Reaction of a Specific Tryptophan Residue in Streptococcal Proteinase with 2-Hydroxy-5-nitrobenzyl Bromide*

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GEORGE W. ROBINSON†
From The Rockefeller University, New York, New York 10021

SUMMARY

The zymogen and enzyme forms of streptococcal proteinase were treated with the tryptophan-specific reagent 2-hydroxy-5-nitrobenzyl bromide (HNB-Br) in 0.46 M phosphate solutions at pH 3.1. The zymogen failed to react with HNB-Br, and none of its potential activity was destroyed. The enzyme incorporated up to 1.8 HNB groups per molecule and lost a maximum of 95% of its potential activity. Four peptides containing the HNB moiety were isolated from a tryptic digest of inactivated enzyme by gel filtration on Sephadex G-25. The HNB-peptides were eluted together in a narrow, retarded band by 0.1 N NH₃OH but were resolved into broad peaks when the eluent was 0.1 M NH₄HCO₃. One HNB-peptide was further purified by filtration on Bio-Gel P-4. Three of these peptides had identical amino acid compositions, and the fourth had a similar composition but 4 less residues. Two peptides contained one HNB group, and the remainder had two HNB groups. Parts of the amino acid sequences of these peptides and of their chymotryptic fragments were determined by Edman degradations and digestions with aminopeptidase M and carboxypeptidases A and B. Apparently, each major HNB-peptide came from the same segment of the enzyme, and a single tryptophan residue underwent both monosubstitution and disubstitution. The proposed sequence around the reactive tryptophan is:

His-Val-Asn-Trp-Gly-Phe-Gly-Trp(HNB₃)ₙ-Gly-Val-Ser-Asn-Gly-Phe-Arg

This proposed sequence is similar to the sequence around a tryptophan residue placed at the active site of papain by x-ray crystallography. Both proteins have a Trp-Gly-X-Gly-Trp-Gly sequence. From the data of this paper, it is proposed that streptococcal proteinase also has a tryptophan residue in its active site.

Streptococcal proteinase is an extracellular protein produced by hemolytic streptococci (1, 2). The single polypeptide chain of the protein has a molecular weight of 44,000 (3), and a volatile mercaptan blocks its single half-cystine residue in a mixed disulfide bond (4). After this disulfide is reduced, the zymogen becomes a proteolytic enzyme (5). In concentrated solutions (5 mg per ml), this active species autodigests and generates a mixture of active enzymes. Controlled digestion with trypsin, however, produces a homogeneous protein of 32,000 molecular weight by removing about 100 residues from the peptide chain of the unreduced, intact zymogen (6). This form of the proteinase, which is generally called "enzyme" or more recently "proteolyzed zymogen" (5), is stable and active after its mixed disulfide bond is reduced. In this study, only the species with molecular weights of 44,000 and 32,000 were used, and the smaller protein is referred to as "enzyme."

Reduced enzyme cleaves peptide substrates that contain hydrophobic residues and splits dipeptides that have bulky NH₄-blocking groups (7). These specificity requirements suggest that apolar residues on the enzyme surface bind substrates at the active site in the proper orientation for hydrolysis. Recent studies with the thiol-enzyme papain have shown that it hydrolyzes a polypeptide substrate at essentially the same residues as streptococcal proteinase (8) and that it has a tryptophan residue in its active site (9). Because the indole side chain of tryptophan could interact strongly with hydrophobic portions of substrates and because of the similarities with papain, I sought to determine by chemical modification whether tryptophan is located in the postulated binding site of streptococcal proteinase. In this investigation, I probed for exposed tryptophan residues by treating unreduced zymogen and enzyme with 2-hydroxy-5-nitrobenzyl bromide. Koshland et al. (10-12) showed that this reagent combined readily and specifically with tryptophan in acidic solutions in the absence of free thiol groups.

MATERIALS AND METHODS

Proteins

Dr. Stuart Elliott kindly provided crystalline suspensions of zymogen that he had obtained by fractionating culture filtrates of streptococci (Group A, type 5707) with ammonium sulfate (2). Limited tryptic digestion converted unreduced zymogen into a homogeneous form of enzyme with its mixed disulfide bond intact (6). This enzyme was stored frozen in 0.15 M sodium phosphate buffer, pH 5.7, or as a crystalline suspension in 60% saturated ammonium sulfate, pH 5.7, at 5°C.

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† Present address, Department of Biochemistry, University of Kentucky, Lexington, Kentucky 40506.
Trypsin (twice crystallized), α-chymotrypsin (three times crystallized), carboxypeptidase A (DFP-treated), and carboxypeptidase B (DFP-treated) were obtained from Worthington. Aminopeptidase M from porcine kidney was purchased from Henley and Company, New York, and Nagarse was bought from the Enzyme Development Corporation, New York. Dr. Robert L. Hill generously supplied a prolidase-rich acetone powder from porcine kidney (see Reference 13).

Reagents

2-Hydroxy-5-nitrobenzyl bromide, phenylisothiocyanate, and anhydrous trifluoroacetic acid were purchased from Pierce Chemicals. Guanidine hydrochloride was obtained from Mann. Pyridine and triethylamine were redistilled before use. p-Dimethylaninobenzaldehyde was recrystallized by the method of Adams and Coleman (14), and 2-nitrophenylsulfenyl chloride from Fisher was recrystallized from dry ethyl ether. All other chemicals were reagent grade and were used without further purification.

Assays

The enzyme was reduced for 30 min at 37° with 1 mM 2-mercaptoethanol in 0.1 M sodium phosphate buffer, pH 6.7, that contained 1 mM EDTA. Hydrolysis of 7.5 mM N-benzoyl-L-phenylalanine-L-leucine (Z-Phe-Leu) dissolved in the same buffer was monitored at 37° by the ninhydrin reaction (7). Potential activity of thezymogen toward the same substrate was determined after the precursor had been digested with trypsin (10% weight of zymogen) at pH 6.7, 37°, for 1 hour and then reduced with 2-mercaptoethanol. Activity against protein substrates was measured by the casein assay (3).

Reaction with HNB-Br

Unreduced zymogen or unreduced enzyme was treated with HNB-Br in phosphate solutions at acid pH. One volume of protein solution (1 to 8 mg per ml) in 0.15 M sodium phosphate buffer, pH 6.7, that contained 1 mM EDTA. Hydrolysis of 0.1 M NaH₂PO₄ and 1 volume of 0.1 M NaCl. A small gel filtration column (1.5 x 15 cm) was used for analytical experiments, and a large one (2.5 x 95 cm) for the preparation of material for peptide isolation studies. The incorporation of HNB groups was measured spectrally on aliquots of the protein solution by the technique of Barman and Koshland (12).

Proteolytic Digestions

Trypsin—The labeled enzyme (about 150 mg) obtained by gel filtration was concentrated to 30 to 40 ml by ultrafiltration. The solution was acidified to pH 3 with formic acid, heated in a boiling water bath for 3 min, and neutralized with NaOH, and this treatment precipitated most of the protein. The denatured enzyme was digested with trypsin (3 to 5% weight of the labeled enzyme) in 0.05 M NaCl at 37°, pH 8.5, for 3 hours. Most of the heavy precipitate dissolved within 30 min; adjusting the solution to pH 10.3 with NH₄OH clarified a slight haze that remained at the end of the digestion. The digest was reduced to 10 to 15 ml by rotary evaporation for gel filtration.

Chymotrypsin—Two trypic peptides (0.2 μmole of HNB-2, 0.3 μmole of HNB-3) were hydrolyzed by chymotrypsin (0.5 mg) in 2 ml of 0.1 N NH₄OH adjusted to pH 8.5 with concentrated formic acid. The digests were kept at 37° for 12 to 15 hours and then were concentrated to 1 ml by rotary evaporation for chromatography.

Carboxypeptidases A and B—One peptide (0.125 μmole of HNB-3) was digested by carboxypeptidase B (15 μg) at 37° in 1 ml of 0.1 M NH₄HCO₃ adjusted to pH 8.3 with 0.1 N NH₄OH. After the solution had stood for 70 min, an aliquot was acidified with formic acid and lyophilized. Carboxypeptidase A (40 μg) was added directly to the digestion mixture after 80 min. An aliquot was removed after an additional 2 hours and was treated as described above. Chymotryptic peptides (0.02 μmole of HNB-2, 0.02 μmole of HNB-3, Ch 1) were digested only with carboxypeptidase A (40 μg) at 37° in 1 ml of 0.1 N NH₄OH adjusted to pH 8.3 with concentrated formic acid. After 2 hours, aliquots of the digests were acidified and lyophilized. Control digestions contained only buffer and enzymes. Commercially available solutions of carboxypeptidase B were diluted before use to 0.6 mg per ml in 0.2 M potassium phosphate buffer, pH 7.8. Carboxypeptidase A was dissolved by the method of Potts et al. (15) as described by Ambler (16).

Aminopeptidase M—Aminopeptidase M (40 μg) completely hydrolyzed one peptide (0.02 μmole of HNB 3, Ch 1) in 1 ml of 0.1 M NH₄HCO₃ adjusted to pH 8.3. The digest was lyophilized after 16 hours at 37°.

Tryptophan Determinations

Several methods were applied for determining the total tryptophan content of the enzyme. The following techniques were used essentially without modification. Titration with INH-D-treat ment in 8 M urea (12), spectral analysis in 6 M guanidine hydrochloride (17), amino acid analysis after hydrolysis in 6 N HCl that contained 4% (v/v) thioglycolic acid (18), and amino acid analysis after alkaline hydrolysis in the presence of starch (19) with 5 N NaOH instead of Ba(OH)₂.

The NPS-Cl method of Scoffone, Fontana, and Rocchi (20) was used as published and was also modified as suggested by Scoffone. Various amounts of NPS-Cl dissolved in 0.05 to 0.2 ml of p-dioxane were added to 2-ml portions of enzyme (36 μg) in 50% acetic acid. A yellow precipitate formed in solutions containing a large excess of reagent and it was removed by centrifugation. Labeled protein was precipitated by addition of 10 ml of ice-cold acid-acetone (1 part 1 N HCl to 39 parts acetone). The precipitate was dissolved in 1 ml of concentrated formic acid and reprecipitated as before. The residue was again dissolved in 1 ml of formic acid, the solution was diluted to 2.5 ml with water, and 125 μl of 2-mercaptoethanol were added. The protein was reduced at room temperature for 2 to 4 hours and then was precipitated with acid-acetone. The residue was 1 The abbreviations used are: DFP, diisopropyl phosphorofluoridate; HNB-Br, 2-hydroxy-5-nitrobenxyl bromide; NPS-Cl, 2-nitrophenylsulfenyl chloride; Z, benzyloxycarbonyl.

G. Stark, personal communication.

E. Scoffone, personal communication.
washed twice with acetone and dried over P2O5. The labeled protein was dissolved in 80% acetic acid, and the NPS content was determined from the absorbance at 365 nm (ε = 4,000 M⁻¹ cm⁻¹).

Total enzymatic digestion of the enzyme was carried out by the general method of Hill and Schmidt (21) with the combination of Nagarse, aminopeptidase M, and prolidase. The colorimetric method of Spies and Chambers (22) for the detection of tryptophan was used after extensive degradation of the enzyme by Nagarse and aminopeptidase M (see References 23, 24, and 25).

**Other Procedures**

**Edman Degradation**—The amino acid sequences of peptides were determined by the subtractive phenylisothiocyanate method of Edman discussed by Konigsberg (26). The coupling step was carried out in 50% pyridine-2% triethylamine. Butyl acetate was used to extract the derivatized amino acid.

**S-Carboxymethylation**—The labeled protein was reduced at 37°C for 30 min in a solution at pH 8.5 that contained 0.3 M Tris chloride, 0.6 mM EDTA, 4 M guanidine hydrochloride, and 85 mM 2-mercaptoethanol. A neutralized solution of iodoacetate was added to a final concentration of 90 mM, and the solution was kept at room temperature in the dark for 15 min. Carboxymethylated protein was isolated by gel filtration on a column (2 x 40 cm) of Sephadex G-25 equilibrated with 50% acetic acid. The protein peak was diluted with water and lyophilized. Unlabeled enzyme was reduced and carboxymethylated by the method of Crestfield, Moore, and Stein (27).

**Alkaline Hydrolysis**—Peptides and proteins were hydrolized with 6 N HCl under reduced pressure at 110°C for 22 to 24 hours. Amino acid analyses were performed by the general procedure of Spackman, Stein, and Moore (28) with an accelerated system (50 ml per hour flow rate) similar to that described by Spackman (29). Tryptophan was determined on a column (0.9 x 10 cm) of Beckman PA-35 resin with the pH 5.28 buffer.

Dried residues from the enzymic hydrolyses were dissolved in 0.2 N citrate buffer, pH 2.2, for amino acid analysis. One aliquot from a digest with carboxypeptidase A that appeared to contain asparagine or serine was hydrolyzed with 2 N HCl under reduced pressure at 110°C for 2 hours. Analysis of control samples containing only carboxypeptidase A and subjected to this treatment showed less than 1 to 2 nmoles of any amino acid. Asparagine was quantitatively converted to aspartic acid under these conditions.

**Protein Determinations**—All protein concentrations of zymogen and enzyme were determined by amino acid analyses of acid hydrolysates.

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**TABLE I**

Comparison of tryptophan determinations

<table>
<thead>
<tr>
<th>Method</th>
<th>Tryptophan/enzyme (residues/molecule)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic digestion [3]</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>Colorimetric reaction [4]</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>Spectrum in guanidine-HCl [8]</td>
<td>5.5 ± 0.1</td>
</tr>
<tr>
<td>Acid hydrolysis with thiol [6]</td>
<td>2.7 ± 0.1</td>
</tr>
</tbody>
</table>

* Hill and Schmidt (21) modified as described in the text.  
* Spies and Chambers (22) on partially degraded enzyme.  
* Edelhoch (17).  
* Drée (19) modified by Stark (personal communication).  
* Matsubara and Sasaki (18).

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**RESULTS**

**Total Tryptophan Content**—Before I examined the reaction of a tryptophan-specific reagent with the proteinase, I analyzed for...
the total number of tryptophan residues in the enzyme. Table I summarizes results from spectral and hydrolytic methods. Total enzymatic hydrolysis released 4 molecules of tryptophan per molecule of enzyme. All other amino acids were recovered in the expected amounts except for 1 less residue of lysine and proline; apparently a single Lys-Pro bond located near the half-cystine residue (30) resisted hydrolysis. The colorimetric method gave 3.8 residues per molecule on samples of partially degraded enzyme, and control analyses of chymotrypsinogen by this same technique yielded 7.7 of the theoretical 8 residues (31). The molar ratio for tryptophan released during alkaline hydrolysis was calculated on the basis of quantitative recovery of aspartic acid, glutamic acid, and proline (6) and was also found to be about 4 residues per molecule. Values determined by the spectral method in guanidine hydrochloride, however, indicated 5 to 6 residues of tryptophan per molecule of enzyme. Less tryptophan was recovered from acid hydrolysates containing a mercaptan than was found by the other methods.

Unreduced enzyme, titrated in 8 M urea with HNB-Br, incorporated a maximum of eight HNB groups in the presence of a vast excess of reagent (Fig. 1). Barman and Koshland (12) recommend only a 20-fold molar excess of HNB-Br over tryptophan residues, but even when these data were plotted with an expanded abscissa scale, no definite break could be seen in the titration curve. Because HND-Br can disubstitute on the indole nucleus (32, 33), the limiting value of eight probably indicates complete disubstitution of 4 tryptophan residues in the proteinase.

The enzyme was titrated with NPS-Cl, and excess reagent was also incorporated into the enzyme when the standard procedure (20) was followed (Fig. 2). Gel filtration of several samples over Sephadex G 25 equilibrated with 50% acetic acid eliminated the possibility that precipitation of labeled enzyme from the reaction mixture had trapped excess reagent. Reduction of the labeled enzyme with 2-mercaptoethanol lowered the maximal number of NPS groups that were bound to four, as also seen in Fig. 2. Side reactions, probably with amino groups, may account for the excess incorporation. The sulfenamide bond that would be formed with amino groups is readily cleaved by mercaptoethanol, whereas the tryptophan product is stable to reduction (34).

The results from these determinations generally point to 4 tryptophan residues in the enzyme, but the possibility of 5 residues cannot be eliminated because if the high values obtained from spectral analyses in a denaturant. The presence of 6 M

![Fig. 3. Effect of HNB-Br reaction. Unreduced enzyme (35 \( \mu M \)) (○) and zymogen (48 \( \mu M \)) (□) were treated in acidic phosphate solutions with various amounts of HNB-Br as described in the text. Hydrolysis of the synthetic substrate Z-Phe-Leu was measured after reduction of the enzyme and after proteolysis and reduction of the zymogen.](http://www.jbc.org/)

![Fig. 4. Incorporation of HNB groups into enzyme. Unreduced enzyme (10 to 100 \( \mu M \)) was treated with various amounts of HNB-Br in acidic phosphate solutions as described in the text. Activity against Z-Phe-Leu was measured, and incorporation of HNB groups was determined by the method of Barman and Koshland (12).](http://www.jbc.org/)

![Fig. 5. Gel filtration of tryptic digest with 0.1 N \( \text{NH}_4\text{OH} \). The tryptic digest of labeled enzyme was adjusted to pH 10.3 with \( \text{NH}_4\text{OH} \) and was filtered through a column (2.5 × 98 cm) of Sephadex G-25 equilibrated with 0.1 N \( \text{NH}_4\text{OH} \). Absorbance of the effluent was measured at 410 nm (●). Aliquots (100 \( \mu l \)) from the fractions were hydrolyzed with alkali and analyzed with ninhydrin by the general method of Hirs, Moore, and Stein (38) as described by Fruchter and Crestfield (39); ninhydrin color was monitored at 570 nm (□). The bar indicates the fractions containing the HNB label that were pooled.](http://www.jbc.org/)
guanidine hydrochloride should normalize tryptophanyl absorption in the enzyme and give the true number of residues, but a spectral method indicated a high value of tryptophan in glyceraldehyde 3-phosphate dehydrogenase (35) that was not confirmed by sequence studies. Thus determination of the amino acid sequence of streptococcal proteinase may be necessary to resolve this question.

Reaction with HNB-Br—The HNB-Br reagent was chosen to test the hypothesis that the substrate-binding site contains tryptophan. The reagent attacks only tryptophan in acidic solutions in the absence of thiol groups and yet hydrolyzes so rapidly that it probes only for residues readily accessible to solvent (12).

Unreduced zymogen and enzyme were treated with HNB-Br in 0.46 M phosphate solutions at pH 3.1, and Fig. 3 shows the effects of reaction upon potential activity of the two species. Up to 95% of the original activity of the enzyme was destroyed, but a residual amount always remained. Control samples of enzyme, acidified in the manner described under "Materials and Methods," but treated only with acetone, had the same specific activity as untreated enzyme. Neither hydrolyzed reagent (HNB-OH) nor acetone inactivated the enzyme in the concentrations used for the reaction.

Zymogen lost none of its potential proteolytic activity when it was treated with HNB-Br (see Fig. 2) and incorporated less than 0.1 of an HNB group per molecule. Activity of the zymogen was measured after it was converted to enzyme by tryptic digestion.

High amounts of phosphate in the buffer prevented precipitation of enzyme upon neutralization for assay. When the solution was only 0.15 M in phosphate, the enzyme was soluble at pH 3.1 but precipitated when the solution was neutralized. In 0.46 M phosphate solution, the enzyme remained soluble during the pH changes. Although no studies were done to check for differences in conformation of the enzyme at neutral and acidic pH, a high concentration of phosphate ions appears to prevent structural changes that may occur in acidic solutions or at least to make them freely reversible. Phosphate ions also protect the enzyme against rapid denaturation by guanidine hydrochloride (36).

During this reaction, the enzyme retained its natural thiol-blocking group; amino acid analysis of a sample of enzyme that was 95% inactivated and then reduced and carboxymethylated in a denaturant indicated the presence of 0.97 residue of S-carboxymethylcysteine per molecule of enzyme. Reduction with 2-mercaptoethanol also failed to release any HNB groups from the labeled enzyme.

In the standard assay, hydrolysis of Z-Phe-Leu was measured, and tryptophan modification also appeared to cause identical losses of activity against protein substrates. For example, a sample of enzyme retaining 60% of its activity toward Z-Phe-Leu also had 60% of its original activity against casein.

Fig. 4 shows the relationship between HNB incorporation into the enzyme and the loss of activity. When the enzyme was 60% inactivated, about 1 HNB group had been bound to the protein. Incorporation of about 1.8 HNB groups corresponded to loss of 95% activity.

Isolation of HNB-Peptides—Because of the complex nature of the HNB-Br reaction, I isolated peptides containing the HNB group to determine whether HNB-Br had substituted at 2 distinct tryptophan residues, disubstituted at a single residue, or modified randomly several tryptophan residues of the enzyme. The unusual interactions of the HNB-peptides with Sephadex gels, reported below, facilitated fractionation of labeled peptides by gel filtration. Ion exchange resins with a polystyrene matrix were avoided because preliminary experiments of this study and of other investigations (37) indicated that the HNB moiety adsorbed tightly to these resins.

Enzyme that contained 1.7 HNB groups per molecule and 10% of its original activity was hydrolyzed by trypsin, and the digest was filtered through a column of Sephadex G-25. The HNB-
The HNB-peptides were filtered again over Sephadex G-25 that was equilibrated with 0.1 M NH₄HCO₃. With this eluent, the HNB-peptides separated into four peaks (Fig. 6). Sephadex extensively retarded these peptides; the first HNB-peptide was eluted near the position expected for dipeptides or free amino acids. The volume of eluent required to remove the final peptide was 5 times the total volume of the column. A solution of 0.1 M NH₄OH adjusted to pH 8.5 with concentrated formic acid yielded a chromatogram essentially identical with that obtained with bicarbonate.

Small unlabeled peptides still contaminated the first HNB-peptide to elute (HNB-1), as evidenced by analysis with ninhydrin in Fig. 6. This peptide was purified on Bio-Gel P-4 as shown in Fig. 7. With this peptide, the polyacrylamide matrix of Bio-Gel did not adsorb the HNB group as much as the dextran of Sephadex. Properties of other labeled peptides on Bio-Gel were not compared.

The amino acid compositions of these four peptides, presented in Table II, indicated that each came from the same segment of the polypeptide chain. Peptides HNB-2, 3, and 4 had identical amino acid compositions, and Peptide HNB-1 contained similar amino acids but 4 less residues. The values for tryptophan were 100% of acid hydrolysates, and the values in parentheses are the as-

### Table II

#### Amino acid composition of HNB-peptides

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Peptide HNB-1</th>
<th>HNB-2</th>
<th>HNB-3</th>
<th>HNB-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>0.0 (0)</td>
<td>0.87 (1)</td>
<td>0.98 (1)</td>
<td>1.07 (1)</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.04 (1)</td>
<td>0.74 (1)</td>
<td>0.83 (1)</td>
<td>0.95 (1)</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>1.17 (1)</td>
<td>2.25 (2)</td>
<td>2.02 (2)</td>
<td>2.02 (2)</td>
</tr>
<tr>
<td>Serine</td>
<td>0.99 (1)</td>
<td>0.97 (1)</td>
<td>1.05 (1)</td>
<td>0.99 (1)</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.82 (1)</td>
<td>3.08 (2)</td>
<td>4.33 (4)</td>
<td>4.13 (4)</td>
</tr>
<tr>
<td>Valine</td>
<td>1.15 (1)</td>
<td>1.78 (2)</td>
<td>1.05 (2)</td>
<td>1.87 (2)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.90 (2)</td>
<td>2.13 (2)</td>
<td>1.85 (2)</td>
<td>1.82 (2)</td>
</tr>
<tr>
<td>HNB groups*</td>
<td>0.6 (1)</td>
<td>1.4 (1)</td>
<td>1.8 (2)</td>
<td>2.4 (2)</td>
</tr>
<tr>
<td>Tryptophanb</td>
<td>(1)</td>
<td>(2)</td>
<td>(2)</td>
<td>(2)</td>
</tr>
</tbody>
</table>

| Total no. of residues | 11 | 15 | 15 | 15 |
| Recovery (%)* | 4 | 11 | 7 | 1 |
| NH₂ terminusb | Gly | His* | His* | His |

- a Calculated from the absorption at 410 mu of the peptide in alkaline solution (ε = 18,000 cm⁻¹ M⁻¹).
- b Assumed from the sequence studies presented in the text.
- c Based on the amount of labeled protein taken for tryptic hydrolysis.
- d Determined by the subtractive Edman procedure described in the text.
- e Determined on chymotryptic fragment HNB-2, Ch-1 (see text).

Each peptide represented the same portion of the polypeptide chain of the protein and to establish whether HNB-Br labeled 1 or 2 tryptophan residues. The paragraphs which follow summarize the sequence data. The values represent molar ratios determined from analysis of acid or enzymatic hydrolysates, and the residues removed by Edman degradations are given in boldface type. Dashes (--) indicate residues not determined, and the numbers in parentheses represent the recovery of peptide at each step. The values under Trp(HNB-3) or Trp(HNB-4) refer to the total number of HNB groups in the peptide; the number was calculated from the absorbance at 410 mu of an aliquot of peptide in 0.1 M NH₄OH (ε = 18,000 cm⁻¹ M⁻¹) (12).

Edman degradation of HNB-3 and HNB-4 showed that both peptides had NH₂-terminal histidine (Table II). Because these peptides had the same end group and composition, they were tentatively considered identical. Additional sequence data on Peptide HNB-3 are outlined below.

#### Fig. 8

Fractionation of a chymotryptic digest of Peptide HNB-3. A chymotryptic digest of Peptide HNB-3 (Fig. 6) was filtered through a column (0.9 × 85 cm) of Sephadex G-15 that was equilibrated with 0.1 M NH₄HCO₃. The peptides were eluted with a linear gradient of 0.1 M NH₄HCO₃ (75 ml) and 0.1 M NH₄OH (75 ml). Absorbance was measured at 410 mu ( ), and the absorbance of 0.4-ml aliquots was determined at 570 mu (○) after alkaline hydrolysis and reaction with ninhydrin. The large breakthrough peak contained chymotrypsin. The bars indicate the fractions that were pooled and lyophilized, and these peptides are called HNB-3, Ch-1 and HNB-3, Ch-2.

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FIG. 9. Fractionation of a chymotryptic digest of Peptide HNB-2. A chymotryptic digest of Peptide HNB-2 (Fig. 6) was applied to a column (0.9 X 58 cm) of Sephadex G-15 that was equilibrated with 0.1 N NH₄OH adjusted to pH 8.5 with concentrated formic acid. After 50 ml of this solvent had been passed through the column, the eluent was changed to 0.1 N NH₄OH. Absorbance was measured at 410 nm ( ), and 280 nm ( ). The fractions indicated by the bars were pooled and lyophilized; these peptides are referred to as HNB-2, Ch-1 and HNB-2, Ch-2.

upon analysis of an aminopeptidase M hydrolysate. Recovery of this peptide from the digest was 22%.

Peptide HNB-3, Ch-1: His - Val-Asn-Trp
Composition: 1.0 0.9 1.1
Aminopeptidase M: 0.9 1.2 0.8
Carboxypeptidase A: <0.1 <0.1 0.8

A second fragment from the chymotryptic digest (HNB-3, Ch-2) contained two HNB groups, and Edman degradations suggested that its NH₂-terminal sequence was Gly-Trp(HNB1)-Gly. The Edman method failed to remove glycine completely after the first cycle, and the corresponding drop in HNB groups after the second degradation could not completely eliminate the possibility of another monosubstituted tryptophan residue in the peptide. There was no additional evidence, however, suggesting that any of the main HNB peptides contained more than 2 tryptophan residues. This peptide was obtained in 20% yield from the digest.

Line Peptide HNB-3, Ch-2: Gly-Trp(HNB2)-
(a) Composition: 2.9 1.6
(b) First Edman degradation (84%): 2.4 1.7
(c) Second Edman degradation (100%): 2.2 1.2
(d) Third Edman degradation (100%): 1.5 0.7

Line Gly(Asx,Ser,Gly,Val,Phe)
(a, continued) 1.0 0.7 1.0 1.0
(b, continued) 1.0 0.8 0.8 0.9
(c, continued) 1.0 0.7 0.7 0.9
(d, continued) 1.0 0.8 0.7 0.8

Sequence analysis of Peptide HNR-1 linked the two chymotryptic fragments of HNB-3 with a Gly-Phe dipeptide. This peptide contained only one HNB group and apparently was formed by a chymotryptic-like hydrolysis of the major 15-residue peptide during the tryptic digestion.

Line Peptide HNB-1: Gly-Phe-Gly
(a) Composition: 3.8 1.9
(b) First Edman degradation (91%): 3.1 2.0
(c) Second Edman degradation (97%): 2.7 1.3
(d) Third Edman degradation (100%): 2.2 1.3

Line Peptide HNB-2 was also hydrolyzed by chymotrypsin; Fig. 9 shows the separation of two labeled peptides on a column of Sephadex G-15. An Edman degradation on Peptide HNB-2, Ch-1 removed histidine, and this result suggested that the intact HNB-2 peptide had the same sequence as Peptides HNB-3 and HNB-4. Carboxypeptidase A released asparagine from the COOH terminus, and this residue was distinguished from serine by analysis of a mild acid hydrolysate of the digest. This peptide was recovered in 22% yield.

Line Peptide HNB-2, Ch-1: His(Asx,Ser,Gly,Val,Phe,Arg)
(a) Composition: 0.7 2.2 0.8 2.7 1.9
(b) First Edman degradation (70%): 0.6 1.2 0.9 2.6 2.1
(c) Carboxypeptidase A:

Line Peptide HNB-2, Ch-2: Gly-Trp(HNB2)-
(a) Composition: 3.1 1.0
(b) First Edman degradation (96%): 2.6 0.9
(c) Second Edman degradation (84%): 2.1 0.4

The second fragment of Peptide HNB-2 (HNB-2, Ch-2) had the same composition as the chymotryptic Peptide HNB-3, Ch-2 except for one less HNB group. Edman degradations on HNB-2, Ch-2 support the sequence data on the HNB-3 peptide. Recovery of this peptide from the digest was 30%.

Line Peptide HNB-2, Ch-2: Gly-Trp(HNB2)
(a) Composition: 1.0 0.9 1.0 1.0
(b) First Edman degradation (91%): 0.9 0.9
(c) Second Edman degradation (81%): 0.8 0.7

Sequence analysis of Peptide HNB-1 linked the two chymotryptic fragments of HNB-3 with a Gly-Phe dipeptide. This peptide contained only one HNB group and apparently was formed by a chymotryptic-like hydrolysis of the major 15-residue peptide during the tryptic digestion.
These sequence studies are summarized in Table III. Although no single HNB-peptide was sequenced completely, all data were consistent with identical sequences for the HNB-peptides. Edman degradations and carboxypeptidase digestions of various fragments provided overlapping sequences for all but a (Val, Ser) dipeptide and 1 glycine residue that was placed by difference analysis of the chymotryptic peptides from HNB-2.

**DISCUSSION**

Streptococcal proteinase hydrolyzes a peptide represented schematically as A-X-Y at the X-Y position when A is a bulky, hydrophobic amino acid such as phenylalanine or leucine. With dipeptide substrates, the NH₂ blocking group serves as the A residue, and its size affects the susceptibility of a dipeptide to hydrolysis. For example, the proteinase does not split N-acetyl-Phe-Leu but readily cleaves N-benzoyloxycarbonyl-Phe-Leu (7). The enzyme appears to have a hydrophobic site that binds the bulky group of substrates, and the results given in this paper on the reaction between HNB-Br and the enzyme are consistent with the hypothesis that the binding site contains tryptophan.

HNB-Br reacts with the unreduced enzyme form of streptococcal proteinase and destroys up to 95% of the original proteolytic activity. The reagent does not attack the essential cysteine of the proteinase and appears to modify only tryptophan.

Three main possibilities could explain the loss of activity. Addition of one or two HNB groups to a tryptophan residue in the postulated hydrophobic binding site could: (a) prevent substrates from binding at the active site, (b) cause nonproductive binding of substrates, or (c) disrupt critical alignments of catalytic and binding residues. It is experimentally difficult to eliminate a fourth possibility, namely, that HNB-Br modifies a tryptophan distant from the active site producing changes in the position of active site residues. Labeled and unlabeled enzyme are eluted together, however, when they are chromatographed on sulfated Sephadex, and, if any essential structures do change their conformation, chromatographic mobilities do not reflect these alterations. Less severe effects of steric hindrance, nonproductive binding, or conformational changes plus the possibility of incomplete reaction could account for traces of activity that persist in samples of modified enzyme.

Yzymogen fails to react with HNB-Br; apparently the sensitive tryptophan residue in the unreducedzymogen is shielded from interaction with solvent components. Bustin et al. (6) have recently shown that thezymogen possesses inherent activity when its natural thiol-blocking group is removed and that no cleavage of peptide bonds seems necessary for expression of activity. They conclude that reduction of the mixed disulfide bond may initiate events that expose the active site in the intactzymogen. Results of these studies suggest that in the enzyme, in contrast to thezymogen, the entire active site is exposed even in the absence of reduction. Part of the large fragment that is removed by trypsin in the conversion ofzymogen to enzyme could easily cover the reactive tryptophan, or loss of these residues could cause the proteinase to rearrange and expose the tryptophan to solvent.

Because mono- and disubstituted enzyme could not be separated, positive identification of the inactive species was impossible. The compositions and partial amino acid sequences of labeled peptides point to a single tryptophan residue that reacts with HNB-Br. The 15-residue segment of the polypeptide chain containing this tryptophan has 4 glycine residues and another tryptophan which is unreactive. One or two HNB groups appear able to bind covalently to the reactive tryptophan, although the sequence data supporting this conclusion do not completely rule out a 3rd tryptophan residue in this part of the polypeptide chain that undergoes monosubstitution with HNB-Br. Such a 3rd residue seems unlikely and no data support its presence.

The total recovery of HNB-peptides from the tryptic digest was only about one-fourth the theoretical amount (Table II), and the ratio of mono- to disubstituted peptides did not correspond to the value expected from the over-all incorporation of HNB groups into the enzyme. A possible cause of the low recoveries may be the behavior of the HNB-peptides on Sephadex G-25. When the gel was equilibrated with solvents of slightly alkaline pH (pH 8 to 8.5), the matrix adsorbed the HNB-peptides. The disubstituted peptides, as seen in Fig. 6, were particularly retarded and were eluted in broad peaks. Some losses undoubtedly occurred when these fractions were pooled. Sephadex adsorbed HNB-peptides differently when the columns were equilibrated with a strong base. At high pH, the HNB group exists as the phenoxide anion, and the peptides were eluted together in a narrow band at a position equal to the total volume of the column (Fig. 5). Although this difference in behavior of the HNB-peptides in the two solvents was not investigated further, it was used to isolate the HNB-peptides from a soluble tryptic digest and to resolve the fractions into pure peptides.

The products of HNB-Br reaction with tryptophan or similar indole compounds are complex (32, 33, 40, 41). The reagent attacks mainly position 3 of the indole nucleus to form diazatricers, and these monosubstituted products can rearrange under appropriate conditions to a single compound labeled at position 2 of the indole ring (32, 33). Disubstituted products occur as evidenced by paper electrophoresis (12) and by determination of a product obtained in low yield from the reaction between 1.9 mmoles of N-acetyl-tryptophan ethyl ester and 2.2 mmoles of HNB-Br (32, 33). Exact structures of disubstituted products

**Papain (Res. 174-188)**  
Lys-Asn-Ser-Trp-Gly-Thr-Gly-Trp-Gly-Glu-Asn-Gly-Tyr-Ile-Arg

**Streptococcal proteinase**  
His-Val-Asn-Trp-Gly-Phe-Gly-Trp-Gly(Val, Ser)Asn-Gly-Phe+Arg

**Fig. 10.** Comparison of a papain fragment to the tryptophan peptide from streptococcal proteinase. The amino acid sequence of residues 174 to 188 of papain (96) is compared to the proposed sequence around the reactive tryptophan in streptococcal proteinase. Dashed lines enclose identical residues. The asterisk indicates the tryptophan in the proteinase that reacts with HNB-Br.
are not known, but the reagent can probably bind covalently to positions 2 and 3 of the same indole side chain. The variety of possible reaction products may cause the complex behavior on Sephadex of peptides with identical amino acid content that was experienced in this investigation and also by Dopheide and Jones (37).

Since the discovery of streptococcal proteinase, this enzyme has been recognized to have many features similar to papain, and Moore has recently compared these properties (42). Both proteinases have an essential —SH group involved in catalysis (3, 43, 44) and a histidine residue located near the reactive thiol (45, 46). When papain is isolated by the method of Kimmel and Smith (47), a disulfide bond with free cysteine blocks its reactive sulfhydryl group (48, 49, 50) in the same way that a small mercaptan obstructs the potential cysteine residue of streptococcal proteinase (4). The essential thiol groups of these enzyme-reactive cysteine (48), but the bacterial proteinase has only a single residue is almost twice the size of papain (44,000 to 23,466), and the inactivate papain (59).

The data presented here provide another similarity. In Fig. 10, the sequence in the segment containing the reactive tryptophan residue of streptococcal proteinase is compared to the sequence in the vicinity of tryptophan-177 of papain (56). In both of these peptides, 2 glycine residues flank 1 tryptophan, and both peptides have a second Trp-Gly sequence. X-ray crystallography of papain places tryptophan-177 in the cleft near the catalytic cysteine and nearby histidine residues and tryptophan-181 on the surface of the molecule possibly at the extreme end of the cleft (9). The peptide from streptococcal proteinase, however, exhibits greater homology to the papain fragment when its reactive tryptophan is aligned with tryptophan-181 rather than with tryptophan-177. In the absence of crystallographic data for the proteinase, it is impossible to determine which tryptophan residue of this peptide is closer to the catalytic residues, but HNB-Br reacts with only 1 of them and inactivates the proteinase. Prior to the x-ray analysis of papain, fluorescence studies suggested that a tryptophan residue interacts with a histidine and that the histidine residue had a pK identical with the pK of a group involved in hydrolysis (57). Recent analyses indicate that the tryptophan residue (residues) responsible for fluorescence is in a polar environment but is not perturbed by solvent changes (58). Preliminary studies also suggest that HNB-Br can react with a tryptophan residue to inactivate papain (59).

Although streptococcal proteinase and papain have many similar features, significant differences in the two proteins cannot be overlooked. The zymogen form of streptococcal proteinase is almost twice the size of papain (44,000 to 23,466), and the enzyme produced by trypsin is still one-third larger than papain (the molecular weight for papain was calculated from Reference 56). The plant enzyme has three disulfide bridges plus a reactive cysteine (48), but the bacterial proteinase has only a single thiol residue (3, 6). The two enzymes also differ in their activity against ester and amide substrates. Streptococcal proteinase cleaves esters about 1000 times faster than amides (60), whereas papain hydrolyzes both types of substrates at about the same rate (61, 62, 63). This kinetic property may reflect different mechanisms for the hydrolytic process in the two enzymes and may indicate the involvement of a carboxyl group in papain (60).

Smith and Margoliash (64) have already noted the possible importance of glycine at identical positions in a series of cytochromes c, and glycine residues probably occupy critical positions in the active sites of papain and streptococcal proteinase. Three of the glycine residues around the reactive tryptophan in the proteinase are homologous to glycine residues in papain and 1 glycine is shifted only one position (Fig. 10). The sequence around the reactive cysteine of both enzymes is Gly–X–Cys, as is the case for all known thiol proteinases as well as an analogous Gly–X–Ser sequence for seryl proteinases (compiled in Reference 65). The limited homologies found between papain and the proteinase could represent converging evolution of active sites that have similar specificities, or they could be the vestiges of a primitive active site conserved during diverging evolution of the two proteins.

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