THE PURIFICATION AND SOME PHYSICAL-CHEMICAL STUDIES OF CRYSTALLINE CHYMOPAPAIN B

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DEDICATION

To my parents

and to Vivian, my loving wife-to-be

ACKNOWLEDGEMENTS

DR. MITSUO EBATA for his generous advice and encouragement during his all too brief stay in our laboratory.

MISS PINKIE GEE for her competent assistance during ultra-centrifugation studies.

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ABBREVIATIONS

B.A.A.	N-Benzoyl-L-Arginine Amide
BAL	2,3 Dimercapto Propanol (British Anti-Lewiscite)
CMC	Carboxymethyl Cellulose
DNP	Dinitrophenyl
E.D.T.A.	Ethylenediaminetetraacetic Acid (Versene)
M	Molar Concentration
mu	millimicrons
NEM	N-Ethyl Maleimide
PCMB	p-Chloro Mercuribenzoic Acid
$s_{20,w}$	Svedberg unit (10^{-13}sec^{-1}), corrected to water and 20°C
-SH	Sulfhydryl Group
TCA	Trichloroacetic Acid
umoles	Micromoles
XE-64	Amberlite IRC-50 (200-400 mesh)

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ABSTRACT

Chymopapain was first isolated and crystallized from fresh papaya latex by Jansen and Balls (J. Biol. Chem., 137, 459, 1951). More recently, a proteolytic component with some of the properties described by these workers was isolated and crystallized from dried papaya latex by Ebata (J. Biol. Chem., 237, 1086, 1962).

During the course of the purification of chymopapain from dried papaya latex, the appearance of several proteolytically-active components was consistently observed during chromatography on Amberlite IRC-50 (XE-64). We have been able to crystallize the protease present in one particular fraction which evidently differs from the chymopapain isolated by Ebata. This crystalline component (designated chymopapain B) is shown to be homogeneous by the criteria of ultra-centrifugation, electrophoresis, amino-terminal analysis and re-chromatography.

Physical studies on chymopapain B indicate that it differs from the chymopapain described by Ebata (designated chymopapain A). Molecular weight determinations by the Archibald procedure yield a molecular weight of 35,000 for component A and 30,000 for component B. Electrophoretic studies indicate that both components are very basic, with their isoelectric points in the neighborhood of pH 10. Furthermore, both show relative stability to heat and acidic pH's.

Like papain and chymopapain A, chymopapain B is also activated by thiol compounds, indicating that it is a sulfhydryl enzyme. Titration of the active enzyme with p-chloro-mercuribenzoate, mercuric chloride and N-ethyl-maleimide reveals the presence of two free -SH groups but extended studies

indicate that only one -SH is essential for activity.

Preliminary investigations into the substrate specificity of this enzyme show that it resembles papain in its ability to hydrolyze a large variety of peptide and amide linkages. Thus far, N-benzoyl-L-arginine amide is found to be the most sensitive substrate for chymopapain B. Moreover, the ability of the chymopapain B to carry out anilide synthesis has been demonstrated, suggesting that this enzyme, like papain, can function as a transferase.

Detailed kinetic studies with N-benzoyl-L-arginine amide as substrate show that chymopapain B hydrolyzes this compound at approximately one-third the rate of papain. Determinations of the Michaelis constant and maximal velocity at various pH's have also been undertaken.

I. INTRODUCTION

A. Historical Background of Studies on Some Protein Constituents of Papaya Latex

The dicotyledonous plant papaw or melon tree (Carica papaya) is indigenous to regions encompassed by the tropical zone. The edible fruit is of commercial interest for basically two reasons: first, the ripened fruit constitutes an agricultural commodity and second, the latex of the fully grown green fruit (with its proteolytic principles) is widely utilized in several industries, among others, in the beer industry (for preventing "chill hazes"), in the tanning industry (to bate skins and hides) and in the meat packing industry (to tenderize meat). (Tauber, 1949; Tainter and Buchanan, 1951).

From a scientific standpoint, it has been the latex which has interested biochemists for over a half century, beginning with the observations of Wurtz and Bouchut (1879) who described a proteolytic principle present in the latex (termed "papain" by these workers). Smith and Kimmel (1960) have given an excellent account of the significant studies concerned with papain.

While studies on papain are well documented in the literature, studies on the other protein components of papaya latex have been relatively lacking. An impetus toward studies on the other components of

papaya latex was provided by Jansen and Balls in 1941 when they successfully isolated and crystallized another proteolytic enzyme (termed "chymopapain") from fresh papaya latex (Jansen and Balls, 1941; Balls, 1941). Their method of isolation is re-stated in Section III.A.1. (Evaluation of the Procedure of Jansen and Balls). Their findings on chymopapain can be summarized as follows:

1. Chymopapain crystallized as broad sabre-like needles and sometimes in obvious plates.
2. The yield of this enzyme in crude latex is considerably greater than is that of papain.
3. Chymopapain has a higher ratio of milk-clotting activity to protein digestion activity when compared to papain. (Activity equal to papain with respect to milk-clotting and one-half of papain with respect to hemoglobin digestion, computed per unit of protein nitrogen.)
4. The proteolytic activity is undiminished for several weeks at pH 2.
5. The enzyme gave a strong -SH test with nitroprusside.
6. Chymopapain is many times more soluble than papain at salt concentrations and pH's where the latter is soluble.
7. It exhibits an apparent pH optimum of 7.0 when assayed with casein, ovalbumin and denatured hemoglobin (Greenburg, 1955).

During the period 1941-1950, relatively few fundamental investigations were carried out with chymopapain. Gottschall (1944) had shown that peptic digests of meat (beef muscle, liver) rapidly activated both papain and chymopapain. This activation was apparently due to the liberation of previously combined -SH groups in the substrate since the activating

properties of peptic digests were destroyed by pre-treatment of the latter with sufficient iodoacetate to abolish the nitroprusside test. His findings were in general accord with the then (as now) prevailing notion that papain and chymopapain (as well as other plant proteases such as ficin, bromelain and ascelepain) were -SH proteases. Some aspects of the activation processes of -SH proteases by thiols, cyanide and H_2S are reviewed by Greenburg and Winnick (1945).

Ito (1950) has demonstrated the formation of an antibody by chymopapain in vitro using diptheria toxin as antigen.

Kimmel and Smith (1954) have reported on the chymopapain content of fresh and dried papaya latex based on the electrophoretic separation of the various constituents present in the crude extract. They have estimated that fresh papaya latex contains 65% chymopapain and that the dried latex contains 53%. This chymopapain fraction has an electrophoretic mobility of +6.9 to +7.2 (x $10^{-5}cm^2$ per volt per second) in acetate buffer, pH 4.0, 0.10 ionic strength. This designation however, has only been tentative. Furthermore, these workers have prepared this component in approximately 90% purity (by the criterion of electrophoresis) and have shown it to possess one-fifth of the activity of papain with respect to the hydrolysis of N-benzoyl-L-arginine amide. Further characterizations of their chymopapain, however, have not been reported to date.

These same workers, it might be pointed out, have carried out some limited studies on chymopapain crystallized from fresh papaya latex by the method of Jansen and Balls. They have reported that the crystalline chymopapain of Jansen and Balls is heterogeneous electrophoretically (Smith and Kimmel, 1960).

In addition to their extensive studies with papain and some limited studies on chymopapain, Emil Smith and co-workers (1955) have successfully isolated and crystallized as a mercury-derivative an enzyme with lytic activity from dried papaya latex (termed "papaya lysozyme" by these workers). The properties of papaya lysozyme are summarized in Table I. On the basis of the activity, these workers have estimated that lysozyme accounts for one-third of the soluble protein in dried papaya latex.

More recently, Cayle and Lopez-Ramos (1961) have reported some properties of chymopapain. Since our information of their findings is based exclusively on an abstract, the method of preparation remains in doubt. However, it is safe to assume that their isolation is based on the method of Jansen and Balls, using fresh papaya latex as starting material. Their observations are summarized below:

1. The pH-activity curve with hemoglobin shows an apparent optimum between pH 2.5 and 4.0. Below and above this optimum, the activity falls off rapidly.
2. Amino acid analysis of chymopapain reveals an abundance of lysine residues, with an isoelectric point of approximately 9.0. A minimal molecular weight, based on this analysis, is approximately 35,000. Analysis also reveals 10.5 cysteine residues, one of which combines readily with PCMB and iodoacetic acid, and therefore, presumably associated with the "active center."
3. The activity of chymopapain towards BAA is approximately one-tenth of that of papain. This enzyme does not hydrolyze N-acetyl-L-tyrosine ethyl ester and N-acetyl-L-tyrosine amide. The hydrolysis of casein indicates that chymopapain has a narrower specificity than papain.

Most recently, Ebata (1962) has isolated, crystallized and characterized chymopapain from dried latex. His method of purification is outlined in Section III.A.1., Table III. Some of the properties of chymopapain are summarized in Table I.

His work constitutes the first study on chymopapain which makes subsequent studies meaningful. In particular, the demonstration of the homogeniety of chymopapain (which is lacking in previous studies, with the possible exception of the work of Smith) is obviously of paramount importance for any extended studies. Furthermore, such properties as the amino-terminal and sedimentation coefficient are requisite insofar as these properties help establish the presence of a particular enzyme present in the latex, since the presence of other proteases has not been altogether excluded. Some of these essential parameters have been obtained for his chymopapain. Thus, a basis for differentiation is available in the event of the isolation of other chymopapain-like components from papaya latex.

B. Summary of Some Properties of Enzymes Isolated from Dried Papaya Latex

Before the initiation of the present study, three enzymes were isolated in crystalline form from dried papaya latex and some of their properties elucidated. Some of the more salient properties of these enzymes are presented in Table I. (It might be stated that the properties represent the results of the most recent work on these enzymes and do not necessarily relegate to a position of lesser importance those studies carried out prior to those cited here.)

It will be noted that the chymopapain purified by Ebata is denoted chymopapain A. This designation arises from the fact that the present study is concerned with the characterization of a still third protease in the latex which resembles the chymopapain isolated by Ebata, but is evidently different in many respects from the latter. We have therefore arbitrarily designated the enzyme isolated by Ebata as chymopapain A and our enzyme, chymopapain B. (This aspect is discussed in more detail in Section I.C. Statement of the Problem.)

The properties of chymopapain B, the result of the present study, are summarized in Table XXVI, Section IV.A.

TABLE I

PROPERTIES OF SOME PAPAYA ENZYMES

PROPERTY	PAPAIN[1]	CHYMOPAPAIN[2]	PAPAYA LYSOZYME[3]
1. $S_{20,w}$	2.42 S	2.71 S	2.57 S
2. $D_{20,w}$	10.23×10^{-7}	----------	9.35×10^{-7}
3. $\bar{v}$	.724	.721	.726
4. Molecular Weight			
a. S and D	21,000	----------	24,300
b. Amino Acid Composition	20,900	34,600*	24,745
c. Archibald	20,700	35,400*	----------
5. Isoelectric Point	8.75	10.00*	10.5
6. Amino-Terminal	Isoleucine	Glutamic	Glycine
7. Carboxyl-Terminal	Asparagine	----------	----------
8. E (1%, 1 cm, 280 mu)	24.00	18.70	----------
9. Nitrogen Content	16.1%	16.1%	17.2%
10. Sulfur Content	1.2%	----------	1.9%
11. Crystalline form (Untreated)	Needles	Rods	Needles (Mercury Derivative)
12. Activation Requirements	Thiols, CN-, H_2S	Thiols, CN-, H_2S	None
13. Specific Activity	7.0 (Casein), 1.30 (BAA)	1.00 (Casein)	0.1 (S. Lutea)
14. Essential -SH	1.0	2.00*	None
15. pH Optimum	7.0 (Casein), 5.0-8.0 (BAA)	7.00 (Casein)	4.65 (S. Lutea)
16. Substrate Specificity	Wide range of peptide, amide and esters	Wide range of peptide and amide	----------

[1] Smith and Kimmel, 1960

[2] Ebata, 1962

[3] Smith, et. al., 1955

* Unpublished observation

C. Statement of the Problem

Our objective for isolating chymopapain was essentially three-fold: first, to extend the previous studies on chymopapain utilizing a highly purified enzyme (i.e. an enzyme which satisfies the criteria of homogeniety) with respect to a more involved physical-chemical characterization; second, to determine the substrate specificity with the hope of finding a protease with a specificity different from that of the well-characterized proteases; and third, to obtain a sulfhydryl enzyme which would permit studies on the possible role of the sulfhydryl group in enzyme catalysis.

At the outset, however, it became readily apparent that the original procedure of Jansen and Balls would not yield crystalline chymopapain if the commercially dried latex (marketed as "crude papain") were used. (This aspect is discussed in greater detail in Section III.A.1.) Furthermore, inasmuch as fresh papaya latex is not readily available, it was obvious that our isolation of chymopapain would necessarily involve the use of this dried latex. Therefore, attempts to obtain crystalline chymopapain from dried latex were initiated and carried through successfully in our laboratory. (See Section III.A.1.b.)

During the isolation of chymopapain A, the occurrence of proteolytic activity in many fractions was readily observed, and this distribution of activity, notably during cation-exchange chromatography, suggested the presence of other proteases. Especially noteworthy were the relatively higher specific activities of some of these fractions when compared to that of chymopapain A. These observations, it might be pointed out, coincided with that of others (Finkle and Smith, 1958). Concerning these observations, Finkle and Smith have stated that "the spread of activity

may be due, in part, to the presence of autolyzed fragments which vary in size and other properties." However, their observations per se do not necessarily exclude the possibility of the presence of a discrete enzyme (or enzymes) in dried latex, physically distinct from papain and chymopapain A. It is our hope that this study will demonstrate that chymopapain B does in fact represent such a distinct enzyme and, as such, merits extended studies.

The general experimental approaches taken during this study are well defined ones: first, the isolation and purification of the enzyme and the demonstration of its relative homogeniety; second, the characterization of the physical and chemical properties of the enzyme, especially the molecular weight, such that subsequent studies may be quantitated; and third, study of the enzymic activity and the possible relationship of different amino acid residues in catalysis. The ramifications of these general approaches are, of course, many and diverse. Furthermore, these studies constitute but the groundwork for the eventual understanding of the "mechanism of enzyme action," a goal of enzymology in general. Realizing that the present study constitutes only the initial attempts at the characterization of this enzyme, the choice of specific approaches to the problem must be dictated by an arbitrary preference for what one might consider to be fundamental. Moreover, since the time available to pursue this fundamental characterization is all too limited, the studies reported here can, at best, represent only that short step in the direction of the elucidation of mechanism. It is our hope, therefore, that future studies which utilize chymopapain B as a model enzyme will use the results of this study advantageously.

II. MATERIALS AND METHODS

A. Materials

1. Materials and Reagents Obtained Commercially:

a. Barnstead Still and Sterilizer Company, Massachusetts

IRA-411

b. California Corporation for Biochemical Research, California

Pentadecadfluorooctanoic acid (Na salt), Lot No. P505595

Sedoheptulose Anhydride (B Grade), Lot No. 500276

c. Carl Schleicher and Schuell Company, New Hampshire

Carboxymethylcellulose

d. Eastman Organic Chemicals, New York

p-Aminoacetophenone, Lot No. 46

Anthrone, Lot No. 11

D (+) Galactose (anhydrous)

Hydrazine Dihydrochloride

Hydroxylamine Hydrochloride (sulfate-free), Lot No. 127

p-Nitrophenylhydrazine

4-Phenylazo-1-naphthylamine

Phenylhydrazine Hydrochloride

e. E.I. Du Pont De Nemours and Company, Incorporated, Delaware

Sodium Lauryl Sulfate (U.S.P., "Duponal C")

f. Evans Chemetics, Incorporated, New York

Thioglycolic acid

g. J. T. Baker Chemical Company, New Jersey

Dowex 50-X8 (200-400 mesh)

h. Mann Research Laboratories, New York

1) Amino Acids, Amino Acid Derivatives and Dipeptides

L-Alanylglycine, Lot No. E1190

Benzoyl-L-Arginine, Lot No. G2336

Benzoyl-L-Arginine Amide.HCl.monohydrate, Lot No. G2060

Benzoyl-L-Arginine Ethyl Ester.HCl., Lot No. F3668

Benzoyl-Glycine Amide, Lot No. C2390

L-Cysteine, Free Base, Lot No. D3361

Glutathione (Reduced), Lot No. G2006

L-Histidine Ethyl Ester.diHCl, Lot No. C2538

L-Lysylglycine Sulfate, Lot No. 2236

N-Cbz-L-Glutamic Acid, Lot No. C2899

N-Cbz-alpha-Glutamyl-L-Phenylalanine, Lot No. G2454

N-Cbz-alpha-Glutamyl-L-Tyrosine, Lot No. F3918

N-alpha-Tosyl-L-Arginine Amide.HCl, Lot No. H1270

L-Phenylalanylglycine, Lot No. F4149

L-Prolylglycine, Lot No. G1296

2) Sugars

L-Fucose, Lot No. J1254

D-Galactosamine.HCl, Lot No. G2328

Alpha-D-Galacturonic Acid, Lot No. H1351

L (+) Rhamnose, Monohydrate, Lot No. G2193

3) Miscellaneous

2,3 Dimercaptopropanol

2,4 Dinitrofluorobenzene, Lot No. G1444

Indole, Lot No. H1013

Iodoacetic Acid, Lot No. G2048

Iodoacetamide, Lot No. G2180

O-Iodoso Benzoic Acid, Lot No. G1681

N-Ethyl Maleimide, Lot No. F3023

i. Matheson, Coleman and Bell, New Jersey

Ammonium Sulfamate, Lot No. 315464

2,4 Dinitrophenyl Hydrazine, Lot No. 364144

Ethylenediaminetetraacetic Acid (di Na salt), Lot No. 12

Semicarbazide Hydrochloride, Lot No. 1

D (+) Xylose, Lot No. 3021210

j. Metal Hydrides, Incorporated, Massachusetts

Sodium Borohydride

k. Nutritional Biochemicals Corporation, Ohio

Casein (Hammersten Quality), Lot No. 8503

p-Chloromercuribenzoic Acid (Na salt), Lot No. 8031

Hippuric Acid

L-Leucinamide.HCl, Lot No. 6534

D (+) Mannose

Orcinol, Lot No. 8031

l. Paul Lewis Laboratories, Incorporated, Wisconsin

Dried papaya latex

m. Pharmacia Fine Chemicals, Incorporated, New York

Sephadex G-25, G-50, G-75, G-100

SE-Sephadex

n. Rohm and Haas Company, Pennsylvania

Amberlite IRC-50 (XE-64)

o. Worthington Biochemical Corporation, New Jersey

Alpha-Chymotrypsinogen, 6X Crystallized

2. Materials Obtained As Gifts:

Porcine Glucagon and Bovine Insulin were kindly supplied by Dr. W. Bromer of Eli Lilly Company, Indianopolis, Indiana.

3. Materials Obtained by Preparative Methods:

Papain was prepared from dried papaya latex as described by Ebata (1962). The enzyme was re-crystallized three times in the absence of cysteine before use.

B. Methods

1. Preparation of Resins

a. Carboxymethyl cellulose, obtained as a dry powder, was suspended in a large volume of water and the fines removed by decantation. Repeated decantations aided in the removal of most of the fines. The wet resin was washed with 2N acetic acid, then with water. Subsequently, the resin was converted to the Na^+ form by washing with an equal volume mixture of 0.5N NaOH and 0.50M NaCl solutions. After repeated washing with water, the resin was buffered until the effluent pH approached the value of the influent pH.

b. XE-64 was prepared according to the method of Hirs (1955), except that the equilibration was carried out with 0.20M phosphate buffer, pH 5.90. After suspension of the resin in the buffer, the pH was adjusted to 5.90 by the addition of 10N NaOH. Final adjustment was effected after allowing the mixture to stand overnight.

c. Dowex-50 was converted to the H^+ form after overnight treatments with 4% (w/v) NaOH and 4% (v/v) H_2SO_4.

d. IRA-411 was converted to the OH^- form by overnight treatments with 4% (v/v) H_2SO_4 followed by 4% (w/v) NaOH.

e. All forms of Sephadex were treated as follows: the powder was suspended in buffer to disperse the aggregates, and the fines removed by repeated decantations. The resin was then introduced to a column and continually washed until salt-free (where water was used as eluent) or until the pH of the effluent was within 0.1 pH units of the influent buffer.

2. Ultra-Centrifugation Studies

a. Purity Determinations

Ultra-centrifugation measurements were made with the Spinco Model E analytical ultra-centrifuge, equipped with an RTIC unit for temperature regulation within .1°C. The conventional 12-mm cell was used with the 4° centerpiece. The speed employed was 59,780 rpm. All runs were carried out at 24°C.

b. Sedimentation-Velocity Method

Sedimentation velocity measurements were made at 59,780 rpm, and the sedimentation coefficient (S) was calculated according to equation 1:

$$S = 2\,(x_2 - x_1)/\,(x_2 + x_1)\,w^2\,(t_2 - t_1) \quad \ldots\ldots\ldots \quad (1)$$

where w is the angular velocity in radians per second, t is the time in seconds, and x the distance of the boundary in centimeters measured from the axis of rotation.

The initial concentrations obtained by serial dilution were corrected for radial dilution, where applicable, during the experiment using the well-known square dilution law of Trautman, et. al. (1954).

The observed sedimentation coefficients were reduced to standard conditions (water as solvent, 20°C) according to Svedberg, et. al. (1940).

Protein concentrations were determined refractometrically with a differential refractometer at 546 mu using a refractive index increment of .00186 for a one per cent solution. This value is probably within two per cent of the correct value. (See Section III.B.5.

The time-dependent sedimentation coefficients ($S_{20,w}$) were averaged (since the magnitude of ds/dt was small, well within the standard deviation) and then plotted against the initial concentration (concentrations corrected for radial dilution are no longer applicable in view of the averaging process for the values) and the line extrapolated to infinite dilution by the method of linear least squares.

c. Archibald "Approach-to-Equilibrium" Method

Molecular weight determinations by the Archibald approach-to-equilibrium method were conducted as outlined by Schachman (1957) and were performed at a speed of 12,590 rpm, with the schlieren phase plate angles ranging from 60° - 70°. No silicone fluid was used. Consequently, the molecular weight was calculated only from the meniscus by using equation 3 with $F = w^2 (dc/dx)_m / x_m c_m$ where the subscript m denotes the position of the meniscus. The concentration at the meniscus (c_m) was computed by the equation of Klainer and Kegeles (1955):

$$c_m = c_o - (1/x_m^2 \int_{x_m}^{X} x^2 (dc/dx)\, dx \quad \ldots\ldots\ldots\ldots (2)$$

where x_m is the distance of the air-solution meniscus measured in centimeters from the axis of rotation, X is an arbitrary x-coordinate in the plateau region (dc/dx = 0), and where c_o, the initial concentration, is determined with a standard 12mm, 4° sector synthetic boundary cell. All concentrations, it might be noted, are expressed in optical units, obtained by trapezoidal integration of the areas under the schlieren curves. In this laboratory, measurement of these curves were done with a Gaertner micro-comparator.

d. Measurement and Calculation of the Partial Specific Volume ($\bar{v}$)

The calculation of the molecular weight by either the sedimentation-velocity-diffusion method or the Archibald procedure

(approach-to-equilibrium method) involves the use of the relationship:

$$M = RT/ (1 - \bar{v}) \times F \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

where R = 8.314×10^7 ergs/degree/mole

T = temperature on the Kelvin scale

$\bar{v}$ = partial specific volume

= density of solution

F = (s/D) where D is the diffusion constant standardized to water and 20°C (Sedimentation-Diffusion Procedure)

$F = w^2 (dc/dx)_{m,b} / x_{m,b} c_{m,b}$ (Archibald Procedure)

Accordingly, the parameter $\bar{v}$ has to be evaluated independently.

The measurement of this parameter was carried out according to the method of densities as outlined by Schachman (1957), in which the densities of the solvent and solution were measured pycnometrically. From these measurements, the apparent partial specific volume could be calculated by means of equation 4 below:

$$v_{app} = 1/d_o - 1/x (d - d_o) / d_o \quad \ldots\ldots\ldots\ldots\ldots (4)$$

where d_o = density of solvent (buffer)

d = density of solution

x = concentration of protein in gm/cc

The concentration of the protein was determined refractometrically as discussed above.

3. Electrophoresis

a. Free-Boundary Electrophoresis

Electrophoresis was conducted at 0° with a Perkin-Elmer electrophoresis apparatus, Model 38, equipped with schlieren

scanning optical system. A record of the electrophoretic behaviour was obtained by use of a Polaroid land-back camera attached to the instrument.

The buffers were prepared by the method of Miller and Golder (1950).

b. Measurement of the Isoelectric Point

Electrophoresis was carried out as described above.

Buffers of one-tenth ionic strength were prepared according to Miller and Golder, previously cited. The pH of the solution was determined at ice-bath temperatures with a Beckman pH-meter with expanded scales.

Conductivity measurements of the buffered protein solutions were measured in a conductivity cell with platinum electrodes, using a Leeds and Northrup Wheatstone bridge. The cell constant was determined using .10N KCl, and the value 1.37 obtained.

Dialysis of the protein solution against the supporting electrolyte was carried out in the cold room for at least 24 hours with continuous stirring. Prior to the measurement of the conductivity, the protein solution was routinely centrifuged to remove extraneous suspensions.

During the electrophoresis run, a minimum of six photographs of the descending boundary patterns was taken at various time intervals.

The mobilities were calculated in the customary manner from the distances of migration of the maximal positions of the individual peaks. The mobilities cited in Table XIV represent the average values of the calculated mobilities obtained from five

separate determinations of the descending limb. The measurement of the distances of migration was made with the aid of a micro-comparator.

4. Measurement of the Refractive Index Increment (dn/dc)

The refractive index increment of chymopapain B was determined at 436 mu and 546 mu using a Brice-Phoenix differential refractometer (Phoenix Instrument Company, Philadelphia, Pennsylvania). The enzyme was de-ionized by passing it through a multi-bed resin containing Dowex 50 (H^+ cycle) and IRA-411 (OH^- cycle). All measurements were made on a de-ionized preparation to minimize contributions from extraneous variables (e.g. necessity of correcting for the weight of the protein in the presence of buffer, differential contribution to the refractive index increment by the solvent due to the Gibbs-Donnan equilibrium). A stock solution of this de-ionized enzyme was used and the measurement of (dn) (de-ionized water as solvent) made on serially diluted solutions. The concentration of the enzyme was determined by taking a suitable aliquot and drying to constant weight in tared weighing bottles over P_2O_5 in a vacuum dessicator. Three-times crystallized papain and six-times crystallized alpha-chymotrypsinogen were also used. The concentrations of these enzymes were determined spectrophotometrically using $E_1^{1\%}{}_{cm}$ (278) = 25.00 for papain,(Glazer and Smith, 1961) and $E_1^{1\%}{}_{cm}$ (282) = 20.0 (alpha-chymotrypsinogen),(Wilcox, et. al. , 1957).

5. Nitrogen Content

Samples of once-crystallized chymopapain B were lyophilized after de-ionizing the enzyme solution by passage through a multi-bed resin

containing Dowex-50 and IRA-411. The lyophilized sample was dried to constant weight in an Aberhalden apparatus. The nitrogen content was determined in quadruplicate by a micro-Kjeldahl procedure.

6. Amino Terminal Analysis

The procedure of Sanger (1945, 1949) was used to determine the amino-terminal acid. In order to identify the dinitrophenyl acids, the solvent system t-amyl alcohol-3% NH_3 and 1.5M phosphate buffer, pH 6.0 was used to examine the ether phase and the solvent system t-amyl alcohol-phthalate buffer (pH 6) used to examine the aqueous phase (Fraenkel-Conrat, et. al., 1958). No correction was applied for either manipulative losses or destruction during acid hydrolysis.

7. Analysis for Carbohydrate Content

Total sugars (hexoses, pentoses, methyl pentoses, 2-desoxypentoses) were determined by the cysteine-sulfuric acid method of Dische (1949).

Hexoses were determined by the Weimer-Moshin modification (1952) of the method of Lustig and Langer (1931).

Pentoses were estimated by the orcinal method of Bial, (1902).

Methyl pentoses were determined by the water effect test using the cysteine-sulfuric acid reaction of Dische and Shettles (1951).

Hexuronic acid was determined by the Dische carbazole reaction (1947).

Heptoses were determined by the (Primary) cysteine-sulfuric acid reaction of Dische (1949).

Hexosamine was determined by examination of a 24-hour hydrolysate (6N HCl) in the automatic amino acid analyzer.

8. Determination of SH Groups

The spectrophotometric method of Boyer (1954) using PCMB was employed for most of the -SH measurements reported here. To a fixed amount of protein (0.05-0.10 umoles) graded levels of PCMB were added, and the final volume adjusted to 4.0 cc with the appropriate buffer. Each tube was read at 255 mu (0.33M acetate buffer, pH 4.60) or 250 mu (0.10M phosphate) buffer,(pH 6.8) with appropriate blanks to correct for the contribution to the total absorbancy by the protein and PCMB. Periodic readings indicated that the reaction between protein and PCMB was essentially complete in thirty minutes. All samples were routinely allowed to stand for ninety minutes and activity measurements subsequently carried out.

A limited number of measurements were made using the spectrophotometric method of Alexander (1958), but a wavelength of 310 mu (molar extinction coefficient of NEM = 589) was utilized in order to minimize the absorption due to the protein, as recommended by Habeeb (1960).

This method was utilized to a very limited extent, being used only to confirm the -SH titer as measured by the PCMB titrations. The serious limitation of this method, it might be pointed out, is the requirement of a fairly large amount of enzyme (at least .1 - .2 umoles -SH/cc). However, when employed, the system was composed as follows: 2.0 cc of enzyme (5 mg/cc), 4 umoles of NEM dissolved in 2.0 cc of 0.10M phosphate buffer, pH 6.8. The absorbancy was read periodically at 310 mu against a blank containing the protein solution without NEM. At the same time, a solution containing only NEM was read against a

buffer blank in order to obtain the absorbancy of the unreacted NEM which tends to decompose with time. (Alexander, 1958) -SH groups were calculated from the difference in absorbancy of the unreacted and reacted NEM at any given time using the molar extinction coefficient of NEM cited above.

9. Methods of Assay and the Measurement of Enzymic Activity

a. Casein Digestion Method

The proteolytic activity of chymopapain and papain was determined by the method of Kunitz (1947) using a 1% solution of casein (Hammersten quality, re-precipitated with glacial acetic acid and repeatedly washed with acetone and ether) in suitable buffers. For most of the assays used in this study, activity measurements were made at 35°C in 0.10M phosphate buffer, pH 7.20 (arbitrarily chosen as standard condition). When necessary, the activation of the enzymes was carried out as follows: to a suitable volume of enzyme containing ca. 200 ug of enzyme was added an equal volume of 0.10M cysteine in phosphate buffer, pH 7.2 containing .01M E.D.T.A. The mixture was incubated in a constant temperature bath at 35° for at least five minutes. The reaction with casein was then carried out in the following manner: to a series of test tubes were pipetted graduated aliquots of this activation mixture up to a volume of 1.0 cc (the adjustment to this volume being brought about by the addition of suitable amounts of buffer) and to this was added 1.0 cc of 1% casein which had been equilibrated at bath temperature. Digestion was then allowed to proceed for ten minutes, after which the reaction was terminated

by the addition of 3.0 cc of 5% trichloroacetic acid. This mixture was allowed to stand at room temperature for a least thirty minutes and subsequently filtered. The absorbancy of the clear supernatant was then measured at 280 mu in a Beckman D.U. spectrophotometer. A unit of enzyme activity was defined as the amount of enzyme necessary to cause a change in absorbancy of .001 unit, corrected for the absorbancy contributions of a non-enzymic nature (this blank being prepared by adding 3.0 cc of T.C.A. to 1.0 cc casein, followed by the addition of 1.0 cc of the enzyme solution). Specific activities (or P.U., proteolytic unit) were expressed as the change in absorbancy at 280 mu per minute per ug of enzyme, the maximum value being estimated from the initial slope of a plot of change of absorbancy versus ug enzyme/ml of reaction mixture (2.0 cc under these conditions).

b. Assay for Amidase Activity

In studies where a more accurate measurement of enzymic activity was necessary, particularly in the kinetic studies, the choice of a readily available, well-defined substrate is limited to amino acid derivatives (e.g. amides, esters, hydrazides, etc.), to dipeptides and their derivatives and to higher order peptides and their derivatives.

Preliminary work on the specificity of chymopapain B with respect to the hydrolysis of some simple amino acid derivatives and some dipeptides (Section III.D.5.) indicated that the amides of arginine, glycine and leucine were hydrolyzed, with N-benzoyl-arginine amide being the most sensitive substrate. Hence, methods for the quantitative determination of the extent of hydrolysis

were necessary. For this purpose, two well-known methods were generally used. For low levels of hydrolysis (up to 10%), the ninhydrin method of Moore and Stein (1954) was used and for higher levels of hydrolysis, the Schwert, et. al., (1948) modification of the Conway (1939) method was utilized.

1) Ninhydrin Method of Moore and Stein (1954)

Ammonia formed during the hydrolysis of BAA was determined by the method of Moore and Stein (1954). The procedure was calibrated with ammonium sulfate standards which contained all of the components of the reaction mixture.

For purposes of assay, 0.10 cc of a stock solution of .05M BAA in 0.10M phosphate buffer, pH 7.2, was added to a series of test tubes and incubated at 35°C for at least five minutes. Subsequently, 0.10 cc of the enzyme solution (containing 10-50 ug of enzyme per ml) was added to each of these tubes. At known intervals of time, 1.0 cc of ninhydrin solution (20 grams ninhydrin, 3 grams hydrindantin, 250 cc 4.0N acetate buffer, pH 5.5, and 750 cc methyl cellosolve) was added to stop the reaction. The resulting mixture was heated in a boiling water bath for twenty minutes (the test tubes were capped with aluminum foil to minimize evaporation) and rapidly cooled in tap water. To this cooled solution, 10.0 cc of 50% ethanol was added and the absorbancy of the solution was determined at 540 mu in a Beckman D.U. spectrophotometer. A blank was prepared by adding 1.0 cc of ninhydrin to .10 cc of substrate followed by the addition of 0.10 cc of the enzyme solution. The subsequent work-up of the blank is, of course, similar to

that of the samples under study. From a plot of absorbancy readings (which can be converted to concentration terms by a standard curve calibrated with $(NH_4)_2\ SO_4$ containing all of the components) versus time, the rate of the reaction (expressed as absorbancy change per unit time, moles per liter ammonia formed per unit time or per cent hydrolysis per unit time) could be calculated.

2) Conway Method for Ammonia

The Schwert modification of the Conway micro-diffusion technique was also used to measure ammonia formed during the hydrolysis of BAA. The micro-diffusion was carried out in an Obrink (1955) modification of the Conway dish (Scientific Products, New York). The inner chamber consisted of 1.0 ml of 2% Boric Acid. 1.5 ml of saturated potassium carbonate was used in the outer chamber to liberate NH_3. After allowing the aliquot and potassium carbonate to react for at least two hours, 1 drop of Tashiro's indicator was added and the contents titrated with .01N HCl delivered by a syringe microburet (Model SB2, Micro Metric Instrument Company, Cleveland, Ohio).

10. Kinetic Analyses

Rate measurements were conducted by means of the ninhydrin method of Moore and Stein (1954), discussed above.

Unless otherwise specified, assays were conducted at 39°C. The reaction mixture consisted of 0.75 ml of N-Benzoyl-L-Arginine amide in a suitable buffer, 0.55 ml of sufficient KCl to adjust the ionic strength of the mixture to 0.20M and 0.20 ml of the enzyme solution,

containing 7.5×10^{-2}M BAL. Immediately after mixing, and at suitable intervals thereafter, 0.20 ml aliquots were removed and added to 1.0 cc of ninhydrin. The tubes were capped with aluminum foil and worked up as described above.

The method was calibrated with ammonium sulfate solutions containing all of the components of the reaction mixture, including the enzyme which was suitably denatured by the ninhydrin solution.

Initial velocities were estimated essentially by the method described by Lumry, et. al. (1951) in their studies with carboxypeptidase A.

Kinetic analyses were made with the Michaelis-Menton scheme of enzyme catalysis:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

Assuming the validity of the steady state approximation, the constants K_M and V_{max} were calculated from plots of the data according to the method of Lineweaver and Burk (1934). The values obtained are linear least squares estimates from a 1/v versus 1/S plot of the velocity data. K_M and V_{max} are defined as $(k_2 + k_3)/k_1$ and $k_3(E)_t$, respectively, where E_t is the total enzyme concentration. The values for k_3 were calculated on the assumption that the enzyme contains one catalytic center per molecule.

III. RESULTS

A. Purification and Crystallization of Chymopapain B

1. Purification Procedures

a. Evaluation of the Procedure of Jansen and Balls

As pointed out earlier in the Introduction, the original observations of the presence of chymopapain in fresh papaya latex was made by Jansen and Balls. Their method for the isolation and crystallization of this acid-stable protease is reproduced in Table II.A.

Because of the unavailability of fresh papaya latex, our studies have employed the commercially dried latex as the starting material. However, with this dried latex the method of these workers failed to yield crystalline chymopapain, a difficulty similarly encountered by Kimmel and Smith (1954).

Table II.B. outlines the method initially used for the purification of chymopapain with the dried latex as the source of enzyme. This method represents but a very slight modification of the method of Jansen and Balls. The results of the purification from the standpoint of specific activities and protein content is given in Table II.C.

On the basis of activity recovered, the yield is approximately 19% and the fraction isolated after step 4 (Table IIB) represents a 1.8 fold purification (considered from step 2, after the removal of papain). Attempts to crystallize chymopapain from the precipitate obtained from step 4 have met with consistent failure.

As previously pointed out, the crystalline chymopapain of Jansen and Balls is heterogeneous electrophoretically. Figure 1 shows the electrophoretic pattern of the uncrystallized precipitate obtained by the method given in Table II.B. Three major components are clearly discernable. Furthermore, chromatography of this fraction on XE-64 shows the presence of several chromatographically distinct fractions (Figure 2). It is to be noted that the chromatographic behaviour of the various components from XE-64 resembles the elution pattern obtained during XE-64 chromatography by the method of Ebata. These experiences have led to the conclusion concerning the non-feasibility of the method of Jansen and Balls for obtaining crystalline chymopapain from dried latex.

By drastically modifying their procedure, Ebata was able to successfully obtain crystalline chymopapain from dried latex. His method of preparation is discussed in the next section.

Figure 1. The electrophoresis of non-crystalline chymopapain prepared by the method of Jansen and Balls from dried papaya latex. Run carried out in .02M phosphate buffer, pH 7.45, u = .17. The temperature was 1°C. (A) ascending pattern at 135 minutes (B) ascending pattern at 185 minutes.

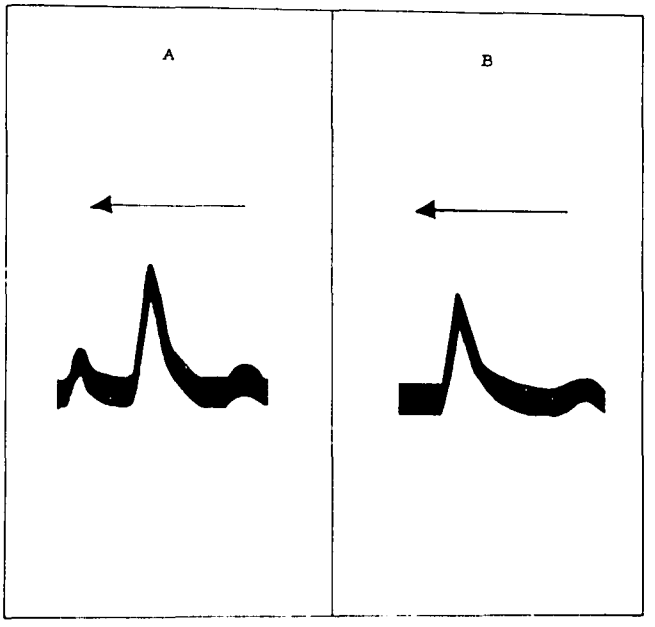

A
B

Figure 2. Chromatography of non-crystalline chymopapain prepared by the method of Jansen and Balls from dried papaya latex on XE-64. 4.20 grams of protein were charged on a 2.5 x 25.0 cm column and elution effected by the buffers indicated. All operations were carried out at 4°C.

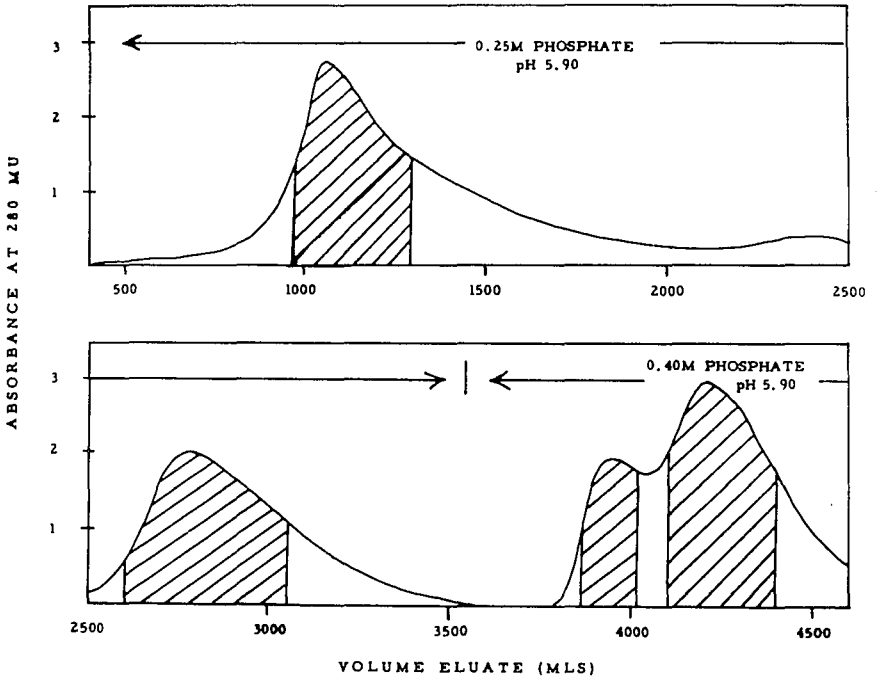

0.25M PHOSPHATE
pH 5.90
0.40M PHOSPHATE
pH 5.90
ABSORBANCE AT 280 MU
VOLUME ELUATE (MLS)
1
2
3
500
1000
1500
2000
2500
3000
3500
4000
4500

TABLE II. A.

METHOD OF JANSEN-BALLS (1941)

FRACTION NUMBER	PROCEDURE
F-1	pH of fresh papaya latex adjusted to 5.0 and filtered. Precipitate discarded.
F-2	The pH of the above supernatant is adjusted to 2.0. NaCl is added to one-half saturation. The resulting mixture is filtered or centrifuged. The precipitate is discarded.
F-3	The pH of the supernatant is adjusted to 4.0 and the precipitate formed is filtered or centrifuged off and discarded.
F-4	The pH of the supernatant is again adjusted to 2.0 and NaCl is added to full saturation. After standing, the precipitate is filtered or centrifuged and the supernatant discarded.
F-5	The precipitate is suspended in a minimal volume of water and solid NaCl added slowly. The pH is carefully adjusted to 2.0. Under these conditions, chymopapain crystallizes from solution.

TABLE II. B

MODIFIED JANSEN-BALLS PROCEDURE

FRACTION NUMBER	PROCEDURE
F-1	Dried papaya latex is extracted with cold .02N acetate buffer, pH 5.0. pH is adjusted to 5.0 and the resulting mixture filtered. The precipitate is discarded.
F-2	The pH of the solution is adjusted to 2.0 and the resulting precipitate centrifuged and discarded.
F-3	The pH of the supernatant is adjusted to 4.0 and NaCl added to one-half saturation. The precipitate is discarded.
F-4	The pH of the supernatant is adjusted to 2.0 again and the precipitate formed discarded. NaCl is added to full saturation. The resulting precipitate is collected by centrifugation. The supernatant is discarded.

TABLE II. C.

PURIFICATION OF CHYMOPAPAIN

BY THE MODIFIED JANSEN AND BALLS PROCEDURE

FRACTION NUMBER	VOL.	PROTEIN (mg/cc)	SPECIFIC ACTIVITY	TOTAL ACTIVITY	TOTAL PROTEIN	RECOVERY (PER CENT)
F-1	117	46.1	2.81	15,146	5.39	100
F-2	122	32.8	1.56	6,240	4.00	41
F-3	125	23.3	1.57	4,570	2.91	29
F-4	29	36.9	2.76	2,953	1.07	19

b. Evaluation of the Procedure of Ebata

The modified procedure developed in our laboratory by Ebata is re-stated in Table III. This procedure incorporates the NaCl fractionations of Jansen and Balls, but in addition, introduces the use of two cation-exchangers (carboxymethyl cellulose and XE-64) as well as two ammonium sulfate fractionations.

The purification of chymopapain A and B by this procedure can be judged electrophoretically, as is shown in Figure 3. The yield of crystalline chymopapain A on the basis of activity is approximately 2% (c.f. 19% by the method of Jansen and Balls). Some of the properties of chymopapain A have already been reported (Ebata, 1962; Ebata and Tsunoda, 1962; Ebata, 1963) and the more notable ones cited in Table I.

The low yield of proteolytic activity (2% for chymopapain A and 15% for papain) suggested the possibility of the presence of other discrete proteases. The appearance of several components during XE-64 chromatography seemed to lend credence to this possibility, especially since the specific activities of some of these components were much higher than that of chymopapain A. Figure 4 shows the chromatographic behaviour of the proteins in the full NaCl precipitate on XE-64, and Table IV summarizes the activities obtained for these fractions. It will be noted that the components eluted at the higher ionic strength (which implies the greater basicity of these components) have higher specific activities than do the components eluted at a lower ionic strength.

Studies with component IX (Figure 4) have led us to conclude that the protease present in this particular fraction constitutes an enzyme different from chymopapain A. The basis for differentiating between chymopapain A and component IX (designated chymopapain B) are the following observations: (1) Upon concentrating this fraction and adding NaCl to full saturation, an obviously crystalline material was obtained. The great bulk of the enzyme crystallized out of solution when the pH of the solution was gradually adjusted to 2.0 by addition of solid citric acid. Examination with a microscope revealed the presence of broad, sabre-like needles (Figure 5) which contrasted in form to that observed with chymopapain A, which crystallizes as obvious rods; (2) Specific activity measurements showed that this crystalline material was at least three times more active than chymopapain A; and (3) amino-terminal analysis

of chymopapain B revealed the presence of tyrosine, whereas glutamic acid occupies the N-terminus position in chymopapain A (Table I).

However, one apparently inconsistent fact still remains: the isolation of chymopapain A during XE-64 chromatography involves the use of 0.40M phosphate buffer, pH 5.90, as eluent, the same buffer used to elute chymopapain B. Figure 6 shows some chromatographic patterns obtained during the preparation of chymopapain A. It will be noted that the elution of this enzyme always involves the use of the 0.40M buffer as eluent. In fact, Figure 5-E represents the published chromatogram (Ebata, 1962).

Several possibilities exist which can reconcile this seemingly inconsistent behaviour of chymopapain on XE-64. If one examines the N-terminal residues of the proteins eluted by the 0.25M buffer in Figure 4, it will be found that glutamic acid accounts for the greatest proportion of the residues. Furthermore, perusal of the chromatograms in Figure 6 indicates that only a negligible fraction of the total protein is eluted by either the 0.25M or 0.30M buffer, whereas in Figure 4 approximately 60% of the total protein is eluted by the 0.25M and 0.30M buffers. This significant difference in chromatography observed in this study in contrast to that observed by Ebata may possibly be due to the following:
(1) differences in the method of preparation of the buffer may lead to the observed discrepancy in chromatographic behaviour since the pH is very critical during XE-64 chromatography and differences of the order of 0.10 pH unit can either enhance or retard elution;
(2) a failure on the part of the other worker to elute extensively

enough with the 0.25M buffer, since according to our observations, approximately 6 liters are required to elute the less basic components with a column of the size cited in Figure 4; or (3) differences in the properties of the resin utilized could lead to either a greater or lesser affinity of the resin for the proteins, and accordingly, manifesting itself in the differences observed during chromatography.

In spite of this apparent discrepancy of preparation in different hands, the reproducible preparation of chymopapain A by Ebata and the preparation of chymopapain B by ourselves are established facts. This discrepancy could conceivably be explained by a third party who prepares chymopapain employing the same procedure and the same resin (XE-64).

Suffice it to note here that the procedure devised by Ebata for the purification of chymopapain A can also be used to prepare chymopapain B. However, the yield of the latter enzyme is relatively low by this procedure, and accordingly, modifications are necessary in order that chymopapain B can be obtained in significantly higher yields.

TABLE III

PURIFICATION OF CHYMOPAPAIN

BY THE METHOD OF EBATA (1962)

STEP NO.	DESCRIPTION
1.	Extraction of Crude Latex with water Filtration to remove insoluble material
2.	pH of extract adjusted to 7.00-9.00 Solid $(NH_4)_2SO_4$ added to 0.45 Saturation (277 gm/liter) Stand 4-6 hours Centrifuge at 20,000 x g
3.	pH of Supernatant adjusted to 2.00 with 1N HCl Small precipitate formed discarded after centrifugation $(NH_4)_2SO_4$ concentration adjusted to 0.65 Saturation (134 gm/liter) Stand 4-6 hours Centrifuge at 20,000 x g
4.	Precipitate dissolved in minimal volume of water and dialyzed exhaustively against water, then 0.02M Acetate buffer, pH 5.00
5.	Buffered fraction introduced onto a column of CMC previously equilibrated with 0.02M Acetate buffer, pH 5.0 Step-wise elution carried out with 0.02M buffer, 0.10M buffer, 0.70M buffer and finally with 1.0M buffer containing 1.0M NaCl. The 0.70M fraction was collected, dialyzed against water.
6.	To the supernatant from Step 5, solid NaCl was added to 0.50 saturation (170 gm/liter) and the pH was adjusted to 2.00 with HCl. After standing overnight, the precipitate was removed by centrifugation. To the supernatant, solid NaCl was added to full saturation (and the pH was again adjusted to 2.00). The resulting precipitate was collected by centrifugation and dissolved in a minimum volume of 0.30M phosphate buffer, pH 5.90, and dialyzed against the same buffer overnight.
7.	The dialysate was introduced onto a column of XE-64 which was equilibrated with 0.30M phosphate buffer, pH 5.90. Elution was carried out step-wise, first with the 0.30M buffer and then with 0.40M buffer, pH 5.90.
8.	The main fraction from the 0.40M elution was collected, concentrated and crystallized by the gradual addition of solid NaCl at pH 2.00.

Figure 3. The course of purification of chymopapain A and B as determined by electrophoresis. (A) crude extract; (B) 0.45$(NH_4)_2SO_4$ saturated supernatant; (C) 0.70M acetate buffer eluent; (D) full NaCl saturated precipitate; (E) chymopapain A; and (F) chymopapain B. (A), (B), and (C) run in .02M acetate buffer, pH 4.0, u = .10; (D), (E), and (F) run in .02M phosphate buffer, pH 7.45, u = .17. The temperature in all instances was 1°C.

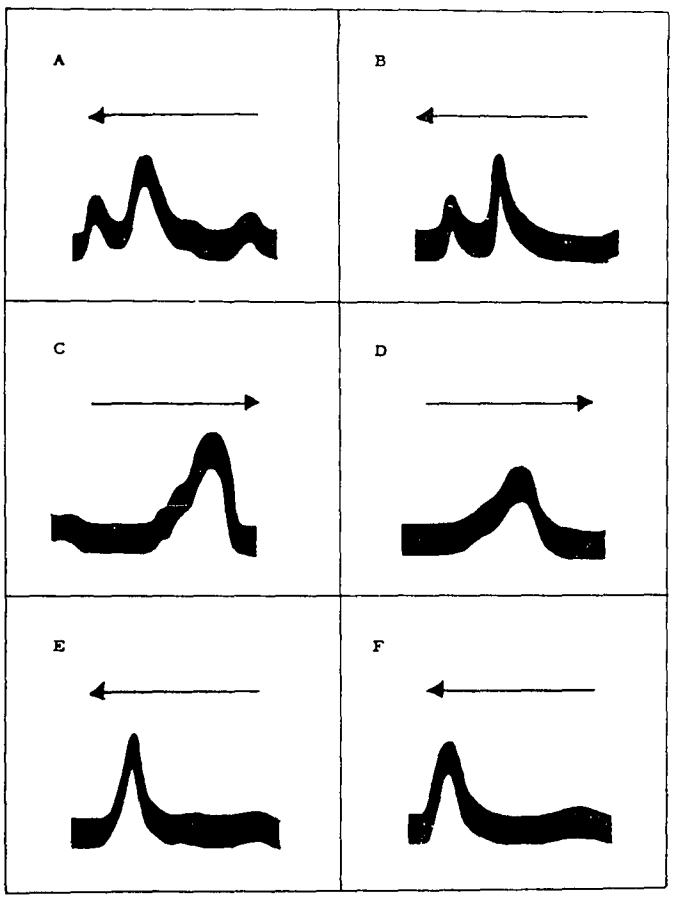

A
B
C
D
E
F

Figure 4. The chromatography of the full NaCl saturated precipitate prepared according to Ebata on XE-64. 1.74 grams of protein were charged on a 2.0 x 25.0 cm column and elution was effected by the buffers indicated. All operations carried out at 4°C. (————) absorbance readings; (▲ ▲ ▲) casein digestion.

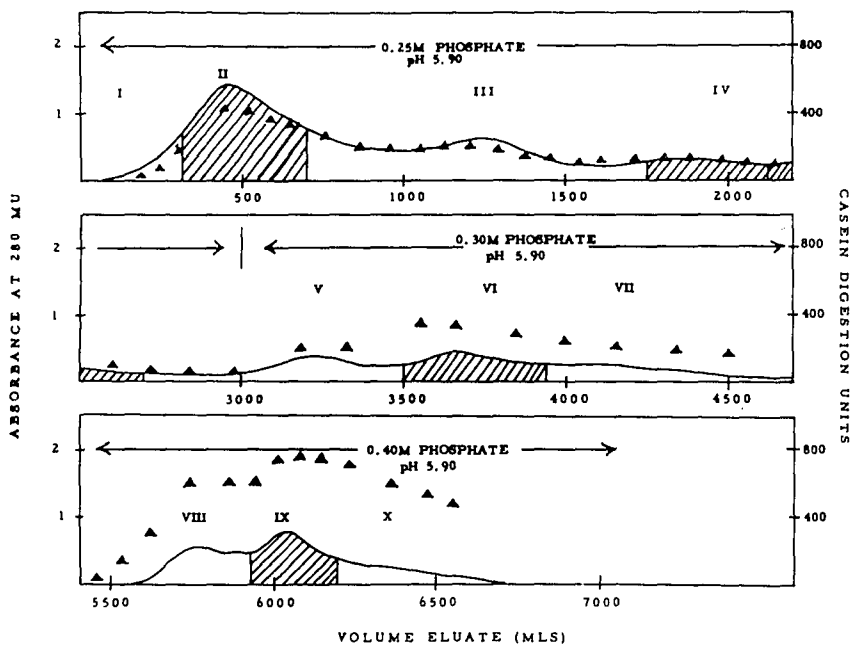

0.25M PHOSPHATE
pH 5.90
I
II
III
IV
0.30M PHOSPHATE
pH 5.90
V
VI
VII
0.40M PHOSPHATE
pH 5.90
VIII
IX
X
ABSORBANCE AT 280 MU
CASEIN DIGESTION UNITS
VOLUME ELUATE (MLS)

TABLE IV

SOME PROPERTIES OF COMPONENTS ELUTED FROM XE-64

DURING THE PURIFICATION OF CHYMOPAPAIN A AND CHYMOPAPAIN B

BY THE METHOD OF EBATA (1962)

COMPONENT NUMBER	PROTEIN (grams)	SPECIFIC ACTIVITY	TOTAL ACTIVITY	RECOVERY (%)	N-TERMINAL
I	.045	.80	36	.9	---
II	.308	1.30	400	10.1	---
III	.245	1.30	319	8.0	---
IV	.121	1.75	212	5.3	---
V	.137	2.10	288	7.2	---
VI	.183	3.00	549	13.8	---
VII	.063	3.00	189	4.8	---
VIII	.060	3.50	210	5.3	tyr
IX	.127	4.50	572	14.4	tyr
X	.073	4.50	329	8.3	tyr

Figure 5. Photomicrograph of once-crystallized chymopapain B.

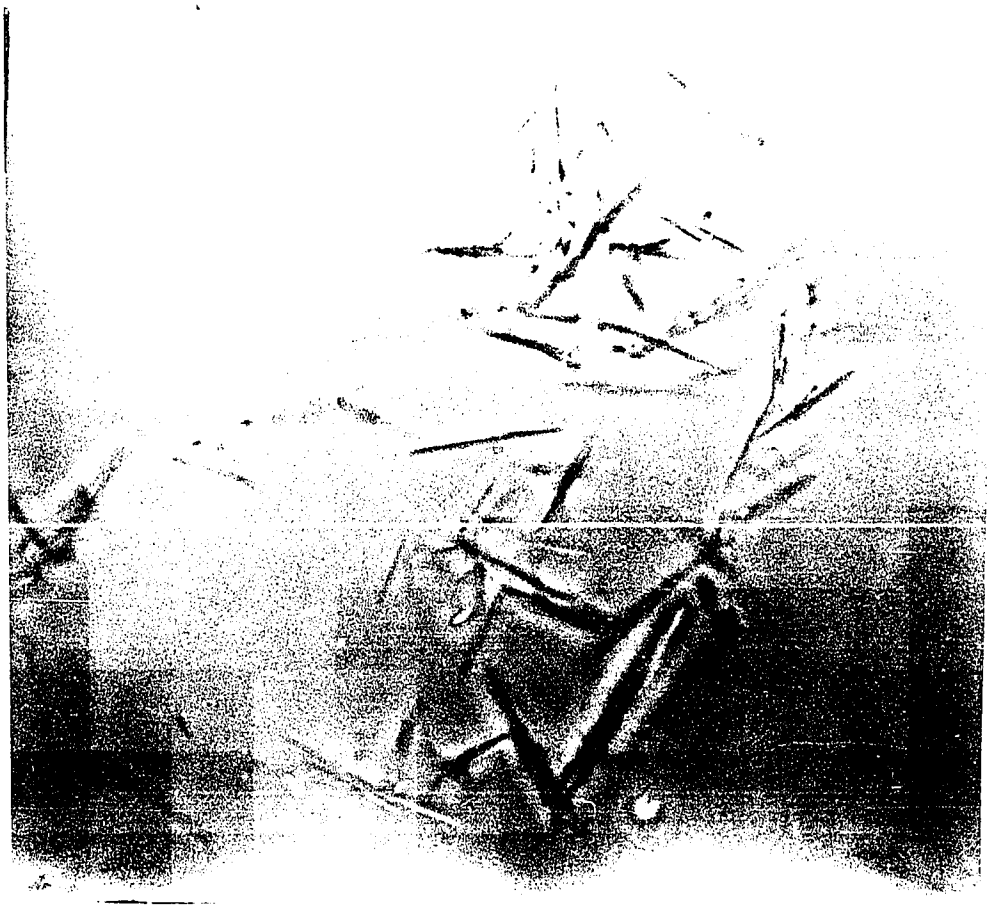
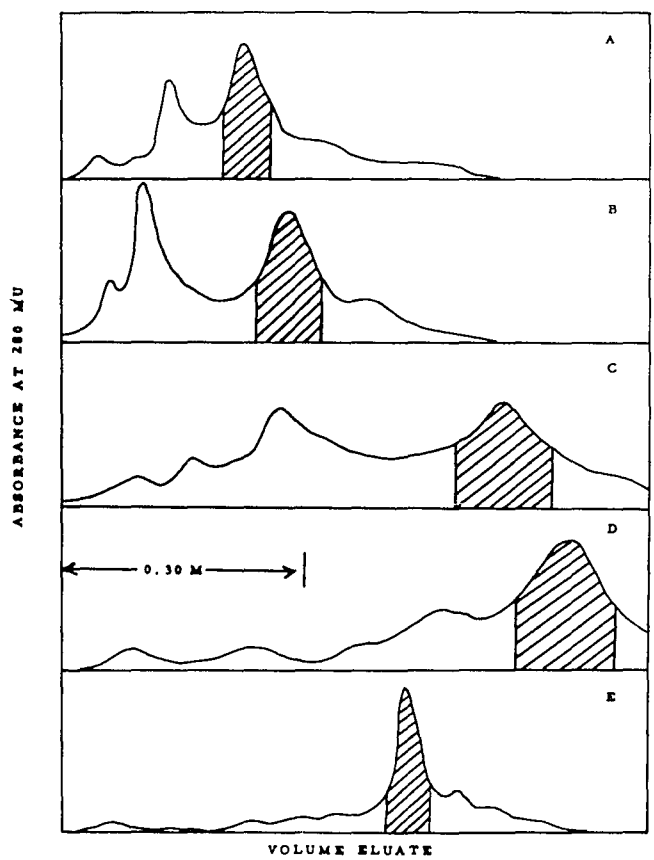
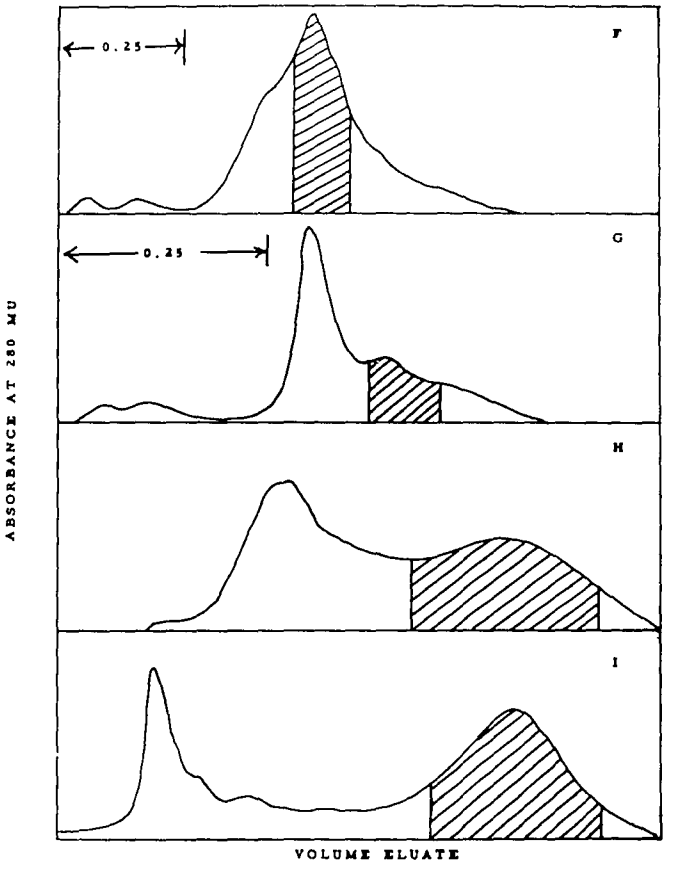


Figure 6. Some elution patterns obtained during the chromatography of the full NaCl precipitate prepared by the method of Ebata on XE-64. Cross-hatched areas indicate the fractions taken to obtain chymopapain A. These chromatograms were taken from the data of Ebata (unpublished).


A
B
C
D
E
0.30 M
ABSORBANCE AT 280 MU
VOLUME ELUATE


0.25
F
0.25
G
H
I
ABSORBANCE AT 280 MU
VOLUME ELUATE

c. Suggested Modifications for the Preparation of Chymopapain B

As pointed out in the previous section, chymopapain B can be prepared by the method of Ebata (component IX in Figure 4). The recovery of activity after XE-64 chromatography is approximately 15% based on the total activity obtained from the full NaCl saturated precipitate (Table V). However, when calculated on the basis of the activity originally present in the starting material, the recovery is only 1%. Table VI summarizes the results of the purification of chymopapain B by the method of Ebata.

Two modifications were incorporated which significantly increased the yield of chymopapain B and a third modification introduced for the purpose of assessing the role (if any) of autolysis during the process of purification.

The first modification (which is adopted in the present study) involves the elimination of the NaCl fractionation. In the preparative scheme, this means that the eluate from CMC chromatography is dialyzed first against de-ionized water and then against 0.25M phosphate buffer, pH 5.90, after which the eluate is directly charged on an XE-64 column and chromatographed. A representative elution pattern is presented in Figure 6. It will be seen that the elution is very similar to that obtained during the chromatography of the full NaCl saturated precipitate and that the recovery of activity (component III, Table V) after XE-64 chromatography is approximately the same (15%). However, the yield of chymopapain B has increased to 5% (on the basis of activity recovered, calculated from the starting material). A 4-fold increase over the un-modified procedure is thus obtained. Table VII summarizes the results for

the purification of chymopapain B by this slightly modified procedure.

Physical studies (molecular weight, N-terminal studies) and chemical studies (specific activity measurements) indicate that component IX (Figure 4) and component IV (Figure 7) are the same species, indicating that the NaCl fractionation merely lowers the yield of chymopapain B and does not engeander gross modifications of the enzyme, insofar as these limited studies indicate. Furthermore, these observations suggest that chymopapain B is also acid-stable since the NaCl fractionations are carried out at pH 2 prior to XE-64 chromatography. (This acid-stability is discussed more fully in Section III.B.3., Stability Studies.)

The second and third modifications, it might be pointed out, are only preliminary and are reported here as possibilities that might be utilized for the preparation of chymopapain B. These two studies were undertaken primarily for two different reasons: the first modification was incorporated to ascertain whether the use of the acid-steps (i.e. adjustment of the pH of the enzyme solution to 2.0) would alter the enzyme in any manner with respect to the activity and chromatographic behaviour and the second modification introduced to determine whether the phenomenon of autolysis would affect the number of chromatographic components present during cation-exchange chromatography.

Concerning the attempts made to answer the first question, i.e. the effect of acid on both the activity and chromatographic behaviour of chymopapain B, the enzyme was prepared by eliminating the two acid-steps from the scheme of Ebata (steps 3 and 6, Table III.)

TABLE V

THE PROPERTIES OF SOME COMPONENTS

CONTAINING N-TERMINAL TYROSINE ELUTED FROM XE-64

FRACTION NUMBER *	PROTEIN (grams)	SPECIFIC ACTIVITY	TOTAL ACTIVITY	% RECOVERY **
0.40 - I	.420	2.00	840	6.9
0.40 - II	.143	2.76	395	3.2
0.40 - III	.550	3.34	1837	14.9
0.40 - IV	.072	4.10	295	2.4

* Keyed to Figure 7

** Expressed as % of Total Activity = 12,170 units

TABLE VI

THE PURIFICATION OF CHYMOPAPAIN B

BY THE METHOD OF EBATA (1962)

PROCEDURE	VOLUME (cc)	PROTEIN (mg/cc)	SPECIFIC ACTIVITY	TOTAL PROTEIN (gm)	TOTAL ACTIVITY	RECOVERY (%)
Crude Extract	830	25.60	2.77	21.22	58,780	100.0
0.45 Supernatant	956	17.89	2.80	17.10	47,880	81.5
0.65 Precipitate	610	14.22	3.08	8.67	26,700	45.4
0.70M CMC-Eluate	720	7.57	2.70	5.44	14,690	25.0
Full NaCl Precipitate	284	6.14	2.42	1.74	4,210	7.2
XE-64 Treated Chymopapain B	480	.27	3.93	.13	510	.9
Once-Crystallized Chymopapain B	8	11.26	3.50	.10	350	.6

TABLE VII

PREPARATION OF CHYMOPAPAIN B

BY MODIFICATION I

PROCEDURE	VOLUME	PROTEIN (mg/cc)	SPECIFIC ACTIVITY	TOTAL PROTEIN	TOTAL ACTIVITY	RECOVERY (%)
Crude Extract	1,785	33.51	2.80	59.82	167,500	100.0
0.45 Supernatant	2,080	24.28	2.50	50.51	126,280	75.4
0.65 Precipitate	1,240	25.50	3.08	31.62	97,390	58.1
0.70 CMC-Eluate	961	12.43	2.70	15.61	42,150	25.2
XE-64 Treated Chymopapain B	1,060	2.14	3.70	2.27	8,399	5.0
Once-Crystallized Chymopapain B	74	21.42	3.40	1.59	5,406	3.2

Figure 7. Chromatography of the 0.70M Acetate buffer fraction on XE-64. 4.50 grams of protein were charged on a 2.5 x 50.0 cm column of XE-64, equilibrated with 0.25M phosphate buffer, pH 5.9. Elution was effected with the buffers indicated. All operations were carried out at 4°C.

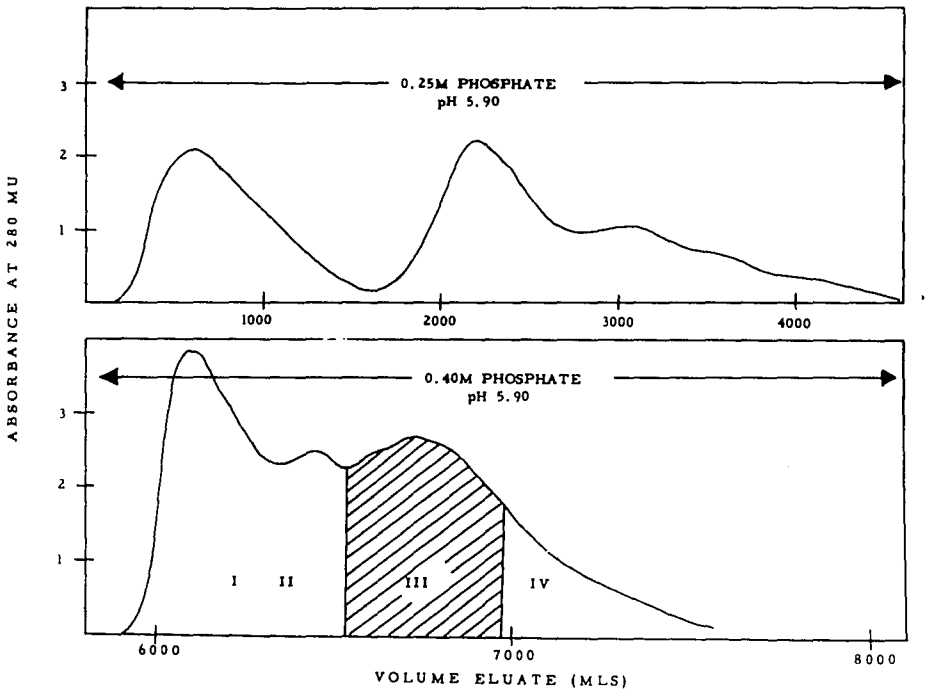

0.25M PHOSPHATE
pH 5.90
0.40M PHOSPHATE
pH 5.90
ABSORBANCE AT 280 MU
VOLUME ELUATE (MLS)
I
II
III
IV
1000
2000
3000
4000
6000
7000
8000

Figure 8.A. shows the behaviour of the 0.45 $(NH_4)_2SO_4$ saturated supernatant on CM-cellulose. Figure 8.B. presents the chromatographic behaviour of the 0.65 $(NH_4)_2SO_4$ precipitate (prepared according to Ebata) and is included as a basis for comparison. It will be noted that when chromatographed under the same conditions, the non-acid treated fraction exhibits slightly more basic behaviour. Furthermore, the elution during XE-64 chromatography reveals that chymopapain B is eluted at a higher ionic strength (0.50M) and a more basic pH (ca. 6.3), indicating its greater basicity. (The fraction corresponding to the cross-hatched area in Figure 8.C. yields tyrosine as the N-terminal and is assumed to be chymopapain B.) While the specific activity of this fraction is relatively unaltered by this modification, the yield is again significantly higher (Table VIII). This fraction could be chromatographed on SE-sephadex as a single component (Figure 8.D.), but attempts to crystallize chymopapain B from this fraction were not successful. Hence, detailed studies were not carried out with this fraction.

The third modification was carried out to see whether progressive autolysis during the course of purification would account for the appearance of some of the proteolytically active components observed during ion-exchange chromatography. To do this, the crude extract was first treated with $HgCl_2$ (after adding cysteine to the extract) to inhibit the proteases, and the resulting inactive mercury derivatives subjected to purification by a slightly modified procedure. The rationale of this undertaking is at once obvious: if it is progressive autolysis which accounts for some of the components

observed during cation-exchange chromatography (particularly with XE-64), the conversion of the active proteases to the inactive mercury derivatives at the outset of purification would prevent this autolysis, thereby manifesting itself in the appearance of fewer components during chromatography.

The procedure employed was the following: an excess of solid $HgCl_2$ was added to the crude extract to which cysteine (10^{-2}M final concentration) had been added with continuous stirring and aliquots removed at various time intervals and assayed for proteolytic activity. After complete inhibition of the proteolytic activity, the extract was dialyzed exhaustively against water and the somewhat turbid solution centrifuged. The resulting supernatant was then dialyzed against .02M acetate buffer, pH 5.0, and the dialyzate subsequently charged on a column of CM-cellulose equilibrated with the same buffer. Figure 9.A. shows the chromatographic behaviour of the mercuri-derivatives on CMC. Fractions 3 and 4 were subsequently separately dialyzed against water overnight and then against .25M phosphate buffer, pH 5.90. This dialyzate was then introduced onto a column of XE-64 and chromatographed (Figure 9.B.). The fractions eluted by 0.40M phosphate buffer, pH 6.53, yielded tyrosine as the N-terminal and were pooled and then chromatographed on SE-sephadex equilibrated with 0.25M phosphate buffer, pH 6.00. The protein passed through the column unretarded with the 0.25M buffer (Figure 9.C.).

Table IX delineates the purification of chymopapain B by this procedure. The yield of this enzyme was 5.2% (sum of steps 3a and

3b). The specific activity of the mercuri-derivative of chymopapain B obtained by this method is approximately 3.5, a value similar to those obtained by the procedures previously described.

Based on this limited study, several facts are apparent: (1) the appearance of the various components during chromatography suggests that many of these components are present in the latex itself and do not reflect the presence of a large number of artifacts caused by autolysis during the preparation under the conditions employed; (2) the specific activity of chymopapain B would not be expected to be diminished by the preparative procedure employing such steps as adjustment of the pH of the enzyme solution to 2.0, chromatography on cation-exchange resins, salt fractionations, since the specific activity of chymopapain under widely varied conditions are essentially the same; and (3) chymopapain is stable at room temperature provided that the so-called "essential" -SH group is blocked by an alkylating reagent.

Figure 8. A. Chromatography of the 0.45 $(NH_4)_2SO_4$ saturated supernatant on CM-cellulose. 16.91 grams of protein were charged on a 3.5 x 39 cm column containing CM-cellulose equilibrated with .02N acetate buffer, pH 5. Elution was carried with 0.70M acetate buffer, pH 5, as described in the text.

B. Chromatography of the 0.65 $(NH_4)_2SO_4$ saturated precipitate on CM-cellulose. 8.67 grams of protein were charged on a 3.5 x 39 cm column of CM-cellulose equilibrated with .02N acetate buffer, pH 5.0. Elution was carried out as described in (A).

C. Chromatography of the 0.70M acetate buffer eluate on XE-64. The eluate from (A) was dialyzed against water and then against 0.25M phosphate buffer, pH 5.9. 3.35 grams of protein were subsequently charged on a 2.5 x 30 cm column of XE-64 and elution effected by the buffers indicated in the figure. The pH gradient was carried out by mixing equal volumes of 0.50M potassium phosphate buffer, pH 6.00 and 0.50M potassium phosphate buffer, pH 7.00 in a closed system.

D. Chromatography of the XE-64 eluate (0.50M gradient fraction) on SE-sephadex. .38 grams of this eluate were dialyzed against water and 0.25M phosphate buffer and charged on a 1.5 x 40 cm column of SE-sephadex equilibrated with the same buffer. After washing the column with 2 liters of this buffer, elution of the enzyme was effected with the buffer indicated.

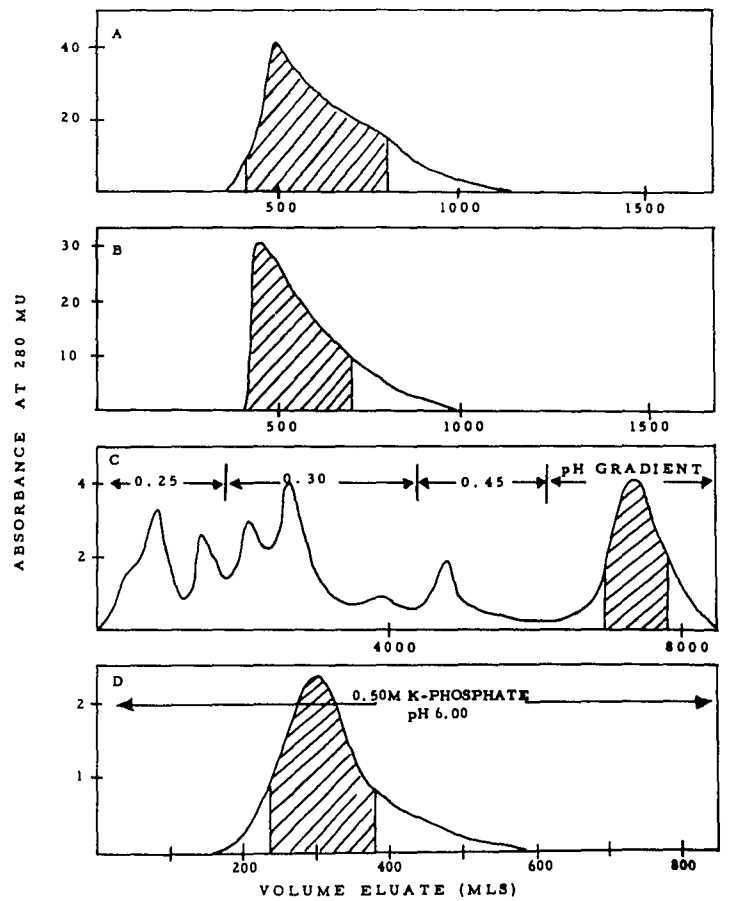

A
40
20
500
1000
1500
B
30
20
10
500
1000
1500
C
0.25
0.30
0.45
pH GRADIENT
4
2
4000
8000
D
0.50M K-PHOSPHATE
pH 6.00
2
1
200
400
600
800
ABSORBANCE AT 280 MU
VOLUME ELUATE (MLS)

TABLE VIII

PREPARATION OF CHYMOPAPAIN B

BY MODIFICATION II

PROCEDURE	VOLUME	PROTEIN (mg/cc)	SPECIFIC ACTIVITY	TOTAL PROTEIN	TOTAL ACTIVITY	RECOVERY (%)
Crude Extract	787	24.28	2.77	19.11	52,900	100.0
0.40 $(NH_4)_2SO_4$ Saturated Supernatant	950	17.80	2.80	16.91	47,300	89.4
0.70 CMC-Eluate	2,085	3.35	2.70	6.99	18,900	35.7
XE-64 Treated Chymopapain B	588	1.39	3.43	.82	2,810	5.3

Figure 9. A. Chromatography of the $HgCl_2$-treated crude extract on CM-cellulose after dialysis against .02M acetate buffer, pH 5. 1.00 gram of this extract was charged on a 1.5 x 15 cm column of CM-cellulose equilibrated with the .02M buffer. Elution was carried out at room temperature (25°C).

B. The chromatography of fractions 0.55 (3) and 0.55 (4) on XE-64 (See 9.A.). 61 mg of 0.55 (3) and 63 mg of 0.55 (4) were charged on a 1.0 x 5 cm column of XE-64 equilibrated with 0.25M phosphate buffer, pH 5.9, after dialysis against this same buffer. Chromatography was carried out as indicated at room temperature.

C. Chromatography of the pooled XE-64 eluates on SE-sephadex. 40 mg of protein (fraction XE-64 0.40M, pH 6.53) was charged on a column of SE-sephadex equilibrated with 0.25M potassium phosphate buffer, pH 6.00. The protein emerged unretarded. Elution was carried out at room temperature.

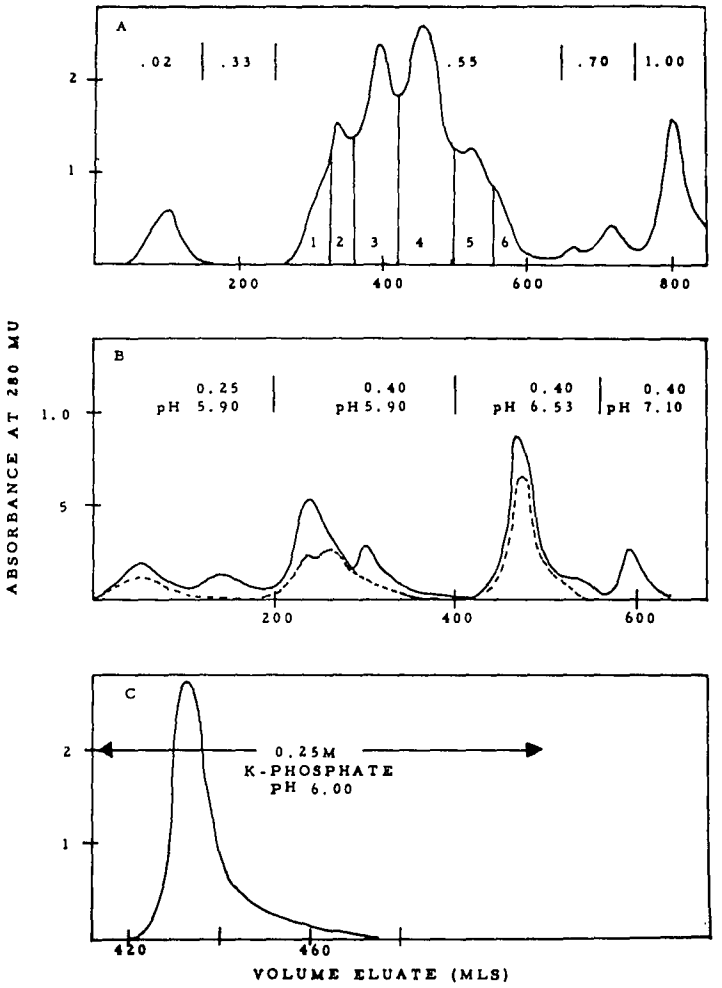

A
.02
.33
.55
.70
1.00
1
2
3
4
5
6
200
400
600
800
B
0.25
pH 5.90
0.40
pH 5.90
0.40
pH 6.53
0.40
pH 7.10
1.0
5
200
400
600
C
0.25M
K-PHOSPHATE
PH 6.00
2
1
420
460
VOLUME ELUATE (MLS)
ABSORBANCE AT 280 MU

TABLE IX

THE PURIFICATION OF MERCURI-CHYMOPAPAIN B

BY MODIFICATION III

PROCEDURE	VOLUME	PROTEIN (mg/cc)	SPECIFIC ACTIVITY	TOTAL PROTEIN	TOTAL ACTIVITY	RECOVERY (%)	N-TERMINAL
1. Crude Extract	20	49.47	2.80	.989	2,770	100.0	----
2a. 0.55 CM-3	49	1.24	2.60	.061	159	5.7	----
2b. 0.55 CM-4	47	1.30	2.70	.063	165	6.0	----
3a. XE-64 (1)	116	.18	3.40	.021	71	2.6	Tyr.
3b. XE-64 (2)	109	.18	3.60	.020	72	2.6	Tyr.
4. SE-Sephadex Treatment	36	.86	3.20	.031	87	3.1	Tyr.

d. Existence of Other Proteases in Dried Papaya Latex

With the wide distribution of proteolytic activity and existence of distinct chromatographic components during XE-64 chromatography, the question arises: are there any other proteases present in dried papaya latex distinct in any manner whatsoever from chymopapain A and B? N-terminal studies of the full NaCl precipitate indicate the presence of only two residues (tyrosine and glutamic) in significant quantities. Hence, the possibility of the existence of other proteases seems improbable. In short, the distribution of activities and components during XE-64 chromatography is suggestive of the presence of autolysis products and/or the existence of chymopapain A and chymopapain B in different forms (varying in activity, net charge). The latter possibility can be rationalized in terms of the differential binding of an as yet undetermined constituent known to be present in the enzyme preparation (Section III.C.3.). The contribution of this factor to the distribution of activities is, however, not determinable in terms of the data available.

2. Purity Determinations

It is often of paramount importance to determine the purity of a protein preparation particularly when a rigorous study of its physical and chemical properties and its biological activity is undertaken. Furthermore, it is a well-documented truism that crystallinity of the protein per se does not indicate its purity in the manner where crystallinity is an index of purity with simple organic compounds (e.g. heart lactic acid dehydrogenase, urease). While the determination of purity in the strictest sense of the word is often a very difficult problem, physical and chemical methods do exist which can be utilized as criteria for indicating relative homogeneity or heterogeneity of a protein preparation.

Penetrating discussions of the problems involved in the determination of the purity of proteins may be found in the literature (Li, 1951; Pirie, 1940).

In the present study, the purity of chymopapain B was examined utilizing the criteria of ultra-centrifugation, free-boundary electrophoresis, amino terminal analysis and rechromatography.

a. Ultra-Centrifugation Studies

Examination of the sedimentation patterns obtained indicates that there are no gross heterogeneity at pH 4.80 (Figure 10). This monodispersity during sedimentation was consistently observed, indicating that chymopapain B is relatively homogeneous with respect to size and shape (i.e. the frictional ratio of the sedimenting species shows little or no distribution). Furthermore, as will be shown later in Section III.B.1., the calculated

sedimentation coefficients ($s_{20,w}$) show a negative concentration dependence, indicative of non-associating system.

b. Electrophoresis Studies

As shown earlier, the extent of purification of chymopapain A and B can be judged electrophoretically (Figure 3). It can be seen that the multi-components of the crude extract are essentially reduced to a single component by the methods of purification outlined in the previous section. A more detailed examination of the electrophoretic migration as a function of pH was carried out, and some of the electrophoretic patterns which were obtained are shown in Figure 11. It is apparent that crystalline chymopapain B is, in general, monodisperse, in the sense that it is free of grossly contaminating proteins. The sample shown in Figure 11.A. contained less than 5% of a minor component which was not present in most of our preparations. However, it should be noted that after prolonged periods of migration (in the order of 120-240 minutes), some of these patterns appeared to be slightly broader than might be expected for a completely homogeneous protein. Especially noteworthy is the trailing evident in most of these patterns. A reversible boundary-spreading experiment carried out at pH 10.40 also reveals heterogeneity of charge (Figure 12). While no quantitative significance is attached to this reversible boundary-spreading, a method for calculating a heterogeneity parameter is available (Alberty, et. al., 1948). Qualitatively assessed, the experiment suggests only minor heterogeniety.

The absence of complete homogeneity does not appear to reflect any gross contamination of chymopapain B which is evidently free

of other proteins by several criteria, e.g. N-terminal analysis as studies by the DNP-method of Sanger (see Section C.) and sedimentation in the ultra-centrifuge, just discussed. It is more likely that the slight electrical heterogeneity in the region of relative stability may be due, in part, to partial binding of ions by this basic protein, to small variations in the amide content and/or to differential binding of some unknown constituent (see Section IV.C.).

c. Amino-Terminal Studies

Table X shows the results of the amino-terminal analyses on different preparations of chymopapain B. On the average, tyrosine accounts for over 90% of the DNP-amino acids found in all preparations. The low levels of DNP-glutamic acid, aspartic acid and threonine (accounting for the remaining 10%) were always present, a situation analogous to that found with the amino-terminal studies with papain (Thompson, 1954). The significance of the presence of these amino acids is as yet undetermined.

The enrichment of the tyrosine fraction as the amino-terminal during the course of purification has previously been cited (Table IV, X). Furthermore, the presence of N-terminal tyrosine in the crude extract can be taken to indicate that chymopapain B is native to the dried latex and, in this respect, rules out the possibility of its formation by autolysis during the course of purification by the methods outlined above.

d. Re-chromatography

The re-chromatography of chymopapain on XE-64 is shown in Figure 13. This enzyme could also be re-chromatographed on SE-sephadex (Figure 8.D.). Its re-chromatography as a single,

relatively symmetrical peak and a high recovery of the activity are strongly suggestive of the relative homogeneity of this enzyme.

Figure 10. Sedimentation patterns of once-crystallized chymopapain B. Unactivated chymopapain B at a concentration of 1.09% in 0.10M NaH_2PO_4 buffer, pH 4.75, was analyzed in a 1-ml cell at 24°. Sedimentation is from left to right at times of 13, 29, 45, 61, 77, 93, 109, 125, 141 and 167 minutes, respectively.

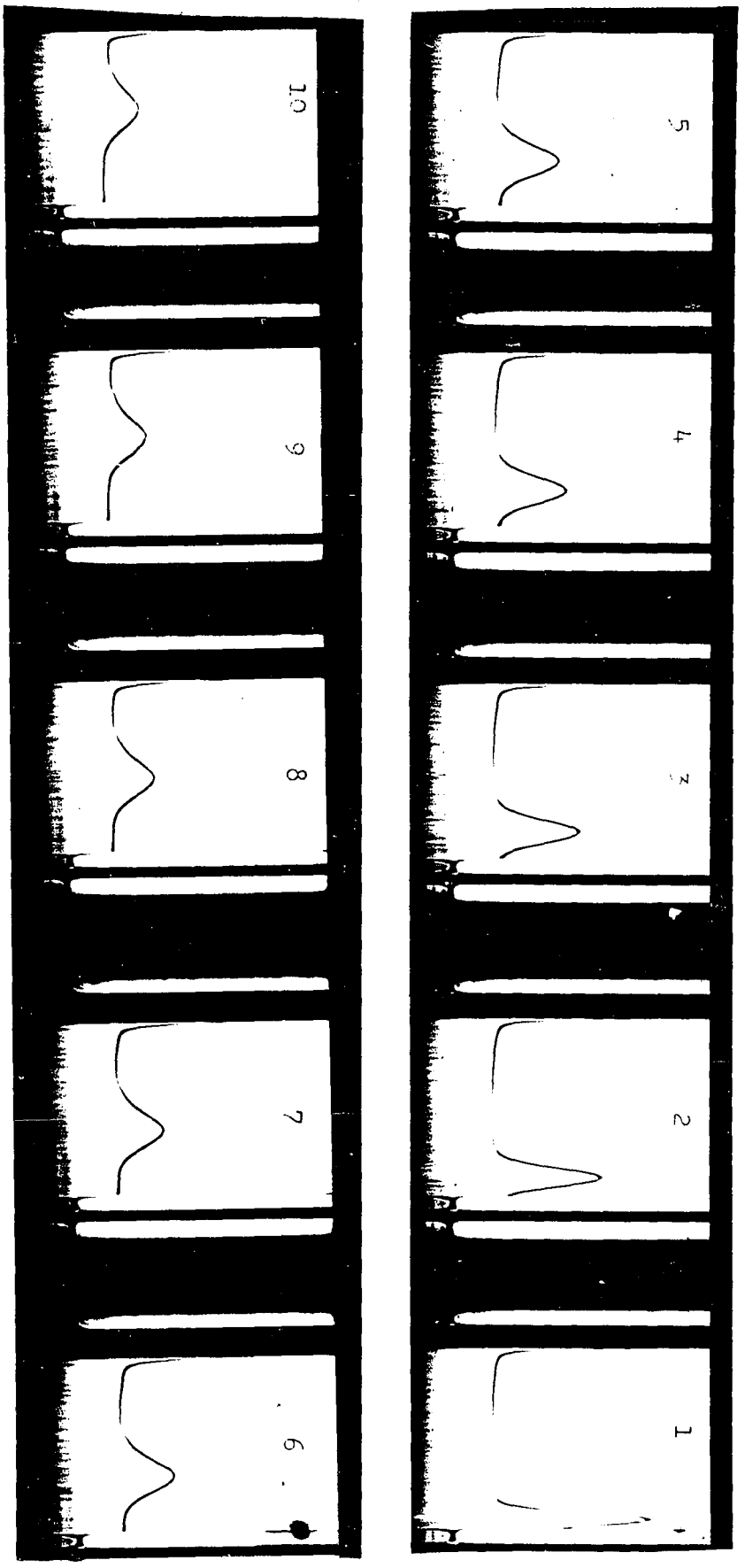

1
2
3
4
5
6
7
8
9
10

Figure 11. Electrophoretic behaviour of once-crystallized chymopapain B at various pH's.

A. In acetate buffer, pH 3.83. (1) time = 46 minutes; (2) time = 81 minutes; and (3) time = 105 minutes. Protein concentration was 1.19%.

B. In phosphate buffer, pH 8.25. (1) time = 31 minutes; (2) time = 45 minutes; and (3) time = 96 minutes. Protein concentration was 0.71%.

C. In glycine-NaOH buffer, pH 10.40. (1) time = 29 minutes; (2) time = 56 minutes; and (3) time = 105 minutes. Protein concentration was 0.58%.

D. In glycine-NaOH buffer, pH 11.10. (1) time = 15 minutes; (2) time = 35 minutes; and (3) time = 71 minutes. Current was reversed at this particular pH. Protein concentration was 0.55%.

The ionic strength of the medium in all cases was 0.10M. The temperature was 5°C. Electrophoretic migration in all instances is from left to right.

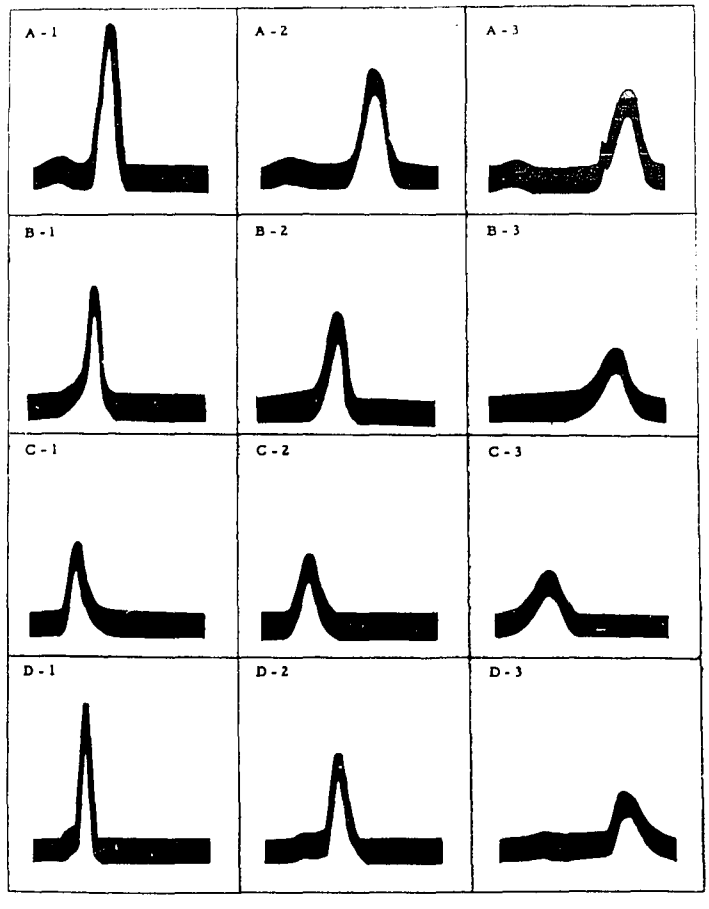

A - 1
A - 2
A - 3
B - 1
B - 2
B - 3
C - 1
C - 2
C - 3
D - 1
D - 2
D - 3

Figure 12. Reversible boundary-spreading of once-crystallized chymopapain B at pH 10.40. (A) Time = 0; (B) 15 minutes; (C) 33 minutes; (D) 63 minutes; (E) 81 minutes; and (F) 105 minutes. The buffer was glycine-NaOH, ionic strength of 0.10. Migration was allowed to proceed from right to left for 60 minutes and then the direction of migration reversed.

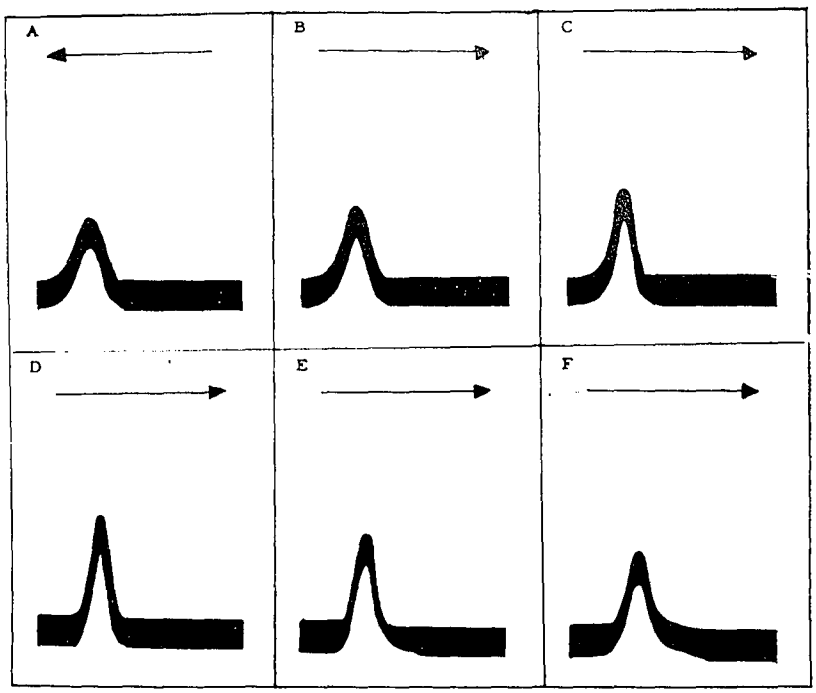

A
B
C
D
E
F

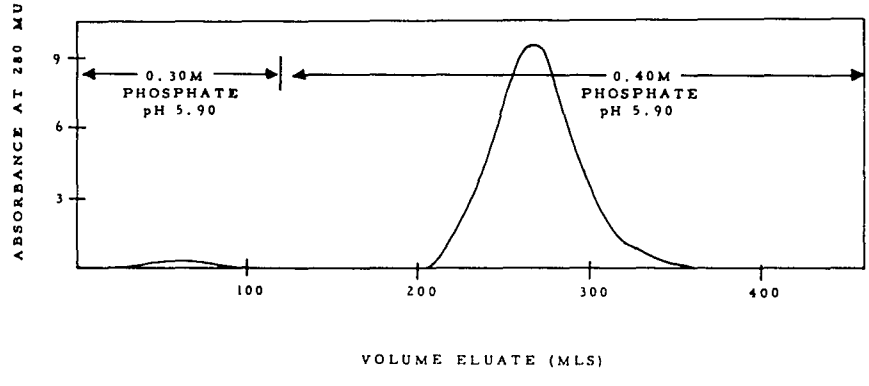
TABLE X

SUMMARY OF SOME AMINO-TERMINAL DETERMINATIONS

PROCEDURE	Amino Acid (% of Total Moles DNP-AA) GLU	SER(THR)	TYR	"X"*	OTHERS
Crude Extract (Hg^{++} Derivative)	30	4	27	40	--
1/2 NaCl Precipitate	26	11	38	--	19
Full NaCl Precipitate	40	13	38	6	--
0.25M Phosphate Buffer (XE-64)	50	15	15	3	--
0.40M Phosphate Buffer (XE-64)	16	1	33	43	6
(a) Fraction 1	12	7	38	43	--
(b) Fraction 2	10	7	32	44	6
(c) Fraction 3: Pooled as Chymopapain (Form B)	10	5	35	43	6
(d) Fraction 4	9	2	34	49	6
Chymopapain B (Re-chromatographed on XE-64)	9	5	35	47	6
Chymopapain B (Once-crystallized)	12	6	40	41	7
Chymopapain B (Prepared by Modification 2)	6	9	5	80	--
Chymopapain B (Prepared by Modification 3)	4	--	72	24	--
Chymopapain A (My sample)	49	4	20	--	27

* "X" probably represents some derivative of di-DNP-tyrosine (probably the dichloro or trichloro derivative). The R_f of this compound coincides with that of di-DNP-tyrosine in the solvent system t-amyl alcohol-3% NH_3 but this compound migrates in the second dimension in the solvent system 1.5M phosphate buffer, pH 6.00.

Figure 13. The re-chromatography of chymopapain B on XE-64. 425 mg. of once-XE-64 treated enzyme was charged on a 1.0 x 20.0 cm column of XE-64 and elution carried out with the buffers indicated. Approximately 85% of the activity was eluted with the 0.40M buffer. All operations were carried out at 4°C.


ABSORBANCE AT 280 MU
9
6
3
0.30M
PHOSPHATE
pH 5.90
0.40M
PHOSPHATE
pH 5.90
100
200
300
400
VOLUME ELUATE (MLS)

B. Physical Properties

1. Weight-Average Molecular Weight Determinations

A large variety of physical methods may be used to determine the molecular weight of a particular protein. The interested reader is referred to Alexander and Block (1961) and Tanford (1962) for a brief treatment of methods which are widely used for the determination of the molecular weight of polymers.

The method adopted in the present study for the determination of the molecular weight of chymopapain B involves the use of the analytical ultra-centrifuge. Schachman has discussed the principles of ultra-centrifugation in an excellent monograph (1959) and has also outlined the experimental aspects of molecular weight determination with the ultra-centrifuge in another article (1955).

a. Sedimentation-Velocity Method

1) Measurement and Calculation of $S_{20,w}$:

Table XI summarizes the values obtained for the sedimentation constants of chymopapain B under various conditions and Figure 14 graphically illustrates these values. Included in this same plot are the sedimentation constants of papain and chymopapain A. The extrapolated values of the sedimentation constants ($S_{20,w}$ at infinite dilution) are:

Papain . . . . . 2.42 $\pm$ .05S

Chymopapain A . . . . . 2.70 $\pm$ .03S

Chymopapain B . . . . . 2.82 $\pm$ .05S

It will be noted that at acid pH's the values of the sedimentation constants of both chymopapain A and chymopapain B

exhibit a negative concentration dependence, whereas papain does not exhibit any concentration dependence. This concentration dependence is indicative of a thermodynamically non-ideal solution, and hence, extrapolation procedures to limiting values must necessarily be adopted. This negative concentration dependence, it might be pointed out, is indicative of a non-associating system.

2) Measurement and Calculation of the Partial Specific Volume:

The value of the partial specific volume of chymopapain B as determined at room temperature in the manner outlined under Methods is .72 $\pm$ .010 cc/gm.

3) Determination of the Weight-Average Molecular Weight by the Method of Sedimentation Velocity-Diffusion:

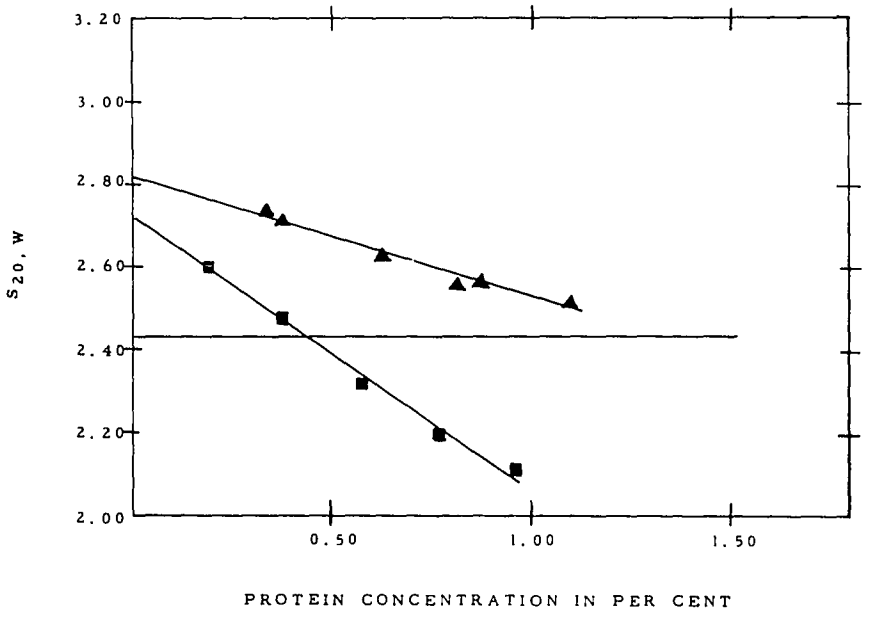
By using equation 2 above with F = s/D, the weight-average molecular weight can be calculated. Unfortunately, the value of the diffusion constant ($D_{20,w}$) is not available. However, an approximate estimate of the molecular weight can be obtained by making certain approximations: if one assumes that the frictional ratio of chymopapain B roughly approximates that of papain and papaya lysozyme (f/f_o = 1.20), then the molecular weight of chymopapain B can be estimated to be in the neighborhood of 31,000 (these proteins are used in the approximation since they are isolated from the same source and the detailed measurements of their physical parameters available). That these assumptions are essentially valid will be shown by a detailed study of the molecular weight by the Archibald procedure.

TABLE XI

SEDIMENTATION CONSTANTS OF CHYMOPAPAIN B

NO.	PROTEIN CONCENTRATION (gm/cc)	$S_{20,w}$
1	0.33	2.70
2	0.37	2.68
3	0.66	2.58
4	0.82	2.55
5	0.88	2.53
6	1.09	2.48

Figure 14. Sedimentation constants of chymopapain A (■—■—■), chymopapain B (▲—▲—▲), and mercuri (——•——) papain as a function of protein concentration. The chymopapains were studied in 0.10M NaH_2PO_4, pH 4.75. Mercuripapain was studied in acetate buffer, pH 3.9, ionic strength = 0.12 containing .02M cysteine and .002M Versene (Smith, et. al., 1954). The straight lines drawn represent a linear least squares representation.


3.20
3.00
2.80
2.60
2.40
2.20
2.00
$S_{20,w}$
0.50
1.00
1.50
PROTEIN CONCENTRATION IN PER CENT

b. Approach-To-Equilibrium Method (Archibald Procedure)

Figure 15 shows the patterns obtained from a typical Archibald run. The calculations of the molecular weight of chymopapain A and B obtained at a series of concentrations and from two separate preparations of the enzyme are summarized in Table XII and Table XIII, respectively.

It can be readily noted that the apparent molecular weight is a function of time of centrifugation at the high concentrations, and that this dependence is almost lacking at the lower concentrations. This is shown in Figure 15.A. where the apparent molecular weight, M_{app} (t), is plotted against the time of centrifugation. This positive dependence of M_{app} with t represents an observation which has not been given any theoretical treatment until recently (Fujita, et. al., 1962). This treatment of a non-ideal, polydisperse system was used in arriving at the molecular weight by the Archibald method.

Very briefly, the method at arriving at the molecular weight by the Archibald procedure in a system which shows a negative concentration dependence (therefore a positive dependence of MW_{app} with time), according to the above authors, is the following: calculated values of MW_{app} are first plotted against time (Figure 16.A.) and the extrapolated value at t = 0 determined. This procedure is carried out with different concentrations of the protein. Since the theory shows that the relationship between the molecular weight and initial concentration can be expressed by equation 5 below, a method of obtaining the weight-average molecular weight and the second virial coefficient is available. (It should be

pointed out that the second virial coefficient derived from the Archibald method has a value two times that obtained by light-scattering, i.e. B = 2A where A is the second virial coefficient as defined in light-scattering). Accordingly, the reciprocal of the extrapolated molecular weight is plotted against the initial concentration (Figure 16.B.). From the y-intercept, the reciprocal of the weight-average molecular weight is obtained. (The second virial coefficient, which can be obtained from the slope according to equation 5, cannot be calculated in this particular case, since the molecular weight apparently reaches a limiting value at low protein concentration.)

$$\frac{1}{(MW)_{app}} = \frac{1}{(MW)} + BC \quad \ldots\ldots\ldots\ldots (5)$$

Harrington and Kielley (1960) have also observed this concentration dependence of the molecular weight in their studies with myosin, also using the Archibald procedure. They have derived a relationship exactly in form to equation 5, although their derivation starts from a different principle.

Treatment of the data obtained with chymopapain B yields a weight-average molecular weight of 30,100 $\pm$ 1000. The deviation arises from a 1% uncertainty in the partial specific volume which gives rise to a 3% uncertainty in the molecular weight.

Figure 15. Archibald "approach-to-equilibrium" run on chymopapain B. (A) Synthetic boundary run at 12,590 rpm in 0.10M NaH_2PO_4 buffer, pH 4.75. (B) Run in 12-mm cell with the 4° sector Kel-F centerpiece. Times after full speed are (a) 66 minutes, (b) 98 minutes, (c) 126 minutes, (d) 158 minutes and (e) 190 minutes. Pictures taken with a bar angle of 70°; temperature, 24°.

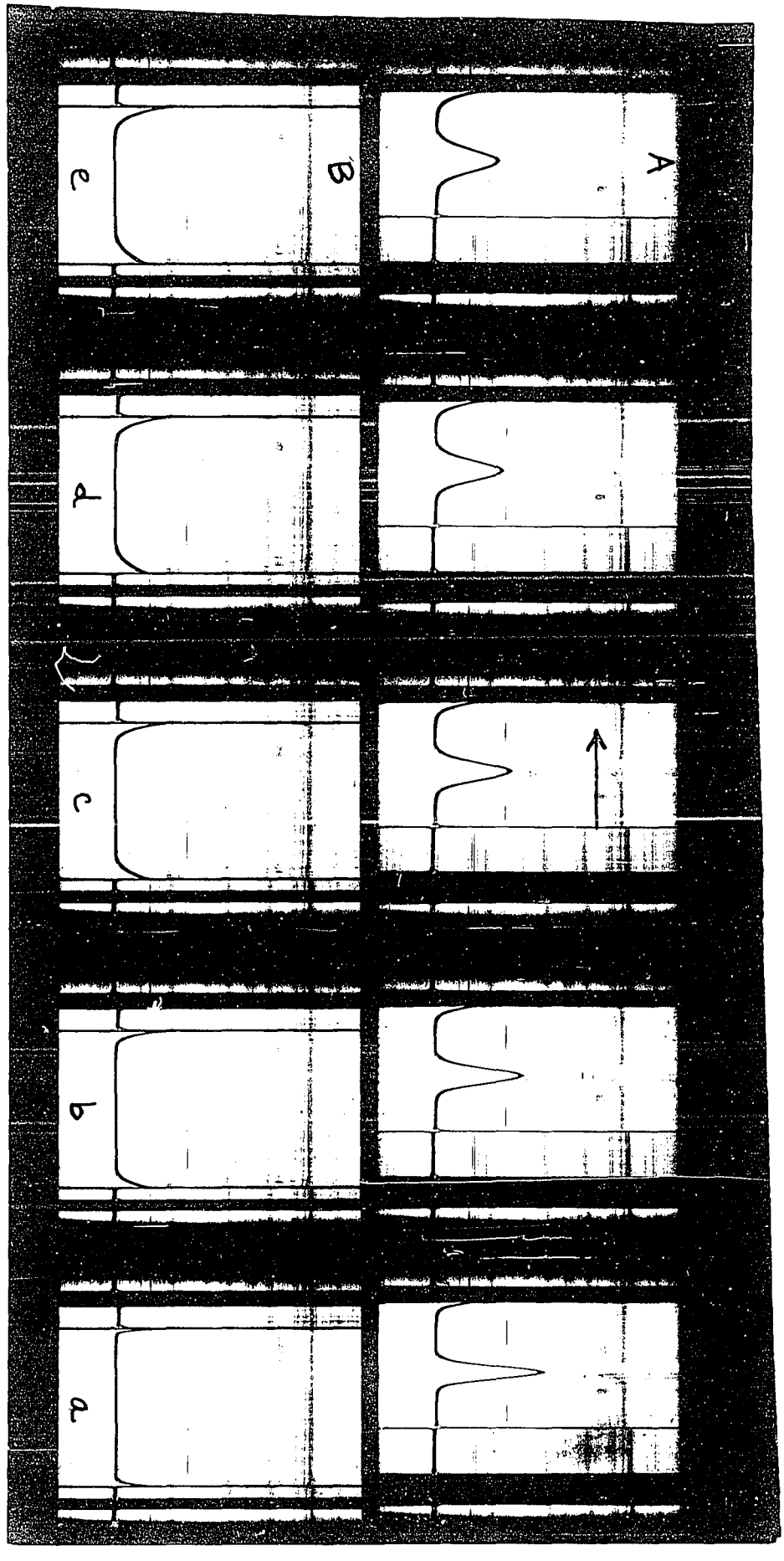

A
B
e
d
c
b
a

TABLE XII

SUMMARY OF ARCHIBALD CALCULATIONS OF CHYMOPAPAIN A

NO.	PREPARATION	BUFFER	PROTEIN (g/100cc)	TIME (MINUTES)	APPARENT M.W.
1	I	0.10M NaH_2PO_4 pH 4.75	0.50	70	31,690
				102	31,350
				134	31,970
				166	31,930
2	DIP-chymopapain (Ebata)	"	0.76	97	28,700
				113	28,840
				129	29,630
				145	29,360
3	I	"	1.18	65	26,470
				97	26.760
				126	26,860
				161	27,020
4	Intact-chymopapain (Ebata)	"	1.26	81	24,110
				97	23,910
				113	24,310
				129	25,360
5	"	" + .005M EDTA	1.37	81	28,040
				97	26,850
				112	27,930
				128	27,190
6	I	"	1.50	73	23,810
				105	24,120
				137	24,580
				169	25,010
				201	25,390

TABLE XIII

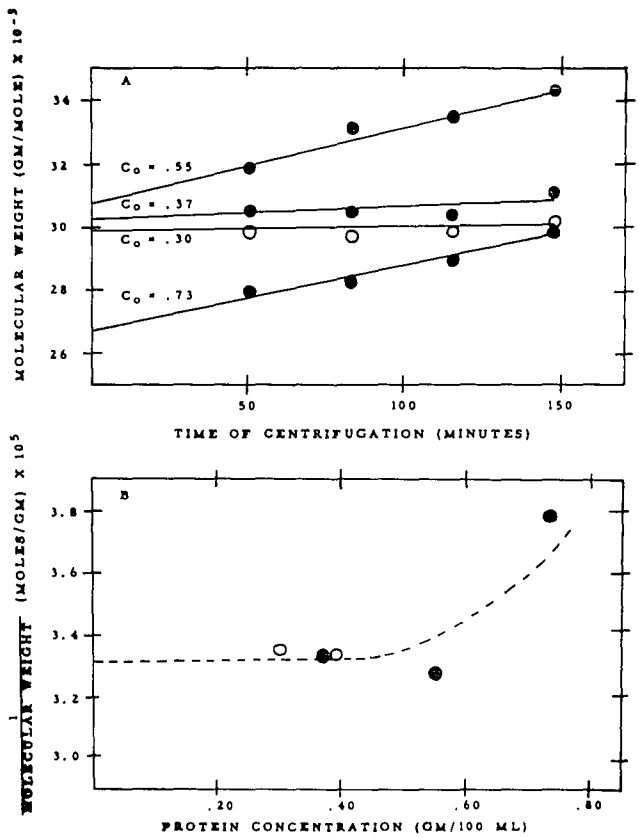
SUMMARY OF ARCHIBALD CALCULATIONS

OF CHYMOPAPAIN B

NO.	PREPARATION	BUFFER	PROTEIN (gm/100cc)	TIME (MINUTES)	APPARENT M.W.
1	I	.10M NaH_2PO_4 pH 4.75	.30	49	29,760
				81	29,616
				113	30,298
				145	30,044
2	I	"	.38	50	29,876
				82	30,321
				114	30,426
				146	30,228
3	II	"	.37	51	30,540
				83	30,476
				115	30,199
				147	31,305
4	II	"	.55	49	31,920
				81	33,241
				113	33,529
				145	34,391
5	II	"	.73	50	27,802
				82	20,324
				115	28,994
				147	30,625

Figure 16. A. Plots of $M_{app}(t)$ versus time of centrifugation for chymopapain B. The values of the initial concentration (c_o) are indicated.

B. Plot of $1/M_{app}$ versus initial concentration (c_o) for chymopapain B. The dashed line indicates an extrapolation of the straight line portion of the curve. The open and closed circles represent two different preparations.


A
MOLECULAR WEIGHT (GM/MOLE) X 10⁻⁵
34
32
30
28
26
C_o = .55
C_o = .37
C_o = .30
C_o = .73
50
100
150
TIME OF CENTRIFUGATION (MINUTES)
B
$\frac{1}{\text{MOLECULAR WEIGHT}}$ (MOLES/GM) X 10⁵
3.8
3.6
3.4
3.2
3.0
.20
.40
.60
.80
PROTEIN CONCENTRATION (GM/100 ML)

2. Electrophoretic Studies

a. The Variation of Electrophoretic Mobility with pH: The Isoelectric Point

Figure 17 plots the calculated mobility as a function of pH. Interpolation of the mobility to u = 0 yields an isoelectric point of 10.42 $\pm$ .10 pH unit.

The mobilities of papain and chymopapain A are included for comparative purposes. It will be readily noted that all these proteases exhibit an alkaline isoelectric point. Papaya lysozyme (not shown) has an isoelectric point of 10.5, as noted in Table I.

While chymopapain B has a more basic isoelectric point than does that of either papain or chymopapain A, its mobility over a wide pH range is relatively low. In fact, its mobility at pH 4.00 approximates its mobility in the pH range 8.00-9.50. In the acid region, the mobility of chymopapain B is even less than that of papain. This is somewhat surprising in view of the fact that chymopapain B has a much higher net charge (sum of the lysine, arginine, histidine residues less the residues of glutamic acid and aspartic acid, corrected for the amide content) than papain. (See section III.C.2., Amino Acid Composition.) This seemingly anamolous behaviour on electrophoresis suggests that chymopapain B either binds negative ions more strongly than does either papain or chymopapain A or that the positive residues (in particular, lysine) are somehow prevented from manifesting its positive electrostatic potential due to the "burying" of these positive charges by a conformational phenomenom, to "shielding" of the charges by the "ion double layer," by bonding of the epsilon-amino group to some

unknown residue (e.g. a sugar) or to a combination of these possibilities.

b. The Variation of Electrophoretic Mobility with Ionic Strength: The "Ion-Atmosphere"

An increase or decrease of the electrolyte concentration which affects the thickness of the "ion-atmosphere" may cause the mobility to change with the ionic strength of the medium. A discussion of the nature of the "ion-atmosphere" is given by Moore (1958).

It may be seen in Figure 18 that the mobility of chymopapain B decreases as the sodium chloride content in 0.02M acetate buffer, pH 4.00, increases. Lactogenic hormone (Li, et al., 1940), egg albumin (Tiselius and Svenssohn, 1940) and hemoglobin (Davis and Cohn, 1939) were some proteins whose electrophoretic mobilities were found to be retarded by an increase in the ionic strength of the medium. With these proteins, the retarding effect seemed to follow the decrease of the thickness of the ion-double layer, the magnitude of which is given by the Debye-Huckel expression:

$$d = (8\ Ne^2/1000DkT)^{-1/2}\ u^{-1/2} \quad \ldots\ldots\ldots (6)$$

where d = diameter of the ion-double layer
N = Avagadro's number
e = charge of the electron
D = dielectric constant of the medium
k = Boltzmann's constant
T = temperature (^{o}K)
u = ionic strength

For a semi-quantitative rationalization of the retardation of electrophoretic mobility with increasing electrolyte concentration, we invoke equation (7) below, which is strictly applicable only for a sphere whose radius is larger than the thickness of the "ion-atmosphere" (Li, 1951):

$$U = zD/6\pi n \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (7)$$

where U = electrophoretic mobility
D = dielectric constant of the medium
n = viscosity of the medium
z = zeta-potential
= q/Dr in the absence of the "ion-atmosphere"
= q/Dr - q/(r + d) in the presence of the "ion-atmosphere"

where q = charge of the sphere
r = radius of the charged sphere
d = diameter of the diameter of the ion-double layer, defined by equation 6 above.

Based on this model of a charged sphere whose electrical potential is influenced by the presence of the ion double layer, it is at once obvious that an increase in the ionic strength of the medium should decrease the electrophoretic mobility of this charged sphere. Therefore, if this model of the charged sphere can be used as an approximate model of a charged protein, then a semi-quantitative explanation of the observed effect is available. However, it is apparent that a rigorous prediction of the magnitude of this retarding effect would not be possible with the available treatment.

Figure 17. Electrophoretic mobility of the three proteases of dried papaya latex as a function of pH. (■—■—■), chymopapain A; (▲—▲—▲), chymopapain B; and (●—●—●), papain. The values for chymopapain A are those of M. Ebata (this laboratory, unpublished), and the values for papain were obtained from Smith, et. al. (1954).

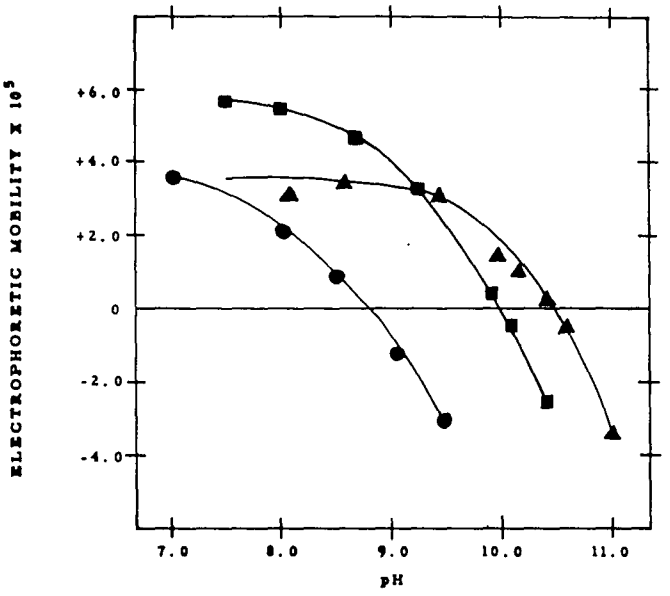

ELECTROPHORETIC MOBILITY X 10^5
+6.0
+4.0
+2.0
0
-2.0
-4.0
7.0
8.0
9.0
10.0
11.0
pH

TABLE XIV

SUMMARY OF ELECTROPHORETIC MOBILITY MEASUREMENTS

RUN NUMBER	BUFFER	pH*	IONIC STRENGTH	F**	u x 10^5***
1	Acetate	3.83	.10	16.88	+2.86
2	Acetate	3.83	.04	16.10	+6.30
3	Acetate	3.83	.02	15.30	+9.17
4	Phosphate	8.25	.10	12.70	+3.05
5	Veronal-HCl	8.57	.10	9.08	+3.50
6	Veronal-HCl	9.33	.10	9.03	+3.39
7	Glycine-NaOH	9.83	.10	13.03	+1.27
8	Glycine-NaOH	10.07	.10	10.28	+ .99
9	Glycine-NaOH	10.40	.10	8.75	+ .26
10	Glycine-NaOH	10.55	.10	11.27	- .33
11	Glycine-NaOH	11.07	.10	8.19	-3.38
12	Glycine-NaOH	11.42	.10	8.77	-5.70
13	Glycine-NaOH	11.65	.10	8.61	-7.82

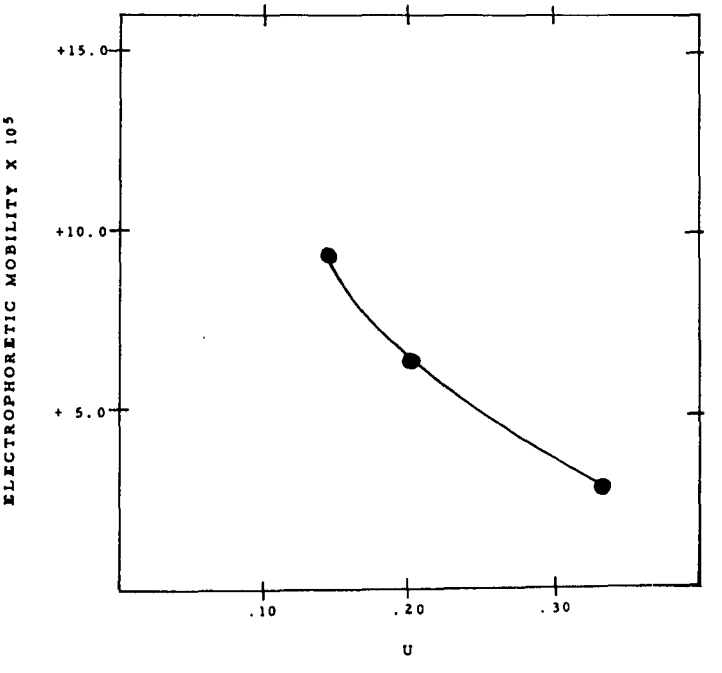
* Measured with a Beckman pH meter with expanded scales at ice-bath temperature.

** Potential gradient = i/Ak (volts/cm)

where i = current (amperes)
A = cross-sectional area of cell
k = specific conductance of solution

*** Electrophoretic mobility in units of square cm. per volt-seconds.

Figure 18. The mobility of chymopapain B as a function of the square root of ionic strength. Electrophoresis was carried out in 0.02M acetate buffer, pH 3.83 containing no NaCl, .02M NaCl and .08M NaCl. All runs made at 0.5°C.


+15.0
+10.0
+ 5.0
ELECTROPHORETIC MOBILITY X 10⁵
.10
.20
.30
U

3. Stability Studies

a. Stability as a Function of pH

An interesting property of chymopapain that was noted in earlier studies was the stability of this enzyme at acid pH's (Jansen and Balls, 1941). In fact, these workers utilized this stability in acid solution as a basis for purifying chymopapain, since at pH 2.0 a considerable amount of inert protein (including denatured papain) can be removed by salt fractionation. Moreover, all of the preparative procedures outlined in Section III.A. also incorporate this "acid step."

Ebata (1962) has shown that chymopapain A is stable at pH 2.0 and has stated that this property might serve to establish the similarity between the chymopapain isolated by Jansen and Balls from fresh papaya latex and the chymopapain which he has isolated from dried papaya latex.

An examination of Figure 19 reveals that chymopapain B is also stable at pH 2.0 for several weeks, but that this stability markedly depends on the temperature at which the enzyme solution is stored. If the enzyme solution is frozen, it rapidly loses 50% of its activity in less than 24 hours, and then is inactivated at a much slower rate. However, if the sample is stored at $4^{o}C$, it will retain over 85% of its activity after a period of two weeks. Papain, it will be noted, is rapidly inactivated after 24 hours.

Further examination of Figure 19 shows that chymopapain B is most stable at slightly acid pH's (stability at pH 5.00 pH 7.30 pH 8.90). This stability apparently parallels the attenuation of

the rate of sulfhydryl oxidation, since this oxidation is known to proceed very rapidly in alkaline solution and much more slowly in acid solution. That this interpretation is roughly correct will be further supported by studies of the stability as a function of temperature.

b. Stability as a Function of Temperature:

Figure 20 and Figure 21 illustrates the stability of chymopapain B at various pH's and temperatures. Inspection of Figure 20 shows that this enzyme is very heat-stable. The calculated half-lives for inactivation of chymopapain B at 75° are 105 minutes (pH 7.34), 120 minutes (pH 8.83) and 139 minutes (pH 4.88). The corresponding half-lives of chymopapain A and papain at pH 7.20 were 75 and 57 minutes, respectively.

Figure 21 presents the results of a more extended study of the stability of unactivated chymopapain B as a function of four pH's and four temperatures. (The unactivated enzyme was utilized since studies of the inactivation as a function of temperature and pH might afford some insight into the effects of these variables on -SH oxidation.) It can be readily noted that the stability of this enzyme generally follows a consistent pattern: at elevated temperatures, the enzyme is irreversibly inactivated; at intermediate pH's (5-9), the inactivation is due to several factors, the most notable being the susceptibility of the -SH groups toward oxidation. This oxidation proceeds relatively fast at the more alkaline pH's and more slowly in slightly acid solution (c.f. the reactions cysteine $\longrightarrow$ cystine, 2 glutathione $\longrightarrow$ $(\text{glutathione})_2$, nBAL $\longrightarrow$ $(\text{BAL})_n$. That oxidation does indeed occur is shown by the capacity

of the enzyme to be re-activated with the addition of cysteine. This can be seen by comparing the inactivation at 75° of the un-activated enzyme (Figure 21.D.) and the activated enzyme (Figure 20). At all pH's, an enhancement of the calculated half-lives is evident when the enzyme is re-activated with cysteine, the ratio $t_{\frac{1}{2}}$ (activated)/$t_{\frac{1}{2}}$ (unactivated) serving as a somewhat crude index of the relative magnitude of reversible oxidation (Table XV).

Figure 19. The stability of chymopapain B at various pH's.

A solution of chymopapain B was allowed to stand in the buffers indicated at either 5°C (closed symbols) or -10°C (open symbols). At the times indicated, 1.0 cc aliquots containing 40 ug/cc enzyme were mixed with 1.0 cc of a .01M cysteine-.005M EDTA solution in 0.10M phosphate buffer, pH 7.2 and 1.0 cc of this mixture assayed for activity using the casein digestion method. (○), .02M HCl-KCl buffer, pH 2.00; (△), .02M acetate, pH 5.0; (□), 1.02M phosphate, pH 7.30 and (×), .02M Tris, pH 8.95. (◒) represents papain in .02M HCl-KCl buffer, pH 2.00 kept at 5°C.

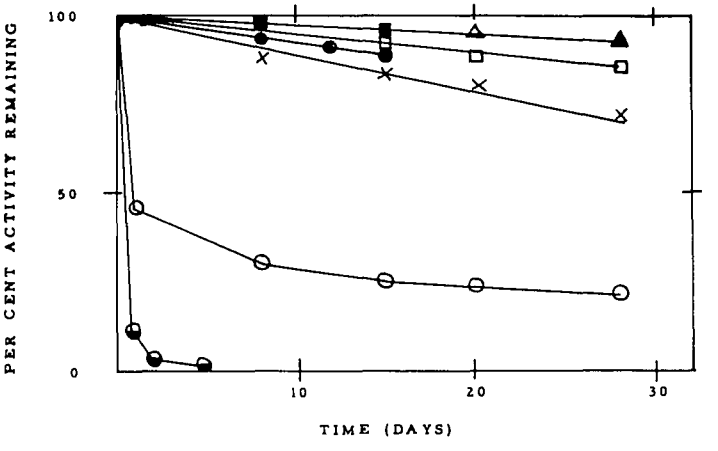

100
50
0
PER CENT ACTIVITY REMAINING
10
20
30
TIME (DAYS)

TABLE XV

HEAT STABILITY OF CHYMOPAPAIN B AT 75°C

pH	$t_{\frac{1}{2}}$ (UNACTIVATED)	$t_{\frac{1}{2}}$ (ACTIVATED)	$\frac{t_{\frac{1}{2}} \text{ (ACTIVATED)}}{t_{\frac{1}{2}} \text{ (UNACTIVATED)}}$
2.33	1 minute	2 minute	2.0
4.88	84 minutes	139 minutes	1.7
7.34	22 minutes	105 minutes	4.8
8.83	12 minutes	120 minutes	10.0

Figure 20. The heat stability of chymopapain A, chymopapain B and papain at 75°C. A solution of the unactivated enzyme solution was incubated at 75°C in the buffers indicated and an aliquot removed periodically and assayed for activity after reducing with cysteine (.05M final concentration). Open symbols represent chymopapain B incubated in .02M HCl-KCl, pH 2.0 (●); .02M acetate, pH 5.0 (△); .02M phosphate, pH 7.3 (□) and .02M Tris, pH 8.95 (X). Papain (◪) and chymopapain A (■) were incubated in .02M phosphate buffer, pH 7.2.

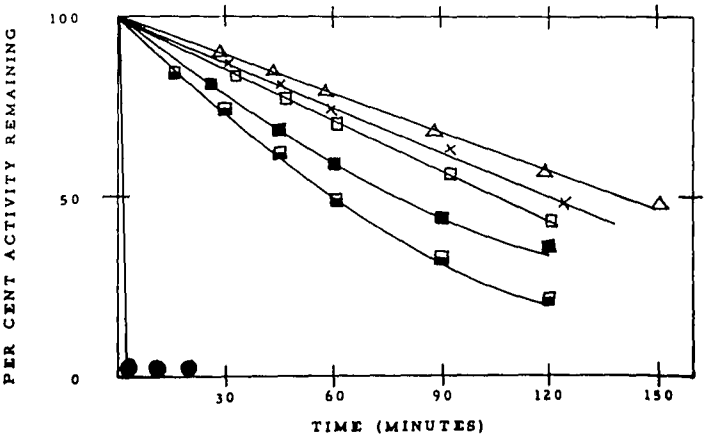
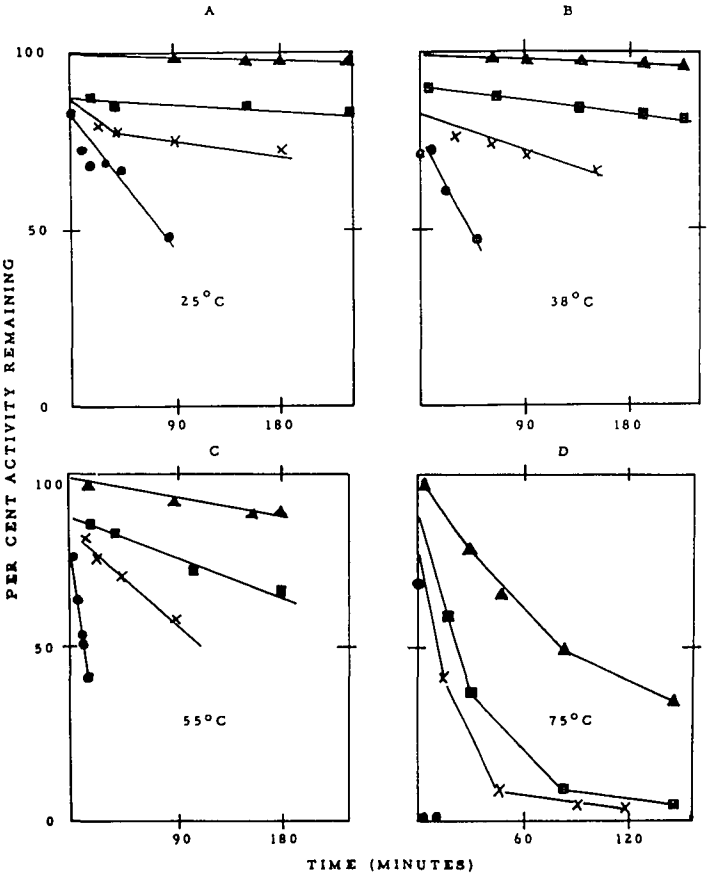

PER CENT ACTIVITY REMAINING
100
50
0
30
60
90
120
150
TIME (MINUTES)

Figure 21. The stability of non-treated chymopapain B as a function of pH and temperature. Solutions of the enzyme were incubated at different pH's and at the temperatures indicated. At various time intervals, 1.0 cc aliquots were withdrawn and added to 1.0 cc casein and digestion allowed to proceed for 10 minutes (casein digestion method). (●), .02M HCl-KCl, pH 2.1; (▲), .02M acetate, pH 5.0; (■), .02M phosphate, pH 7.5; and (X), .02M Tris, pH 9.0.


A
B
C
D
100
50
0
90
180
60
120
25°C
38°C
55°C
75°C
PER CENT ACTIVITY REMAINING
TIME (MINUTES)

4. The Spectra and $E^{1\%}_{1\ cm}$ (280 mu) of Chymopapain B

Examination of the spectrum of chymopapain B in the ultra-violet and near visible indicates that the spectra of this protein resembles that of a typical protein, exhibiting a maximum at approximately 280 mu and a minimum at approximately 250 mu. (Figure 22.A.). The examination of the spectrum in the far visible does not reveal the presence of non-protein chromophores as would be evident with proteins containing certain prosthetic groups. The spectrum in 0.10M NaOH shows the typical bathochromic shift arising from the ionization of the tyrosyl residues (Goodwin and Morton, 1946).

Determination of the $E^{1\%}_{1\ cm}$ (280 mu) was done by relating the absorbancy to a known weight of protein (as determined refractometrically as outlined before). The values obtained apparently depended on the pH of the solution (Figure 22B).

Some of the quantitative aspects of the analysis of the spectra and the measurements of $E^{1\%}_{1\ cm}$ (280 mu) are summarized in Table XVI.

TABLE XVI

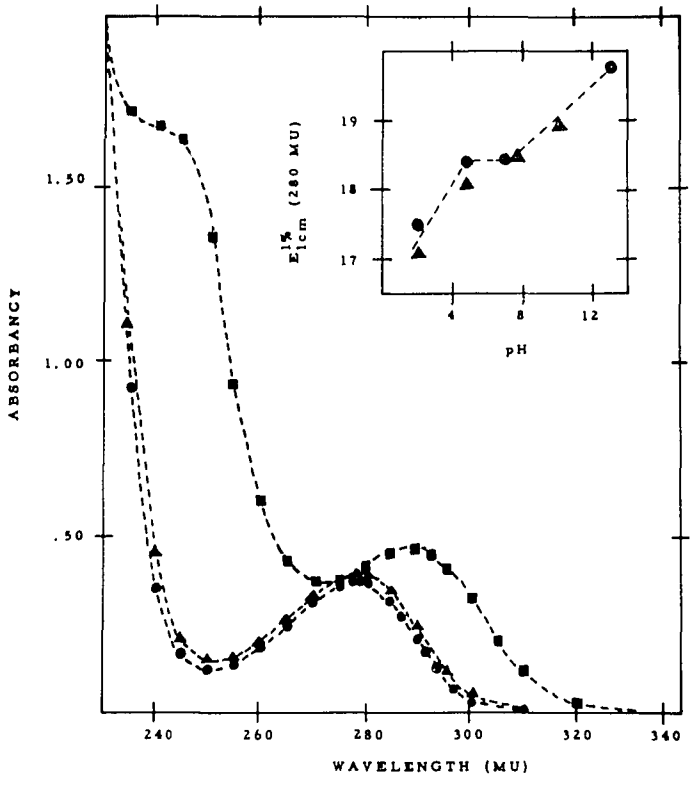
SUMMARY OF SOME SPECTRAL MEASUREMENTS

AND THE CALCULATION OF E(1%, 1 cm, 280 mu)

NO.	SOLVENT	pH	$E^{1\%}_{1\ cm}$	E(280/260)	E(280/292)
1	.01N HCl	2.10	17.04	1.952	2.226
2	.01N HCl	2.10	17.50	1.891	2.171
3	.10M Acetate	4.70	18.38	1.985	2.046
4	De-ionized H_2O	7.03	18.37	1.888	1.945
5	.10M Phosphate	7.38	18.52	2.015	2.026
6	.10M NaOH	13.00	19.72	.700	.936

Figure 22. A. The spectrum of chymopapain B in various solvents. (●—●—●), 0.01N HCl, pH 2.08; (▲—▲—▲), 0.10M phosphate buffer, pH 7.03; (■—■—■), 0.10M NaOH, pH 13.00.

B. The variation of $E^{1\%}_{1\ cm}$ (280 mu) as a function of pH. The different symbols represent values obtained with a separate preparation.


ABSORBANCY
1.50
1.00
.50
240
260
280
300
320
340
WAVELENGTH (MU)
$E^{1\%}_{1cm}$ (280 MU)
19
18
17
4
8
12
pH

5. Optical Properties

a. Measurement of the Refractive Index Increment (dn/dc)

The physical parameter (dn/dc) occurs in the calculation of molecular weight using the technique of light-scattering. The accuracy of the measurement of dn (difference between the refractive index of a solute and its solvent) is generally limited by the necessarily stringent temperature controls which must be observed.

The availability of a differential refractometer in our laboratory makes it possible to measure the refractive index increment of a protein with an accuracy sufficient for estimating protein concentrations, and it was for this purpose that a measurement of the refractive index increment was undertaken.

The measurement of the increment was done by determining dn as a function of concentration. The average values obtained for several proteins are summarized in Table XVII.

This method for determining protein concentrations has two distinct advantages over the methods conventionally used for this purpose, such as dry weight measurements, nitrogen analysis by the Kjehldahl procedure or turbidimetric estimations: (1) it is more rapid, limited only by the time required for temperature equilibration and (2) it is more accurate since the parameter dn/dc at any particular wavelength does not vary by more than $\pm$ 3%. Its main disadvantages are its relative insensitivity to low concentrations of proteins (for all practical purposes, concentrations greater than 1 mg/cc are required) and the non-applicability, in certain instances, of one particular value of the refractive index increment

in different buffer systems, particularly where the solute is equilibrated with the solvent by dialysis (the Gibbs-Donnan equilibrium effects a non-equilibrium condition between the solvent in the two phases).

TABLE XVII

THE REFRACTIVE INDEX INCREMENT OF

CHYMOPAPAIN B, PAPAIN AND ∝-CHYMOTRYPSINOGEN

PROTEIN	$(dn/dc)^{436}$ Calculated	$(dn/dc)^{546}$ Calculated
Chymopapain B	.193	.186
Papain	.193	.186
∝-Chymotrypsinogen	.193 (194)*	.184 (.185)*

* Values cited by Wilcox, et. al., 1957.

C. Chemical Properties

1. Elementary Composition

a. Nitrogen Analyses

The nitrogen content, uncorrected for residual moisture and ash gave an average value of 15.83%, with an average deviation of $\pm$ .15%.

2. Amino Acid Composition

a. Analysis Using the Automatic Amino Acid Analyzer

The values cited here represent a preliminary determination of the amino acid composition of chymopapain B. A detailed investigation of the composition is in progress at the moment, but unfortunately will not be completed during the writing of this thesis. Hence, the values cited can only serve to give an approximate indication ($\pm$ 10%) of the composition of the enzyme.

A 24-hour hydrolysate was analyzed with the Beckman/Spinco Model 120 Automatic Amino Acid Analyzer. Calculations were carried out according to standard methods (Spinco Instruction Manual and Handbook) and the values obtained summarized in Table XVIII.

From this preliminary determination, several facts are at once apparent. The relatively low recovery of the amino acid residues (ca. 90%) indicates the possibility of non-amino acid groups attached to the protein (e.g. carbohydrates). That the recovery of amino acid residues was not complete is not surprising in view of the fact that crystalline chymopapain B exhibits a faint yellow-brown color in concentrated solutions. While some of this color can be removed by re-chromatography on XE-64 or chromatography on SE-sephadex (as evidenced by the attachment of a brownish color at

the top of the resin), the crystallization of this two-times, column-treated enzyme has never been realized. Furthermore, a significant loss of enzyme activity was evident in most instances. The significance of this observation, however, is not known at present.

The abundance of lysine residues is at once evident. Furthermore, the basicity of chymopapain B (its very high isoelectric point has been already cited) becomes obvious by a consideration of the excess positive residues (sums of lysine and arginine less the sums of the dicarboxylic acids corrected for amide content).

The most interesting fact that will probably emerge from a complete amino acid composition determination is the marked similarity between chymopapain A and B. Examination of the compositions of A and B indicates that the residues parallel one another in occurence. While an exact duplication of residues is not at once evident, the likelihood that they are very similar exists nontheless. A more thorough discussion of the comparison of the properties of chymopapain A and B will be given at the conclusion of this thesis (see Summary).

TABLE XVIII

AMINO ACID COMPOSITIONS OF CHYMOPAPAIN A, CHYMOPAPAIN B,

PAPAIN AND PAPAYA LYSOZYME

	CHYMOPAPAIN B (24-HOUR HYDROLYSATE)		CHYMOPAPAIN	PAPAIN	LYSOZYME
RESIDUE	gm Amino Acid Residue/100 grams protein	Calculated Number of Residues/Molecular Weight of 30,000	* (1)	** (2)	*** (3)
Lysine	10.52	25	29	9	10
Histidine	1.54	3	4	2	3
Ammonia	1.73	32	40	19	21
Arginine	3.06	6	4	10	13
Aspartic	7.78	20	25	17	22
Threonine	4.23	12	19	7	13
Serine	3.70	13	21	11	16
Glutamic	8.98	21	26	17	11
Proline	4.30	13	17	8	18
Glycine	5.90	31	37	23	26
Alanine	3.89	16	19	13	21
Cysteine	3.46	10	11	6	8
Valine	6.16	19	21	15	8
Methionine	.44	1	1	0	4
Isoleucine	3.55	9	10	10	11
Leucine	5.25	14	16	10	12
Tyrosine	8.65	16 (18)****	20	17	13
Phenylalanine	3.07	6	8	4	12
Tryptophan	(---)	5****	6	5	7
TOTAL	86.21	242	294	184	228

* From M. Ebata (unpublished observations), calculated on the basis of molecular weight = 34,000.

** From Smith, et. al. (1960)

*** From Smith, et. al. (1955)

**** Estimated by the method of Morton and Goodwin (1946)

3. Carbohydrate Composition

The relatively low recovery of amino acid residues in the 24-hour hydrolysate and the occurence of a brownish-yellow tinge in the crystalline preparations of chymopapain B prompted an investigation into the possible nature of this constituent. The presence of carbohydrate seems likely, in view of the nature of our starting material, and therefore a determination of the sugar content was undertaken.

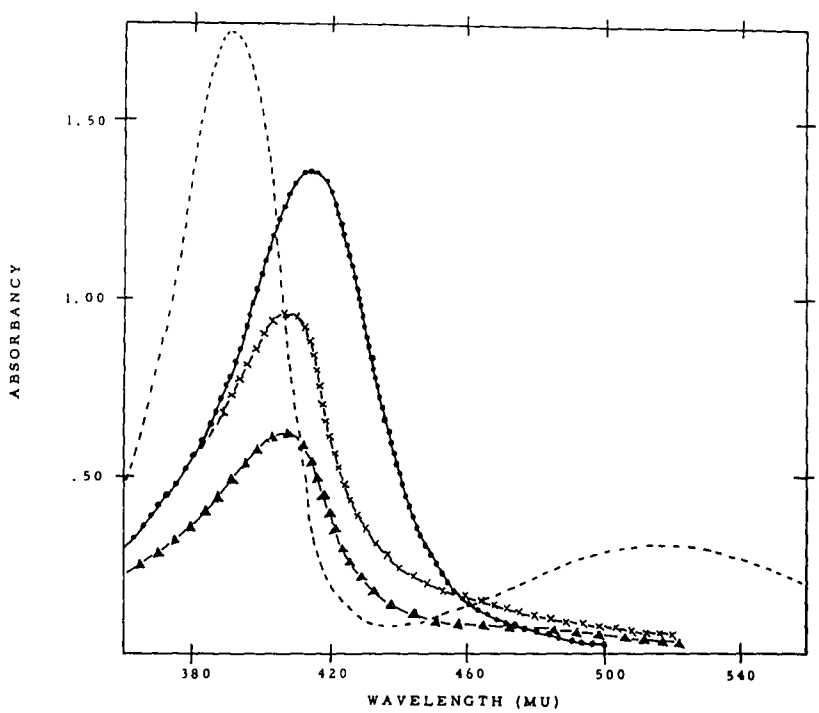
Reaction of chymopapain B with cysteine and sulfuric acid gave a yellow product indicative of hexoses (Dische, 1955), but the absorption maxima did not coincide with that of a typical hexose (mannose) or that of a pentose (xylose). (Figure 23.)

The maxima at ca. 405 mu indicated a methyl pentose, but the mixture failed to yield the water effect test, characteristic of methyl pentoses. Moreover, it was found that the protein gave this yellow product without the addition of cysteine. However, it was felt that the presence of free -SH groups in the protein might have accounted for the formation of this addition product, and a more detailed analysis for true sugars was undertaken with the other well-known tests.

The presence of a hexosamine was ruled out by the failure to detect this sugar in the amino acid analyzer (this constituent is present between the appearance of leucine and tyrosine using the 150 cm column in a Beckman Model 120 automatic amino acid analyzer).

Tests for hexoses, pentoses, hexuronic acids were also negative. Hence,the presence of true sugars is not indicated.

Figure 23. The visible spectra of chymopapain B and some simple sugars treated with 85% sulfuric acid and cysteine hydrochloride according to Dische (1955). (✕—✕—✕—✕), chymopapain B, re-chromatographed on SE-sephadex, 1100 ug/cc; (▲—▲—▲—▲), once-crystallized chymopapain B, 790 ug/cc; (●—●—●—●), D (+) xylose, 27 ug/cc and (- - - - - - -), D (+) mannose, 84 ug/cc.


1.50
1.00
.50
ABSORBANCY
380
420
460
500
540
WAVELENGTH (MU)

4. Studies of the Sulfhydryl Groups of Chymopapain B

a. Introduction

The essentiality of a single thiol group for the activity of papain (Balls and Lineweaver, 1939; Finkle and Smith, 1958; Sanner and Pihl, 1963) and ficin (Liener, 1961) has been well established. On the basis of indirect thermodynamic data, Smith (1958) suggested that the sulfhydryl group of papain was present in the form of an internal thioester, but this hypothesis for the probable participation in the catalytic mechanism of this thiol group has been questioned by several workers (Boyer, 1960; Hartley, 1960; Sanner and Pihl, 1963). More recently, Smith, et. al., (1962) have modified the original thioester hypothesis.

Hypotheses to explain the findings that the so-called "sulfhydryl" proteases require the addition of certain types of activators for maximal activity are predicated upon three general considerations: (1) that these activating agents reduce a disulfide bond to form a free, active thiol; (2) that these activators remove inhibitory metals; and (3) that these activators function as a coenzyme. These hypotheses have been reviewed in great detail by Kimmel and Smith (1957). Suffice it to note here that while an extensive number of studies have been brought to bear on the nature and reactivity of this essential -SH group in papain, mechanistic considerations are equivocal, and the nature of the mechanism of activation is not yet fully understood. The most recent study on the mechanism of activation by thiols with papain suggests that the mechanism involved is one hitherto unsuspected, i.e. that an activator increases the rate of cleavage of the enzyme-substrate

complex via an equilibrium reaction involving this thiol to form an enzyme-activator-substrate complex (Pihl and Sanner, 1963).

Chymopapain, which is also present in papaya latex, has also been recognized as an -SH enzyme (Greenburg, 1955).

The activation requirements of crystalline chymopapain A have been reported (Ebata, 1962) and unpublished observations in our laboratory indicate that two -SH groups per mole of chymopapain A react with PCMB.

The availability of a homogeneous preparation of chymopapain B makes it possible to evaluate the number and reactivity of its thiol groups in a quantitative manner. The purpose of this particular study was to undertake a detailed investigation of the -SH groups with particular regard to their possible involvement in enzymic catalysis.

b. Method of Obtaining Reducing Agent-Free Enzyme

While chymopapain B can be obtained in an active form (40% - 75% of maximum) by the preparative methods cited earlier, it was of interest to study the enzyme under conditions where it exerted maximal activity in the absence of the activator. This was accomplished by passing cysteine-activated chymopapain B through a multi-bed resin containing Dowex 50 (H^+ cycle) and IRA-411 (OH^- cycle) and obtaining an activator-free enzyme with an enhanced activity.

As shown in Figure 24, activator-free chymopapain B eluted from this multi-bed resin possessed approximately 85% of the activity of the enzyme assayed in the presence of the activator, but that the level of this activity markedly depends on the pH of

the medium in which the enzyme is stored. In slightly acid solution (pH 5.10), the enzyme is very stable, maintaining 70% of maximal activity even after 24 hours at room temperature. However, in slightly alkaline solution (pH 8.90), the enzyme was rapidly and reversibly inactivated, its activity falling rapidly to the level of the unactivated enzyme incubated at the same pH (c.f. stability as a function of pH and temperature). This is further evidence indicating the lability of the essential -SH group to oxidation, especially in alkaline solution. The marked stability of the activator-free enzyme in slightly acid solution again indicates the probable conversion: 2 -SH $\longrightarrow$ -S-S- in the inactivation process. However, the failure to completely re-activate the enzyme after 24 hours implies that irreversible oxidation and/or autolysis are concomitant processes.

c. Reaction with p-chloro-mercuribenzoate (PCMB)

1) Reaction with Native Chymopapain

Unactivated and activator-free chymopapain B was used. Initially the molar extinction coefficient of mercaptide formation was determined by titrating a known amount of protein (ca. .10 umole protein) with increasing amounts of PCMB. From the break in a plot of the change in absorbancy at 255 mu (corrected for protein and PCMB absorbances) versus (PCMB/Protein), the -SH titer can be calculated. (Figure 25.) The calculated molar extinction coefficient determined in this manner was 6.85×10^3 per mole protein per liter (0.33M acetate buffer, pH 4.6, 255 mu). Thereafter, -SH titers were calculated by

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measuring the maximal change in absorbancy at 255 mu by titrating a known amount of protein with an excess of PCMB and using this measured extinction coefficient to relate the absorbancy change to the -SH titer.

Figure 26 shows that with an excess of PCMB, the reaction with chymopapain B is very rapid, reaching 95% of the maximal change within one minute. In general, readings and activity measurements were made at 90 minutes. The data of Figure 25 also indicates that the inhibition closely parallels mercaptide formation, indicating the essentiality of the thiol groups for the catalytic function of the protein. The non-linearity of the inhibition is presumably due to differential oxidation in the different aliquots and therefore, the linear portion of this curve was extrapolated to 100% inhibition. The values so obtained parallel the -SH titer. The non-coincidence of the inhibition of amidase activity (using BAA as substrate) and protease activity (using casein as substrate) was observed in all instances and should be regarded as significant. A reasonable explanation for this non-coincidence may reside in the ability of casein to reverse inactivation to a small extent by virtue of -SH groups presumably present in this substrate. It might be pointed out that this non-coincidence between the inhibition of the esterase activity (using BAEE as substrate) and protease activity (using casein as substrate) has also been observed in the reaction between PCMB and ficin. (Liener, 1961)

Figure 24. The stability of reducing agent-free chymopapain B. The enzyme was reduced with .05M cysteine and the reducing agent removed as described in the text. Aliquots of the reducing agent-free enzyme were incubated at various pH's at room temperature and assayed for activity at the times indicated. (●), .02M acetate buffer, pH 5.0 containing .05M cysteine; (▲), .02M acetate buffer, pH 5.0; (■), .02M phosphate buffer, pH 7.30 and (X), .02M Tris, pH 9.0. (- - - - - -) indicates activity measurements made in the presence of .05M cysteine.

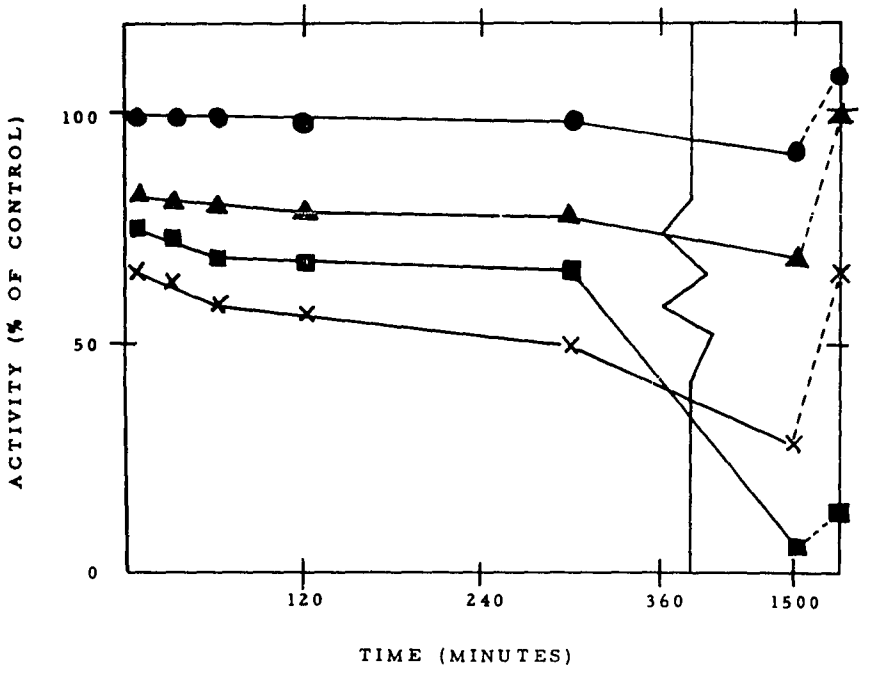

ACTIVITY (% OF CONTROL)
100
50
0
120
240
360
1500
TIME (MINUTES)

Figure 25. The reaction of chymopapain B with PCMB. Aliquots of the enzyme were treated with various levels of PCMB and after allowing the mixture to stand at room temperature for 90 minutes, the absorbancy measured at 255 mu (A) or 250 mu (B) (————). Activity measurements were made on casein (●—●—●) and BAA (▲—▲—▲). The activity of the enzyme in the absence of PCMB is arbitrarily given as 100% activity. The reactions were carried out in 0.33M acetate buffer, pH 4.60 (A) and in 0.10M phosphate buffer, pH 7.00 (B).

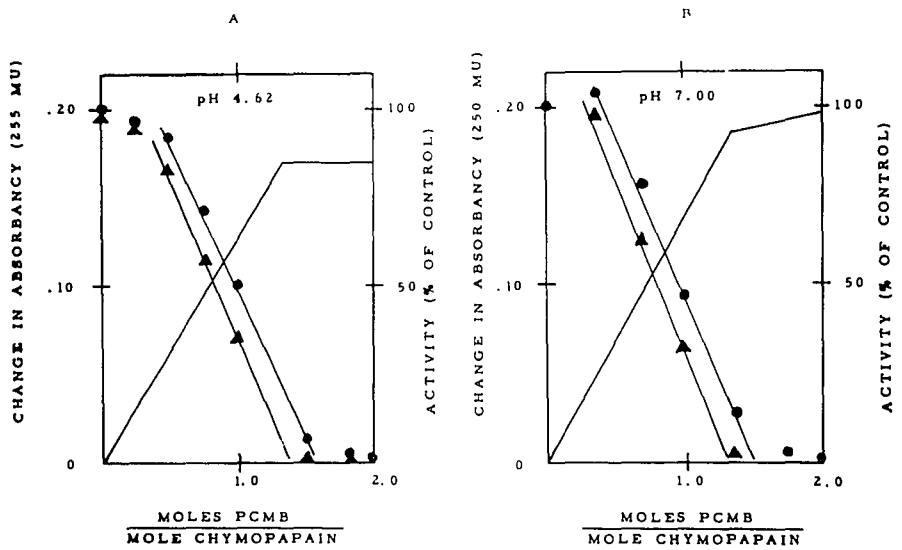
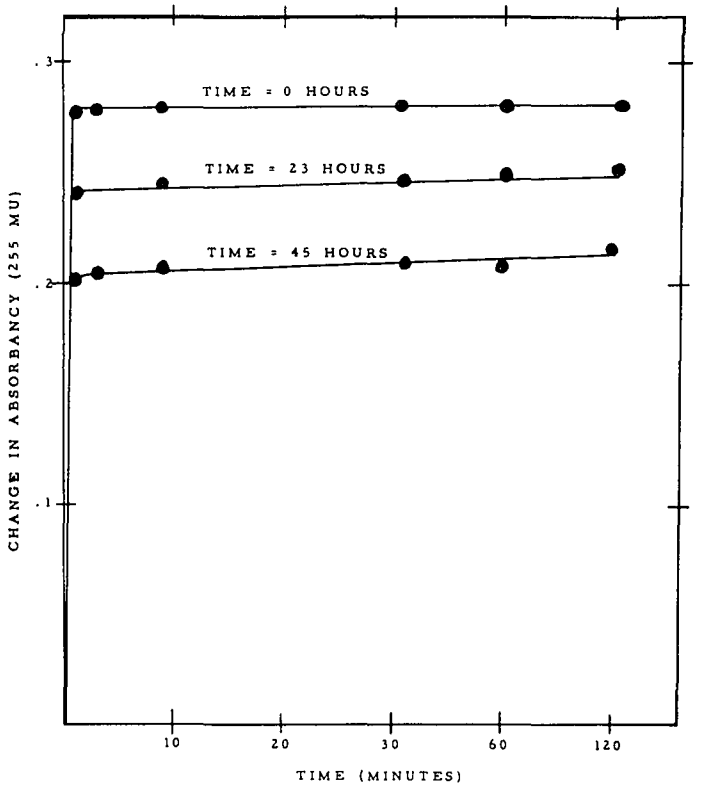

A
pH 4.62
CHANGE IN ABSORBANCY (255 MU)
ACTIVITY (% OF CONTROL)
.20
.10
0
100
50
1.0
2.0
MOLES PCMB
MOLE CHYMOPAPAIN
B
pH 7.00
CHANGE IN ABSORBANCY (250 MU)
ACTIVITY (% OF CONTROL)
.20
.10
0
100
50
1.0
2.0
MOLES PCMB
MOLE CHYMOPAPAIN

Figure 26. Time course of mercaptide formation involving chymopapain B and PCMB. To a solution of the enzyme, an excess of PCMB was added and the change in absorbancy at 255 mu measured as a function of time of incubation. The reaction was carried out in 0.33M acetate buffer, pH 4.60 at 25°C. The different curves represent the reaction with PCMB of samples left standing at room temperature for the times indicated.


TIME = 0 HOURS
TIME = 23 HOURS
TIME = 45 HOURS
CHANGE IN ABSORBANCY (255 MU)
.3
.2
.1
10
20
30
60
120
TIME (MINUTES)

Data obtained from several different preparations (unactivated, activated, unactivated and activator-free which were deliberately allowed to oxidize for varying periods) show different amounts of reactive thiol, and that the amount of reactive -SH is proportional to the specific activity (P.U.). (Figure 27.) The highest value obtained for the amount of reactive thiol was 1.42 -SH/mole chymopapain B, where the specific activity was 2.55 (10 ug-level). If an extrapolation to 2 -SH groups/mole is made (the nearest integer), a maximal value of (P.U.) = 3.20 is indicated. The most active enzyme preparation (assayed in the presence of the activator) gave (P.U.) = 3.30. Therefore, it is apparent that chymopapain B purified by the preparative methods discussed above represents an enzyme which possesses the activity of the "native" enzyme, or requires a maximum of more than 2 -SH groups/mole.

In this connection, it might be of interest to cite the data obtained with papain under essentially similar conditions. Finkle and Smith (1958) have also extrapolated the titratable -SH content to the nearest integer (one in the case of papain) and find the maximal predicted activity to be 2.2 (measured as C_1, the proteolytic coefficient). Their preparations had maximal C_1 values of ca. 1.3. By isolating papain from freshly prepared papaya latex (shipped via air from the Belgian Congo), these workers isolated papain which had a C_1 = 1.8 with a correspondingly higher -SH titer. On the basis of these studies, Finkle and Smith drew the most obvious conclusion, viz., that

the absolute value of the activity obtained depends upon the history of the dried latex used. A situation analogous to this could also be possible with the chymopapains, which would suggest, then, that more than 2 -SH groups/mole are required. However, the possibility that activation enhances proteolytic activity (the conditions under which maximal activity is obtained) by a mechanism that involves more than the mere liberation of free -SH groups presents a way in which the data obtained with chymopapain B would still be in harmony with the prediction that 2 -SH groups would yield maximal activity. The maximal activity predicted by the data of Figure 27 would be that of the enzyme activity in the absence of the activator. In the presence of the activator, one would expect to see an enhancement in the activity. (This aspect is discussed again in Section IV. Discussions.)

Although the data of Figure 27 predicts that the maximal activity is attained in the presence of 2 -SH groups, it is apparent that only one -SH is essential for activity. The values which deviate from the straight line indicate that while -SH's can be titrated with PCMB, the enzyme has essentially no activity. These values were obtained with samples which had been left standing at room temperature for periods greater than six days. The data would be consistent with an aggregation phenomenom primarily involving the essential -SH's. This hypothesis is supported to some extent by the observation that approximately 60% of the activity is recovered by reactivation with cysteine. The lack of total recovery of activity is

suggestive of other processes contributing to the irreversible loss of enzymic activity. (Ninhydrin color yields of the various PCMB-treated fractions, standing from five hours to seven days did not show observable differences, which would be indicative of no autolysis.)

The reactivity of this unessential -SH is, therefore, as great as that of the so-called "essential" -SH, and hence the observed curves of Figure 26.

2) Reaction with Denatured Chymopapain B

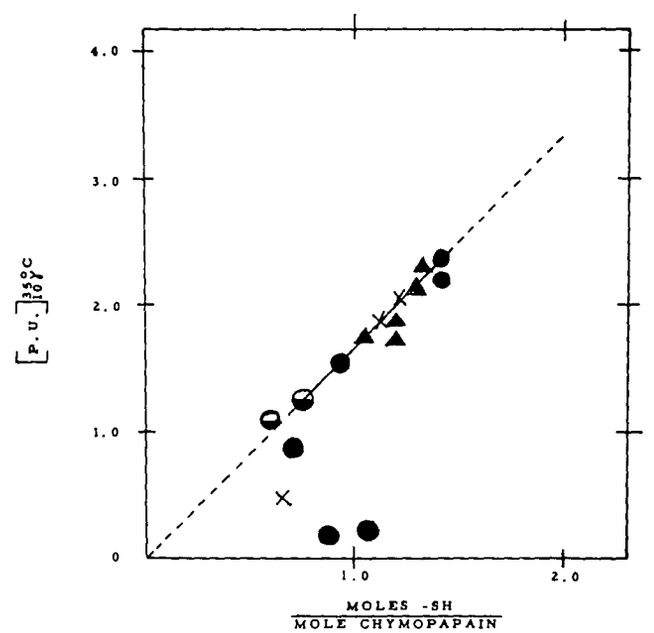
Experiments were conducted to determine if additional thiol groups could be exposed by the action of a denaturant, a technique useful for the detection of so-called "buried" residues. Liener (1961) has found an additional thiol group in ficin upon the addition of sodium dodecylsulfate (Duponal).

Addition of 1.5% sodium dodecylsulfate to unactivated and activator-free chymopapain B did not reveal the presence of "buried" thiol groups. Titrations with PCMB revealed essentially the same amount of reactive -SH as in the native enzyme.

3) Reactivity of Chymopapain B -SH

Some insight into the reactivity of the -SH group of chymopapain B is indicated by its rapid reaction with PCMB. To characterize further the reactivity of the essential thiol groups of chymopapain B, the sensitivity of the enzyme to hydrogen peroxide was investigated, since such studies have been informative in the case of papain (Sanner and Pihl, 1963).

Figure 27. The specific activity of chymopapain B as a function of sulfhydryl reactive with PCMB for different preparations of the enzyme. (◒), Preparation I, unactivated; (X), Preparation I, reduced; (▲), Preparation II, reduced; (●), Preparation III, reduced. In all cases, samples were deliberately left standing at 25°C for varying periods of time (to a maximum of seven days) and both the -SH titer and specific activity measured.


4.0
3.0
2.0
1.0
0
1.0
2.0
[P.U.] 35°C 10'
MOLES -SH
MOLE CHYMOPAPAIN

The results in Figure 28 indicate that native chymopapain B, like papain, is inhibited by low concentrations of hydrogen peroxide. Moreover, it may be noted that an appreciable fraction of the enzyme (ca. 30%) remained in a form that could be partially reactivated by cysteine over a wide range of (H_2O_2)/(chymopapain B). It is reasonable to assume, as was done in the case of papain, that the form capable of being reactivated is the disulfide form of the enzyme which is presumably resistant to further oxidation by hydrogen peroxide. That this situation is approximately correct is supported by studies in which increasing levels of hydrogen peroxide are added to different aliquots of chymopapain B in which varying proportions of the reactive -SH's are protected with PCMB. (Figure 28.) Furthermore, it is to be noted that the amount of enzyme capable of being reactivated increases with the ratio (PCMB)/(enzyme). Accordingly, it rules out the possibility that the inactivation by means of oxidation involves groups other than the active thiol group, since when all the -SH groups available for reaction with PCMB have actually been alkylated, there is almost complete protection against inhibition by hydrogen peroxide. This is shown more clearly in Figure 29.

However, the fact that chymopapain B inactivated by hydrogen peroxide cannot be completely reactivated by cysteine strongly indicates that the oxidation products other than disulfides are also formed.

Figure 28. The inhibition of native and PCMB-treated chymopapain B by hydrogen peroxide. The enzyme (2.0×10^{-5}M) was incubated for sixty minutes in .05M phosphate buffer, pH 6.8 at 4°C with increasing concentrations of hydrogen peroxide. The native enzyme contained 1.42 -SH per molecule. (▲), native chymopapain B, no cysteine present during measurement of activity; (●), PCMB-treated chymopapain B (molar ratio PCMB: enzyme as indicated), cysteine present during measurement of activity.

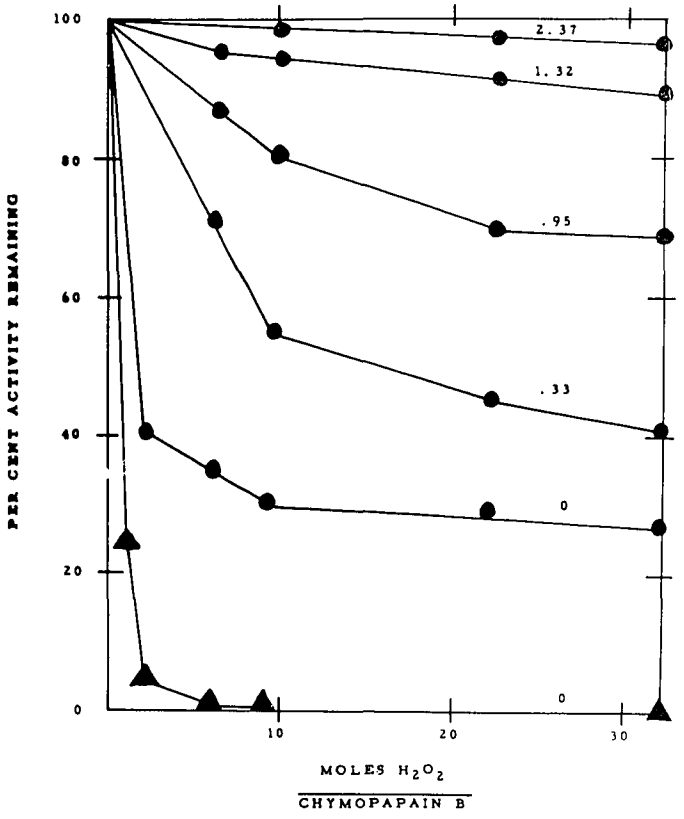
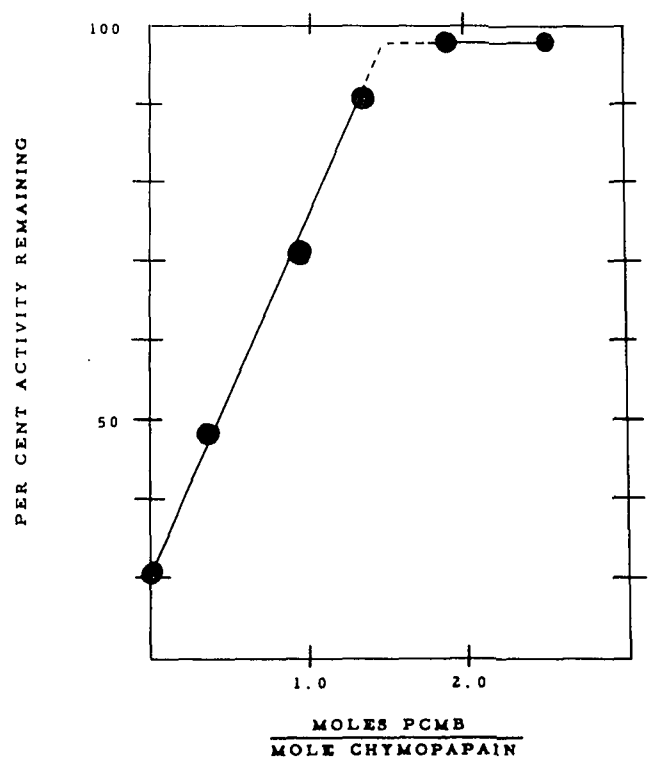

100
80
60
40
20
0
PER CENT ACTIVITY REMAINING
2.37
1.32
.95
.33
0
0
10
20
30
MOLES H_2O_2
CHYMOPAPAIN B

Figure 29. The protection of chymopapain B by PCMB against the inhibiting effect of hydrogen peroxide. The enzyme (2.0×10^{-5}M) was incubated for thirty minutes at 25°C in .05M phosphate buffer, pH 6.80 with increasing concentrations of PCMB and subsequently for thirty minutes with a 20-fold molar excess of hydrogen peroxide at 4°C. The enzyme activity was determined in the presence of cysteine. The chymopapain preparation contained 1.42 -SH group per molecule.


100
50
PER CENT ACTIVITY REMAINING
1.0
2.0
MOLES PCMB
MOLE CHYMOPAPAIN

On the basis of the above data and those reported by others on the hydrogen peroxide oxidation of the -SH groups of glyceraldehyde-P-dehydrogenase (Pihl and Lange, 1962) and papain (Sanner and Pihl, 1963), it can be calculated that the -SH groups of chymopapain B are approximately as sensitive to oxidation by hydrogen peroxide as the -SH group of papain and five times more sensitive than those of glyceraldehyde-P-dehydrogenase.

On the basis of all studies carried out thus far on the reversible inactivation of chymopapain B (pH inactivation, H_2O_2 oxidation), the obvious inference that can be drawn is that the reaction:

$$\text{chymopapain } (\text{-SH})_2 \longrightarrow \text{chymopapain } (\text{-S-S-})$$

and/or

$$2 \text{ chymopapain } (\text{-SH})_2 \longrightarrow \underset{(\text{SH})}{\text{chymopapain-S}}\text{-S-}\underset{(\text{SH})}{\text{chymopapain}}$$

best accounts for the inactivation process and that the reduction of the inter- or intra- molecular disulfide bond yielding free -SH groups essentially characterizes the activation process.

4) Reductive Cleavage of Disulfide Bonds

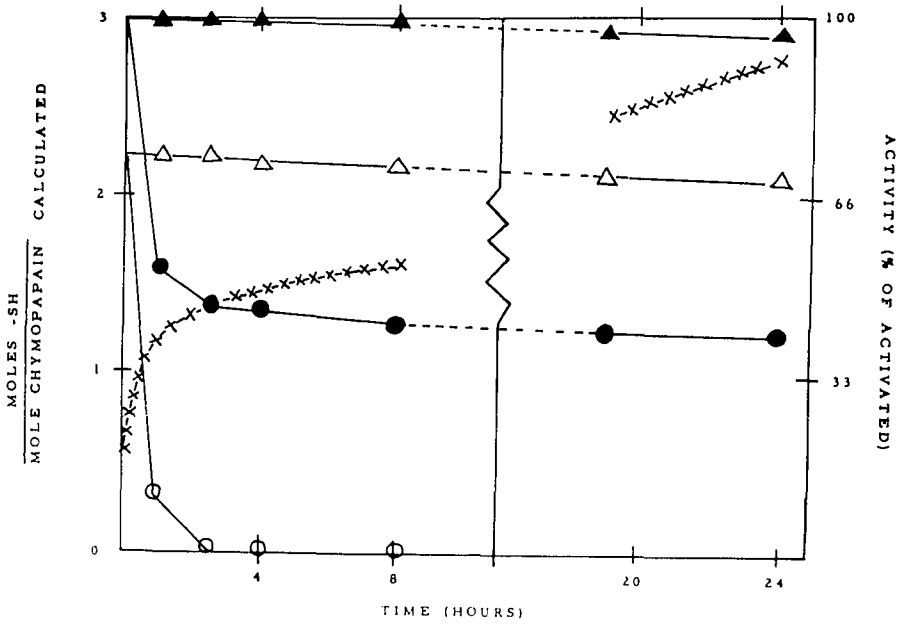
In addition to experiments conducted with the purpose of uncovering "buried" -SH groups, experiments were also carried out to determine whether additional thiol groups could be exposed by the combined action of a reducing agent, such as $NaBH_4$ and a denaturant, a technique which Moore, et. al. (1958)

have shown to be effective in cleaving the disulfide bonds of proteins. In this connection, unactivated chymopapain was treated with 2% SDS and 5% $NaBH_4$, and the pH adjusted to 10.5 with N NaOH. After four hours at room temperature, the pH was adjusted to approximately pH 8 (to decompose excess borohydride) and aliquots titrated with PCMB. Under these conditions, a total of 5.40 -SH groups could be titrated. Therefore, two additional disulfide bonds (in addition to the 1.40 -SH available in the native enzyme) are reduced under these circumstances.

d. Reaction with N-Ethylmaleimide (NEM)

The rate at which NEM reacts with chymopapain B containing 1.40 PCMB-titratable -SH groups is shown in Figure 30. It can be easily noted that the reaction with NEM is much slower than with PCMB. At approximately three hours of incubation 1.40 SH groups are titrated with NEM with complete inhibition of proteolytic activity, in agreement with the results obtained by PCMB titration of the same sample. However, a secondary reaction apparently occurs, with the titration of approximately one additional residue over the next 24 hours. The rate of breakdown of NEM during the titration of this additional residue proceeds at approximately one-tenth as fast as the breakdown of NEM during the titration of the 1.40 -SH groups available for reaction with PCMB. This secondary reaction probably represents the breakdown of NEM by means of a group different from an -SH, probably a tyrosyl residue.

Figure 30. Reaction of chymopapain B with NEM. To 1.0 ml solution containing 3.3×10^{-4}M chymopapain, 2.0 ml of .002M NEM in 0.10M phosphate buffer (pH 6.8) was added and the final volume adjusted to 4.0 ml with buffer. SH-groups were calculated from absorbancy readings at 310 mu as described in Methods, (✕—✕—✕), the activity of a suitably diluted aliquot was measured with casein as substrate. (○—○—○), protein with NEM added assayed in the absence of cysteine, (●—●—●) and in the presence of cysteine; protein without NEM assayed in the absence of cysteine (△—△—△), and in the presence of cysteine (▲—▲—▲). All measurements were made at 25°C.


MOLES -SH / MOLE CHYMOPAPAIN CALCULATED
3
2
1
0
4
8
20
24
TIME (HOURS)
100
66
33
ACTIVITY (% OF ACTIVATED)

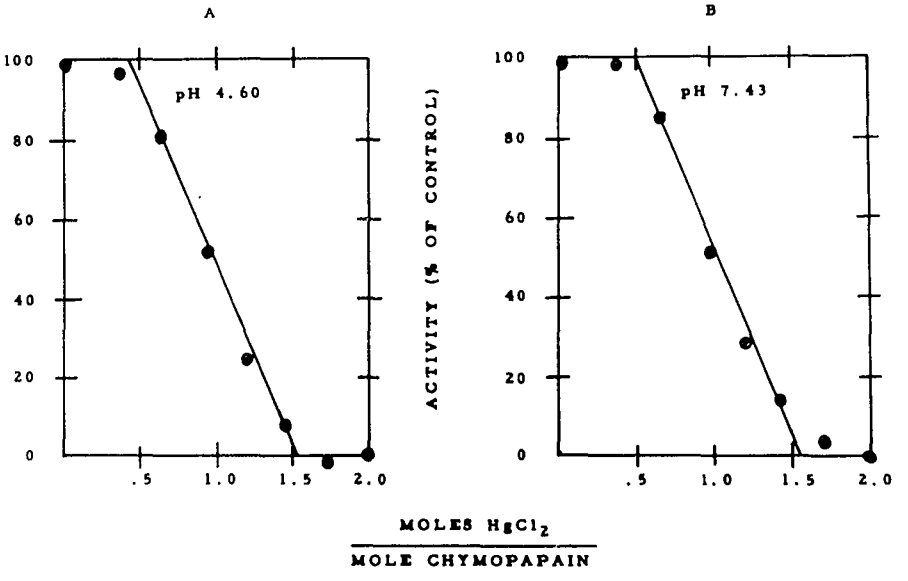
Examination of the reactivation process indicates that approximately 50% of the -SH groups are in a form that can be converted by a reducing agent (cysteine in this particular case) into an active -SH. The hymothesis that the process: -S-S- 2 -SH accounts for the liberation of additional -SH's essential for catalytic activity seems warranted on the basis of its simplicity and consonance with the observed facts.

e. Reaction with Mercuric Chloride

When chymopapain B solutions were treated with various levels of mercuric chloride at pH 4.62 (.02M acetate buffer) and pH 7.46 (.02M Tris buffer), complete loss of activity with respect to casein digestion was observed. This loss of activity was observed at a level essentially equal to the titratable -SH. (Figure 31.) This inhibition was reversible, the enzyme being completely reactivated in the presence of .05M cysteine containing .005M E.D.T.A.

The observed inhibition is indicative of a 1:1 (moles Hg^{++} to moles enzyme) reaction since it would seem fortuitous that a one-fold excess of the inhibitor would be required for complete inhibition, especially when considered in the light of the inhibition by P.C.M.B. This situation (the exact ratio of combination must await a mercury analysis which was not undertaken in these studies) differs from the reaction with mercuric chloride with papain (Kimmel and Smith, 1954) and ficin (Liener, 1961) where the combining ratio Hg^{++} to enzyme was 1:2.

Figure 31. Effect of mercuric chloride on the proteolytic activity of chymopapain. Graded levels of $HgCl_2$ in .01M acetate buffer, pH 4.62 (A) and in .01M Tris buffer, pH 7.46 (B) were added to a fixed amount of chymopapain B in de-ionized water. The reaction mixture was allowed to stand at 25°C for sixty minutes and the activities measured on casein in the absence of a reducing agent.


A
B
pH 4.60
pH 7.43
100
80
60
40
20
0
.5
1.0
1.5
2.0
ACTIVITY (% OF CONTROL)
MOLES $HgCl_2$
MOLE CHYMOPAPAIN

D. Enzymic Properties

1. Enzymic Activity

a. Casein Digestion

The digestion of casein by the proteases of dried papaya latex is illustrated in Figure 32. The specific activities of these enzymes calculated by the method outlined above are tabulated in Table XIX.

TABLE XIX

SPECIFIC ACTIVITIES OF THE PROTEASES OF DRIED PAPAYA LATEX ASSAYED WITH CASEIN

ENZYME	ASSAY CONDITIONS	SPECIFIC ACTIVITY
Papain	0.10M phosphate buffer, pH 7.20	2.20*
Papain	.05M Cysteine + .005M E.D.T.A. in 0.10M phosphate buffer, pH 7.20	7.10*
Chymopapain A	0.10M phosphate buffer, pH 7.20	0.25**
Chymopapain A	.05M cysteine + .005M E.D.T.A. in 0.10M phosphate buffer, pH 7.20	1.03**
Chymopapain B	0.10M phosphate buffer, pH 7.20	2.70***
Chymopapain B	.05M cysteine + .005M E.D.T.A. in 0.10M phosphate buffer, pH 7.20	3.80***

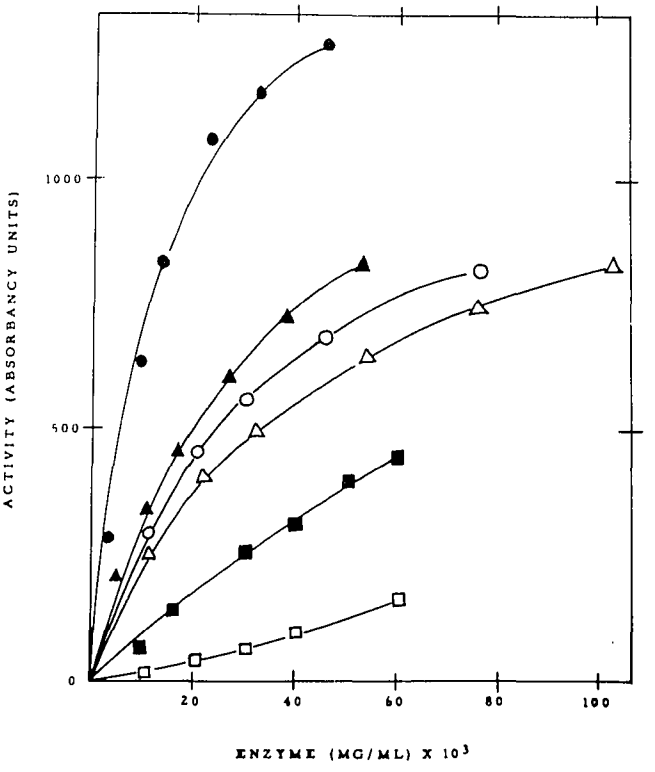
* Concentration determined by using $E^{1\%}_{1\ cm}$ (280) = 24.03

** Values cited by Ebata (1962)

*** Concentration determined by using $E^{1\%}_{1\ cm}$ (280) = 18.4

It will be noted that papain is approximately two times more active than chymopapain B and seven more active than chymopapain A, the comparison being made of their maximal specific activities. Comparison between the chymopapains indicate that the B form is approximately four times more active than the A form. In view of the above data it is difficult to understand the observations of Ebata (1962) that "in agreement with the studies of Jansen and Balls, chymopapain hydrolyzed casein approximately one-half as rapidly as papain," for the comparison of the S.A. of papain and chymopapain A clearly indicates that papain is at least seven times more active. In fact, chymopapain B more closely resembles the chymopapain described by Jansen and Balls, particularly on the basis of activities. However, it is also very likely that the chymopapain of Jansen and Balls may have actually consisted of a heterogeneous mixture of several components, including the A and B forms isolated in our laboratory.

Figure 32. The digestion of casein by the proteases of dried papaya latex. The activity measurements were carried out under standard conditions as described in the text. (●-○-●-○), papain; (■-□-■-□), chymopapain A; and (▲-△-▲-△), chymopapain B. Closed symbols represent assay carried out with the enzyme activated with .01M cysteine, and open symbols represent values obtained with the unactivated enzyme.


ACTIVITY (ABSORBANCY UNITS)
1000
500
0
20
40
60
80
100
ENZYME (MG/ML) X 10^3

2. Activation and Inhibition

a. Activation

Table XX shows the relative effect of various thiol-activators on the activity of native and PCMB-treated chymopapain B. It can be noted that a wide variety of thiol compounds activates the enzyme. The degree of activation, however, is not the same with all of the compounds tested. Cysteine, 2,3 dimercaptopropanol, glutathione and thioglycolic acid yields maximal activation, while mercaptoethanol and cyanide are less effective. The enhancement of activation in the presence of the chelating agent, E.D.T.A. is especially noteworthy.

b. Inhibition

1) Inhibition by Metals

The inhibition of chymopapain B by the well-known -SH reagents such as PCMB, NEM, $HgCl_2$ and iodoacetate has already been described. It was also of interest to determine the relative effect of certain cations on the activity of the enzyme. To this end, twelve different metals were incubated with the enzyme, and their effect on the activity of the enzyme noted. (Table XXI.)

Under the conditions employed, the data seem consistent with the general behaviour of -SH proteases toward various metals. Besides Hg^{++} and Ag^{+}, the enzyme is also markedly inhibited by Cu^{++}, Zn^{++}. The inhibition at high concentrations of Co^{++} and Ni^{++} is also to be noted. The lack of any inhibition by Fe^{+++} seems surprising when considered in the light of the high oxidation potential of this particular cation.

TABLE XX

EFFECT OF VARIOUS THIOL-ACTIVATORS

ON THE ACTIVITY OF NATIVE AND PCMB-CHYMOPAPAIN B

ACTIVATOR	ENZYME ACTIVITY IN PER CENT	
	NATIVE	PCMB
Cysteine	97	93
Cysteine + EDTA	103	99
BAL	88	90
BAL + EDTA	100	100
Mercaptoethanol	91	93
Mercaptoethanol + EDTA	91	94
Glutathione	97	99
Glutathione + EDTA	105	102
Thioglycolic Acid	100	90
Thioglycolic Acid + EDTA	102	91
NaCN	69	81
NaCN + EDTA	79	85
None	42	0
EDTA	49	9

Activator Concentration: .05M.
EDTA Concentration: .005M.

TABLE XXI

THE INHIBITION OF CHYMOPAPAIN B BY SOME METALS

The reaction mixture contained 2.0 cc enzyme (3.2 milliumoles) and 2.0 cc of the inhibitor in 0.10M Tris buffer, pH 7.20. After incubation for 10 minutes at room temperature, 1.0 cc aliquots were added to 1.0 cc 1% casein. To another 1.0 cc aliquot, 1.0 cc of a solution containing .10M cysteine and .02M E.D.T.A. was added, and 1.0 cc of this mixture added to 1.0 cc 1% casein. The assays were carried out at 35°C, and digestion was allowed to proceed for 10 minutes. Activities are calculated as described in the text.

INHIBITOR	(INH)/(ENZ)	INHIBITION (% OF CONTROL) 1x4	1x20	1x200	REACTIVATION (% OF CONTROL)
None Added		0	0	0	100
$AgNO_3$*		99	99	100	95
$AlCl_3$		0	0	0	100
$BaCl_2$		0	0	0	99
$CoCl_2$		0	2	21	100
$CuSO_4$		91	95	100	97
$FeCl_3$		0	0	0	95
$FeSO_4$		0	0	0	100
$HgCl_2$		100	100	100	100
$MgCl_2$		0	0	0	100
$MnCl_2$		0	0	0	98
$NiSO_4$		0	0	30	97
$PbNO_3$*		0	0	0	99
$ZnCl_2$		30	73	100	100

* Incubation of enzyme and metal in de-ionized water.

2) Inhibition by Aldehyde Reagents and Some Carbonyl Compounds

The effect of various aldehyde reagents (hydrazine, phenyl hydrazine, hydroxylamine and semi-carbazide), two carbonyl compounds (pyruvic acid and p-amino acetophenone) and two carboxylic acids (benzoyl-L-arginine and L-glutamic acid) on unactivated chymopapain B is shown in Table XXII.

It can be seen that hydroxyl amine and phenyl hydrazine inhibits the enzyme. The other compounds tested show no effect under the conditions employed.

The inhibition by these reagents suggests the presence of an aldehyde group as an active functional group, but the limited studies carried out here do not offer any suggestions as to the nature of this inhibition.

3. Apparent Temperature Optimum

The digestion of casein as a function of temperature is shown in Figure 33. Incubations were carried out at two pH's, corresponding to the dual maxima observed during the studies of activity as a function of pH. (Section 4 below.)

It can be seen that chymopapain B exhibits a very high temperature optimum (75^{o} - $90^{o}C$). Furthermore, the hydrolysis of casein at the two different pH's employed indicates that the dual maxima observed (pH optimum) probably reflect the heterogeneity of the substrate rather than the presence of two separate enzymes with different pH optima, since the activities at these two pH's parallel one another over the whole temperature range.

TABLE XXII

THE INHIBITION OF CHYMOPAPAIN B

BY SOME ALDEHYDE REAGENTS, CARBONYL-COMPOUNDS

AND SOME CARBOXYLIC ACIDS

The reaction mixture contained 1.0 cc of the unactivated enzyme (1.6 milliumoles) and 1.0 cc of the inhibitor (1.0 umole) in 0.10M Tris buffer, pH 7.20. After incubating for 30 minutes at room temperature, 1.0 cc aliquots were added to 1.0 cc 1% casein incubated at 35°C. After digestion was carried out for 10 minutes, the reaction was terminated by the addition of 3.0 cc 5% T.C.A. Activity is expressed as the absorbancy of the filtered supernatant at 280 mu.

REAGENT ADDED	INHIBITION (% OF CONTROL)
-None-	0
Hydroxyl amine	95
Phenyl hydrazine	94
Hydrazine	0
Semicarbazide	0
p-amino acetophenone	0
Pyruvic acid	0
Benzoyl-L-arginine	0
L-Glutamic Acid	0

The high temperature optimum exhibited and the heat stability of of the enzyme are strongly suggestive of a low degree of helical content which are characteristic of heat-stable enzymes. The relatively high proline content supports this hypothesis. Further support of this hypothesis must await further studies of the tertiary structure of this enzyme, particularly by studies of the optical rotary dispersion properties.

4. Apparent pH Optimum

The hydrolysis of 1% casein as a function of pH by the three proteases of dried papaya latex is shown in Figure 34. It is of interest to note that all three proteases exhibit a dual optima, one optimum occurring at ca. pH 7.0 and the other near pH 9.0. Moreover, these proteases exhibit maximal activity over a wide range of pH's, suggestive of their wide pH stability.

It might be of interest to note that all of the chromatographically distinct fractions of bromelain also exhibit multiple optima with casein as substrate, indicative of the heterogeneity of the substrate (El-Gharbawi and Whitaker, 1963). That this dual optima observed is due to the heterogeneity of the substrate is indicated by studies just cited on the temperature optimum and will be further supported by studies of the hydrolysis of a homogeneous substrate (B.A.A.), in which case only a single broad optimum is observed. (Section D.6. Kinetic Studies.)

Figure 33. The hydrolysis of casein as a function of temperature. Solutions of 1% casein were equilibrated at the temperatures indicated. At the same time, 1 ml. solutions of chymopapain in water were also brought to bath temperature and left standing for one minute. Immediately thereafter, 1.0 ml. of the casein solution was added to each of the enzyme solutions and the reaction allowed to proceed for ten minutes. The reaction was terminated by the addition of 3.0 cc of 5% TCA. Activities are expressed as the differences in absorbancy of the filtered supernatant (corrected for the blank) at 280 mu where an absorbancy change of .001 unit corresponds to one activity unit. Reactions were run in .10M phosphate buffer, pH 7.34 (●), and in .10M Tris, pH 9.10 (▲).

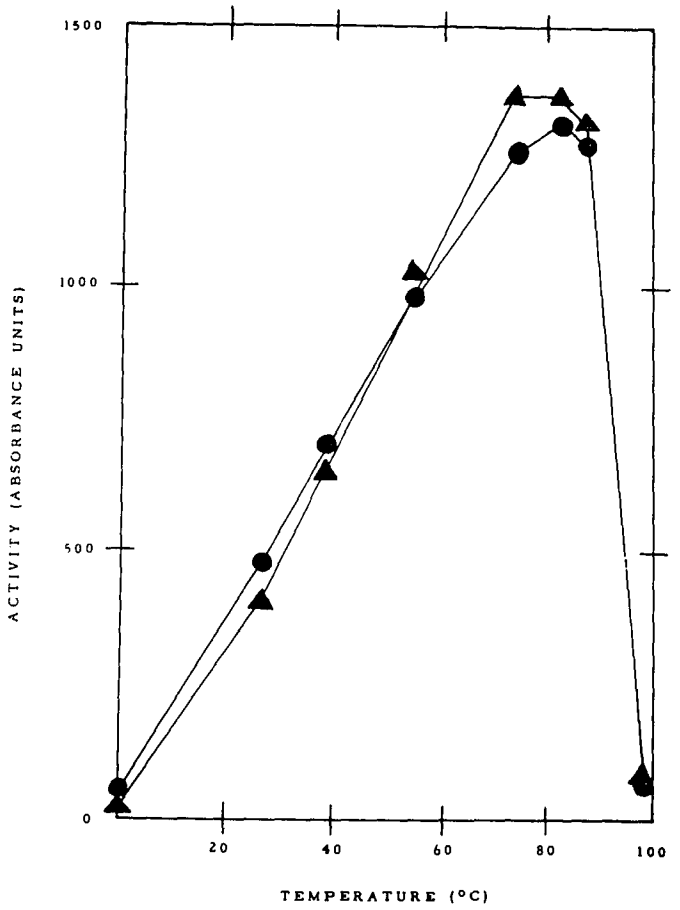
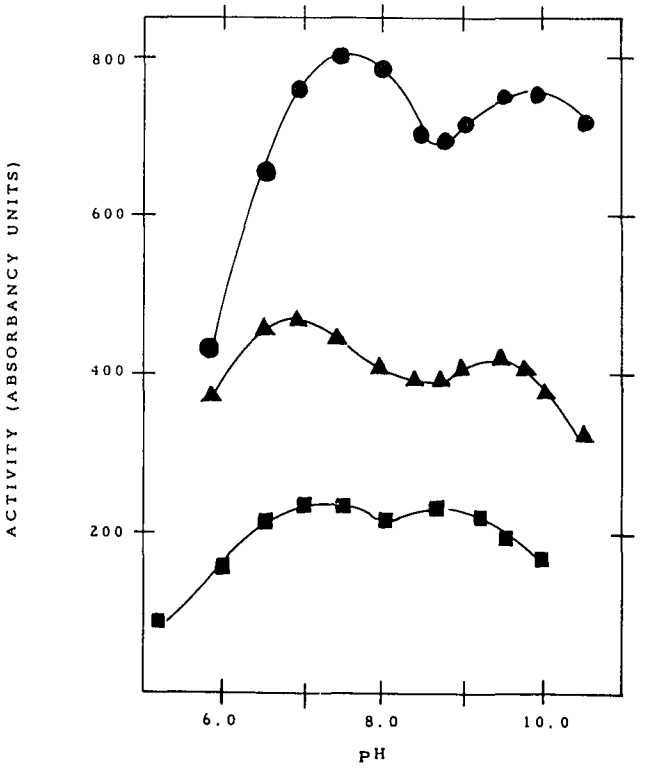

1500
1000
500
0
ACTIVITY (ABSORBANCE UNITS)
20
40
60
80
100
TEMPERATURE (°C)

Figure 34. The apparent pH optimum for the digestion of casein by the proteases of dried papaya latex. (● ● ●), papain, 18ug/cc; (■ ■ ■), chymopapain A, 25 ug/cc; and (▲ ▲ ▲), chymopapain B, 20 ug/cc. The substrate was 0.5% casein. Activity measurements were made under standard conditions as described in the text. 0.10M phosphate buffers were used in the pH range 5.3 - 8.5; 0.10M Tris buffers used in the pH range 7.5 - 9.5 and 0.10M carbonate-bicarbonate buffers in the pH range 9.5 - 10.5.


ACTIVITY (ABSORBANCY UNITS)
800
600
400
200
6.0
8.0
10.0
pH

5. Specificity

a. Action on Some Peptide and Amide Bonds

Earlier work, mainly by Bergmann and Fruton (1941) demonstrated that partially purified papain hydrolyzes many kinds of synthetic amide and peptide substrates. Kimmel and Smith (1954) have demonstrated this wide hydrolytic action with highly purified papain.

Sanger, Thompson and Tuppy (1952) have shown that crystalline papain has a broad action on the oxidized A chain of insulin.

In this connection, therefore, it was of interest to determine the specificity of crystalline chymopapain B utilizing some simple, well-defined synthetic compounds. Table XXIII gives the data for a representative selection of compounds. Thus far, the most sensitive substrate found is B.A.A., which is also a substrate for trypsin, papain, ficin and bromelain. In addition, low levels of hydrolysis are observed with cbz-L-glutaminyl-tyrosine and cbz-L-glutaminyl-L-phenylalanine (carboxypeptidase substrates), leucinamide (leucineaminopeptidase substrate). Other compounds, not susceptible to the purified enzymes of animal origin are also hydrolyzed (alanyl-glycine, prolyl-glycine).

While this study should be regarded as preliminary to a more detailed kinetic analysis, some interesting points can be noted nontheless. In its action of a wide variety of peptide and amide bonds, chymopapain B closely resembles papain. While the data presented here is too incomplete for comparative purposes, it is of interest to note that their activities toward many substrates parallel one another with respect to the types of bonds hydrolyzed

and to their rate of hydrolysis of these bonds. Particularly significant is their mutual preference for B.A.A.

However, differences in their modes of action are evident. In particular, their rates of hydrolysis (calculated on a mole basis) are very different, papain exhibiting greater rapidity in its ability to hydrolyze peptide and amide bonds. Furthermore, papain probably shows a wider specificity toward the hydrolysis of protein substrates (e.g. casein) where its activity is nearly two times greater than is that of chymopapain B. However, papain does not display significant milk-clotting activity with casein, while chymopapain B exhibits strong milk-clotting activity.

b. Synthetic Action (Anilide Synthesis)

The papain-catalyzed synthesis of an amide bond from an acylamino acid and a suitable amine is a well-documented phenomenom (Abernethy and Kilday, 1960).

Chymopapain, prepared according to Jansen and Balls, has been shown to catalyze the synthesis of a benzoyl-tripeptide anilide from a benzoyldipeptide and an amino acid anilide (Tollin, et.al., 1959).

Chymopapain A has been shown to catalyze the synthesis of carbobenzoxy-DL-alanine analide, carbobenzoxy-L-tyrosine anilide and carbobenzoxy-L-leucine anilide (Ebata, 1962).

In the present study, chymopapain B was used to catalyze anilide synthesis using the amino acids benzoyl-L-arginine, carbobenzoxy-L-glutamic acid and hippuric acid. Table XXIV summarizes the observations.

TABLE XXIII

THE HYDROLYSIS OF SOME SYNTHETIC SUBSTRATES
BY CHYMOPAPAIN B AT pH 6.50

SUBSTRATE	SUBSTRATE CONC. (M/L)	ENZYME CONC. (M/L)	HYDROLYSIS 20 MIN. (%)	40 MIN. (%)	60 MIN. (%)	80 MIN. (%)
N-BZ-ARG-NH_2	.020	1.16×10^{-6}	5.3	10.8	16.1	20.9
N-BZ-GLY-NH_2	.030	"	.3	.5	1.0	1.3
GLY-TYR	.025	"	-	-	-	3.0
N-BZ-LEU-NH_2	.010	"	.9	1.8	2.8	3.5
LEU-GLY	.025	"	-	-	-	1.8
CBZ-GLU-TYR	.025	"	.2	.4	.6	.8
CBZ-GLU-PHE	.025	"	.4	.6	.7	1.0
N-ACETYL-TYR-TYR	.010	"	0	0	0	0
LYS-GLY	.025	"	0	0	0	0
PHE-GLY	.025	"	-	-	-	2.0
ALA-GLY	.025	"	-	-	-	6.1
PRO-GLY	.025	"	.2	.5	.6	.8

TABLE XXIV

AMINO ACID ANILIDE SYNTHESIS CATALYZED

BY CHYMOPAPAIN B

The reaction mixture contained 2.5 millimoles of the acylamino acid, 0.50 cc. of redistilled aniline and 3.24 mg of BAL-activated chymopapain B, all in 0.10M citrate buffer, pH 5.0. The final volume of the mixture depended upon the respective solubilities of the acylamino acid. After incubation for 24 hours at 35°C, the mixture was transferred to the cold room where it was left for 24 hours. After filtration, the anilides were washed with water and subsequently dried in a vacuum dessicator. After the drying was complete, the anilides were weighed.

ACYLAMINO ACID	REACTION VOLUME	YIELD (%)
N-Benzoyl-L-arginine	75 cc	(Non-observable)
Carbobenzoxy-L-glutamic	20 cc	85% *
Benzoyl-L-glycine	20 cc	9

* Calculated as the monoanilide

6. Kinetics of Hydrolysis of N-Benzoyl-L-Arginine Amide

a. Effect of pH

Figure 35 shows a detailed study of the effect of pH on the rate of hydrolysis of .04M B.A.A. by chymopapain B in the presence of .05M BAL and E.D.T.A. At pH 7.0 the velocity is at a maximum. It will be noted that specific ion effects are missing. Furthermore, the presence of a single optimum is to be noted, as compared with the two optima observed during the digestion of casein.

b. Effect of Ionic Strength

At pH 6.0 and 39°C, there was no significant effect on the velocity of hydrolysis over the range of ionic strength .05 to .30. Consequently, all studies were routinely performed at .20 ionic strength.

c. Effect of Enzyme Concentration

The influence of enzyme concentration on the rate of hydrolysis of B.A.A. under pseudo-zero order kinetic condition is shown in Figure 36. The rate is proportional to the enzyme concentration up to 5 ug.

d. Effect of Substrate Concentration

Figure 37 shows a typical plot of the ammonia produced as a function of time of incubation at various substrate concentrations. It can be seen that under the experimental conditions employed, the initial reaction velocity is essentially zero order.

1) Measurement of the Michaelis-Menton Constants and of the Maximal Velocity

The data which were obtained are in accord with normal Michaelis-Menton kinetics. When 1/v is plotted against 1/(S),

a linear relationship exists. (Figure 38.B.)

Similar measurements were made at various pH's in the range 4.0 - 9.0, and the values of K_m and V_{max} obtained by graphical treatment of the data summarized in Table XXV. Similarly, the data obtained at the different pH's are in general accord with Michaelis-Menton kinetics.

2) Variation of log V_{max} and pK_M with pH

Figure 39 shows the relationship existing between log V_{max} and pK_M as a function of pH. This particular plot is due to Dixon (1953) and provides a means whereby information about the pK values for groupings at the "active center" of the enzyme might be deduced.

While the data presented here do not unequivocally determine the pKs of the essential groupings present in chymopapain B, they do suggest the possibility of the presence of at least two residues in the "active center," a grouping with an acidic pK and another with a slightly alkaline value. These groupings are consonant with those of a beta- or gamma- carboxyl and sulfhydryl group, respectively.

The assignment of pKs to a particular group must be considered provisional, however, pending a more detailed kinetic analysis (in particular, a detailed examination of the heats of ionization) as well as a definitive chemical characterization of the essential residues. (The present study has implicated the sulfhydryl group by chemical means, but has not established the essentiality of a carboxyl group.)

Figure 35. Effect of pH on the hydrolysis of B.A.A. by chymopapain. The hydrolysis of .04M B.A.A. by 3.0×10^{-7}M chymopapain was followed by the ninhydrin method as described in the text. The reaction was carried out at 39°C in the following buffers: (●), .04M citrate; (▲), .05M phosphate; (■), .10M Tris and (X), .05M carbonate-bicarbonate. The ionic strength of the medium was adjusted to 0.20M by the addition of KCl.

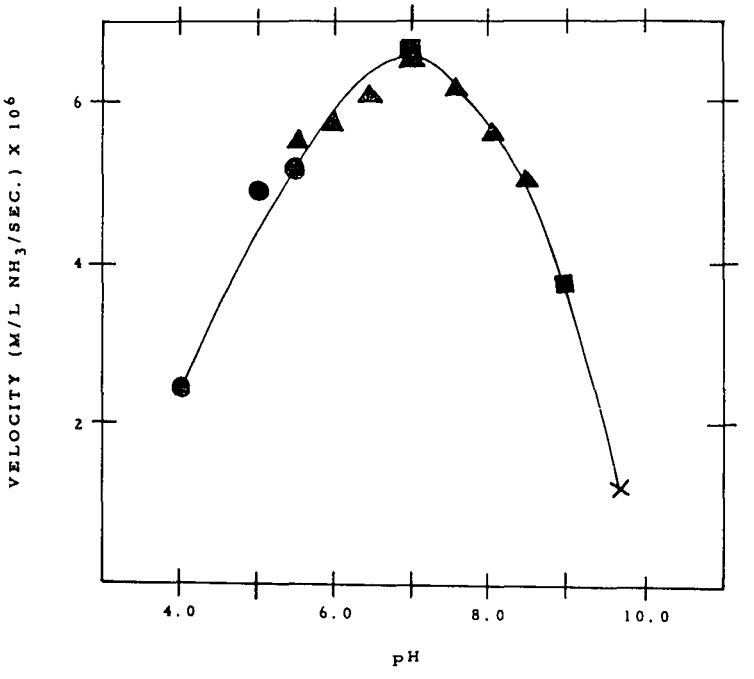

VELOCITY (M/L NH_3/SEC.) X 10^6
6
4
2
4.0
6.0
8.0
10.0
pH

Figure 36. Velocity of hydrolysis of 0.02M B.A.A. as a function of chymopapain concentration. The reaction was run in .05M phosphate buffer, pH 6.0 and 39°C.

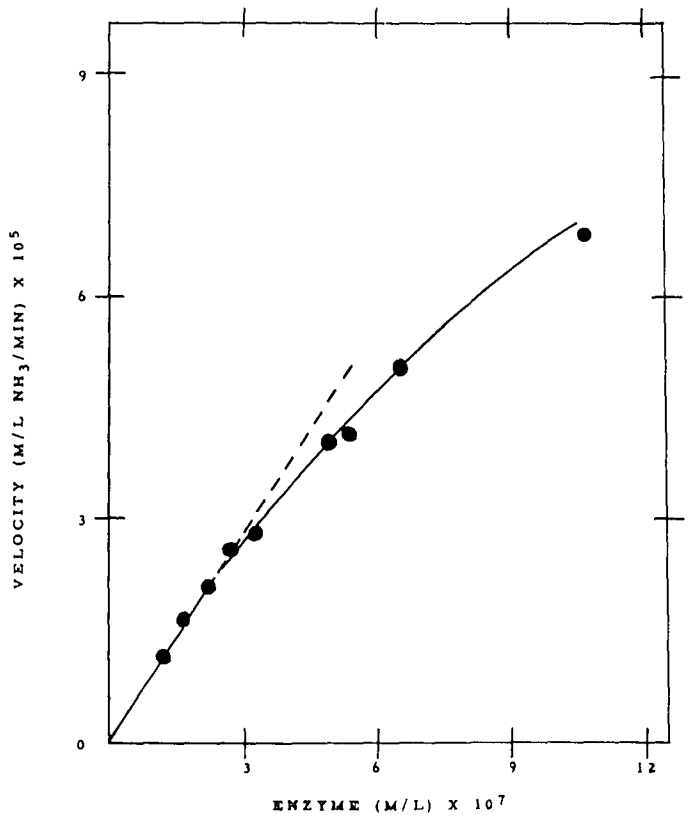

VELOCITY (M/L NH_3/MIN) X 10^5
9
6
3
0
3
6
9
12
ENZYME (M/L) X 10^7

Figure 37. The production of ammonia by chymopapain B as a function of time at various concentrations of B.A.A. The reactions were performed in .04M citrate buffer, pH 5.50, and at 0.20M ionic strength.

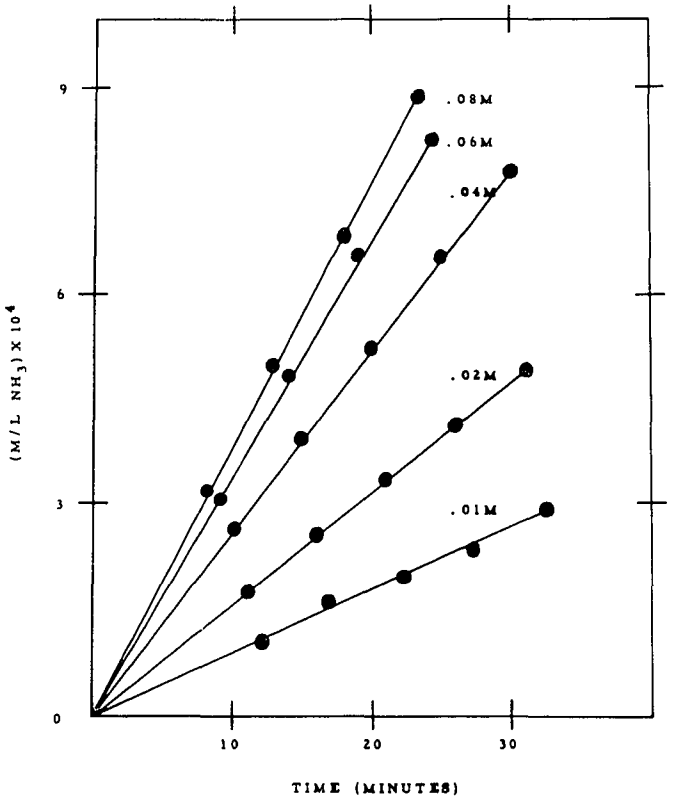

9
6
3
0
10
20
30
.08M
.06M
.04M
.02M
.01M
(M/L NH3) X 10^4
TIME (MINUTES)

Figure 38. A. Plot of velocity versus substrate concentration for the hydrolysis of B.A.A. The reaction mixture contained in 1.5 ml: citrate buffer, pH 5.50, 60 umoles; KCl, 48 umoles; enzyme, 0.45 milliumoles containing 75 umoles BAL and 5 umoles E.D.T.A. Incubation at 39°C.

B. Lineweaver-Burk plot of the same data.

$V_{max} = 13.12 \times 10^{-7}$

$K_M = .061M$

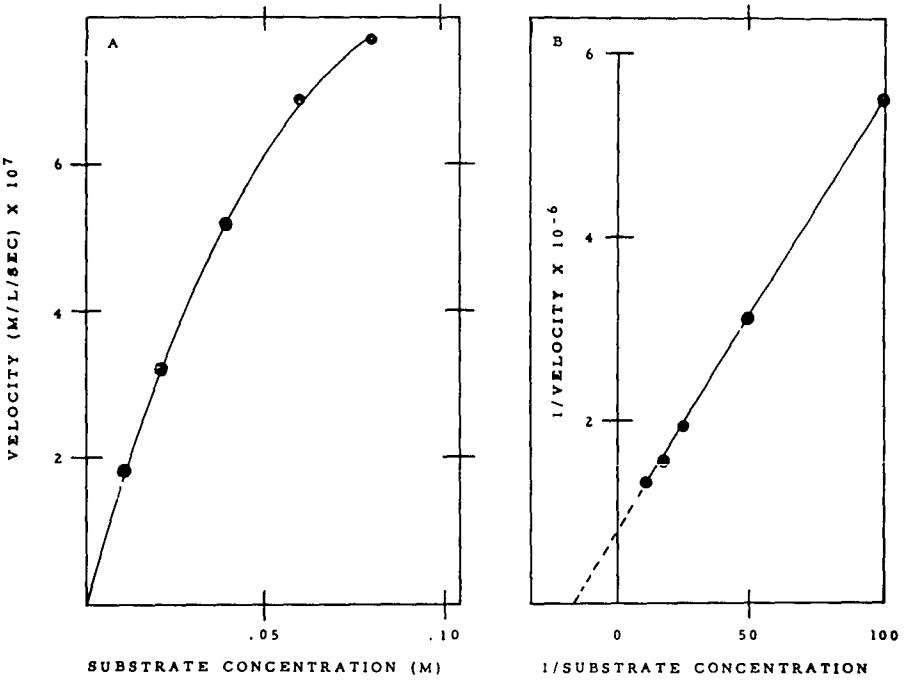

A
B
VELOCITY (M/L/SEC) X 10^7
1/VELOCITY X 10^{-6}
6
4
2
.05
.10
0
50
100
SUBSTRATE CONCENTRATION (M)
1/SUBSTRATE CONCENTRATION

TABLE XXV

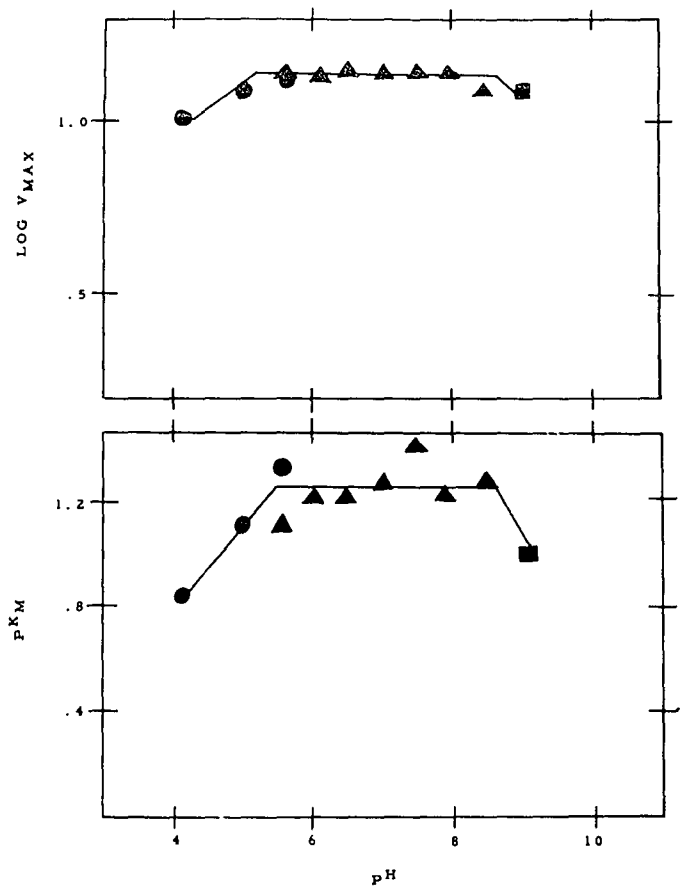
SUMMARY OF KINETIC PARAMETERS OBTAINED FOR THE HYDROLYSIS OF B.A.A.

BUFFER*	pH	K_m	V_{max}	k_3	k_1 **
.04M Citrate	4.03	.141	11.10	4.02	28.51
" "	4.98	.071	12.50	4.53	63.80
" "	5.50	.045	12.80	4.63	102.80
.05M Phosphate	5.53	.081	13.76	4.99	61.60
" "	5.98	.059	13.59	4.92	83.38
" "	6.50	.054	14.40	5.22	96.66
" "	7.00	.050	14.00	5.07	101.40
" "	7.49	.036	14.10	5.08	140.83
" "	7.90	.060	13.70	4.96	82.66
" "	8.43	.049	12.30	4.46	91.02
.10M Tris	8.95	.100	12.34	4.47	44.70

* Ionic strength adjusted to 0.20M by the addition of KCl

** Calculated as k_3/K_m

Figure 39. Variation of log V_{max} and pK_M with pH for the hydrolysis of B.A.A. (●), .04M citrate buffer; (▲), .05M phosphate buffer and (■), .10M Tris buffer.


LOG V_{MAX}
1.0
.5
pK_M
1.2
.8
.4
4
6
8
10
pH

Examination of the variation of k_1 (Table XXV, calculated by assuming $k_2 = 0$) does not provide any definitive basis as to the nature of K_m (i.e. whether $K_m = k_3/k_1$, the stationary state constant, e.g. as observed with peroxidase, catalase and pancreatic carboxypeptidase, or whether $K_m = k_2/k_1$, the equilibrium constant for the enzyme-substrate complex or whether K_m is explicitly equal to its definition). However, examinations of the assumed values reveal that it exhibits a bell-shaped curve with pKs of approximately 4.5 and 9.0, respectively. This assignment, as pointed out, must await further studies which will either show that the heats of ionization of the groupings involved approximate the heats of ionizations of known groupings or that $K_m = k_3/k_1$.

Extended kinetic studies should include the effects of temperature, dielectric constant, ionic strength and various inhibitors on the values of K_m and V_{max}. From such studies it is conceivable that clues as to the nature of the binding of substrate to the enzyme may be obtained.

IV. DISCUSSION

A. Comparison of Chymopapain B with Chymopapain A. Conjectures Concerning the Wide Distribution of Proteolytic Activity.

A comparison of chymopapain B with chymopapain A seems warranted with the available data. This thesis has assumed that these proteases are different and, accordingly, it was deemed profitable to characterize the second chymopapain (Form B).

Among the evidences available at the outset which tended to indicate that the chymopapains were different were the following: (1) the specific activity of chymopapain B was three to four times greater than the activity of chymopapain A; (2) untreated chymopapain B crystallized in the form of sabre-like needles whereas chymopapain A crystallized as obvious rods; (3) tyrosine occupies the N-terminus position in Form B whereas glutamic acid occupies the N-terminus position in Form A and (4) the occurrence of elution from XE-64 is different; chymopapain A is eluted at a lower ionic strength than is chymopapain B, judged by the analysis of the N-terminal amino acid residues.

On the basis of the evidences derived from the studies comprising this dissertation, it seems very probable that the chymopapains are very similar in both their physical and chemical properties. The possibility that one is derived from the other by some degradative process is very strong on the basis of the approximate similarities in their amino acid compositions. However, as pointed out in Section III.A., the occurrence of these two proteases in the crude extract (as determined by the presence of N-terminals glutamic acid and tyrosine in this extract) can be taken to indicate that these two proteases are "native" to the dried latex and as such, represent

two distinct enzymes. Table XXVI lists some of the more salient properties of chymopapain B which can be used to compare them with those of chymopapain A as given in Table I.

Conjectures concerning the nature of the possible conversion of one chymopapain to yield the other by autolysis or proteolysis must await a detailed study of the amino acid sequences of these two chymopapains. However, in the absence of this information, it is still possible to propose an interpretation with the available data, specifically by referring to their molecular weights. The molecular weight of chymopapain B has been determined to be approximately 30,000. The molecular weight of chymopapain A has been cited to be 34,300. Assuming that the contributions from non-amino acid residues are approximately the same, the higher molecular weight of Form A indicates that the B form represents the degradation product. But if this situation is correct, then it is difficult to understand how the autolyzed component can be more active than the native form. A re-examination of the molecular weight of chymopapain A seems warranted, especially since the value obtained was obtained with a preparation of dubious purity, at least when considered by the criterion of N-terminal analysis. (Table X.)

The presence of a non-amino acid constituent in the enzyme preparation may serve to explain the broad distribution of proteolytic activity observed during chromatography. This study has given only token attention to the possible nature of this constituent. Specifically, determination of the carbohydrate content was undertaken and the results indicate the absence of true sugars. However, further examinations for other possible constituents may commence with the following observations: (a) reaction of the enzyme with sulfuric acid yields a colored derivative with an absorption

maximum at 403 mu; the crude extract undergoes a reversible color change in acidic (yellow-green) and basic (brown) media. Therefore, the presence of quinone-like compounds (e.g. polyphenols) seems very probable. By assuming that these compounds are bound to the enzyme by means of ionic or covalent linkages, the differential binding of these substances to the enzyme offers a reasonable explanation for the differences in net charge existing among the same enzyme and accordingly, for differences in chromatographic behaviour. The low yields of the chymopapains are very likely a reflection of this differential binding. If the nature of this unknown constituent can be elucidated, then a careful quantitative examination of this constituent in different fractions of the same enzyme will undoubtedly reveal differences in binding.

Another factor probably contributing to the spread of activity is probably the aggregation of the chymopapains into complexes which behave differently during chromatography, again contributing to a decreased yield of the monomer.

Finally, the contribution of autolysis to the spread of activity must be considered. Preliminary studies have indicated that there is no detectable change in the ninhydrin color when chymopapain B is allowed to stand at room temperature. However, there is really no corrobative data available that might be used to assess the role of autolysis in the preparative scheme.

TABLE XXVI

SUMMARY OF SOME PROPERTIES

OF CHYMOPAPAIN B

PROPERTY	
1.	$S_{20,w}$	2.82
2.	$D_{20,w}$	----
3.	$\bar{v}$	.72
4.	Molecular Weight	
	a. S and D	31,000 (Estimated by assay f/fo = 1.20
	b. Amino Acid Composition	30,000 (Preliminary Analysis)
	c. Archibald	30,100
5.	Isoelectric Point	10.42
6.	Amino-Terminal	Tyrosine
7.	$E^{1\%}$	18.40
8.	Nitrogen Content	15.83
9.	Crystalline Form (Untreated)	Broad, sabre-like needles
10.	Activation Requirements	Thiols, CN^-
11.	Specific Activity	3.80 (casein)
12.	Essential -SH	1.0
13.	pH Optimum	7.0 (casein), 5.0 - 8.0 (B.A.A.)
14.	Substrate Specificity	Wide range of peptide and amide linkages

B. Considerations of the Mechanism of Enzyme Action

Any discussion of the mechanism of enzyme action must have as its basis the availability of a wide body of evidence which suggests plausible hypotheses. The evidence derived from this dissertation is certainly only suggestive of possible routes and processes which may describe the hydrolytic action of chymopapain B. The involvement with preparative procedures and physical-chemical characterizations has left little time for extended studies which might have been brought to bear on some aspects of the mechanism of action. It is hoped that these preliminary studies will stimulate extended investigations of mechanism.

For the purposes of general discussions, however, considerations of the mode of action of chymopapain B are simplified somewhat by an examination of the considerable body of data available which relates to papain. Preliminary studies strongly indicate striking similarities existing in the enzymic properties of chymopapain B and papain. Some of these properties include the essentiality of a single -SH group, the possible involvement of an ionized carboxyl group, a wide specificity with respect to the hydrolysis of peptide and amide bonds, the ability to effect anilide synthesis and possibly in their ability to function as transferases. The mapping of the "active center" of chymopapain, presently in progress in our laboratory, will probably reveal this similarity of the mode of action by disclosing a resemblance in the composition of this "active center." Perhaps this similarity, if such is the case, has its basis in a common biosynthesis of these two proteases.

However, the focusing of attention to their similarities should not be taken to imply that these proteases are exactly alike in their mechanism

of action, since differences are obviously evident. Among these differences, their milk-clotting activities are clearly different. More important, papain can hydrolyze every single substrate tested so far at a much faster rate (as compared to chymopapain B on a mole-to-mole basis), implying a difference in their catalytic properties. Finally, the examination of the specificity of chymopapain B is much too limited to warrant definitive conclusions regarding its similarity with papain.

Any speculations of mechanism, however, must be consonant with the following observations: chymopapain B can, like papain, hydrolyze a wide variety of substrates containing peptide and amide bonds involving both ionic and hydrophobic side-chains; the enzyme, like papain, can carry out anilide synthesis and can effect a transfer reaction; the enzymic activity is enhanced in the presence of a suitable "activator;" the un-ionized sulfhydryl group and possibly an ionized carboxyl group are probably involved in the catalytic process; chymopapain B shows strong milk-clotting activity with casein and exhibits maximal activity at neutral pH's.

In this connection, a summary of some observations relating to papain may help to illumine the possible mode of action of chymopapain B. Smith, et. al. (1962), have elucidated the amino acid sequence around the "active" sulfhydryl group of papain. This decapeptide has the sequence:

-Glu-Leu-Leu-Asp-CySH-Asp-Arg-Arg-Ser-Tyr

An "active center" with this arrangement of amino acid residues is consistent with the well-known specificity of papain, and the possible correlations between this "active center" and some observations of the enzymic aspects of papain action are summarized in Table XXVII.

TABLE XXVII

THE RELATION OF STRUCTURE TO THE ACTIVITY OF PAPAIN

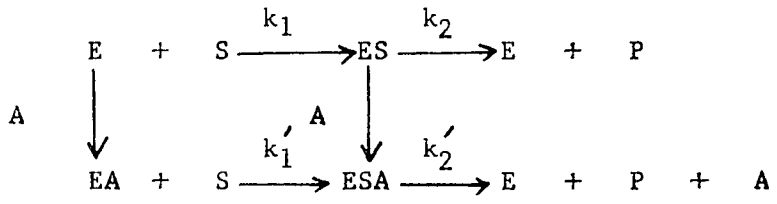
TYPE OF STUDY	OBSERVATIONS	INTERPRETATION AND CORRELATION
SPECIFICITY	1. Suitable substrates are acylamino acid amides, esters or other derivatives, with the effect due primarily to the type of amino acid residue. The best substrates are those of lysine and arginine.	1. If electrostatic forces are responsible for the attraction of the substrate to the "active center," the existence of anionic groups (one glutamic acid, two aspartic acid) would furnish the appropriate anionic groups.
	2. Carbobenzoxy-L-isoglutamine and acyl dipeptides are not hydrolyzed at pH's where the carboxyl group is fully ionized.	2. Electrostatic repulsions between anionic groupings existing in the "active center" and in the substrate would be a reasonable explanation.
	3. Carbobenzoxy-L-glutamic acid and carbobenzoxy-L-aspartic acid are powerful non-competitive inhibitors (1:1 combination).	3. Binding to the cationic Arg-Arg sequence offers an attractive possibility.
	4. Many acylamino acid amides or esters with hydrophobic side chains are good substrates.	4. The side chains of Leu-Leu could provide such a hydrophobic region for substrate binding.
KINETIC	1. The curve relating k_1 to pH is bell-shaped; only the alkaline limb is displaced when the temperature of reaction is changed.	1. Data are consistent with groupings of pK = 4.3 (carboxyl) and 8.13 (sulfhydryl).
	2. Studies with BAEE indicate that the values of k_3 are constant between pH 4.2 and 8.5.	2. An ionized carboxyl group is involved in the nucleophilic attack of the acyl-enzyme.

In addition to determining the specific residues involved in the catalytic process, any considerations of the mechanism of action of thiol proteases must incorporate a description of the activation process, since it is a fact that the addition of certain "thiol-activators" enhances the activity of these proteases. The question to be answered, of course, is the role of this activator.

The simplest description ascribed to the activation process is the liberation of "essential" -SH groups from a previously oxidized (e.g. as a disulfide) or alkylated form (e.g. as a mercury or PCMB-derivative). According to this description, the enzyme is "activated" when a reducing agent can convert this inactive form to the active form with the liberation of free -SH groups.

There is no reason to doubt this process as being the primary basis for the activation observed. Correlation of the relative activity with the titer of the free -SH groups is an observed fact. However, it is also quite conceivable that the activation process involves more than the mere liberation of free and "active" -SH groups.

One possibility for the mechanism of activation of thiol proteases is that suggested by Sanner and Pihl (1963). Based on their kinetic studies utilizing papain as enzyme, these workers obtained evidence which are consistent with the mechanism shown schematically below.



According to this mechanism, activators may enhance the activity of the enzyme by increasing the rate of decomposition of the enzyme-substrate

complex. In their studies with cysteine as activator, k_2' is approximately four times greater than k_2 and the ratio (k_2'/k_2) remains essentially constant in the pH range 4.5 to 7.6. Furthermore, calculated values of k_1 (assuming $k_{-1} = 0$) assumes approximately the same value in the absence and presence of the activator.

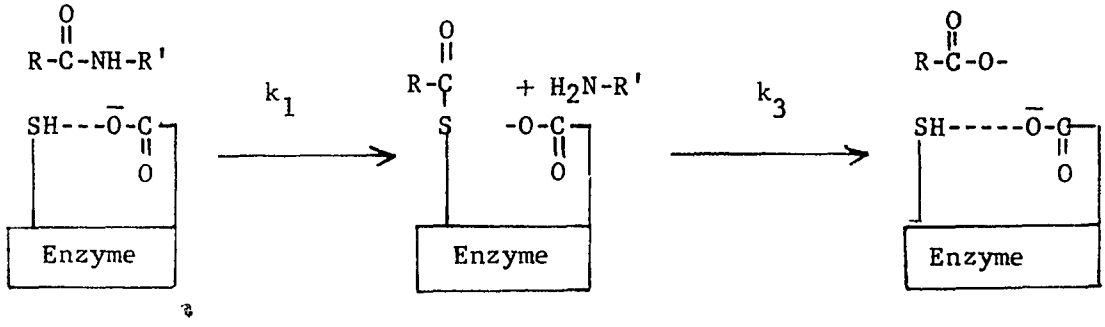
A literal interpretation of these findings would suggest that thiols are involved in the deacylation step and not in the binding process, as the mechanism advocated by Smith, et. al. (1962) would suggest. (See below.)

Exactly how a thiol would enhance this deacylation step remains an open problem. Furthermore, the nature of the binding of substrate to the enzyme is unknown and evidence to indicate the particular residue involved in this binding has never been presented. Hence, it is apparent why mechanistic studies have been equivocal and the nature of the activation process not clearly understood.

A possibility worth considering is the following: if thiols are involved in the deacylation step, it is quite possible that the activator that is introduced into the system is merely providing more -SH's than the enzyme itself can provide. But it can only do this provided the enzyme can first activate the substrate by means of the enzyme-substrate complex. The possibility that the carboxyl group is involved in the binding of the substrate via some anhydride-type linkage should also be considered.

Finally, an aspect of mechanism which should also be taken into account is the energetics of the reaction. In short, what is providing the enzyme with the energy necessary to activate the substrate? An obvious source of this energy would be chemical bonding energy, for example, hydrogen-bonding, hydrophobic bonding, Van der Waals exchange energy. But exactly how the enzyme can utilize this energy and with an expenditure of energy to again

re-form this bond seems very esoteric at the present. In this connection, the mechanism advocated by Smith, et. al. (1962) seems attractive.



In conclusion, the problems involved in the elucidation of the mechanism of enzyme action are very formidable ones. However, a great number of inroads into the problems involved are being made and presumably, therefore, an understanding will eventually arise out of the diverging approaches to the problem. With the so-called thiol proteases (of which chymopapain B is only one), the degree of any accomplishments made to date is measured strictly against the progress made with papain, undoubtedly the most extensively studied plant protease. Any insight gained into the mode of action of papain could, in all likelihood, be found applicable to most of the other plant proteases. The few attempts made to interpret the mode of action of chymopapain B in this study have been effected along such considerations. Future studies with chymopapain will also depend strongly upon the nature of the successes obtained with papain. It is hoped, however, that a breakthrough in our concepts of mechanism of action of thiol proteases will somehow involve chymopapain B as a model system.

C. Summary of the Properties of Chymopapain B

Chymopapain B represents the third protease isolated and crystallized thus far from dried papaya latex. Optimal yields have been obtained by a modification of the procedure of Ebata (1962).

The enzyme is homogeneous by several criteria: it exhibits monodisperse behaviour during ultra-centrifugation and free-boundary electrophoresis. N-terminal studies reveal a single polypeptide chain with tyrosine occupying the N-terminus position.

Untreated chymopapain B exhibits monomeric sedimentation behaviour at pH 5.0 and yields a sedimentation constant at infinite dilution ($s_{20,w}$) of 2.82 Svedberg units. The values of the sedimentation constants show a negative concentration dependence. The molecular weight, as determined by the Archibald "approach-to-equilibrium" technique, is 30,100 $\pm$ 900.

Electrophoresis indicates an isoelectric point of 10.5. The mobility curve at one-tenth ionic strength has a broad zone between pH 3.9 and 9.0 over which the mobility remains essentially constant. At acid pH's, the mobility is very sensitive to the ionic strength of the medium.

The enzyme appears to be relatively stable with respect to changes in pH and temperature. This stability apparently depends upon the enzyme's susceptibility to -SH oxidation. Hence, the enzyme is most stable at slightly acid pH's. Furthermore, the stability of chymopapain B at pH 2 has been demonstrated implying the similarity of this enzyme with the chymopapain isolated by Jansen and Balls (1942).

Preliminary analysis of the amino acid composition of chymopapain B indicates an abundance of lysine residues and a large excess of positive charges, consistent with the basic isoelectric point observed during electrophoresis. An incomplete recovery of the amino acids after acid

hydrolysis is suggestive of the presence of other non-protein moieties on the enzyme. Analyses for "true sugars" have yielded no information regarding the possible nature of the carbohydrate content, but such studies have strongly indicated the occurrence of non-protein constituents.

Chymopapain B has been shown to be an -SH enzyme on the basis of the following evidences: (1) the proteolytic activity is enhanced in the presence of the so-called "thiol-activators," such as cysteine, glutathione, 2,3 dimercaptopropanol, cyanide and thioglycolate; (2) the -SH groups in the enzyme can be reacted stoichiometrically with PCMB, NEM, Hg^{++}, Ag^{+}, Cu^{++}, Zn^{++}, iodoacetate with concomitant loss of enzymic activity; reversal of inhibition can be effected in the case of the mercurials and heavy metals by use of a suitable reducing-agent, such as cysteine; (3) the specific activity of the enzyme is found to be proportional to the available -SH; it is shown that two free -SH groups are present in the unactivated enzyme with only one -SH essential for activity.

Besides being inhibited by -SH reagents, unactivated chymopapain B can be irreversibly inhibited by a large excess of the aldehyde reagents hydroxylamine and phenylhydrazine. The nature of this inhibition has not been elucidated.

The pH optimum of proteolytic activity is at pH 7.0 when assayed with casein and B.A.A. (A dual optima is observed with casein as substrate, the other optimum occurring at pH 9.0), and the temperature optimum (under the conditions of assay) is found to be in the neighborhood of $80^{o}C$.

Some limited studies on the specificity of this enzyme have been carried out with some simple, synthetic compounds. These preliminary studies indicate that chymopapain B resembles papain in its ability to

hydrolyze a wide variety of peptide and amide bonds. N-Benzoyl-L-Arginine Amide is found to be the most sensitive substrate tested to date.

The ability of chymopapain B to catalyze anilide synthesis has been demonstrated.

Kinetic studies with B.A.A. indicate that chymopapain B can hydrolyze this compound at approximately one-fourth to one-third the rate of papain. Examination of the V_{max} versus pH curve indicates that at least two possible groups might be involved in the catalytic process: a group with a pK = ca. 4, most likely corresponding to a beta- or gamma- carboxyl group and a group with pK = ca. 9, assumed to be a sulfhydryl group. The kinetic data are amenable to treatment by the classical Michaelis-Menton equation, and the observed values of K_m are essentially constant in the range pH 4.5 to pH 8.5.

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