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THE STUDY OF THE ACTIVE CENTER OF
CHYMOPAPAIN.

University of Hawaii, Ph.D., 1966
Chemistry, biological

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DEDICATION

To my mother
and to my husband, Peter.
ACKNOWLEDGMENTS

To Dr. Kerry T. Yasunobu
   for his advice, encouragement and support.

To the National Science Foundation
   for support, 1960-1964.
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<tr>
<td>BAA</td>
<td>N-Benzoyl-L-Arginine Amide</td>
<td></td>
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<tr>
<td>BAL</td>
<td>2,3-dimercapto Propanol (British Anti-Lewisite)</td>
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<tr>
<td>CMB</td>
<td>p-Chloro Mercuribenzoic Acid</td>
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<td>CPAse</td>
<td>Carboxypeptidase</td>
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<tr>
<td>DDPM</td>
<td>N-(4-dimethylamino-3,5-dinitrophenyl) maleimide</td>
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<td>DDPS-</td>
<td>N-(4-dimethylamino-3,5-dinitrophenyl) succinimido-</td>
<td></td>
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<td>DFP</td>
<td>Diisopropylfluorophosphate</td>
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<td>1-Dimethylaminonaphthalene-5-sulfonyl chloride</td>
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<td>FDNB</td>
<td>1-Fluoro-2,4-dinitrobenzene</td>
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<tr>
<td>LAP</td>
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<td>M</td>
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<td>μm</td>
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<tr>
<td>NEM</td>
<td>N-Ethyl Maleimide</td>
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<tr>
<td>P-Ser</td>
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<td>PTC</td>
<td>Phenylthioisocyanate</td>
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<td>PTH-</td>
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<tr>
<td>-SH</td>
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<tr>
<td>TCA</td>
<td>Trichloroacetic Acid</td>
<td></td>
</tr>
<tr>
<td>μmoles</td>
<td>Micromoles</td>
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</tr>
<tr>
<td>XE-64</td>
<td>Amberlite IRC-50 (200-400 mesh)</td>
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ABSTRACT

The proteolytic enzyme chymopapain was first isolated from fresh papaya latex by Jansen and Balls (J. Biol. Chem., 137, 459, 1951). Subsequently, a modified procedure for purification of chymopapain from dried papaya latex was developed by Ebata (J. Biol. Chem., 237, 1086, 1962).

Chymopapain was shown to be a sulfhydryl enzyme on the basis of its susceptibility to sulfhydryl reagents and its requirement for "thiol activators" such as cysteine and cyanide for maximum activity.

Titration of cyanide activated chymopapain with p-chloro mercuribenzoate (CMB) revealed the presence of 1.4-1.6 moles of SH per mole of enzyme (m.w., 30,000).

The controlled addition of the colored sulfhydryl reagent, N-4-dimethylamino-3,5-dinitrophenyl)-maleimide (Witter and Tuppy, Biochim. Biophys. Acta, 45, 429, 1960), and enzymic hydrolysis of the S-DDPS-chymopapain thus formed led to the isolation of a deca-peptide containing the labeled half cysteine residue. The amino acid sequence of this peptide was found to be:

Lys-Arg-Val-Pro-Asp-Ser-Gly-Glu-Cys-Tyr

(Cys = the labeled half of cysteine residue).

Possible reasons for this difference in the sulfhydryl peptides from papain and ficin and that from chymopapain are discussed.

Preliminary studies (Ebata et al., Biochem. Biophys. Res. Comm., 2, 173, 1962) indicated that chymopapain was inhibited by diisopropylfluorophosphate (DFP) under certain conditions. The present investigation showed that there was a corresponding loss of the CMB-titratable SH group and enzymic activity with increasing concentration of DFP added. This corroborated the work of Gould and Liener (Biochemistry, 4, 349, 1965) who reported that an impurity in the DFP preparation reacted with the essential SH group of ficin thus inactivating the enzyme.

However, cyanide-activated chymopapain was phosphorylated by DFP (organic phosphorus/protein, 0.89, mole/mole). Unactivated chymopapain was not phosphorylated under the same conditions. The site of phosphorylation was the hydroxyl group of a serine residue. The partial structure of the phosphorylated peptide from tryptic digest of DIP-chymopapain was

\[ \text{P} \quad \text{Ser-Gly(Cys,Asp,Thr,Ser,Glu,Ala)-Lys} \]

Methylene-blue catalyzed photooxidation of CMB-chymopapain resulted in the loss of all three histidine residues of chymopapain with only 50% loss of enzymic activity.

A tentative mechanism for the reaction between chymopapain and substrate is proposed on the basis of the available data.
I. INTRODUCTION

A. Background

Chymopapain is a proteolytic enzyme found in the latex of the papaya (Carcia papaya). This enzyme was first isolated from the fresh papaya latex (Jansen and Balls, 1941; Balls, 1941).

Ebata (1962) in our laboratory developed a modified procedure for the isolation and crystallization chymopapain starting with the commercial preparation of purified dried latex, "Purified Papain", Paul-Lewis Laboratories, Milwaukee, Wisconsin.

During the subsequent purification of chymopapain from the crude papaya latex (the partially purified latex was no longer available), Kunimitsu (1964) observed the appearance of several proteolytically active components on chromatography on Amberlite IRC-50 (XE-64). Furthermore, Kunimitsu was able to crystallize the protease present in one particular fraction (0.40M acetate, pH 5.90). This component, designated as chymopapain B, differed from the chymopapain described by Ebata (chymopapain A) in several aspects (Kunimitsu, 1964): (1) The specific activity of chymopapain B is two to three times greater than that of chymopapain A; (2) Tyrosine was found to be the NH₂-terminal residue in chymopapain B, while glutamic acid was found in the NH₂-terminal position in chymopapain A; (3) Chromatographic behavior on XE-64 was different; chymopapain A was eluted at a lower ionic strength than was chymopapain B; (4) Chymopapain B crystallized in the form of needles while chymopapain A crystallized as rods; (5) Molecular weight as determined by the Archibald procedure was 30,000 for B and 35,000 for A.
However, both forms were similar to each other in the overall amino acid composition, susceptibility to sulfhydryl reagents, substrate specificity, electrophoretic behavior, and greater heat and pH stability as compared to papain.

Kunimitsu was able to show also that neither chymopapain A nor B is an autolysis product of one form to the other. Both forms do not undergo autolysis when left standing at room temperature for a number of days. Furthermore, both DNP-glutamic acid and di-DNP-tyrosine were detected when the crude latex itself was dinitrophenylated, indicating that chymopapain A and B are present in the starting material.

B. The Sulfhydryl Group(s) of Chymopapain

Chymopapain, like many other proteases of plant origin, has been shown to be a sulfhydryl enzyme. Like papain, it is inactivated by various SH group reagents and it requires "thiol activators" such as cysteine, glutathione, cyanide and thioglycolate for maximum catalytic activity. However, unlike papain which contains one active sulfhydryl group after activation with mercaptans, activated chymopapain was found to contain 1.4-1.6 moles of SH per mole of enzyme when titrated with CMB. In the unactivated form, however, less than one SH can be detected by the CMB titration method. Reaction with N-ethylmaleimide also revealed a maximum of two titratable SH groups in the thiol-activated chymopapain. Similar results were observed with iodoacetate. The amino acid composition of the carboxymethylated chymopapain (CMC-chymopapain) showed that in the unactivated chymopapain there was 0.85 residues of CMC-cysteine per mole of chymopapain B (m.w. 31,500) and in the activated form, 1.43 residues (Kunimitsu, unpublished).
Whether one or both of the two titratable SH groups are necessary for activity has not been definitely determined. Preliminary studies by Kunimitsu indicated that perhaps only one of the SH groups is essential. Kunimitsu allowed chymopapain to stand at room temperature for periods greater than six days and then measured both the SH titer and specific activity. He observed that while a maximum of one SH could be titrated with CMB, the enzyme had essentially no activity.

Also necessary for an understanding of the mechanism of action of chymopapain is the knowledge of the immediate environment around the active SH group. The detailed structure around the active sulfhydryl group of papain and the position of this "active site" in relation to the entire papain molecule have been reported by Light (1964). The essential SH group in papain was found attached to the cysteine residue at position 25 (counting from the NH$_2$-terminal end of the molecule) and this cysteine residue was located within a small loop formed by the disulfide bonds between half cystine residues 22 and 159 and between 43 and 152, Fig. 1. The sequence of the peptide containing the active SH group was determined, and a remarkable similarity was noted between this sequence and that of the active site peptide from ficin (Wong and Liener, 1964):

- Papain: -Pro-Val-Lys-Asn-Gln-Gly-Ser-Cys-Gly-Ser-Cys-
- Ficin: -Pro-Ileu-Arg-Gln-Gln-Gly-Gln-Cys-Gly-Ser-Cys-

(The underlined cysteine residue contains the essential SH group). The papain sequence represents residues 15-25. The position of the active site peptide in the overall structure of ficin is not yet known. The similarity in the sequence of the active centers suggests that this unique chemical structure around the reactive sulfhydryl group is essential for proteolytic activity in both enzymes.
FIGURE 1.

A diagramatic representation of the structure of papain, taken from paper by Light et al., (1964). The relative positions of the reactive SH group, the three disulfide bridges and the aspartyl group as position 163 are shown.
Inasmuch as chymopapain and papain were isolated from the same source and both were shown to be sulfhydryl enzymes which display similarities in their enzymic properties, it was of interest to compare the sequence of the active center peptide of chymopapain with that of papain. Neither the complete amino acid sequence of the chymopapain nor the locations of the ten half cystine residues in chymopapain are known at the present time. However, a comparison of the amino acid sequence of the active center peptide of chymopapain with those of papain and ficin would aid in the ultimate understanding of the mechanism of action of chymopapain and other plant proteases.

C. Reaction of Chymopapain with Diisopropylfluorophosphate

The inhibition of sulfhydryl proteases such as papain, ficin, bromelain and chymopapain by the organophosphorus reagent, diisopropylfluorophosphate (DFP), has been a controversial subject for some time. Preliminary studies in our laboratory (Ebata et al., 1962) indicated that chymopapain was inhibited by DFP provided that a large excess of the DFP was used (DFP/enzyme = 100, mole/mole). The DFP inhibition of chymopapain was also found to be dependent on several other factors. These were: (1) The source of DFP (DFP obtained from Aldrich Chemical Co., was found to be more potent than that from Mann Research Lab); (2) The nature and the concentration of the activating reagent (chymopapain must be activated and the activator must be removed prior to DFP treatment); (3) The pH of the reaction mixture (the optimum pH for inactivation was pH 7.2).

Furthermore, Ebata (1963) was able to isolate and partially characterize the enzymatically inactive, crystalline DIP-chymopapain which
contained one gram atom of phosphorus per mole of enzyme. The initial 
and obvious deduction drawn from the results of these preliminary studies 
was that a specific serine residue was necessary for the activity of 
chymopapain, as it is in the case of trypsin and chymotrypsin.

However, Murachi (1963) reported that sulfhydryl enzyme such as 
papain and bromelain could be non-specifically phosphorylated by DFP 
without loss in enzymatic activity, and that this pH dependent phos-
phorylation leads to the non-specific alkylation of the tyrosine resi-
dues.

More recently, Liener and co-workers (1963, 1965) reported that 
commercial samples of DFP contained an impurity which combined irrever-
sibly with the SH groups of ficin, cysteine, and glutathione. This im-
purity was reported to be found in the non-distillable residue after 
fractional distillation of DFP. The inhibition of ficin by DFP was pre-
vented by prior activation of ficin with cysteine, which lead to the 
conclusion that cysteine exerts its protective effect by reacting prefer-
tentially with the inhibitor. The chemical identity of this inhibitor 
remains unknown at the present time.

Because of these conflicting reports concerning the inhibition of 
plant proteases by DFP, a part of the present dissertation on the active 
center of chymopapain involved repeating the work of Ebata in order to 
obtain more definitive data on the relationship between DFP-inhibition, 
phosphorylation of a specific amino acid residue, and SH content. Also, 
if phosphorylation does take place, an attempt would be made to identify 
the site of phosphorylation by investigating the structure of the pep-
tide.
D. Role of Histidine in Chymopapain

Various chemical and kinetic evidences have led to the suggestion that histidine residues have functional roles in the mechanism of action of trypsin and chymotrypsin (Koshland et al., 1962. Schoellmann and Shaw, 1962.)

More recent studies (Walsh et al., 1964; Nieman, 1964) have established the interesting fact that in both trypsin and chymotrypsin the two histidine residues are present in the identical positions in a decapeptide. The structure of this decapeptide is shown below:

\[
\text{His-Phe-Cys-Gly-Gly-Ser-Leu} \\
\text{S} \\
\text{S} \\
\text{Ala-His-Cys-}
\]

Therefore, the similarity in the active centers of chymotrypsin and trypsin extend beyond the common tetrapptide, Gly-Asp-Ser-Gly-, which contains the active serine residue.

The amino acid composition of chymopapain indicated that this enzyme contained three histidine residues per mole. Therefore it was of interest to determine whether any of these imidazole groups have any functional role in the mechanism of action of chymopapain, particularly since it appeared from the DFP studies that an active serine residue might constitute a part of the active center.

Methylene blue catalyzed photooxidation studies in conjunction with quantitative amino acid analyses of the oxidized forms of the enzyme were carried out in order to correlate the changes in enzymatic activity with alteration in the histidine content of the enzyme. A more direct and ideal method for determining the functional role of histidine in chymopapain would be to use a specific reagent for labeling only the
histidine residue. Such a compound was used for chymotrypsin (Schoellmann and Shaw, 1962). Their reagent, L-1-tosyl-amido-2-phenyl-ethyl chloromethyl ketone (TPCK), was found to react only with the enzymatically active chymotrypsin causing the loss of one histidine residue with concommitant loss of all enzymatic activity. However, this reagent is not a general "histidine reagent", but a substrate-like reagent specific for chymotrypsin. The N-methyl derivative of TPCK, for example, failed to inactivate chymotrypsin. Thus in order to use a similar reagent for chymopapain, it would be necessary to obtain a derivative specific for chymopapain, such as the arginyl derivative. If the results of the photooxidation studies indicated that histidine was an essential amino acid, a more extended study of this amino acid in chymopapain would be carried out.

E. Statement of the Problem

The ultimate goal of this study with chymopapain was to gain an understanding of its function and mechanism of action. This required a detailed knowledge of the structure of the enzyme, particularly that of the chemical environment around the amino acid residue(s) which have functional roles in catalysis. As mentioned earlier, remarkable similarities have been demonstrated in the sequence of the active center peptides of papain and ficin, as well as in chymotrypsin and trypsin. The objective of the present study was the identification of the amino acid(s) involved in the catalytic activity of chymopapain and the elucidation of the chemical structure of the active center peptide.

The experimental procedure used are theoretically simple. These are: first, the use of proper reagents to follow changes in enzymic
activity with corresponding changes in the content of a particular amino acid; second, the labeling of the essential amino acid(s) with a reagent specific for that particular amino acid; third, the isolation, purification and characterization of the labeled peptide by established procedures.

However, preliminary studies indicated several complicating factors. First of all, it had not been clearly established as to whether or not the inhibition of chymopapain by DFP was due to the specific phosphorylation of an essential amino acid. (The large excess of DFP required to bring about inhibition and the studies of Liener casted doubts on the results of the preliminary study). Secondly, unlike papain and ficin which contained only one freely reactive sulfhydryl group per molecule, chymopapain in the activated form was found to contain a maximum of two reactive SH groups. Although evidence had been obtained indicating that only one of the SH groups is necessary for activity, the preferential labeling of only the essential SH group posed a difficult problem.

The results of the present studies constitute only a part of the data necessary for the elucidation of the mechanism of action of chymopapain. A final solution of this problem must await more detailed kinetic studies as well as the determination of the complete amino acid sequence and the three-dimensional structure of the enzyme.
II. MATERIALS AND METHODS

A. Materials

1. Materials and Reagents Obtained Commercially:
   a. Aldrich Chemical Company, Inc., Milwaukee, Wisconsin
      Diisopropylfluorophosphate
   b. California Corporation for Biochemical Research, California
      DL-O-Phosphoserine
      Trypsin, pancreatic
      Pronase (Streptomyces griseus protease) Lot No. 109080
   c. Carl Schleicher and Schuell Co., New Hampshire
      Carboxymethylcellulose
   d. Eastman Organic Chemicals, New York
      Isatin
      Ninhydrin
      Hydrazine
      p-Semedine hydrochloride (N-phenyl-p-phenylenediamine monohydrochloride) Lot No. 2043
   e. J. T. Baker Chemical Co., New Jersey
      Dowex 50-X8 (200-400 mesh)
      Dowex 50-X2 (200-400 mesh)
      Dowex 50-X4 (200-400 mesh)
   f. Mann Research Laboratories, New York
      Diisopropylfluorophosphate
      DL-O-Phosphoserine
      N-(4-Dimethylamino-3, 5-dinitrophenyl)-maleimide
      Benzoyl-L-arginine amide.HCl.monohydrate, Lot No. G2060
      L-Cysteine, free base, Lot No. D3361
2,4-Dinitrofluorobenzene
Iodoacetic Acid
g. Nagase Saigyo Co., Amagasaki, Japan
Nagarse (Crystalline Bacterial Al-Proteinase)
h. Nuclear Chicago
P-32 labeled Diisopropylfluorophosphate, No. PB-1
i. Nutritional Biochemicals Corporation, Ohio
Casein (Hammersten Quality), Lot No. 8503
p-Chloromercuribenzoic Acid (Na salt), Lot No. 8031 (CMB)
Pepsin (3 x Crystallized), Lot No. 2215
j. Pharmacia, Uppsala, Sweden
Sephadex G-25, G-50, G-75
k. Rohm and Hass Company, Pennsylvania
Amberlite IRC-50 (XE-64)
l. Worthington Biochemical Corporation, New Jersey

2. Materials Obtained as Gifts:
Pineapple stem bromelain, Dr. Ralph Heinicke, Dole Corp., Honolulu, Hawaii

3. Materials Obtained by Preparative Methods:
   a. Papain was purified from dried papaya latex as described by Ebata (1962).
   b. Chymopapain was purified from dried papaya latex as described by Ebata (1962).
   c. S-DDPS-Cysteine was synthesized according to the method of Witter and Tuppy (1960). To 181 mg of cysteine-HCl suspended in 10 ml of deionized and deoxygenated water, (308 gm of DDPM dissolved in 10 ml of acetone was slowly
added. The pH of the reaction mixture was adjusted to 5 by the addition of solid NaHCO₃. The mixture was stirred at room temperature for 1½ hours and dried in a vacuum dessicator. The orange residue was washed with a little water and redried. The residue was suspended in 95% ethanol, dissolved by the careful addition of 1 N HCl in the cold and reprecipitated by the slow addition of sodium acetate. The orange product was washed in minimal amount of cold water and dried over P₂O₅ in a vacuum dessicator. Yield: 248.6 mg (44%), m.p. 168-170°C, Rf in BAW (4:1:5) was 0.60.

B. **Methods**

1. **Preparation of Chymopapain:**

   a. Chymopapain was isolated from the commercially obtained crude papaya latex according to the method of Ebata (1962) and Kunimitsu (1963).

2. **Methods of Assay and the Measurement of Enzymic Activity:**

   a. **Casein Digestion Method**

   The proteolytic activity of chymopapain 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 using 0.10 M phosphate buffer, pH 7.20 (arbitrarily chosen as the standard condition). When necessary, the activation of the enzymes was carried out as follows: To a suitable volume of
enzyme containing about 200 μg of enzyme was added an equal volume of 0.10 M NaCN in phosphate buffer, pH 7.2. The mixture was incubated in a constant temperature bath at 35ºC 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 time 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 at least thirty minutes and subsequently filtered. The absorbancy of the clear supernatant was then measured at 280 μm in a Beckman DU 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 TCA to 1.0 cc of 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 μm per minute per μg of enzyme, the maximum value being estimated from the initial slope of a plot of change of absorbancy versus μg enzyme per ml of reaction mixture (2.0 cc under these conditions).
b. **Hydrolysis of Benzyol-L-Arginine Amide**

Preliminary work on the specificity of chymopapain B by Ebata and Kunimitsu with respect to the hydrolysis of some simple amino acid derivatives or dipeptides indicated that N-benzyol-L-arginine amide was the most sensitive substrate. Thus, this substrate was used in the measurement of residual enzymic activity after photooxidation, where a more accurate measurement of enzymic activity was necessary. For purpose of assay 0.75 cc of 0.06 M BAA in pH 7.5 phosphate buffer was added to 0.20 cc of activated chymopapain (containing 10-50 μg of enzyme per ml). The final volume was made to 1.5 ml with 0.1 M phosphate pH 6.5. At proper time intervals aliquots of 0.2 - 0.4 ml were taken and added to 1.0 ml of ninhydrin solution (20 g ninhydrin, 3 gm hydrindantin, 250 ml 4.0 M acetate buffer, pH 5.5, and 750 ml methyl cellosolve) to stop the reaction. The resulting mixture was heated in a boiling water bath for twenty-minutes and rapidly cooled with tap water. To this cooled solution 10.0 ml of 50% ethanol was added and the absorbancy of the solution was read at 550 μm in a Beckman DU spectrophotometer.

3. 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 2 N acetic acid, then with water. Subsequently,
the resin was converted to the Na\textsuperscript{+} form by washing with an equal volume mixture of 0.5 M NaOH and 0.50 M NaCl solutions.

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

c. Dowex-50 was converted to the H\textsuperscript{+} form after overnight equilibration with 0.5 N NaOH and 0.5 N HCl.

d. 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 washed continually until salt-free (where water was used as eluent) or until the pH of the effluent was within 0.1 pH unit of the influent buffer.

e. Talc (talcum powder, USP, Merck & Co., N.J.) 30-40 gm was suspended in a mixture of 2 N HCl and 95% ethanol (1:1, v/v), heated in a steam bath for two hours and repeatedly washed with water by decantations until the wash water was no longer acidic. In order to increase the flow rate of the talc column, "Solka-Floc" (cellulose powder, Brown & Co., Berlin, N.H.) which was previously washed with 0.5 N NaOH and water and then steam treated in an ethanol 2 N HCl mixture was mixed with talc, 1:1 (v/v).
4. Determination of SH Groups:

The spectrophotometric method of Boyer (1954) was used for SH measurements. To 1.0 cc of enzyme solution (containing 0.05 - 0.10 umoles of protein) graded levels of CMB were added and the final volume was adjusted to 4.0 cc with an appropriate buffer. Each tube was read at 255 μ (0.33 M acetate buffer, pH 4.60) with appropriate blanks to correct for the contribution to the total absorbancy by the protein and CMB. All samples were allowed to stand for ninety minutes prior to the absorbency readings.

The colored derivative of NEM, N-(4-dimethylamino-3, 5-dinitrophenyl)-maleimide, was first used successfully as a sulfhydryl reagent by Witter and Tuppy (1960). They were able to isolate the S-DDPS-cysteine peptides from human and bovine serum albumin by following the yellow color of the labeled peptides as a guide during the purification procedure.

DDPM was purchased from Mann Research Laboratories (New York) and used without further purification.

Titration of chymopapain with DDPM was carried out as follows: To 1.0 cc of cyanide activated chymopapain dissolved in 0.05 M phosphate buffer, pH 7.2 was added varying volumes (0-0.2 cc) of DDPM dissolved in a mixture of equal volumes of 95% ethanol and 0.33 M acetate buffer, pH 4.6 (DDPM/Enzyme, 5/1). After standing at room temperature for 2½ hours, an aliquot was removed from each tube, diluted with phosphate buffer and the residual enzymic activity was determined by the casein digestion method. The remainder was titrated.
spectrophotometrically with excess CMB (3.0 cc in 0.33 M acetate buffer, pH 4.6) to determine the amount of unreacted SH groups.

5. Determination of Phosphorus:

Phosphorus content was determined by the method of Bartlett (1959).

For the determination of organic phosphorus, 1.0-2.0 ml of sample was mixed with 0.5 ml of 10 N H_2SO_4 and heated in an oven at 150-160° for three hours. The tubes were cooled, 2-3 drops of 30% H_2O_2 were added and the tubes were returned to the oven for another 1.5 hours. After cooling, 4.40 ml of H_2O, 0.20 ml of 5% ammonium molybdate solution and 0.20 ml of p-semidine hydrochloride solution (50 mg in 100 ml of 1% NaHSO_3) were added. The mixture was heated for seven minutes in a boiling water bath, cooled and absorbancy read at 770 mp in a Beckman DU spectrophotometer. In the cases where the amount of sample for analysis was very small, a semi-micro method was used in which 0.10-0.20 ml of sample, 0.30 ml of 10 N H_2SO_4, 0.65 ml H_2O, 0.20 ml of 5% ammonium molybdate and 0.10 ml of p-semidine solutions were used and the absorbancy was read in a cuvette requiring 1 ml of solution.

For the determination of inorganic phosphorus, the sample was made to 4.1 ml with H_2O, mixed with 0.5 ml of 10 N H_2SO_4, 0.20 ml of 5% ammonium molybdate and 0.20 ml of p-semidine solution and then heated in a boiling water bath for seven minutes.

Standard curves were prepared using glucose-1-phosphate and KH_2PO_4.
6. Determination of Radioactivity:

P-32 labeled diisopropylfluorophosphate (Nuclear Chicago Corporation No. PB-1) was used to label chymopapain. Radioactivity was measured by plating the samples on aluminum planchettes and counting with a Nuclear Chicago model D47 counter equipped with an automatic sample changer (model C110A).

Radioactivity on paper chromatograms were detected by radioautography using large sheets of medical X-ray films (Eastman Kodak Company, New York). The presence of radioactive compounds was detected by the appearance of dark bands or spots on the films after development.

7. Performic Acid Oxidation:

Performic acid oxidation was carried out by the method of Moore (1963). The lyophilized protein or peptide sample was dissolved in a suitable volume of a mixture consisting of 88% formic acid and methanol (8/1, v/v) and cooled in the freezer. To this cooled mixture was added 4-5 times the volume of performic acid reagent (88% formic acid / 30% hydrogen peroxide, 9/1) which had been allowed to stand at room temperature for 80 minutes, then cooled in the freezer for at least 30 minutes prior to use. The reaction mixture was allowed to stand in the freezer for 3-5 hours, diluted (1:1) with cold deionized water and then the whole mixture was lyophilized.

8. Enzymatic Hydrolysis:

a. Trypsin

Because of the large number of lysine and arginine residues found in chymopapain (21 lysine and 6 arginine per
molecular weight of 30,000) trypsin was the first enzyme used for hydrolyzing the labeled derivatives of chymopapain.

In order to eliminate any contaminating chymotryptic activity, trypsin was incubated at pH 2.0 in the presence of .01 M Ca^{++} at 35° for 24 hours prior to use. A suspension or solution of the protein or peptide was adjusted to pH 8.0 by addition of 1% trimethylamine. Two to three drops of toluene were added to prevent bacterial contamination. Pre-treated trypsin equivalent to 2% of the weight of protein or peptide was then added at zero time and the pH was maintained at 8.0 by periodic addition of trimethylamine, and hydrolysis was allowed to proceed at room temperature until the theoretical number of susceptible bonds were cleaved. (This can be calculated from the concentration and the volume of the base added). Often, a second addition of trypsin (about 10-20% of the original amount added) was necessary for complete hydrolysis to occur. The reaction was stopped by the addition of anhydrous formic acid to bring the pH of the reaction mixture to 3.0. Any precipitate formed was centrifuged out and the supernatant was lyophilized.

**b. Pepsin**

Pepsin equivalent to 2% of the weight of protein to be hydrolyzed was dissolved in 0.01 N HCl and added to the protein solution which had been adjusted to pH 2.0 with 0.1 N HCl. Usually the protein to be hydrolyzed was rendered more susceptible to hydrolysis by performic acid oxidation
or by heat denaturation (heating in a 100°C water bath for 2-3 minutes).

c. Nagarse

Nagarse (crystalline bacterial Al-Proteinase, Nagase Saigyo Company, Japan, Lot No. 10610, PU 125 x 10⁴) was used to obtain smaller fragments of the active-center peptide. In a typical hydrolysis experiment, 1.0-1.5 ml of the peptide solution containing 0.6-1.0 micromoles of the peptide was adjusted to pH 7.7 with 1% NaHCO₃ solution. Nagarse (substrate/enzyme, 20/1, w/w) was added and hydrolysis was allowed to proceed at 37°C for 2-20 hours. The reaction was terminated by adjusting the pH of the digestion mixture to 1.0 with glacial acetic acid. The clear digestion mixture was dried and subjected to high-voltage paper electrophoresis at pH 4.7 (10 ml pyridine, 10 ml glacial acetic acid brought to 1,000 ml with deionized water). The peptides thus isolated were eluted and studied.

d. The COOH-terminal amino acid residue was identified by the use of carboxypeptidase. The method used was a modification of the procedure described by Fraenkel-Conrat (1958). The enzyme was suspended in deionized water and solubilized by the dropwise addition of 1% NaHCO₃ solution. The pH of the solution was quickly adjusted to 8.0 with 0.2 M acetic acid. The concentration of the enzyme was determined from the absorbancy at 278 μm on the Beckman DU spectrophotometer (E₁%ᵢ₇₈₇₈ = 19.4). An aliquot of the enzyme solution was added to a solution of peptide or protein to be
hydrolyzed, such that the ratio of the substrate to the exopeptidase would be 100:1 or 50:1 (w/w). The reaction mixture was incubated at 36°C. At various time intervals aliquots were removed from the reaction mixture, heated in a 100°C bath for 2-3 minutes to terminate the reaction and any precipitate formed was centrifuged. The supernatant was dried and the amino acid(s) released were determined quantitatively on a Spinco model 120 amino acid analyzer.

e. Leucine Aminopeptidase

Leucine aminopeptidase was used to supplement the results obtained by the dinitrophenylation and Edman's phenylisothiocyanate method for the determination of the NH$_2$-terminal sequence. The method used was essentially that of Hill and Smith (1955, 1957). The enzyme was dissolved in 0.05 M Tris (hydroxymethyl) aminomethane-HCl buffer, pH 8.5, containing 0.01 M Mg$^{++}$. The substrate was dissolved or suspended in the same buffer. The two solutions were separately pre-incubated for 30 minutes at 40°C and then an aliquot of LAP solution was added to the substrate such that the molar ratio of substrate to LAP of 50:1 would result. Aliquots were removed from the reaction mixture at various time intervals and the amino acids released were determined on the Spinco amino acid analyzer.

9. Methods of Peptide Purification:

Following enzymic hydrolysis, the labeled peptide was purified from the hydrolysate by combination of column chromatography, high-voltage paper electrophoresis and paper
chromatography. The various resins used for column chromatography and the methods of preparation of these resins for use are described in Section 3B. Both stepwise and gradient elution methods were employed. Fractions containing peptides were determined by the ninhydrin method of Moore and Stein (1954). For the purpose of assay, 0.10 ml of sample was added to equal volume of 4.0 N acetate buffer, pH 5.5 and to this mixture was added 0.2 ml of the ninhydrin reagent (see B-2b for composition). The resulting mixture was heated in a boiling water bath for 15 minutes, rapidly cooled and diluted with 6.0 ml of 50% ethanol. The absorbancy of the solution was determined at 550 mp in a Coleman Jr. Spectrophotometer. Standard ninhydrin curves were prepared with chromatographically pure leucine hydrochloride.

10. Determination of the Amino Acid Composition:

The amino acid compositions of the isolated peptides were determined quantitatively on a Spinco model 120 amino acid analyzer. Calculations were carried out according to standard methods (Spinco Instruction Manual and Handbook). To a limited extent quantitative amino acid composition determinations were carried out with the "Technicon Auto-Analyzer, (Ser. No. 62A 16, 252). Samples for analysis were hydrolyzed in 6 N HCl for 12 to 72 hours.

Tryptophan was determined by the method of Goodwin and Morton (1946) and by the method of Spies and Chambers (1948).

Whenever necessary qualitative colorimetric tests were used to detect the presence of various amino acids in the peptides.
The preparation and the conditions for the use of the reagents are described by Smith (1960).

11. **NH$_2$-terminal Analysis:**

   a. **Dinitrofluorobenzene (FDNB) Method**

      The method of Sanger as described by Fraenkel-Conrat (1958) was used for the determination of the NH$_2$-terminal amino acid residue. For the identification of the DNP-amino acids, the ether phase was chromatographed in the upper phase of the solvent system, tertiary-amyl alcohol/3% NH$_3$ in the first dimension and the 1.5 M tertiary-amyl alcohol/phthalate buffer (pH 6) system.

   b. **Phenylisothiocyanate (PTC) Method**

      The amino terminal residue and sequence were determined by the phenylisothiocyanate method of Edman (1956) as modified by Konigsberg and Hill (1962). The coupling reaction was carried out in N-ethylmorpholine-acetic acid buffer, pH 8.8 (6.0 ml of N-ethylmorpholine, 0.15 ml glacial acetic acid, 50 ml 99% ethanol, 50 ml water, where the pH of the buffer was checked before the addition of ethanol) at 40° for 2.5 hours. Excess phenylisothiocyanate was extracted with benzene and the reaction mixture was thoroughly dried under a stream of nitrogen. The cyclization step was carried by adding 2.0 ml of anhydrous trifluoroacetic acid (TFA) to the dried sample and allowing the reaction to proceed for one hour at room temperature. When PTH-glycine was the NH$_2$-terminal amino acid, the cyclization was carried out in glacial acetic acid-anhydrous HCl mixture.
Two chromatographic systems (Solvents A and C) as described by Fraenkel-Conrat (1958) were used for the identification of the PTH-amino acid. Localization of the phenylthiohydantoins was accomplished by use of the modified iodide-acide spray reagent in which the starch was incorporated into the spray reagent (Smith, 1960). After the extraction of the PTH-amino acid into the organic phase, an aliquot was removed from the aqueous phase, hydrolized for hours in 6 N HCl and the amino acid composition of the residual peptide was determined on the automatic amino acid analyzer.

12. Photooxidation Experiments:

Photooxidation in the presence of methylene blue has been widely used as a method for destroying histidine and tryptophan, as well as tyrosine, methionine and cysteine. For our studies, the CMB- or the Hg-derivatives of the enzymes were used to protect the essential -SH groups. Photo-inactivation experiments were carried out in the GM-Lardy Warburg apparatus equipped with 15x30 watt lamps, a shaker, and a constant temperature bath which was maintained at 30° during the experiment. The final methylene blue concentration was 0.01% and the concentration of the enzyme solution used was 15-20 mg in final volume of 3-5 cc. Aliquots were taken at various time intervals, and the methylene blue removed by filtration through a micro-column packed with Dowex-50X2. The residual enzymatic activity was assayed by both casein digestion and BAA hydrolysis. Aliquots were also taken at certain time intervals,
hydrolyzed in 6 N HCl for 24 hours and the amino acid compositions of the photo-oxidized samples were determined on the automatic amino acid analyzer. Tryptophan content was determined by the analysis of the ultraviolet spectra of the protein in 0.1 N NaOH (Goodwin and Morton, 1946).
III. RESULTS

A. Purification of Chymopapain

Chymopapain was purified from crude papaya latex (Crude Standardized Papain, Paul Lewis Laboratories, Lot No. 20211010) according to the method of Ebata (1962). A typical elution pattern during XE-64 column chromatography is given in Figure 2. Studies on the chemical and physical properties of the four main proteolytic components from the XE-64 column chromatography were carried out by Kunimitsu (1964) who found that all four fractions have similar amino acid compositions but differ in the NH$_2$-terminal residues, chromatographic behavior, and specific activities. The NH$_2$-terminal residue of Fractions I and II was predominantly glutamic acid, while tyrosine occupied the NH$_2$-terminal position in Fractions III and IV.

In spite of the differences in chromatographic behavior, it was concluded from Kunimitsu's studies that chymopapain A and B are different forms of the same enzyme. Thus, the chemical environment around the active sites of both forms of chymopapain should be similar, if not identical. A preliminary check on this point was made by comparing the "fingerprint patterns" obtained from the two-dimensional paper chromatography of the peptic digests of the DDPS-derivatives of Fractions I, III and IV. The DDPS-derivatives are prepared by treating chymopapain with the colored sulfhydryl reagent, N-(4-Dimethylamino-3,5-dinitrophenyl)-maleimide. (The details on the reaction of this reagent with chymopapain will be given later). Fractions I, III and IV (300-500 mg) were activated with NaCN, passed through columns of Dowex-50-x8 (H$^+$ form) to remove
FIGURE 2.

Chromatography of the 50% NaCl saturated supernatant on XE-64. 13.9 g of protein was charged on a 2.8 x 50 cm column of XE-64, equilibrated with 0.25 M phosphate buffer, pH 5.9. Elution was carried out with the buffers indicated. All operations were carried out at 4°C. Protein concentration, OD 280 μm, (---) and specific activity (▲▲▲) are represented. Cross-hatched areas represent fractions pooled.
the excess CN\(^-\) and then reacted with five-fold molar excess of DDPM at room temperature. Aliquots were removed at various time intervals to check the enzymatic activity. All three fractions were inactivated after 12 hours. The inactive, colored derivatives were then precipitated with ten-fold excess (v/v) of ethanol, the precipitate collected by centrifugation, thoroughly dialyzed and subjected to peptic digestion for 24 hours. Aliquots of the digestion mixtures were examined by high voltage paper electrophoresis and by two-dimensional paper chromatography. The results are shown in Figure 3. All three fractions yielded similar fingerprint patterns as far as the location of the colored fraction was concerned. If there are any slight differences in the amino acid composition or sequence in the labeled peptide from each fraction, these differences will be found later in the determination of amino acid sequence of the peptide. The overall patterns, however, indicate that in spite of the differences in \(\text{NH}_2\)-terminal residue, specific activities and chromatographic behavior, chymopapain A and B possess similar structure in the active site as far as the essential sulfhydryl group is concerned.

Only chymopapain B was used for our present studies on the active center since this fraction was obtained in much better yield and more recent physical-chemical studies have been carried out on this component.

B. Inhibition by Diisopropylfluorophosphate

1. Studies on the P-32 Labeled DIP-chymopapain:

P-32 labeled DFP was used initially to prepare the enzymatically inactive, radioactively labeled derivatives of
FIGURE 3.
Two-dimensional fingerprint patterns of the peptic digest of DDPS-chymopapain. The solvents systems used were: n-Butanol/Acetic acid/Water (4/1/5) in the first dimension; n-Butanol/Pyridine/Water (1/1/1) in the second dimension. The cross-hatched spots represent the yellow-colored fractions.
3(a) Pattern obtained from DDPS-chymopapain prepared from protein fraction I (Chymopapain A), (XE-64 chromatography).
3(b) Pattern from DDPS-chymopapain prepared from protein fraction III (Chymopapain B).
3(c) Pattern from DDPS-chymopapain prepared from protein fraction IV (Chymopapain B).
chymopapain. To 1.0 g of cyanide-activated chymopapain (final cyanide concentration, \(2 \times 10^{-3} M\)) dissolved in 90 ml of 0.05 M phosphate buffer (pH 7.2), 0.04 g. (8.4 millicuries) of DFP-32 diluted with 1.0 g. of non-labeled DFP in 3.0 ml anhydrous isopropanol was added. The mixture was incubated for 90 minutes at 35\(^\circ\). The inactive, radioactively labeled protein was precipitated with ammonium sulfate and the precipitate was thoroughly dialyzed against several changes of distilled water and aliquots were plated on aluminum planchettes and counted. After correction for decay and scattering effects, 48% of the original radioactivity was found to be incorporated into the protein. No attempt was made to run phosphorous analysis on the radioactive protein.

For comparative purpose DIP-32-papain and DIP-32-bromelain were prepared by similar procedures.

The radioactive derivatives were oxidized with performic acid, then hydrolyzed with trypsin. Aliquots of the tryptic digests were subjected to two-dimensional paper chromatography and the resulting chromatograms were examined by ninhydrin spray and radioautography. As shown in Figure 4, the fingerprint patterns obtained from tryptic hydrolysate of DIP-32-chymopapain and DIP-32-papain were identical, but that from DIP-32-bromelain differed.

The rest of the tryptic hydrolysate of DIP-32-chymopapain was separated by column chromatography on Dowex-50X2 which had had been equilibrated with ammonium formate, pH 3.0. Stepwise elution was carried out beginning with 0.01 M formic acid and
FIGURE 4.
Fingerprint patterns of typtic digest of DIP-32-chymopapain, DIP-32-papain and DIP-32-bromelain. (1) High voltage paper electrophoresis in pyridine-acetate buffer (pH 6.5) at 1 kv. for 1.5 hours. (2) Paper chromatography in Pyridine/Isoamyl Alcohol/Water (3/3/3.5). The chromatograms were examined by ninhydrin spray (0.2% in acetone) and by radioautography. Represented are the ninhydrin-positive, radioactive spots.
ending with 0.05 M NH₄OH. Most of the radioactivity came through with 0.01 M formic acid as shown in Figure 5.

With 0.05 M NH₄OH, a dark yellow radioactive eluate was obtained (Fraction H). This fraction turned out to be very heterogeneous, and when subjected to paper chromatography, over 90% of the ninhydrin-positive material from this fraction remained at the origin in all of the solvent systems tried. The amino acid composition of the insoluble components from fraction H indicated that this fraction maybe an undigestible "core" of the DIP-chymopapain. However, a radioactive fraction (H₄c) was isolated from fraction H by a combination of high voltage electrophoresis and paper chromatography (Figure 6.). Amino acid composition indicated a dipeptide, Ser (0.98), Gly (1.00). NH₂-terminal analysis by the FDNB method showed that DNP-Ser was the main component found after hydrolysis of the dinitrophenylated peptide in 6 N HCl for 24 hours. A yellow spot which corresponds to that for DNP-O-phosphoserine was also detected (Figure 7).

The fractions eluted by 0.025 M formic acid were pooled and further purified by high voltage electrophoresis and paper chromatography. The main radioactive component obtained from fraction had an R_f=0.1 in butanol-HOAc-Water (4:1:5), and had the composition Lys (1.0), CySO₃H and/or P-Ser* (1.63),

*Both CySO₃H and P-Ser appear at the same position on the Spinco amino acid analyzer under the condition used for this experiment, thus making it difficult to quantitate each residue.
FIGURE 5
Chromatography of the tryptic digest of DIP-32-Chymopapain on Dowex-50X2 (200-400 mesh), NH₄⁺ form, 50 x 2.6 cm. Stepwise elution was carried out, beginning with 0.01 M formic acid. Represented are the radioactive fractions which were eluted with 0.025 M formic acid, 0.50 M ammonium formate (pH 5.0) and 0.10 M NH₄OH.
Tryptic Digest of DNAChymotryptic

Reductase

100 200 300 400 (nm)

Fraction No.
FIGURE 6.
Purification of the DIP-chymopapain peptide H4c. The radioactive fraction H eluted from Dowex-50 with 0.05 M NH₄OH was subjected to (1) high voltage paper electrophoresis in pyridine-acetate buffer, (pH 6.5) at 1 kv. for 2 hours, followed by paper chromatography in (2) Pyridine/Isoamyl alcohol/Water (3/3/3.5) for 18 hours and (3) n-Butanol/Acetic acid/Water (4/1/5) for 18 hours. The cross-hatched spots represent radioactive fractions.
FIGURE 7.

NH$_2$-terminal analysis of peptide fraction H4c. The dinitrophenylated peptide was hydrolyzed in 6 N HCl for 24 hours at 105°. The solvent systems used were: (1) Tertiary-amyl alcohol/3% NH$_3$ and (2) 1.5 M phosphate buffer (pH 6). The yields (micromoles) of spot 2 (DNP-serine), spot 1 (DNP-O-phosphoserine) were estimated from the absorbance readings at 360 mp (millimolar extinction, 17x10$^3$) to be 0.024 and 0.007 respectively.
Asp (1.0), Thr (0.95), Ser (1.0), Glu (2.1), Gly (3.1), Val (1.0).

The yield of the peptide was 0.5 μmoles or less than 1% of the original amount of protein used. Lack of sample prevented further study beyond the preliminary amino acid composition of the peptide.

2. The Effect of DFP on the SH Group(s) of Chymopapain:

Concurrent with our studies on the DFP inhibition of chymopapain Liener and co-workers (1963, 1965) reported that commercial samples of DFP contained a contaminant which inactivated ficin, a protease isolated from the fig plant, by irreversibly combining with its essential SH group.

Since the requirement of one or more free SH groups in chymopapain for activity had been established, it was necessary to check the effect of DFP, not only on the enzymic activity of chymopapain, but also on the SH content of chymopapain. Chymopapain was treated with varying concentrations of DFP (Aldrich, Lot No. K 230) and the corresponding changes in enzymatic activity and SH content were determined. As shown in Table I, Aldrich's DFP did cause a loss in SH content of chymopapain which paralleled the loss in activity.

3. Phosphorylation of Chymopapain by DFP:

Despite the effect of DFP on the SH group of chymopapain, the fact still remained that chymopapain was phosphorylated by DFP as indicated by the isolation of crystalline DIP-chymopapain by Ebata and the incorporation of P-32 into chymopapain.
Thus the preparation of DIP-chymopapain was repeated using non-labeled DFP. (The preparative procedure is summarized in Table II).
### TABLE I

THE EFFECT OF DFP ON SH CONTENT AND ENZYMIC ACTIVITY OF CHYMOPAPAIN

<table>
<thead>
<tr>
<th>(DFP) W/L</th>
<th>DFP / E (mole/mole)</th>
<th>% Inhibition</th>
<th>-SH / E (mole/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 0</td>
<td>--------</td>
<td>0</td>
<td>1.08</td>
</tr>
<tr>
<td>5 x 10^{-6}</td>
<td>1.1</td>
<td>14</td>
<td>1.08</td>
</tr>
<tr>
<td>1 x 10^{-5}</td>
<td>2.3</td>
<td>17</td>
<td>1.07</td>
</tr>
<tr>
<td>5 x 10^{-5}</td>
<td>11.5</td>
<td>24</td>
<td>0.99</td>
</tr>
<tr>
<td>5 x 10^{-4}</td>
<td>115.0</td>
<td>57</td>
<td>0.44</td>
</tr>
<tr>
<td>1 x 10^{-3}</td>
<td>230.0</td>
<td>72</td>
<td>0.26</td>
</tr>
<tr>
<td>5 x 10^{-3}</td>
<td>1000.0</td>
<td>79</td>
<td>----</td>
</tr>
</tbody>
</table>

1.0 ml (1.97 mg) of chymopapain in 0.05 M cacodylate buffer (pH 7.2) was activated with 1.0 ml of 5 x 10^{-2} M NaCN for 60 min. at 35°. The activated enzyme solution was diluted to 15 ml (final enzyme concentration = 0.13 mg ml^{-1}) with 0.05 M cacodylate buffer. 1.0 ml fractions of the diluted enzyme solution was treated with varying concentrations of DFP and incubated for 90 min. at 35°. Aliquots were taken from each reaction mixture and the residual enzymic activity determined by casein digestion. The rest of the samples were titrated with excess CMB (2.8 cc in 0.33 M acetate buffer, pH 4.6). The SH content was determined from the absorbancy reading at 255 μm using \( E_m = 5.80 \times 10^3 \).
TABLE II

PREPARATION OF DIP-CHYMOPAPAIN

Chymopapain B (1.57 gs) dialyzed against 0.05 M cacodylate buffer (pH 7.2)

1.0 M NaCn was added (final cyanide concentration = 2 x 10^{-3} M). The reaction mixture was incubated for 60 minutes at 37\(^\circ\).

Cyanide activated chymopapain

1.0 g DFP (Aldrich, DFP/enzyme, 100/1, mole/mole) in 53 cc anhydrous isopropanol was added. The mixture was incubated for 90 minutes at 37\(^\circ\).

DFP-treated chymopapain

Residual activity was checked by the casein digestion method. 74% of the original activity was still detected. The solution was dialyzed for 24 hours in the cold against several changes of 0.05 M cacodylate buffer, pH 7.2.

The dialyzed enzyme solution was reactivated with 2.7 x 10^{-3} M cyanide.

1.0 g DFP was added. The mixture was incubated for 90 minutes at 37\(^\circ\).

DFP-treated chymopapain

Residual activity was 20% of the original. The mixture was lyophilized and redissolved in minimal amount of water and thoroughly dialyzed in the cold against several changes of deionized water.

Dialyzed DIP-chymopapain
The purpose of re-isolating DIP-chymopapain was three-fold: First, to re-determine the stoichiometry of phosphorus incorporation; second, to determine the site of phosphorylation; and third, to determine the amino acid composition, and if possible, the amino acid sequence of the peptide containing the phosphorylated residue.

In order to show that the phosphorus being determined was in the protein bound form, and not due to the excess DFP, an aliquot of the DIP-chymopapain was exhaustively dialyzed against deionized water, then precipitated by saturation with ammonium sulfate. The resulting precipitate was dissolved in a small amount of water and dialyzed against deionized water. An aliquot from the dialysate was analyzed for organic phosphorus content. The remainder of the dialysate was re-precipitated with ammonium sulfate, redialyzed and an aliquot was again taken for phosphorus analysis. This process of precipitation, dialysis, and phosphorus analysis was repeated until the value of the organic phosphorus content of the DIP-chymopapain became constant 0.89 moles of phosphorus per 30,000 gs of protein. This is in good agreement with Ebata's result (1963) of 0.85-0.94 moles per 34,000 gs of protein.

4. Difference Spectrum of Diisopropylphosphoryl-chymopapain Versus Chymopapain:

Difference spectra of diisopropylphosphoryl-\(\alpha\)-chymotrypsin (DIP-\(\alpha\)-chymotrypsin) versus chymotrypsin, and of DIP-trypsin versus trypsin have been studied by Hess and co-workers (1963) who showed that these difference spectra are
brought about by the changes in the environment around the tyrosine and tryptophan residues in the molecule caused by the conformational changes in the protein molecule. These conformational changes in turn are induced by the specific phosphorylation of chymotrypsin or trypsin by DFP. If chymotrypsin or trypsin is replaced by its zymogen, no difference spectrum was observed, thus indicating that the difference spectrum is not caused by an unspecific reaction of DFP with the side chains of trypsin or chymotrypsin.

Analogous experiments were carried out with cyanide-activated, DFP-treated chymopapain versus cyanide-activated chymopapain. As shown in Figure 8, a difference spectrum appeared at 290 and 278.2 μ. But no difference spectrum was observed with non-activated, DFP-treated chymopapain versus non-activated chymopapain. Our preliminary studies on DFP inhibition of chymopapain (Ebata, 1963) showed that non-activated chymopapain was not affected by DFP. Thus, it is assumed that the difference spectrum observed for the activated, DFP-treated chymopapain versus activated chymopapain is caused by the specific phosphorylation of active chymopapain by DFP. The data obtained from this experiment also showed that the DFP sample used was contaminated with some substance which absorbs beyond 260 μ.

5. The Isolation and Identification of O-phosphoserine:

31 mg (1 micromole) of DIP-chymopapain was thoroughly dialyzed, dried, and hydrolyzed in 2 N HCl for 24 hours. An aliquot of the hydrolysate was analyzed for phosphoserine on
FIGURE 8.

Ultraviolet difference spectra: DFP-treated, cyanide-activated chymopapain read against cyanide-activated chymopapain (-----). 1.0 ml of cyanide (final concentration, 0.1 M) was added to 1.0 ml of chymopapain (1.60 mg of protein) in 0.1 M cacodylate buffer (pH 7.2), and the mixture was incubated for 60 minutes at 35°. Then 0.1 ml of DFP in isopropanol was added to the reaction mixture and the final volume was made to 3.0 cc with 0.1 M cacodylate buffer (Aldrich DFP, final concentration, 3.3 x 10^{-3} M, DFP/enzyme, 188). After further incubation at 35° for 30 minutes, difference spectrum was read against control consisting of 2.0 ml of cyanide-activated chymopapain and 1.0 ml of buffer.

DFP-treated, non-activated chymopapain read against non-activated chymopapain (-----). 1.0 ml of chymopapain and 1.0 ml of buffer was preincubated for 60 min. at 35°; DFP was added, incubation was continued for 30 minutes and the difference spectrum read against non-activated chymopapain.

DFP vs. buffer (-----). 0.1 ml DFP in 2.9 ml buffer read against 3.0 ml buffer.

All readings were made on a Cary Model 14, Self-Recording Spectrophotometer.
The Spinco automatic amino acid analyzer. As shown in Figure 9 (b), a peak which corresponded to that for the standard O-phosphoserine was found. (Since the DIP-chymopapain had not been oxidized by performic acid at this point, this peak could not have been that for cysteic acid). The amount of phosphoserine detected was equal to 0.026 micromoles (based on color factor of 11.5 calculated from the analysis of standard O-phosphoserine) per 0.060 micromoles of DIP-chymopapain (dry weight).

The remainder of the acid hydrolysate was dried, redissolved in 2.0 ml of deionized water and placed on a column of Dowex 50X8 in the $H^+$ form and eluted with 0.05 N HCl. As shown in Figure 10, the phosphorus containing, ninhydrin-positive fraction appeared between 10 to 20 ml of the eluant. This fraction was designated as Fraction A. A 3.5-ml aliquot of Fraction A was quantitatively analyzed on the automatic amino acid analyzer. The only peak found was that corresponding to 0.055 micromoles of phosphoserine (Figure 9c).

As a further confirmation of the presence of phosphoserine, aliquots of Fraction A were co-chromatographed with standard O-phosphoserine in three different solvent systems. The results shown in Figure 11, provided direct evidence for the presence of the phosphoserine residue in DIP-chymopapain.

6. Isolation and Partial Characterization of the DIP-peptide:

1.14 gs of DIP-chymopapain was oxidized with performic acid and hydrolyzed with trypsin for 24 hours. The DIP-peptide was isolated from the digestion mixture according to the procedure summarized in Table III.
The amino acid composition of the isolated peptide after 24-hour and 48-hour hydrolysis in 6 N HCl was:

Phosphoserine + CyCO₃H (2.0), Asp (1.0), Thr (0.71), Ser (0.96)
Glu (1.08), Gly (1.80), Ala (0.67) Lys (1.03).
FIGURE 9.

Chromatogram of O-phosphoserine on Column 3 (0.9 x 48 cm column) of Spinco model 120 automatic amino acid analyzer. Operating conditions were: Temperature, 60°; Flow rate, 45 ml per hour; Buffers, pH 3.25, 0.2 N sodium citrate with a change to pH 4.25, 0.2 N sodium citrate at 67 ml (89 minutes).

(a) Standard O-phosphoserine, 0.25 μmoles.

(b) Amino acid analysis of 2 N HCl-hydrolysate of DIP-chymopapain.

(c) Amino acid analysis of Fraction A obtained from Dowex-50X8 chromatography of the 2 N HCl-hydrolysate of DIP-chymopapain.

The arrow denotes the point at which buffer change took effect.
Isolation of O-phosphoserine from 2 N HCl hydrolysate of DIP-chymopapain. 30 mg of DIP-chymopapain was hydrolyzed in 2 N HCl for 24 hours at 105°. The hydrolysate was dried, redissolved in 2 ml of deionized water and charged on a column of Dowex-50X8 (H+ form) 1.7 x 14 cm. Fractions of 3 ml each were eluted with 0.05 N HCl. Ninhydrin analysis (-----) and phosphorus analysis (-----) were run on each tube.
MILLILITERS OF 0.05N HCL

Δ OBSERVED

0.00 0.05 0.10 0.15 0.20

0.00 0.05 0.10 0.15 0.20

MILLILITERS OF 0.05N HCL
FIGURE 11.

Paper chromatography of Fraction A: Identification of O-phosphoserine. Aliquots of Fraction A (phosphorus-positive fraction from Dowex-50 chromatography of 2 N HCl hydrolysate of DIP-chymopapain) were co-chromatogrammed with standard O-phosphoserine in three different solvent systems: (1) n-Butanol/Acetic acid/H₂O (4/1/5); (2) Ethanol/NH₃/H₂O (36/3/61); (3) Ethanol/NH₃/H₂O (8/1/1). The spots were identified by the use of ninhydrin spray (0.2% in acetone) and phosphorus spray (Hanes, et al., 1949).
TABLE III

ISOLATION OF THE PHOSPHOPEPTIDE FROM TRYPTIC DIGEST OF DIP-CHYMOPAPAIN

<table>
<thead>
<tr>
<th>Description</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptic digest of performic acid oxidized DIP-chymopapain</td>
<td></td>
</tr>
<tr>
<td>Dowex-50X2 (200-400), NH$_4^+$ form (2.4x55 cm)</td>
<td>eluted with 0.01 M formic acid. 10-ml fractions collected.</td>
</tr>
<tr>
<td>Fraction 1 (Tube No. 13-22, 100 ml, ninhydrin-positive, organic phosphorus-positive)</td>
<td></td>
</tr>
<tr>
<td>Rechromatographed on Dowex 50X2, H$^+$, (1.8x40 cm) eluted with 0.05 N HCl.</td>
<td>7.0-ml fractions collected.</td>
</tr>
<tr>
<td>Fraction 1-1. (Tube No. 3-7, 35 ml, ninhydrin-positive, organic phosphorus-positive)</td>
<td></td>
</tr>
<tr>
<td>Paper chromatography in n-Butanol/HOAc/H$_2$O (4/1/5).</td>
<td></td>
</tr>
<tr>
<td>Band-2 ($R_f=0.17$, ninhydrin-positive, yellowish-blue with ammonium molybdate spray)</td>
<td></td>
</tr>
</tbody>
</table>
NH$_2$-terminal analysis by the FDNB method indicated that one residue of either phosphoserine or CySO$_3$H had disappeared upon the analysis of the aqueous phase. Chromatography of the ether phase in the solvent system, tertiary-amyl alcohol/3% NH$_3$/1.5 M phosphate buffer (pH 6), showed that the main yellow spot corresponded to that for the synthetic DNP-O-phosphoserine, Figure 12b.

The NH$_2$-terminal residue was checked also by the subtractive Edman procedure. After the first cyclization, 0.71 residues of phosphoserine or CySO$_3$H had disappeared. Attempt to identify the PTH-amino acid was not conclusive because of the lack of standard PTH-derivative of O-phosphoserine. However, when chromatographed in solvent A a clear spot ($R_f$=0.05) which turned bluish-yellow upon spraying with ammonium molybdate spray was detected. Based on this result, and that from the FDNB run, the NH$_2$-terminal residue of the DIP-peptide isolated was assumed to be the phosphorylated serine itself.

When the peptide was incubated with carboxypeptidase B for 2 hours, 0.022 micromoles of lysine was released per 0.036 micromoles of the peptide. This is in agreement with the specificity of trypsin which cleaves bonds on the carboxyl end of lysine and arginine.

7. Discussion:

Although it was determined that DFP phosphorylates activated chymopapain to the extent of approximately one mole of phosphorus per mole of enzyme and that the site of phosphorylation is the hydroxyl group of a serine residue, the results
of the present study did not show that this phosphorylation is necessarily the cause of the inactivation of chymopapain by DFP. In fact, the loss of the reactive SH group and the parallel loss of enzymic activity with increasing concentrations of DFP indicated that inactivation is more than likely due to the loss of the essential sulfhydryl group.

Studies involving the ultraviolet difference spectrum of the activated, DFP-treated chymopapain versus activated chymopapain indicated that the activation of the enzyme by reducing agents such as cyanide leads to conformational change in the protein molecule. This conformation change was shown by the appearance of a group reactive to DFP upon activation. Whether this conformation change also exposes that portion of the protein molecule containing the reactive SH groups is not definitely known. This could be the case since activation increases the SH titer as well as the specific activity of chymopapain (Kunimitsu, 1964).

It also appears that chymopapain does not resemble the other "serine proteases" such as trypsin and chymotrypsin in the amino acid sequence around the phosphorylated serine residue. The common peptide sequence, Gly-Asp(or Glu)-Ser-Gly-, which occurs around the specific serine in the active center of the "serine proteases" does not seem to occur in chymopapain. Since trypsic digestion yielded a peptide with NH₂-terminal phosphoserine, the amino acid residue adjacent to the phosphorylated serine could not have been an aspartic acid or glutamic acid because of the specificity of tryptic cleavage.
FIGURE 12.
The NH$_2$-terminal residue of the phosphopeptide: Identification of DNP-O-phosphoserine.

(a) Two dimensional paper chromatogram of synthetic DNP-O-phosphoserine. (0.1 μmole spotted).

(b) Two dimensional paper chromatogram of the ether-extractable DNP-amino acid from 6 N HCl hydrolysate of DNP-phosphopeptide. The main yellow spot (cross-hatched) corresponds approximately to 0.05 umoles of DNP-amino acid as estimated from the absorbancy reading at 360 μm.

(c) Rechromatography of the major yellow spot from (b) in 1.5 M phosphate buffer, pH 6. The yellow spot was cut from the chromatogram, eluted with 3% NH$_3$, the eluate acidified and re-extracted into the ether phase. This ether fraction (6) was co-chromatogrammed with the following standard samples: (1) DNP-OH, (2) DNP-CySO$_3$H, (3) DNP-aspartic acid, (4) DNP-glutamic acid, (5) DNP-O-phosphoserine.
The amino acid adjacent to the phosphoserine on the carboxyl side could not be identified by the phenylisothiocyanate method. However, in an earlier run with the radioactive DFP, a radioactive dipeptide fragment with the sequence, Ser-Gly was found indicating that the DIP-peptide may have the partial structure:

-P-Ser-Gly-(Cys, Asp, Thr, Ser, Glu, Gly, Ala)-Lys-.

C. Role of Histidine in Active Center of Chymopapain

1. Photooxidation Studies:

Methylene-blue catalyzed photooxidation experiments were carried out on CMB-chymopapain as previously described under Methods. Aliquots were removed from the reaction mixture at various time intervals and the residual enzymatic activity was determined both by casein digestion and by the hydrolysis of BAA. Amino acid compositions of the photooxidized protein at various time intervals were determined on the Spinco model 120 amino acid analyzer. Tryptophan content was determined by the procedure of Goodwin and Morton (1946).

For comparative purpose similar photooxidation studies were carried out on CMB-papain and CMB-bromelain.

The results of the changes in the histidine content for the three enzymes are given on Table IV. According to the studies of Weil et al., (1951, 1952, 1953) on the relative rates of disappearance of amino acids during methylene-blue catalyzed photooxidation, histidine was found to be the most sensitive, followed by tyrosine and tryptophan. Because of
the experimental errors present in the determination of cysteine, methionine and tyrosine by the chromatographic method used and in the determination of tryptophan by the ultraviolet spectrum, no attempt will be made to interpret the results obtained from this preliminary photooxidation studies except in terms of the loss of histidine residues with corresponding changes in enzymatic activity.

The original histidine content of the untreated chymopapain was three residues of histidine per molecular weight of 30,000. After two minutes of photooxidation all three residues of histidine were no longer detected. The corresponding changes in the enzymatic activity after varying periods of photooxidation are given in Figure 13(a). Chymopapain still retained 50% of the initial enzymatic activity at two-minute photooxidation time, at which point there was a complete loss of the three histidine residues. Even after one hour of exposure to light and methylene blue, 20% of the original enzymatic activity could still be detected.

Bromelain lost its sole histidine residue after 2 minutes of photooxidation. But, like chymopapain, it still retained 50% of the original activity at this point and 20% of the initial activity after one hour. Figure 13(b).

For papain, one of its two histidine residues was lost after 30 minutes along with 6 of the tyrosine residues. At this point, papain was found to be completely inactivated. Figure 13(c).
<table>
<thead>
<tr>
<th>Photooxidation Time (minutes)</th>
<th>Residues of Histidine per Mole of Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>Chymopapain 3.25  Bromelain 1.0  Papain 2.0</td>
</tr>
<tr>
<td>0</td>
<td>Chymopapain 2.80  Bromelain ---  Papain ---</td>
</tr>
<tr>
<td>2</td>
<td>Chymopapain 0.00  Bromelain 0.00  Papain ---</td>
</tr>
<tr>
<td>10</td>
<td>Chymopapain 0.00  Bromelain 0.00  Papain ---</td>
</tr>
<tr>
<td>30</td>
<td>Chymopapain 0.00  Bromelain 0.00  Papain 1.0</td>
</tr>
<tr>
<td>60</td>
<td>Chymopapain 0.00  Bromelain 0.00  Papain ---</td>
</tr>
</tbody>
</table>
FIGURE 13(a).
Changes in enzymic activity of photooxidized chymopapain. Ali­
quotes were removed from the reaction mixture at various time
intervals, filtered through a small column of Dowex-50X2(H+ form)
to remove the methylene blue and reactivated with 2,3-dimercapto­
propanol (BAL). BAA hydrolysis (a) was carried out in 0.1 M
phosphate buffer (pH 6.5) and casein digestion (b) in 0.1 M
phosphate buffer (pH 7.2) as described in the text. Proper con­
trol were run to determine the effects of light alone and
methylen blue alone on enzymic activity.
FIGURE 13(b).

Changes in enzymic activity of photooxidized bromelain. Conditions and procedures used were same as those of photooxidation of chymopapain.

(a) BAA hydrolysis. (b) Casein digestion.
FIGURE 13(c).

Changes in enzymic activity of photooxidized papain. Only casein digestion was used to assay for the residual activity after photooxidation. Otherwise, the conditions and procedures used were same as those for chymopapain.
2. Discussion of the Results:

The various theories proposed for the mechanism of action of trypsin and chymotrypsin involve the concerted action of the specific serine residue with the histidine residue (Cunningham, 1957; Westheimer, 1957; Spencer and Sturtevant, 1959; Bruice, 1961, Whitaker and Bender, 1965).

The results of the photooxidation studies on chymopapain showed that although all of the histidine residues were destroyed, the enzyme still retained 50% of the initial enzymic activity. This loss of half of the original activity could be due to factors such as the alteration of the secondary and tertiary structure of the enzyme and the irreversible oxidation of the essential SH group (even though the CMB-derivative was used to protect the SH group, this protection may not have been adequate). Whatever the cause of the partial loss of enzymic activity, the important point here is that histidine appears to be non-essential for the action of chymopapain. This result, along with the finding on DFP inhibition, suggest that chymopapain is not a typical "serine protease" in the same category as trypsin and chymotrypsin.

D. The Sulphydryl Group(s) of Chymopapain

1. The Reaction Between DDPM and Chymopapain:

In order to label the essential SH group(s) in chymopapain and to facilitate the isolation of the cysteinyl-peptide, the colored derivative of N-Ethylmaleimide, N-(4-dimethylamino-3,5-dinitrophenyl)-maleimide (DDPM), was used. This reagent was
first used successfully by Witter and Tuppy for the isolation and investigation of the cysteine-containing peptides from human and bovine serum albumin (1960). Because of the yellow color of the reagent, the labeled peptide can be identified visually during purification. Furthermore, the DDPS-peptides are readily adsorbed on talc from aqueous solutions, making it possible to separate the colored peptide from the bulk of the non-essential peptides in one step.

a. Titration of Chymopapain with DDPM.

Chymopapain was activated with $1 \times 10^{-2}$ M NaCN for 30 minutes and then passed through a multibed resin consisting of Dowex-1 (OH$^-$ form) followed by Dowex-50WX8 (H$^+$ form) and finally of Sephadex G-50 at the bottom. The resulting activator-free chymopapain was immediately reacted with 0.00-0.60 ml of DDPM (0.01% mixture of equal parts of 95% ethanol and 0.33 M acetate buffer, pH 4.6). The mixtures were left at room temperature for four hours, then aliquots were taken and diluted to proper enzyme concentration (about 100 μg ml$^{-1}$) for the determination of residual enzymatic activity. The remainder of the reaction mixtures was titrated with excess CMB to determine the amount of unreacted SH remaining. Figure 14 shows the plot of the amount of DDPM added versus the amount of enzymic activity and SH groups remaining. The control sample contained in place of the DDPM, 0.60 ml of the solvent used to dissolve the DDPM.
FIGURE 14.
Titration of chymopapain with DDPM. 3.0 ml of chymopapain (11.2 mg ml\(^{-1}\)) was activated with NaCN (final concentration, \(1 \times 10^{-3}\) M). The mixture was passed through a multibed resin (Dowex 50X8, Dowex-1, Sephadex-G-25) to remove excess CN\(^-\). To test tubes containing 1.0 ml each of the activated chymopapain solution (protein concentration, 2.8 mg ml\(^{-1}\)) were added 0.00-0.60 ml of DDPM (0.00-0.42 micromoles, DDPM/protein = 0-4.5). The reaction mixture was allowed to stand at room temperature for 4 hours. Aliquots of 0.2 ml were then taken from each tube, diluted to 2.0 ml with 0.1 M cacodylate buffer, pH 7.2, and assayed for residual enzymic activity. Remainder of the solution was titrated with excess CMB in 0.33 M acetate buffer, pH 4.6. Concentration of SH remaining calculated from \(E_M\) at 255 m\(\mu\) equal 5.8 \(\times\) \(10^3\). (-----) residual activity, and (-----) residual SH content were plotted against the moles of DDPM added per mole of chymopapain. The arrow denotes the point at which 0.6 equivalents of DDPM was taken up. (See section C-1b, results, for details).
EQUIVALENTS OF OOPM AODED
FIGURE 15.
Rate of reaction of chymopapain with DDPM. To cyanide-activated chymopapain (enzyme concentration, 1.27 mg ml⁻¹) was added a 10-fold molar excess of DDPM (0.43 mg) dissolved in 50% ethanol-2% HOAc. The reaction was allowed to proceed at room temperature. Aliquots were removed at various time intervals and titrated with 3.0 ml of CMB in 0.33 M acetate buffer, pH 4.6. Residual SH content was calculated from $E_M$ at 255 nm $= 5.8 \times 10^3$. At the same time intervals, 0.2 ml aliquots were also removed, diluted to 2.0 ml with 0.1 M cacodylate buffer, pH 7.2 and residual activity was determined by the casein digestion method.
Per cent of the original SH remaining (-----) and per cent inhibition (-----) at various time intervals were plotted.
The initial specific activity of the cyanide-activated chymopapain was 2.0; the initial SH content was 1.22 moles SH per mole of protein.
FIGURE 16.
Absorption spectra of chymopapain (▲ ▲▲), DDPS-chymopapain (---), DDPS-cysteine (-----) and DDPM (----). Chymopapain (1 x 10^{-5} M) was dissolved in 0.1 M cacodylate buffer, pH 7.2, (enzyme concentration, 0.28 mg/cc). DDPS-chymopapain (1 x 10^{-5} M) was prepared by treating the enzyme with 10-fold molar excess of DDPM, by precipitation with ethanol and by thoroughly dialyzing as described in the text. DDPS-cysteine (2 x 10^{-4} M) was prepared according to the method of Tuppy (1960), and dissolved in 50% ethanol-2% HOAc solvent (final concentration, 2.20 x 10^{-4} M). DDPM (2 x 10^{-4} M) was dissolved in 50% ethanol-2% HOAc solvent. All readings were made on Cary Model 14 Self-Recording Spectrophotometer.
When the amount of SH group was decreased to 0.3 moles per mole of chymopapain, the enzyme had lost all of its enzymic activity. This break in the titration curve also corresponds to 0.60 equivalents of DDPM taken up by the enzyme which represents approximately 50% of the SH originally detected by CMB titration of the activated chymopapain. This value of the equivalents of DDPM taken up was estimated from the absorbance reading at 424 μm and the calculated value of the absorbancy index at 424 μm for DDPM. (For details, see section on the absorption spectra of DDPM and its derivatives).

The rate of reaction of chymopapain with DDPM was followed by incubating the enzyme with 10-fold molar excess of DDPM at room temperature and checking the residual enzymic activity and the corresponding changes in SH content at various time intervals. The results are presented in Figure 15. It is seen that immediately upon the addition of the DDPM, 58% of the original SH was lost along with 65% of the activity. When the enzyme was completely inactivated (about 40 hours), 20% of the original SH group could still be detected. Since the SH titer of cyanide-activated chymopapain immediately after passage through the multibed resin column was 1.22 for this experiment, the loss of 80% of the initial SH corresponds to the loss of about one mole of SH per mole of enzyme, at which point there was a concomitant loss of all enzymic activity.

The absorption spectra of DDPM, DDPS-cysteine, DDPS-chymopapain and chymopapain, measured on the Cary Model 14 spectrophotometer, are shown in Figure 16.

The absorbancy index of DDPM at its absorption maximum (424 m\(\mu\)) was calculated to be 1.35x10^{-3}. This value was used to estimate the equivalents of DDPM incorporated into chymopapain at the point on the DDPM-chymopapain titration curve (Figure 14) at which all enzymic activity was lost.

In order to estimate the amount of DDPM incorporated into the protein during the titration of chymopapain with DDPM (Figure 14), the following experiment was run:

For each chymopapain-DDPM titration mixture a control was run using 1.0 ml of 0.05 M cacodylate buffer in place of the enzyme solution. To these buffer solutions were added 0.0-0.6 ml of DDPM and the controls were incubated for the same length of time as the titration mixtures. Then the absorbancy at 424 m\(\mu\) (absorbancy maximum for DDPM) was read for the control samples as well as for the corresponding titration mixtures. Since neither chymopapain nor DDPS-chymopapain absorbs at this wave length (see Figure 16) the absorbance at 424 m\(\mu\) was assumed to be that due to the DDPM in solution. Therefore, the absorbance at 424 m\(\mu\) for the control is equal to the amount of DDPM added; the absorbance at 424 m\(\mu\) for the reaction mixture is equal to the amount of DDPM
left; thus

\[
\text{Absorbancy (control)} - \text{Absorbancy (reaction mixture)} = 424 \text{ m} \mu = \text{reacting with enzyme}
\]

From the standard curve of absorbancy at 424 m\(\mu\) versus the concentration of DDPM, the molar extinction coefficient for DDPM at 424 m\(\mu\) was calculated to be 1.35\(\times\)10\(^3\). This value was used to convert the difference in absorbancy readings between the control and the reaction mixture to obtain the micromoles of DDPM reacting with the enzyme.

2. Preparation of DDPS-Chymopapain:

Cyanide-activated chymopapain with specific activity of 2.0-2.5 and SH titer of 1.2-1.4 was used for all the experiments. A 5-10 fold molar excess of DDPM was added very slowly to a solution of cyanide-activated chymopapain in .01 M phosphate buffer (pH 7). The mixture was allowed to stand at room temperature for 10-12 hours, then the protein was precipitated by the addition of 5-10 fold excess (v/v) of absolute ethanol. After standing overnight in the cold, the orange-colored precipitate was collected by centrifugation, redissolved in a small amount of deionized water and the solution was dialyzed thoroughly. The yield of the DDPS-derivative was 90-97% of the original amount of protein used (rough dry weight determinations).

3. Isolation of DDPS-peptide:

The dialysate of DDPS-chymopapain was denatured by heating in a 100\(°\) water bath for 2-3 minutes. The pH of the cooled
solution was adjusted to 2 with 0.1 N HCl and pepsin, equivalent to 2% of the weight of chymopapain used, was dissolved in 0.01 N HCl and added to the protein solution. The mixture was allowed to incubate at 37° for 24 hours with periodic adjustments to pH 2 with 0.1 N HCl. The solution was then taken to dryness in a vacuum dessicator and applied to a column of Sephadex G-25 which had been washed with water. Fraction of 10-15 ml were eluted with water. Ninhydrin analysis (Moore and Stein, 1954), 280 μ and 440 μ absorbancy (absorption maximum of DDPS-cysteine) determinations were carried out on each fraction (Figure 17). The 440-μ absorbance material was eluted with the first main ninhydrin-positive peak and contained 70-80% of the 440-μ absorbing material and 20-30% of the total ninhydrin value. This fraction was pooled and subjected to a second pepsin digestion with amount of pepsin equivalent to 1% of the original weight of chymopapain. After 24 hours digestion was terminated by heating the mixture for 2-3 minutes in a boiling water bath. The digest was then applied to a talc column (2x20 cm) and washed with several volumes of water until no more ninhydrin positive material was detected in the aqueous eluate. Then, the adsorbed, orange-colored fraction was eluted with a mixture of 50% ethanol-2% acetic acid (1/1) mixture. In a typical purification step using the talc column, the orange-colored material eluted with the ethanol-HOAc eluent comprised 10-20% of the original ninhydrin positive material applied. The colored fraction was concentrated and subjected to high-voltage paper electrophoresis in pyridine-acetate
FIGURE 17.
Sephadex G-25 chromatography of first pepsin digestion mixture. Fractions of 15 ml were collected. Represented are the 440 μ absorbance, (-----), the 550 μ (ninhydrin) absorbance (-----) and the 280 μ absorbance (-.-.-). Cross-hatched bar represents the fraction pooled for second pepsin digestion.
buffer, pH 6.5, at 1.5 kv for 2 hours (Figure 18). The main yellow, ninhydrin-positive band was eluted, concentrated and subjected to descending paper chromatography with the upper phase of the solvent system consisting of n-butanol/acetic acid/water (4/1/5) (Figure 19). The two main yellow-colored bands isolated were arbitrarily designated as Peptide-3 ($R_f$ 0.35) and Peptide-2 ($R_f$ 0.30). Both fractions were eluted, concentrated and an aliquot from each was subjected to high-voltage electrophoresis at pH 4.7 (Figure 20b). Both Peptide-2 and Peptide-3 exhibited the same mobility in this buffer system, and single yellow spot was observed for each. The ionophoretic behavior of both peptide fractions at pH 4.7 indicated that they are neutral peptides.

4. Characterization of the DDPS-peptide:

a. Amino Acid Composition.

The amino acid compositions of Peptide-2 and Peptide-3 were determined on the Spinco automatic amino acid analyzer after hydrolysis in 6 N HCl for 24-hour and 48-hour periods. The results are summarized in Table V. There was no tryptophan present as determined by the colorimetric test using dimethylaminobenzaldehyde (Spies and Chambers, 1949). The presence of an intact disulfide bond in the peptide was checked with the nitroprusside and the iodide-azide reagents. Both tests were negative.

The yields of the two peptides were 3.0% for Peptide-3 and 2.7% for Peptide-2.
FIGURE 18.
Electrophoresis (pH 6.5) of talc eluate. Electrophoresis was carried out at pH 6.5 (pyridine-acetate buffer) at 1500 v., for 2 hours. The main yellow band, indicated by the arrow, was cut and eluted. The remaining bands represent non-yellow colored, ninhydrin-positive fractions.
Slightly yellow ninhydrin positive

Fast yellow, fast and slow ninhydrin

Dark yellow, brown with ninhydrin
FIGURE 19.

Paper chromatography of the major yellow band from pH 6.5 electrophoresis. Solvent system: n-Butanol/HOAc/Water (4/1/5). The chromatogram was developed for 36 hours. The two major yellow ninhydrin-positive bands, indicated by the arrows, were eluted.
FIGURE 20.

Determination of the purity of the two major yellow fractions obtained from paper chromatography.

(a) Rechromatography in the upper phase of the solvent system, Butanol/Acetic acid/Water (4/1/5). The $R_f$ values for Peptide-3 and Peptide-2 were 0.35 and 0.30 respectively.

(b) High voltage paper electrophoresis at pH 4.7 (10 ml pyridine, 10 ml glacial acetic acid, 980 ml water) for 1 hour at 1000 volts.
Peptide-3 and Peptide-2 were similar in their amino acid composition, except for the definitely higher amount of tyrosine in the former. As it will be shown in detail later, tyrosine was released from the COOH-terminal end of Peptide-3 by carboxypeptidase A followed by the slow release of DDPS-cysteine upon the addition of carboxypeptidase B. From Peptide-2, DDPS-cysteine was released with combination of carboxypeptidase A and B. Both Peptide-3 and Peptide-2 were shown to have lysine as the NH$_2$-terminal residue. Thus, it was assumed that Peptide-2 was a partial hydrolysis product of Peptide-3, and not an entirely different peptide. All sequence work was carried out on Peptide-3 since this fraction appeared to be more pure according to the amino acid composition data.

On the basis of the electrophoretic mobilities (pH 4.7) of the two peptides and of the fragments obtained from Nagarse digestion of Peptide-3 (see section D-4d) it was concluded that both the aspartyl and glutamyl residues were present as the dicarboxylic acids.

The possibility of a second half cysteine residue in the peptide was checked by subjecting the DDPS-peptide to performic acid oxidation, then determining the amino acid composition of the oxidized peptide.

If a second half cysteine residue were present in the peptide, it would be converted to cysteic acid, which is also the main degradation product of performic acid-
### TABLE V

**THE AMINO ACID COMPOSITION OF DDPS-PEPTIDES**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Peptide-3</th>
<th>Peptide-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>1.19</td>
<td>1.09</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.86</td>
<td>0.78</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>1.25</td>
<td>1.10</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.15</td>
<td>0.43</td>
</tr>
<tr>
<td>Serine</td>
<td>1.08</td>
<td>0.96</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1.14</td>
<td>1.00</td>
</tr>
<tr>
<td>Proline</td>
<td>0.95</td>
<td>0.88</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.00</td>
<td>1.10</td>
</tr>
<tr>
<td>Valine</td>
<td>0.97</td>
<td>1.00</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.81</td>
<td>0.43</td>
</tr>
<tr>
<td><em>DDPS-cysteine</em>*</td>
<td>0.94</td>
<td>1.08</td>
</tr>
<tr>
<td>(Cysteic acid)</td>
<td>(0.04)</td>
<td>(0.02)</td>
</tr>
<tr>
<td>(2-amino-2-carboxyethyl mercaptosuccinic acid)</td>
<td>(0.43)</td>
<td>(0.48)</td>
</tr>
<tr>
<td>(Half cysteine)</td>
<td>(0.18)</td>
<td>(0.34)</td>
</tr>
<tr>
<td>(Peak 2)</td>
<td>(0.29)</td>
<td>(0.24)</td>
</tr>
<tr>
<td>% Yield</td>
<td>3.0%</td>
<td>2.7%</td>
</tr>
</tbody>
</table>

*The yield of DDPS-cysteine was estimated from the sum of the amount of the various hydrolysis products (in parenthesis). The color factor of 2-amino-2-carboxyethyl mercaptosuccinic acid was calculated to be 4.13; the color factor for phenylalanine was used for the estimation of the yield of "Peak 2".*

Phenol was added to each hydrolysis mixture to minimize the loss of tyrosine due to halogenation.
FIGURE 21.

Chromatogram of DDPS-cysteine and related products on Column 3 (0.9 x 48 cm column) of Spinco model 120 automatic amino acid analyzer. Chromatography was carried out at 60° with a flow rate of 45 ml per hour. Elution was started with pH 3.25, 0.2 N sodium citrate buffer with a change to pH 4.25, 0.2 N sodium citrate buffer at 89 minutes. The arrow denotes the point at which buffer change actually took effect.

(a) Untreated DDPS-cysteine (0.12 μmoles).
(b) DDPS-cysteine which was allowed to stand in pH 8 medium for 12 hours at 37°.
FIGURE 22.
Chromatogram of (a) 6 N HCl hydrolyzed DDPS-cysteine and (b) performic acid oxidized DDPS-cysteine on Column 3 (0.9 x 48 cm column) of Spinco model 120 automatic amino acid analyzer. Elution conditions are the same as that described for Figure 21.
(a) DDPS-cysteine (0.12 µmoles) was hydrolyzed in 6 N HCl for 24 hours at 110°. The color factor, C, for 2-amino-2-carboxylethyl mercaptosuccinic acid was calculated from this chromatogram to be 4.13.
(b) Performic acid oxidation was carried out as described under Methods.
<table>
<thead>
<tr>
<th>Products</th>
<th>Molar Ratios</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hydrolysis in</td>
<td>Performic acid oxidation, followed by hydrolysis in 6 N HCl.</td>
</tr>
<tr>
<td></td>
<td>6 N HCl</td>
<td>6 N HCl</td>
</tr>
<tr>
<td>Half Cysteine</td>
<td>0.18</td>
<td>0.00</td>
</tr>
<tr>
<td>CySO_3H</td>
<td>0.04</td>
<td>0.78</td>
</tr>
<tr>
<td>2-amino-2-carboxyethyl mercapto-succinic acid</td>
<td>0.43</td>
<td>0.001</td>
</tr>
<tr>
<td>&quot;Peak 2&quot;</td>
<td>0.20</td>
<td>0.003</td>
</tr>
<tr>
<td>Total residues</td>
<td>0.94</td>
<td>0.78</td>
</tr>
</tbody>
</table>

0.06 micromoles of Peptide-3 was treated with 2.0 ml of cold performic acid reagent (9.0 cc 88% formic acid + 1.0 cc 30% H_2O_2). The mixture was allowed to stand in the freezer (-40°) for four hours, dried under nitrogen and hydrolyzed in 6 N HCl for 24 hours. The amino acid composition was determined on the Spinco amino acid analyzer.
oxidized DDPS-cysteine (Figure 22(b)). Then the sum total of cysteic acid should approach the value of 2 residues per "mole" of peptide. However, as shown in Table VI, performic acid oxidation indicated that the labeled cysteine was the only cysteine present.

b. NH$_2$-terminal Analysis.

(1) FDNB Method

The amino-terminal residues of Peptide-3 and Peptide-2 were determined by the FDNB method of Sanger as described by Fraenkel-Conrat (1955). The DNP-peptides were hydrolyzed in 6 N HCl for 16-24 hours. The ether-soluble DNP-amino acids were examined by two-dimensional paper chromatography using the upper phase of the solvent system, tertiary-amyl alcohol/3% NH$_3$ (1/1), in one direction and 1.5 M phosphate buffer, pH 6, in the other. The aqueous phase was either subjected to quantitative amino acid analysis to determine the amino acid which disappeared upon dinitrophenylation, or subjected to descending paper chromatography in tertiary-amyl alcohol/phthalate system of Blackburn and Lowther (1951).

The main yellow spot which appeared in the 2-dimensional chromatography of the ether phase from both Peptide-3 and Peptide-2 corresponded to that for di-DNP-lysine, Figure 23. Amino acid analysis of the aqueous phase indicated that lysine was the only amino acid which decreased in significant amount. An aliquot of the same aqueous phase,
when subjected to descending paper chromatography as described earlier, yielded a very faintly yellow spot which was negative to Sakaguchi's reagent and thus assumed to be a small amount of \( \varepsilon \)-DNP-lysine. It was not possible to quantitate the amount of this \( \varepsilon \)-DNP-lysine because of the very small amount of it formed.

(2) Subtractive Edman Method

The sequential arrangement of the first 4 amino acid residues at the amino-terminal end of Peptide-3 was determined by the subtractive Edman procedure (Konigsberg, 1962). After the extraction of the PTH-amino acid into the organic phase, the remaining aqueous phase was dried, re-suspended in a known volume of deionized water from which an aliquot was withdrawn, dried, dissolved in 6 N HCl and hydrolyzed for 24 hours at 110\(^\circ\). The hydrolysate was subjected to quantitative amino acid analysis on the automatic amino acid analyzer. No attempt was made to clean up the aqueous phase by passage through a Dowex-50 column because the nature of the peptide was such that the peptide was not readily eluted from the resin. The results of the 5 cycles of the PTC reaction are summarized in Table VII. The direct identification of the PTH-amino acid was attempted with solvent A (70 ml heptane and 30 ml pyridine) and C (40 ml heptane, 40 ml n-butanol, 20 ml formic acid) were utilized. The tracings of the chromatographic patterns are shown in Figure 24.
FIGURE 23.

\( \text{NH}_2 \)-terminal analysis of Peptide-3 by the FDNB method. Peptide-3 (0.1 \( \mu \)moles) was reacted with FDNB and hydrolyzed in 6 N HCl as described under Methods. The ether-soluble extract of the hydrolysate was chromatogrammed in the solvents systems: (1) Tertiary amyl alcohol/3% \( \text{NH}_3 \) (1/1, v/v) and (2) 1.5 M phosphate buffer, pH 6. Spot 1 (0.033 \( \mu \)moles, uncorrected) corresponded to di-DNP-lysine. Spots 2 and 5 were dinitrophenol and dinitroaniline respectively. The color yields of spots 3 and 4 were less than 1% of that for and the spots were not identified. Spot 6, red in color, did not correspond to any known DNP-amino acid, and was assumed to be a degradation product of DDPS-cysteine.
### TABLE VII

**FRACTION OF AMINO ACIDS REMAINING AFTER FIVE CYCLES OF THE EDMAN DEGRADATION**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>1st</th>
<th>2nd</th>
<th>3rd</th>
<th>4th</th>
<th>5th</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>0.36</td>
<td>0.18</td>
<td>0.25</td>
<td>0.17</td>
<td>0.14</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.81</td>
<td>0.10</td>
<td>0.15</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Valine</td>
<td>0.93</td>
<td>0.75</td>
<td>0.35</td>
<td>0.27</td>
<td>0.28</td>
</tr>
<tr>
<td>Proline</td>
<td>0.98</td>
<td>0.89</td>
<td>0.70</td>
<td>0.29</td>
<td>0.26</td>
</tr>
<tr>
<td>DDPS-cysteine</td>
<td>1.00</td>
<td>0.92</td>
<td>1.06</td>
<td>1.05</td>
<td>0.97</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.95</td>
<td>0.93</td>
<td>0.94</td>
<td>0.91</td>
<td>0.86</td>
</tr>
<tr>
<td>Serine</td>
<td>1.00</td>
<td>0.97</td>
<td>0.85</td>
<td>0.84</td>
<td>0.76</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1.06</td>
<td>0.96</td>
<td>0.96</td>
<td>0.96</td>
<td>0.96</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.91</td>
<td>0.84</td>
<td>0.70</td>
<td>0.64</td>
<td>0.62</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.56</td>
<td>0.42</td>
<td>0.48</td>
<td>0.54</td>
<td>0.44</td>
</tr>
</tbody>
</table>
FIGURE 24.

Direct identification of PTH-amino acids released from Edman degradation of Peptide-3 using Solvent C (40 ml heptone, 40 ml n-butanol, 20 ml formic acid). Development time was 6 hours following a 12-hour equilibration period. Benzene-ethyl acetate extracts of PTH-amino acids were dried, taken up in small amount of acetone and spotted on Whatman No. 1 paper for descending chromatography. The chromatogram was air-dried and sprayed with iodide-azide spray containing 0.5% starch. PTH-amino acids appeared as white spots on background of brownish purple color.
From the above data, and from the result of the DNFB reaction, the amino terminal sequence of Peptide-3 was deduced to be: (Lys-Arg-Val-Pro-).

c. COOH-Terminal Analysis.

As a preliminary experiment about 0.1 μmoles of Peptide-3 was incubated with a mixture of carboxypeptidase A and B (Peptide/enzyme, 20/1, w/w) at 37°C. Aliquots were removed after one-half, six and 43 hours and subjected to ascending paper chromatography in the upper phase of the solvent system, n-Butanol/Acetic Acid/Water, for 12 hours. As shown in Figure 26, the approximate rate of release of the ninhydrin-positive spots were 1>2>3>4. Spot 2 which was yellow in color and turned brownish-purple upon spraying with the ninhydrin spray was assumed to be the unhydrolyzed DDPS-peptide minus the tyrosine residue. Spots 3 and 4 were identified as tyrosine and DDPS-cysteine respectively from co-chromatography with standard compounds. Spot 1 was later identified as the peptide from which tyrosine and DDDS-cysteine residues were removed by the exopeptidase.

For quantitative determination of the amino acids released, Peptide-3 (0.4 μmoles) was incubated with carboxypeptidase A and aliquots removed after 1 hour and 6 hours of incubation were subjected to quantitative amino acid analysis on the Spinco automatic amino acid analyzer. After 11 hours carboxypeptidase B was added and incubation was continued for a total of 24 hours. It was found that even after 11 hours of incubation with carboxypeptidase A
only 0.25 equivalents of tyrosine was released per mole of peptide. It was not possible to quantitate the amount of DDPS-cysteine and tyrosine released after the addition of carboxypeptidase B. This was because when DDPS-cysteine was allowed to stand for 1 hour or longer at pH 8 (condition used in CPAse digestion) it appeared on the amino acid analyzer chromatogram as broad peaks overlapping those of tyrosine and phenylalanine (Figure 21b). The interpretation of the quantitative amino acid composition data was further complicated by the presence of peptides in the enzymatic hydrolysis.

Carboxypeptidase A failed to release any amino acid in significant amount from Peptide-2. The addition of carboxypeptidase B and subsequent incubation for 20 hours led to the release of DDPS-cysteine as identified by paper chromatography.

The data obtained thus far suggest that in Peptide-3 tyrosine might be the COOH-terminal residue followed by the labeled cysteine. In order to confirm this result, 0.1 μmole of Peptide-3 was incubated with carboxypeptidase A and B for 3 hours. After terminating the reaction by the addition of acetic acid to pH 2, the reaction mixture was dried, taken up in 0.2 ml of deionized water and streaked on a sheet of Whatman 3MM paper and subjected to descending chromatography in n-Butanol/HOAc/H₂O (4/1/5) for 18 hours. Subsequently three ninhydin-positive bands with Rf values roughly corresponding to those for Spot 1, 2 and 3 (Figure
were eluted, hydrolyzed and analyzed for amino acid composition on the automatic amino acid analyzer. The results as presented on Table VIII.

The low yield of the tyrosine released from the carboxyl-terminal end indicate that the peptide is resistant to hydrolysis by CPAse under the conditions employed. The reason for this is not known. Gold and Segal (1964) have observed that carboxypeptidase A was unable to release DDPS-cysteine from the peptide, Ala-Ser-(DDPS-Cys)-Thr-Thr-Asn, obtained from the rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. It is possible that the presence of DDPS-cysteine adjacent to tyrosine prevents the latter from being quantitatively released by carboxypeptidase under the conditions employed. That the presence of a resistant residue adjacent to the carboxyl terminal residue prevents the latter from being liberated has been observed in a few cases, the most prominent of which is in the study of the amino acid sequence of ribonuclease (Hirs, et al., 1960). Here the presence of glutamic acid which was found to be resistant to CPAse digestion, prevented the release of serine from the sequence, Val-His-Glu-Ser-Leu.

**d. Hydrolysis with Nagarse.**

Peptide-3 (1 μmole) was dissolved in deionized water and the pH of the solution was adjusted to 7.7-7.8 with 1% NaHCO₃. To this peptide solution, 0.1 ml of 0.1% solution of Nagarse in 0.1 M phosphate buffer, pH 7.7, was added
FIGURE 25.
Carboxypeptidase digestion of Peptide-3. Peptide-3 (0.1 μmole) was incubated at 37°C with a mixture of carboxypeptidase A and B (peptide/CPAse, 20/1, w/w). Aliquots containing approximately 0.03 μmoles of peptide were removed at one-half, six and 43 hour intervals and spotted on Whatman No. 1 paper. Ascending chromatography was run in the upper phase of the solvent system, n-Butanol/HOAc/H₂O, 4/1/5, for 12 hours. The chromatogram was developed with 0.1% ninhydrin in acetone.
TABLE VIII

AMINO ACID COMPOSITIONS OF THE FRACTIONS OBTAINED FROM HYDROLYSIS OF PEPTIDE-3 WITH CARBOXYPEPTIDASE A AND B

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Micromoles</th>
<th>C-1</th>
<th>C-2</th>
<th>C-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine</td>
<td>0.00</td>
<td>0.00</td>
<td>0.054</td>
<td></td>
</tr>
<tr>
<td>DDPS-Cysteine</td>
<td>0.00</td>
<td>0.020 (0.72)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>0.013 (0.65)*</td>
<td>0.033 (1.15)</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>0.020 (1.00)</td>
<td>0.028 (1.00)</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.020 (1.00)</td>
<td>0.028 (1.00)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.020 (1.00)</td>
<td>0.028 (1.00)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>0.020 (1.00)</td>
<td>0.020 (0.72)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>0.020 (1.00)</td>
<td>0.020 (0.72)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>0.016 (0.80)</td>
<td>0.020 (0.72)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>0.016 (0.80)</td>
<td>0.020 (0.72)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

% Yield | 10 | 14 | 25

*Amino acid residues per mole of peptide.
and the mixture was incubated for 8 hours at 37\(^\circ\). The reaction was terminated by the addition of 2-3 drops of glacial acetic acid. The digestion mixture was then dried under nitrogen and the residue was dissolved in minimal amount of deionized water. A small amount of white precipitate which formed at this stage was centrifuged and discarded. The orange-colored supernatant was subjected to high-voltage paper electrophoresis in pyridine acetate buffer, pH 4.7, for 2 hours at 1000 volts (40 v./cm). Several ninhydrin positive bands were found and subsequently eluted with water. The yields of various fractions varied from 0.7-10\% of the original amount of Peptide-3 used. Where the yield of the peptide was low, the FDNB reaction was run directly on the eluate without further purification. The results are summarized in Table IX.

The evidence which led to the reconstruction of the sequence of Peptide-3 is summarized below.

\[
\text{P} \rightarrow \rightarrow \rightarrow \text{A} \quad \text{S} \quad \text{C} \quad \text{C} \quad \text{P}
\]

\[
\text{Lys-Arg-Val-Pro-Asp-Ser-Gly-Glu-Cys-Tyr-}\text{C}_1\text{C}_2
\]

\[
\begin{align*}
\text{S}_1 & : \text{Lys-Arg-Val-Pro-Asp-Ser} \\
\text{S}_2 & : \text{Asp-Ser-Gly-Glu-Cys} \\
\text{S}_3 & : \text{Gly-Glu-Cys}
\end{align*}
\]

P, S, and C represent sites of cleavage by pepsin, Nagarse, and carboxypeptidase respectively. Fragments \(S_1\) to \(S_5\)
represent fractions obtained from high-voltage paper electrophoresis of the Nagarse digest of Peptide-3. Solid arrows show sequences determined by the Edman degradation method and dashed arrows by carboxypeptidase. The residues which are underlined represent those identified by the FDNB method.
TABLE IX

PEPTIDE FRAGMENTS OBTAINED FROM NAGARSE DIGESTION OF PEPTIDE-3

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Electrophoretic Mobility (cm)</th>
<th>( R_f ) (BAW)</th>
<th>Color with Ninhydrin</th>
<th>Amino Acids Released on 6 N HCl Hydrolysis, 24 hours. (Molar Ratios)</th>
<th>NH₂-Terminal Residue</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>( S_1 )</td>
<td>2.5</td>
<td>0.05</td>
<td>Dark purple</td>
<td>Lys(0.56), Arg(0.84), Val(1.00), Pro(0.94), Asp(0.90), Ser(1.01), Tyr(0.30)</td>
<td>Lys</td>
<td>10</td>
</tr>
<tr>
<td>( S_2 )</td>
<td>+5</td>
<td>0.26</td>
<td>(Yellow). Brown with ninhydrin</td>
<td>Asp(0.97), Ser(0.61), Gly(0.83), Glu(1.00), DDPS-Cys(0.83)</td>
<td>Asp</td>
<td>5</td>
</tr>
<tr>
<td>( S_4 )</td>
<td>+8</td>
<td>0.24</td>
<td>Light purple</td>
<td>Asp(0.40), Ser(1.00), Gly(0.78), Glu(0.35)</td>
<td>--</td>
<td>1</td>
</tr>
<tr>
<td>( S_5^* )</td>
<td>+10</td>
<td>--</td>
<td>Faintly purple</td>
<td>Asp(0.40), Ser(0.16), Gly(0.68), Glu(1.00)</td>
<td>Ser ?</td>
<td>1</td>
</tr>
<tr>
<td>( S_3 )</td>
<td>+20</td>
<td>0.23</td>
<td>Faintly purple</td>
<td>Glu(1.00), DDPS-Cys(0.50), Gly(0.68)</td>
<td>Gly</td>
<td>3</td>
</tr>
</tbody>
</table>

*The fraction marked with the asterisk was dinitrophenylated directly after elution from paper. The amino acid composition reported are those of the free amino acids present in the aqueous phase after the extraction of the ether-soluble DNP-residues.
IV. DISCUSSION

A. Amino Acids in the Active Center of Chymopapain: A Summary of the Results of the Present Investigation

One of the most widely used approaches to the elucidation of the active site of an enzyme has been to identify the functionally important amino acids by the use of specific labeling reagents. However, this specific labeling technique has its limitations in that only the amino acids with chemically reactive side chains can be located. Reagents for labeling the amino acids with saturated aliphatic side chains such as glycine and alanine have yet to be discovered. The lack of reactivity of aliphatic side chains does not exclude these amino acids from being functional components of the active site. It is possible that the aliphatic side chains play a role in enzyme action by attracting the substrates through hydrophobic bonding or repelling the substrates by steric hindrance. The determination of the sequence of amino acid residues around the labeled group is an indirect but nevertheless feasible method of placing the less reactive but functionally important amino acid residues in the active center. For example, the repeated occurrence of glycine adjacent to the reactive serine residue in the "serine proteinases" may be indicative of the functional importance of glycine in these enzymes.

Thus the immediate goal of the present investigation was to identify the amino acids involved in the active center of chymopapain by labeling with specific reagents and to determine the amino acid sequence around the labeled groups. The ultimate goal
of this dissertation was the elucidation of the mechanism of action of chymopapain.

The studies on the active site of chymopapain was started with the preliminary observation that this proteolytic enzyme may possess both an "active serine" residue, as in the case of trypsin and chymotrypsin, and one or more essential sulfhydryl groups.

Although preliminary investigation indicated that chymopapain was inhibited by DFP, the large molar excess of the organophosphorus reagent necessary to bring about inhibition and the very low recovery of the DIP-peptide from enzymatic hydrolysate of DIP-chymopapain raised some doubts as to the presence and requirement of an "active serine" in chymopapain.

The incubation of cyanide-activated chymopapain with increasing concentrations of the Aldrich DFP showed a steady decrease in the CMB-reactive SH groups in chymopapain which paralleled the loss of enzymic activity. Thus, as Liener (1964) has shown in the case of ficin, the "DFP inhibition" of chymopapain could probably be a consequence of the irreversible destruction of the essential SH group in the enzyme by some impurity in the DFP sample.

However, chymopapain was found to be phosphorylated by DFP to the extent of 0.89 moles of organic phosphorus per mole of chymopapain. That this phosphorylation was not just an unspecific reaction between DFP and some side chain groups of chymopapain was shown by the following experimental results: (1) DFP inhibition is observed only with activated chymopapain. The specific activity of the unactivated chymopapain was not altered by incubation with DFP. (2) Difference spectrum was observed for the DFP-treated,
activated chymopapain versus activated chymopapain, while none was found in the case of DFP-treated, unactivated chymopapain versus unactivated chymopapain. (The significance of the difference spectrum was discussed earlier under Results). (3) The presence of phosphoserine in DIP-chymopapain was demonstrated by various chromatographic techniques using synthetic O-phosphoserine as a standard. (4) A peptide containing the phosphoserine residue was isolated from tryptic digest of DIP-chymopapain. Partial characterization of the phosphoserine containing peptide showed that the reactive serine residue in chymopapain was not part of the sequence, -Gly-(Asp/Glu)-Ser-Gly-, common to the DFP-sensitive "serine proteases" such as trypsin and chymotrypsin. However, other enzymes which are not directly related to the "serine proteases" have been shown to be phosphorylated at a particular serine residue. The sequence around the phosphoserine residue have been determined for some of these enzymes which include phosphoglucomutase (Milstein and Sanger, 1961), phosphorylase-a (Nakatsu, 1959) and pepsin (Bixler and Nieman, 1959). In all of these three enzymes the sequence around the phosphoserine residue was different from the well known tetrapeptide sequence mentioned above. Thus it seems possible that chymopapain is one of those enzymes which are not "serine proteases" in the strict sense that trypsin and chymotrypsin are, but which contain a serine residue sufficiently reactive enough to be phosphorylated by DFP. Whether this phosphorylation of the serine residue is directly responsible for all or part of the changes in enzymatic activity of chymopapain cannot be ascertained at this time. However, the experimental data
obtained thus far suggest that the loss of the essential SH groups in chymopapain probably is the primary cause of the loss of enzymic activity.

In the reactions catalyzed by trypsin and chymotrypsin the imidazole group of a histidine moiety has been implicated along with the functionally important serine hydroxyl group (Schoellmann and Shaw, 1963). The possibility that one or more histidine residues may be involved in the active site of chymopapain led to the preliminary photooxidation studies on chymopapain. The result of this photooxidation study was that the loss of all three histidine residues of chymopapain caused only a partial inactivation of the enzyme. This indicated that histidine is probably not essential for the mechanism of action of chymopapain.

Titration of cyanide-activated chymopapain (specific activity, 2.55) with CMB at pH 4.6 indicated the presence of 1.2-1.4 moles of SH per mole of chymopapain. Corresponding CMB titration on the unactivated chymopapain (specific activity, 0.9-1.0) showed less than one mole of SH per mole of enzyme. When these values were extrapolated to 2 moles of SH per mole of enzyme, a maximal activity of 3.20 was obtained which agreed with Kunimitsu's data (1964) that the most active preparation of chymopapain B was assayed to have a specific activity of 3.30. Thus it was assumed that chymopapain contains two reactive SH per mole of enzyme even though the maximum value of SH titer was not obtained by the CMB titration method.

When cyanide-activated chymopapain was titrated with DDPM complete loss of enzymic activity was observed at the point at
which 0.3 moles of SH was still detected by the CMB-titration method. The number of equivalents of DDPM incorporated into the enzyme at this point was estimated to be 0.6 moles per mole of chymopapain, or about half of the initial SH titer. The lower value obtained for the SH group remaining can be accounted for by the fact that this value was obtained by the titration of the reaction mixture with CMB. As mentioned just previously, CMB titration tends to give values of SH lower than that actually present. This, in addition to the fact that CMB titration was carried out in the presence of unreacted DDPM remaining in the reaction mixture, probably accounts for the low value of residual SH obtained. But, after taking everything into consideration it appears that when half of the initial SH was titrated with DDPM, chymopapain lost its catalytic property. This is in agreement with Kunimitsu's observation that when chymopapain was allowed to stand at room temperature for several days the enzyme lost all of its enzymic activity, but one mole of SH per mole of chymopapain could still be detected by CMB titration.

On the basis of these preliminary titration data, the preparation of DDPS-chymopapain was carried out by titrating the enzyme with DDPM until half of the original SH was substituted and the enzyme lost all of its activity. Thus it was assumed that one of the two SH groups in chymopapain was preferentially labeled with DDPM. This assumption was substantiated by the fact that only one labeled peptide was isolated from the peptic digest of DDPS-chymopapain.
The amino acid sequence of the peptide containing the labeled SH group of chymopapain was found to be different from those for papain (Light, et al., 1964) and for ficin (Wong and Liener, 1964) as shown below:

Chymopapain: Lys-Arg-Val-Pro-Asp-Ser-Gly-Glu-Cys*-Tyr
Papain: Pro-Val-Lys-Asn-Gln-Gly-Ser-Cys-Gly-Ser-Cys*-Tryp
Ficin: Pro-Ileu-Arg-Gln-Gln-Gly-Gln-Cys-Gly-Ser-Cys*

(Cys* represents the labeled half cysteine residue).

A possible explanation for this difference in the amino acid sequence between the peptide containing the labeled SH group of papain or ficin with that isolated from DDPS-chymopapain may lie in the difference in the number of freely reacting SH groups in chymopapain and in papain and ficin. Both papain and ficin were shown to have one CMB-reactive SH group per mole of enzyme. (The treatment of ficin with sodium dodecylsulfate (Liener, 1961) led to the uncovering of an additional SH group. However, this "buried SH" would not be normally available for reaction under the standard assay conditions). Chymopapain, on the other hand, contains a maximum of two moles of SH per mole of enzyme. Although it has just been demonstrated that one of the two SH groups in chymopapain is preferentially labeled by DDPM with concomitant loss of enzymic activity, the essential nature of the second SH group has not been completely ruled out. It is possible that when the more reactive of the two SH group is substituted by a sulfhydryl reagent, the enzyme is no longer able to continue on the stepwise catalytic process and consequently the enzyme is inactivated.
Whitaker and Bender (1965) have presented kinetic data which support a three-step mechanism for hydrolysis of BAA or BAEE (AX) by papain (E):

\[
E + AX \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} EAX \overset{k_2}{\longrightarrow} EA + X \overset{k_3}{\longrightarrow} E + A \quad \text{(eq. 1)}
\]

where X represents the alcohol portion of an ester substrate or the amine portion of an amide substrate, EAX is the initial enzyme-substrate complex, and EA is the acyl enzyme intermediate. The same mechanism had been proposed earlier for ficin by Hammond and Gutfreund (1959).

If a similar mechanism is assumed for chymopapain (this assumption is justified in view of the similarities in the kinetic constants obtained for the three enzymes, as shown in Table X) it is possible that the more reactive of the two SH groups maybe involved in the formation of the initial enzyme-substrate complex and the second SH group maybe directly involved in the hydrolytic mechanism by the formation of the acylenzyme intermediate. Since it was demonstrated for papain (Whitaker and Bender, 1965) that the rate-limiting step in the hydrolysis of the amide substrate is the formation of the acyl-enzyme complex, any interference with the formation of this intermediate would have a great effect on the overall mechanism.

It is also conceivable that the substitution of one of the SH groups by a large molecule such as DDPM prevents the effective approach of the substrate to the enzyme so that catalysis cannot take place. This problem of steric hinderance of the attached group is discussed by Boyer (1959).
Either of the above mentioned possibilities could account for the observed loss of enzymic activity with the loss of one of the two SH groups in chymopapain. However, in the absence of any supporting data, kinetic or otherwise, these hypotheses are merely suggestions, not explanations for the results obtained for chymopapain.

Theoretically it is possible to label all or part of the second SH group by extended reaction of chymopapain with DDPM. But, as shown in Figure 15, 20-30% of the initial SH group could still be detected by CMB titration even after chymopapain had been incubated with DDPM for 44 hours. Kunimitsu (1964) showed that if the reaction of chymopapain with NEM is continued for an extended period of time, residues other than the SH group seem to be substituted. Thus incubation of chymopapain with DDPM for longer than 44 hours at room temperature may lead to multiple substitution of the side chain groups of chymopapain. This would make the primary goal of specific labeling of the reactive SH group meaningless and present technical difficulties in the subsequent purification of the DDPS-cysteine containing peptide.

In spite of the similarities in the overall properties of chymopapain and papain, there are several definite differences which cannot be overlooked. The ability of papain to hydrolyze every substrate tested at much faster rate, the seemingly narrower substrate specificity of chymopapain and the greater milk-clotting activity of chymopapain reflect some differences in either the basic mechanism or the substrate binding ability of the two enzymes. These differences in turn could be due to the difference
in the amino acid sequence of the peptide fragments containing the reactive SH groups. That enzymes with similar properties have different sequences in the active center have been shown by Jolles (1960) for the active centers of hen's egg white lysozyme and of dog's spleen lysozyme. Both of these enzymes possess similar chemical, physical and enzymic properties including identical NH$_2$-terminal and COOH-terminal residues. However, the amino acid composition and sequence around the reactive histidine residues in the two lysozymes were found to be very different:

(Ala, Cys$_{1-2}$, Asp, Glu, His) Arg for dog's spleen lysozyme.

(His-Gly-Leu-Asp-Asn)-Tyr-Arg for hen's egg white lysozyme.

Finally, the most striking difference between the sequence of the labeled peptide from chymopapain and those from ficin and papain is the fact that the second half cysteine residue which is located two amino acid residues (-Ser-Gly-) away from the labeled half cysteine is missing in the chymopapain peptide. The role of this second half cysteine in papain appears to be that of bringing together residues 165 (Asp) and 25 (the essential Cys) which are located at different parts of the enzyme molecule. (See Figure 1). Although the corresponding information for ficin is not available, it is likely that the second half cysteine residue in ficin has similar function. Also significant is the fact that in both ficin and papain no free carboxyl group was located within the peptide studied. But in chymopapain a free glutamic acid was located adjacent to the labeled half cysteine. In the absence of independent data, it cannot be concluded that this glutamic acid contributes the kinetically important grouping with the pK of 4.5. But
if this were so, then the amino acid residues kinetically implicated in the hydrolytic mechanism of the plant sulphydryl proteases (the SH group and the carboxyl group) are found within the same peptide fragment in chymopapain. Thus a specially placed disulfide bridge is not needed to bring the essential amino acids together.

B. Consideration of the Mechanism of Action of Chymopapain

Limited kinetic studies on chymopapain were carried out by Kunimitsu (1964). Chymopapain, like papain, was found to hydrolyze a wide variety of amide bonds involving both ionic and hydrophobic side chains. The most sensitive substrate for both enzymes was N-benzoyl-L-arginine amide (BAA). Like papain, chymopapain was able to catalyze anilide synthesis. Kinetic studies on the hydrolysis of BAA by chymopapain indicated that two groupings on the protein with pK values of 4.5 and 9 were involved. In the absence of more detailed kinetic analyses definite assignment of these pK values were not made. However, comparison of some of the kinetic constants obtained for the hydrolysis of BAA by papain, ficin and bromelain with those of chymopapain under similar conditions indicate that, at least with BAA as the substrate, the plant sulphydryl proteases studied thus far seem to have similar mechanism of action.

The mechanism for the reaction between chymopapain and substrate shown in Figure 26 is postulated on the basis of data available for chymopapain and using the schemes proposed for papain (Smith, 1962) and ficin (Hammond and Gutfreund, 1959) as models.
Kinetic studies of the papain-catalyzed hydrolysis of BAA and BAEE (Whitaker, 1965) showed that a carboxylic acid group with $pK$ of 4.25 and a sulfhydryl group with $pK$ of 8.35 are involved as an acid-base pair in the acylation process. It is assumed that for chymopapain the groupings with $pK$ of 4.5 and 9 are also the carboxylic acid and a sulfhydryl group respectively.

The probable form of the initial enzyme-substrate complex (EAX) postulated here takes into account the possible roles of the two SH groups in chymopapain. An analogous hydrogen-bonded intermediate between the reactive SH group of the enzyme and the carboxyl group of the substrate was proposed for papain (Soejima, 1962) on the basis of the studies on the interaction between papain and p-benzoquinone, a strong competitive inhibitor of papain.

In the "serine proteases", chymotrypsin and trypsin, the acyl-enzyme intermediate (EA) was isolated and characterized to be the serine ester (Bender and Kaiser, 1962. Bender and Kezdy, 1964). The corresponding acyl-enzyme intermediate for the sulfhydryl proteases was thought to be the thiol-ester. This theory was substantiated by the spectral observations of dithioacylpapain intermediate in the papain-catalyzed hydrolysis of methyl thionohippurate (Lowe and Williams, 1964) and of a trans-cinnamoylpapain in the hydrolysis of N-transcinnamoylimidazole (Bender and Brubacher, 1964).

For papain, $k_3$ (deacylation) was found to be dependent upon a group with $pK$ of 3.90 (carboxyl group) which acts as a base in the deacylation step. Analogous mechanism is proposed for chymopapain
TABLE X

KINETIC CONSTANTS FOR PAPAIN, FICIN, BROMELAIN AND CHYMOPAPAIN

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Temperature (°C)</th>
<th>(k_1) (M^{-1} \text{sec}^{-1})</th>
<th>(K_m) (M)</th>
<th>(k_3) (sec)</th>
<th>pK</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papain</td>
<td>BAA</td>
<td>38</td>
<td>280</td>
<td>0.040</td>
<td>11.0</td>
<td>3.9, 8.2</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>268</td>
<td>0.032</td>
<td>8.70</td>
<td>4.25, 8.35</td>
<td></td>
</tr>
<tr>
<td>Ficin</td>
<td>BAA</td>
<td>25</td>
<td>46</td>
<td>0.048</td>
<td>2.2</td>
<td>4.4</td>
<td>(2)</td>
</tr>
<tr>
<td>Bromelain</td>
<td>BAA</td>
<td>25</td>
<td>2.9</td>
<td>0.0012</td>
<td>0.0035</td>
<td>4.5</td>
<td>(3)</td>
</tr>
<tr>
<td>Chymopapain</td>
<td>BAA</td>
<td>40</td>
<td>102.8</td>
<td>0.045</td>
<td>4.63</td>
<td>4.5, 9.0</td>
<td>(4)</td>
</tr>
</tbody>
</table>

(1) Smith & Parker, 1958  
(2) Hammond & Gutfreund, 1959  
(3) Inagami & Murachi, 1963  
(4) Kunimitsu, 1964
FIGURE 26. A POSSIBLE MECHANISM FOR THE REACTION BETWEEN CHYMOPAPAIN AND SUBSTRATE

- Activated enzyme (EA)
- Acylated enzyme (EA(H\textsubscript{2}O))
- Probable intermediate during the decomposition of the acyl-enzyme
- Possible intermediate for the initial enzyme-substrate complex

Chemical reactions and intermediates are represented in the diagram.
in which a carboxyl group (perhaps the same group involved in the acylation step) makes a nucleophilic attack upon the proton of the water making the OH⁻ available for promoting the removal of the leaving group, A. Deuterium isotope effect was observed for both the acylation and decaylation steps in the papain-catalyzed reaction, but more prominent in the latter, implying a rate-determining proton transfer step in the deacylation mechanism.

The mechanism thus proposed for chymopapain is a tentative one. It may be subject to modification when more detailed kinetic data as well as information on the exact role of the second SH group are made available.

C. A Comparison of the Active Center of Chymopapain with those of Other Proteolytic Enzymes

At the start of this investigation it appeared that chymopapain was an unusual enzyme which, in addition to being a sulfhydryl enzyme, was inhibited by DFP. This susceptibility to DFP inhibition implied that a specific serine residue may be a part of the active site, as it is in trypsin and chymotrypsin. The results of the present investigation, however, ruled out this possibility of chymopapain being a "serine protease" and placed chymopapain among the class of sulfhydryl proteases.

It would be interesting at this time to list some of the proteolytic enzymes obtained from various sources and to compare what is known about the active sites of these enzymes. The list shown in Table XI is far from being complete. However, it helps to point out the variety of proteolytic enzymes found in nature and emphasizes the fact that each of these enzymes seem to fall into one of
## TABLE XI

A COMPARISON OF THE ACTIVE SITES OF VARIOUS PROTEOLYTIC ENZYMES

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>pK of Groups Involved in Amino Sequence Around the Essential Amino (Acid(s))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>Bovine pancreas</td>
<td>6.3, 7.2 Ser Asn-Ser-Cys-Gln-Gly-Gly-Asp-Ser-Gly-Pro-Val-Cys-Ser-Gly-Lys</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ser Asn-Ser-Gly-Tyr-His-Phe-Cys-Gly-Gly-Ser-Leu</td>
<td>(2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>His Val-Val-Ser-Ala-Ala-His-Cys-Tyr-Lys-Ser-Gly-Ileu-Glu</td>
<td></td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>Bovine pancreas</td>
<td>6.7, 7.2-7.4 Ser Gly-Asp-Ser-Gly-Gly-Pro-Leu</td>
<td>(3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>His Gly-His-Phe-Cys-Gly-Gly-Ser-Leu</td>
<td>(4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>His Ala-His-Cys</td>
<td></td>
</tr>
<tr>
<td>Thrombin</td>
<td>Plasma, ox</td>
<td>7.1 Ser Gly-Asp-Ser-Gly</td>
<td>(5)</td>
</tr>
<tr>
<td>Cholinesterase</td>
<td>Horse serum</td>
<td>--- Ser Phe-Gly-Glu-Ser-Ala-Gly</td>
<td>(6)</td>
</tr>
<tr>
<td>Enzyme</td>
<td>Source</td>
<td>pK of Groups Involved in Catalysis</td>
<td>Essential Amino Acids</td>
</tr>
<tr>
<td>-------------------</td>
<td>-----------------</td>
<td>-----------------------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>Carboxy-peptidase</td>
<td>Bovine pancreas</td>
<td>---</td>
<td>2 Tyr, Cys, NH₂-terminal Asn</td>
</tr>
<tr>
<td>Papain</td>
<td>Papaya latex</td>
<td>4.3, 8.0</td>
<td>Cys, Asp</td>
</tr>
<tr>
<td>Chymo-papain</td>
<td>Papaya latex</td>
<td>4.5, 9.0</td>
<td>Cys, Glu†</td>
</tr>
<tr>
<td>Ficin</td>
<td>Fig</td>
<td>4.4, 8.5</td>
<td>Cys, Glu/Asp</td>
</tr>
<tr>
<td>Brome-lain</td>
<td>Pineapple stem juice</td>
<td>4.5, 9.0</td>
<td>Cys, Glu/Asp</td>
</tr>
</tbody>
</table>
TABLE XI (Continued)

A COMPARISON OF THE ACTIVE SITES OF VARIOUS PROTEOLYTIC ENZYMES

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>pK of Groups Involved in Catalysis</th>
<th>Essential Amino Acids</th>
<th>Sequence Around the Essential Amino (Acid(s))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcal</td>
<td>Bac-teria</td>
<td>Cys, His?</td>
<td>Val-Lys-Pro-Gly-Glu-Gln-Ser-Phe-Val-Gly-Gln-Ala-Ala-Thr-Thr-Ala-Val-Cys-His-Gly</td>
<td>(13)</td>
<td></td>
</tr>
<tr>
<td>Proteinase</td>
<td></td>
<td>---</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPN' subtilisin (Nagarse)</td>
<td>Bac-teria</td>
<td>Ser</td>
<td>DIP-Ser</td>
<td></td>
<td>(14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>---</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. subtilis</td>
<td>Bac-teria</td>
<td>6.4, (Zn)</td>
<td>His?</td>
<td>------</td>
<td>(15)</td>
</tr>
<tr>
<td>neutral protease</td>
<td></td>
<td>7.3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

of the several general categories based on the nature of their active sites. The proteolytic enzymes of bacterial origin are interesting in that these enzymes are not restricted to any one of the general categories as demonstrated by the three bacterial proteinases shown here. The *Streptococcal* proteinase was shown to be a sulfhydryl enzyme, the active center peptide of which has an entirely different sequence from those of the plant sulfhydryl enzymes. The BPN' subtilisin represents the "serine proteases" and the *B. subtilis* neutral protease seems to be a metal (Zn) requiring enzyme.

The occurrence of papain and chymopapain in the papaya latex raises an interesting question as to the role of these two *in vivo*. That papain, the smaller of the two, is a partially hydrolyzed product of chymopapain, is ruled out on the basis of all the data available on the differences in physical, chemical and enzymic properties of the two enzymes, including now the difference in the amino acid sequence around the active SH group. It seems redundant that two enzymes of similar general properties and mechanism of action are found in the same source for the purpose of carrying out identical functions. Thus each of the two enzymes probably has an unique and definite function in the papaya latex. The question as to what these functions are may be answered only when more thorough studies (including the determination of the complete amino acid sequence as well as the secondary and tertiary structures of the enzymes) of the two enzymes are carried out.
V. BIBLIOGRAPHY


