. SMITH, in D. GLICK, *Methods of Biochemical Analysis*, Vol. 2, Interscience, New York, 1955, p. 215.
11 F. SANGER, *Biochem. J.*, 39 (1945) 507.
12 H. FRAENKEL-CONRAT, J. I. HARRIS AND A. L. LEVY, in D. GLICK, *Methods of Biochemical Analysis*, Vol. 2, Interscience, New York, 1955, p. 359.
13 K. RANDERATH, *Thin Layer Chromatography*, Academic Press, New York, 1964, p. 85.
14 S. MOORE, *J. Biol. Chem.*, 238 (1963) 235.
15 H. STEGEMANN, *Z. Physiol. Chem.*, 312 (1958) 255.
16 A. L. CHANEY AND E. P. MARBACK, *Clin. Chem.*, 8 (1962) 130.
17 G. L. MILLER AND R. H. GOLDER, *Arch. Biochem. Biophys.*, 29 (1950) 420.
18 P. D. BOYER, *J. Am. Chem. Soc.*, 76 (1954) 4331.
19 D. K. KUNIMITSU, *Ph. D. Thesis*, University of Hawaii, 1964.

20 T. CAYLE AND B. LOPEZ-RAMOS, *Abstr. Papers 140th Meeting Am. Chem. Soc.*, Chicago, 1961, p. 19C.
21 J. R. KIMMEL AND E. L. SMITH, *J. Biol. Chem.*, 207 (1964) 515.
22 E. O. P. THOMPSON, *J. Biol. Chem.*, 207 (1954) 563.
23 EL-GHARBAWAI AND J. R. WHITAKER, *Biochemistry*, 2 (1963) 476.
24 V. C. SGARBIERI, S. M. GUPTE, D. E. KRAMER AND J. R. WHITAKER, *J. Biol. Chem.*, 239 (1964) 2170.
25 D. E. KRAMER AND J. R. WHITAKER, *J. Biol. Chem.*, 239 (1964) 2178.
26 S. OTA, S. MOORE AND W. H. STEIN, *Biochemistry*, 3 (1964) 180.
27 B. J. FINKLE AND E. L. SMITH, *J. Biol. Chem.*, 239 (1958) 669.
28 A. K. BALLS AND H. LINEWEAVER, *J. Biol. Chem.*, 130 (1939) 669.
29 T. W. GOODWIN AND R. A. MORTON, *Biochem. J.*, 40 (1946) 628.