Chemical Properties of Streptococcal Proteinase and Its Zymogen*

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Group A streptococci elaborate an extracellular zymogen, the general properties of which have been described by Elliott (1). In order to transform the protein to an active proteinase, both proteolysis and exposure to reducing agents are necessary. Proteolytic digestion of the zymogen may take place in the culture medium or may be brought about by trypsin (2), although it is not known whether or not the two modes of hydrolysis yield products that are identical. Both the zymogen and the enzyme have been isolated in crystalline form (3). The enzyme may be activated by sulfhydryl compounds or cyanide, and the reduced enzyme is irreversibly inactivated by iodoacetate; in these respects the enzyme resembles papain. Previous reports have described the serological specificities (3) and the electrophoretic mobilities (4) of the zymogen and the enzyme and the substrate specificity of the latter (5).

The present communication deals with chromatographic studies on the two proteins, amino acid analyses, end-group determinations, and data on molecular weights. The results define more fully the nature of the zymogen-enzyme transformation and the chemical properties of the purified proteinase.

EXPERIMENTAL PROCEDURE

Protein Samples—The preparations of streptococcal proteinase and zymogen were twice crystallized materials, suspended in 0.4 or 0.5 M (NH₄)₂SO₄, and were obtained from culture filtrates of a Group A streptococcus, strain 5797 (3). The lots were Elliott's preparations Nos. 57 and 58 of the zymogen and preparation No. 51 of the proteinase. To estimate the amount of protein in a suspension, a portion of the well shaken mixture was diluted 20-fold with water or buffer and the absorbancy of the solution was measured. An absorbancy of 1.37 (1 cm; 280 m), corresponds to 1 mg of zymogen per ml; the corresponding specific absorbancy of the proteinase is approximately 1.64.

Method of Assay—In the presence of reducing agents such as cysteine or mercaptoethanol, the streptococcal proteinase exhibits hydrolytic activity not only toward proteins but also toward synthetic substrates such as benzoyl-L-arginine amide (5); in the present studies the enzyme has also been found to cleave benzoyl-L-arginine-p-nitroanilide and carbobenzoxyglycyl-L-phenylalanine. Hydrolysis of the latter was followed by a measurable amount of hydrolytic activity toward these synthetic substrates is approximately 50 times the amount needed when the substrate is a protein. The method of assay used in the following experiments was based on that described by Kunitz (6) for the assay of trypsin, which employs casein as substrate. The main modification of the method involves the addition of a reducing agent to activate the proteinase. For this purpose, 200 µl of enzyme solution containing 10 to 15 µg of protein were incubated with 800 µl of 0.12 M thiocetanol in 0.1 M phosphate buffer, pH 7.6, for 30 minutes at 40°C. Measurements of the potential activity of the zymogen were made after 60 minutes of incubation, during which time proteolytic autodigestion takes place. One milliliter of 1% casein in 0.1 M phosphate, pH 7.6, was added to the activated enzyme and the mixture was held at 40°C for 20 minutes. Enzymic action was stopped by the addition of 3 ml of 5% trichloroacetic acid and, after centrifugation, the absorbancy of the supernatant solution was measured at 280 m. Appropriate blanks were run in the same fashion, except that the enzyme was added directly to the trichoroacetic acid solution before precipitation of the casein. The proteolytic unit, u, defined as described by Kunitz (6), is the activity that gives rise to an increase of one unit of absorbancy at 280 m per minute of digestion.

Preparation of Samples for Chromatography—Suspensions of the crystalline proteinase or the zymogen in ammonium sulfate, approximately 50 mg in 5.0 ml, were centrifuged for 30 minutes at 30,000 × g. The supernatant solution was removed, and the residue was dissolved in 0 to 8 ml of 0.02 M phosphate buffer, pH 5.0, and recentrifuged for 10 minutes to remove insoluble material. The clear enzyme solution thus obtained was separated from residual ammonium sulfate and any other low molecular weight contaminants by gel filtration on a 2-× 150-cm column of Sephadex G-75 (7) equilibrated with 0.02 M phosphate buffer, pH 5.20 for the zymogen and pH 5.50 for the enzyme. Fractions of 3 ml were collected at a flow rate of 6 ml per hour; the separation was performed at 4°C in the cold room. The pro-

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teinase and zymogen, both of which penetrate the gel only slightly, emerged at 120 to 150 ml and were located by measurement of the absorbancy of the effluent at 280 m\(\mu\). The salt, as detected by a drop test with BaCl\(_2\), was contained in the fractions from 290 to 320 ml.

**Chromatography on Amberlite IRC-50**  A 0.9 × 30-cm column of the cation exchanger, Amberlite IRC-50, was prepared by the general procedure of Hirs, Moore, and Stein ((8), cf. (7)), with a 0.1 M phosphate buffer, pH 6.40. The column was operated at room temperature. A 5-ml sample of the proteinase was taken for chromatography. The protein concentration in the effluent samples was determined by the ninhydrin procedure after a 2-hour alkaline hydrolysis (7). The percentage recovery of protein was calculated by reference to the color obtained on hydrolysis of a 50- or 100-\(\mu\)l portion of the sample solution added to the column. The proteolytic activity of the enzyme in the effluent fractions was determined on aliquots containing 10 to 20 \(\mu\)g of protein. Conditions have not been found under which the zymogen can be chromatographed on Amberlite IRC-50.

**Chromatography on Carboxymethyl-cellulose**—The carboxymethyl-cellulose used in these experiments was obtained from Bio-Rad Laboratories and had a capacity of 0.51 meq per g. The material was screened in wet form to obtain a fraction with 200 to 400 mesh particles. The exchanger was eluted through acid and base as described by Peterson and Sober (9), and was equilibrated with 0.02 M phosphate buffer, pH 6.0, before the column was prepared. Samples of protein solution (1 to 2 ml containing 3 to 6 mg per ml) obtained from the Sephadex G-75 column were introduced into the equilibrated column (0.9 × 15 cm) of CM-cellulose.\(^2\) Gradient elution was performed by allowing 0.05 M phosphate buffer, pH 6.0, containing 2 M NaCl to flow into a 250-ml constant volume mixing chamber containing 0.02 M phosphate buffer, pH 6.0. Fractions of 2 ml were collected. The column was operated in the cold room (4\(\degree\)) at a flow rate of 5 ml per hour.

**Chromatography on Sulfonethyl-Sephadex**—The SE-Sephadex (fine mesh size, 2.0 meq per g, Pharmacia) used for chromatography was first screened in dry form to obtain a 200 to 325 mesh particles. Fines were carefully removed by decantation after suspension of the powder in buffer (cf. (10)). Columns were poured in 5-cm sections; the pressures used for packing were not allowed to exceed those used during subsequent elution. The length of each column was 45 to 60 cm with a diameter of 0.9 cm. Flow rates of approximately 6 ml per hour were obtained with air pressures of approximately 10 cm of Hg. Before introduction of the sample, the column was washed with the eluting buffer until the pH of the effluent and influent coincided to within 0.02 unit. Chromatography was carried out at room temperature. The zymogen was chromatographed with a 0.10 M phosphate buffer at pH 5.20. Chromatography of the proteinase was carried out with 0.10 M phosphate buffer at pH 5.50. The effluent fractions were analyzed for both protein content and enzymic activity. For rechromatography of the proteinase, the selected effluent fractions were pooled and equilibrated with 0.02 M phosphate buffer, pH 5.50, by dialysis or on Sephadex in the cold.

**Amino Acid Analyses**—Salt-free preparations were used for acid hydrolysis, performic acid oxidation, tryptophan determination, and amide analysis. Desalting was performed by gel filtration on a column of Sephadex G-25 equilibrated with 50\% acetic acid (cf. (7)). Acid hydrolysates of representative samples were carried out with 6 N HCl for 22 hours at 110\(\degree\) in evacuated, sealed tubes (11, 12). The amino acid contents of the hydrolysates were determined by the method of Spackman, Stein, and Moore (13). Performic acid oxidation for the determination of half-cystine as cysteic acid was performed by a modification (14) of the procedure of Schram, Moore, and Bigwood (15). Tryptophan was measured spectrophotometrically by the method of Goodwin and Morton (16) and chromatographically after hydrolysis with Ba(OH)\(_2\) by a modification of the method of Drèze (17) in which a Teflon vial is used for the alkaline hydrolysis. Reduction and carboxymethylation were carried out as described by Crestfield, Moore, and Stein (12). The amide-NH\(_2\) value was determined by a modification (18) of the procedure of Laki et al. (19). High speed equilibrium ultracentrifugation (20) was performed by Dr. David A. Yphantis. The amino-terminal residues were estimated by the cyanate method of Stark and Sinyth (21).

**Buffer Solutions**—The phosphate buffers were prepared from 1 M stock solutions of NaHPO\(_4\) and NaH\(_2\)PO\(_4\) diluted just before use. If 0.05\% phenol is needed to prevent mold growth in the dilute buffers, protein concentration can still be measured by absorbancy if the Zeiss spectrophotometer is zeroed against the appropriate blank.

**RESULTS**

**Stability of Proteins**—Solutions of both the enzyme and the zymogen, after removal of (NH\(_4\))\(_2\)SO\(_4\) with Sephadex G-75, were stable for several days in the cold room at 4\(\degree\). The activity of the proteinase obtained from the Sephadex G-75 column was 2.0 ± 0.2 units per mg. There was no indication of loss of potential enzymic activity or of change in chromatographic behavior when solutions of (NH\(_4\))\(_2\)SO\(_4\) suspensions of the two preparations were stored at −20\(\degree\) for a period of 10 months. If, however, the zymogen was kept as a crystalline suspension in half-saturated ammonium sulfate at room temperature, there was a gradual modification to yield activatable forms.

**Properties of Zymogen**—The crystalline zymogen isolated by Elliott is essentially homogeneous in several chromatographic systems. On chromatography on CM-cellulose, the single peak obtained at approximately 20 ml past the start of the gradient contained more than 90\% of the protein applied to the column and all of the potential proteolytic activity. On IRC-50, with 0.1 M phosphate buffer at pH 6.40 as eluent, the zymogen emerged as a single, unretarded peak at 8 to 10 ml. The zymogen was also essentially homogeneous by chromatography on SE-Sephadex. Under the conditions (0.1 M phosphate buffer, pH 5.50) referred to below for the chromatography of the proteinase, the zymogen was not retarded and emerged at 12 ml from the 0.9- × 45-cm column. The effects of changing the concentration and the pH of the phosphate buffer were studied, and it was found that the chromatographic behavior was extremely sensitive to the pH and the ionic strength of the eluent. Lowering the concentration of the buffer from 0.1 M to 0.05 M and keeping the pH at 5.50 resulted in retention of the protein (a trailing peak at 38 ml) but also a lower recovery (56\%) of protein compared to the quantitative recovery achieved when 0.1 M buffer was used. Chromatograms obtained at pH 5.00 with 0.15 M phosphate buffer gave a peak at 20 ml and a higher recovery of protein (93 ± 3\%). The most satisfactory chromatography of the zymogen preparation was achieved when the

\(^2\) The abbreviations used are: CM-cellulose, carboxymethyl cellulose; SE, sulfoethyl.
column was eluted with a 0.1 M phosphate buffer at pH 5.20 (Fig. 1). The retention volume was 32 ml and the recovery of protein was 96 ± 3%. The elution pattern showed a single main peak for which protein and protease activity (after reduction and autodigestion) coincided.

The fully activatable protein obtained from 39 to 36 ml (Fig. 1) was desalted on a Sephadex G-25 column (2 × 40 cm) in 50% acetic acid. The salt-free protein solution was evaporated to dryness, and the residue was subjected to amino acid analysis before and after performic acid oxidation. Tryptophan and amide-NH₂ were also determined. The results of these experiments are shown in Table I. High speed equilibrium ultracentrifugation of a sample from the solution obtained from 44 ml to 56 ml (Fig. 2C) indicated that the protein preparation was homogeneous and had a molecular weight of 43,500 ± 800. The minimal molecular weight calculated from the amino acid residue weights is 44,347.

Properties of Proteinase—In contrast to the crystalline zymogen, the crystalline proteinase isolated from the culture medium was found to contain several components. When subjected to chromatography on SE-Sephadex in 0.1 M phosphate buffer at pH 5.50, the elution pattern exhibited three major peaks (Fig. 2A) with a 94% total recovery of protein. Assay for the potential proteolytic activity of the various fractions gave the results indicated on the figure. In addition, the fractions were tested against carbobenzoxyglycylphenylalanine. The ratio of this carboxypeptidase-like activity to that against casein was the same for all of the peaks on the curve. When Fractions A and B were rechromatographed under the same conditions (Fig. 2B, C), each appeared as a single peak in its original position on the chromatogram with excellent recovery of protein. The recovery of activity was also complete. An almost identical result was obtained on chromatography on IRC-50 in 0.1 M phosphate buffer at pH 6.40.

Portions of the protein solution recovered from 28 to 36 ml (Fig. 2B), 44 to 56 ml (Fig. 2C), and 92 to 120 ml (Fig. 2A) were desalted on a 40- × 2-cm column of Sephadex G-25 in 50% acetic acid. The salt-free protein solutions were evaporated to dryness, and the residues were subjected to amino acid analysis before and after performic acid oxidation. The value obtained for cysteic acid with the amino acid analyzer was checked by chromatography on Dowex 1-X8 (found, 0.94 residue).

The fully activatable protein obtained from 32 to 36 ml (Fig. 1) was desalted on a Sephadex G-25 column (2 × 40 cm) in 50% acetic acid. The salt-free protein solution was evaporated to dryness, and the residue was subjected to amino acid analysis before and after performic acid oxidation. The results for the labile amino acids were corrected approximately for decomposition during 22 hours of acid hydrolysis. The factors applied were: serine, 0.90; threonine, 0.95; tyrosine, 0.95 (cf. Crestfield et al. (7)).

The value obtained for cysteic acid with the amino acid analyzer was checked by chromatography on Dowex 1-X8 (found, 0.94 residue).

Determined as S-carboxymethylcysteine after iodoacetate treatment of the reduced zymogen.

Determined as methionine sulfone after performic acid oxidation.

Determined as methionine on a hydrolysate of the unoxidized protein.

Determined spectrophotometrically (16).

Determined chromatographically after hydrolysis by alkali (17).

Analysis before and after performic acid oxidation. The results are shown in Table II. High speed equilibrium ultracentrifugation of a sample from the solution obtained from 44 ml to 56 ml (Fig. 2C) indicated that the protein preparation was homogeneous and had a molecular weight of 32,000 ± 1,300. The molecular weight calculated from the amino acid composition of this fraction is 31,682 (tryptophan not included).

Reduction and Carboxymethylation—The first attempts at reduction and carboxymethylation of the zymogen preparation

![Fig. 1. Chromatography of the zymogen on sulfoethyl-Sephadex. Column, 0.9 × 45 cm. Eluent, 0.1 M phosphate buffer at pH 5.20. Load, 4.75 mg of protein in 4 ml of 0.02 M phosphate buffer.](image-url)
obtained from SE-Sephadex chromatography met with difficulty. Further experiments revealed that in the presence of 8 M urea, EDTA, and mercaptoethanol, at pH 8.5 (the conditions used for reduction), sufficient active enzyme was formed to cause an appreciable amount of autodigestion over a period of 4 hours at room temperature. The effluent curves obtained by gel filtration on Sephadex G-75 was therefore rather complex (Fig. 3A).

It was found, however, that zymogen could be irreversibly inactivated by treatment with 0.1 N HCl at pH 1.0 ± 0.2 for two hours at room temperature. When this inactivated protein was reduced and carboxymethylated, a single peak was obtained on gel filtration (Fig. 3B). The amount of carboxymethylcysteine formed corresponded to 0.95 ± 0.05 residue per molecule of the zymogen (Table I). Carboxymethylcysteine was not obtained when either the activatable zymogen or thezymogen protein inactivated by HCl was alkylated with iodoacetic acid in the absence of reducing agent but in the presence of 8 M urea and EDTA at pH 8.5.

When the enzyme was exposed to iodoacetate, iodoacetamide, or N-ethylmaleimide, in the absence of urea and without prior reduction, potential activity was unimpaired. No carboxymethylcysteine was formed with iodoacetate. If, however, the reduced enzyme was treated with iodoacetate, still in the absence of urea, activity was lost, and approximately one residue of carboxymethylcysteine was formed.

**NH₂-terminal Groups**—A portion of the zymogen solution, 10 to 15 mg in 4 ml, obtained from SE-Sephadex chromatography was carbamylated, and the NH₂-terminal amino acid was determined by the procedure of Stark and Smyth (21). The special procedures required for the determination of NH₂-terminal cysteine and tryptophan were not applied. Two analyses yielded 0.94 and 1.04 of a residue of NH₂-terminal aspartic acid per molecule of protein. Only traces (less than 0.05 of a residue) of other terminal residues were found except for glutamic acid, for which a value of 0.19 residue was obtained from the culture medium and for Fractions A, B, and C obtained chromatographically (Fig. 2). The data on the unfractionated sample, including determination of amide-NH₂ but not tryptophan, accounted for 97% of the weight and 98% of the nitrogen of the sample on an ash- and moisture-free basis. The results are expressed as the calculated number of residues per molecule, based on leucine equals 17 in this case, for a molecular weight of 31,682, which is compatible with the ultracentrifugally determined value of 32,000 for Fraction B. The most active of the three fractions (Fraction B) contains nearly 1 residue of half-cystine per molecule on this basis.

**Amino acid composition of streptococcal proteinase**

Results are given for the crystalline enzyme isolated from the culture medium and for Fractions A, B, and C obtained chromatographically (Fig. 2). The data on the unfractionated sample, including determination of amide-NH₂ but not tryptophan, accounted for 97% of the weight and 98% of the nitrogen of the sample on an ash- and moisture-free basis. The results are expressed as the calculated number of residues per molecule, based on leucine = 17 in this case, for a molecular weight of 31,682, which is compatible with the ultracentrifugally determined value of 32,000 for Fraction B. The most active of the three fractions (Fraction B) contains nearly 1 residue of half-cystine per molecule on this basis.

**Table II**

<table>
<thead>
<tr>
<th>Amino acid**</th>
<th>Crystalline enzyme</th>
<th>Fraction A</th>
<th>Fraction B</th>
<th>Fraction C</th>
<th>Expressed to nearest integer for Fraction B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid.</td>
<td>38.8</td>
<td>40.0</td>
<td>40.1</td>
<td>39.2</td>
<td>40</td>
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<tr>
<td>Glutamic acid.</td>
<td>28.9</td>
<td>29.2</td>
<td>29.0</td>
<td>28.6</td>
<td>29</td>
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<tr>
<td>Glycine.</td>
<td>37.2</td>
<td>37.4</td>
<td>37.4</td>
<td>37.6</td>
<td>37</td>
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<tr>
<td>Alanine.</td>
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<td>22.1</td>
<td>22.2</td>
<td>24.2</td>
<td>22</td>
</tr>
<tr>
<td>Valine.</td>
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<td>20.2</td>
<td>19.5</td>
<td>20.5</td>
<td>20</td>
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<tr>
<td>Leucine.</td>
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<td>17.0</td>
<td>17.0</td>
<td>17.0</td>
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<tr>
<td>Isoleucine.</td>
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<td>12.3</td>
<td>12.0</td>
<td>12.4</td>
<td>12</td>
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<tr>
<td>Serine**</td>
<td>20.1</td>
<td>26.3</td>
<td>20.3</td>
<td>25.4</td>
<td>26</td>
</tr>
<tr>
<td>Threonine**</td>
<td>11.4</td>
<td>11.5</td>
<td>11.5</td>
<td>11.4</td>
<td>12</td>
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<tr>
<td>Half-cystine**</td>
<td>0.70</td>
<td>0.67</td>
<td>0.93</td>
<td>0.41</td>
<td>1</td>
</tr>
<tr>
<td>Methionine**</td>
<td>5.00</td>
<td>5.00</td>
<td>5.00</td>
<td>5.10</td>
<td>5</td>
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<tr>
<td>Proline.</td>
<td>14.2</td>
<td>14.8</td>
<td>14.0</td>
<td>14.5</td>
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<tr>
<td>Phenyalanine.</td>
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<td>13.1</td>
<td>13.0</td>
<td>12.2</td>
<td>12</td>
</tr>
<tr>
<td>Tyrosine**</td>
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<td>18.9</td>
<td>19.3</td>
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<td>8.00</td>
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</tr>
<tr>
<td>Lysine.</td>
<td>16.9</td>
<td>17.3</td>
<td>17.2</td>
<td>17.4</td>
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<tr>
<td>Arginine.</td>
<td>8.57</td>
<td>8.64</td>
<td>8.92</td>
<td>8.70</td>
<td>9</td>
</tr>
</tbody>
</table>

**A** Tryptophan not determined.

**b** The results for the labile amino acids were corrected approximately for decomposition during 22 hours of acid hydrolysis. The factors applied were: serine, 0.90; threonine, 0.95; tyrosine, 0.95. (cf. Crestfield et al. (7)).

**c** Measured as cysteic acid or methionine sulfone after performic acid oxidation. The methionine values obtained on the four hydrolysates of the oxidized proteins were 4.50, 4.50, 4.55, and 4.91, respectively.

**Fig. 2.** Chromatography of the proteinase on sulfoethyl-Sephadex. Column, 0.9 X 60 cm. Eluent, 0.1 M phosphate buffer at pH 5.50. Load: A, 32 mg of protein in 14 ml of 0.02 M phosphate buffer; B, 3.85 mg of the protein eluted between 30 and 40 ml in A were rechromatographed; C, 3.54 mg of the protein eluted between 40 and 50 ml in A were rechromatographed.
acid. The significance of the small quantity of NH₂-terminal glutamic acid is not known.

Analysis of a proteinase preparation purified on Sephadex G-75 showed it to contain 0.77 of a residue of NH₂-terminal glutamic acid. This sample of proteinase had not been fractionated on SE-Sephadex and also contained 0.12 of a residue each of NH₂-terminal valine and glycine. It should be noted, however, that the relative quantities of the three NH₂-terminal amino acids are not similar to the relative areas of the peaks seen in Fig. 2A.

**DISCUSSION**

**Zymogen to Enzyme Transformation**—Although the zymogen is chromatographically homogeneous, the enzyme derived from it by proteolytic action in the culture medium is not. Several chromatographically distinguishable, activatable components are present in crystalline preparations of the proteinase. These components, although they differ in specific activity, cannot be distinguished on the basis of amino acid composition, other than by the small difference in half-cystine content. There is also a possibility that the components differ in amide content. In the formation of these activatable species, proteolysis has probably occurred at both ends of the peptide chain of the zymogen to reduce the molecular weight from 44,000 to approximately 32,000. Evidence for proteolysis at the amino end is provided by the disappearance of the NH₂-terminal aspartic acid that is characteristic of the zymogen, and the appearance of NH₂-terminal valine, glycine, and large quantities of glutamic acid. This hydrolysis may be a result of the presence of other proteolytic enzymes in the culture medium, or it may arise by autodigestion, as in the spontaneous transformation of trypsinogen to trypsin. Proteolysis at the carboxyl end of the zymogen could also arise by autodigestion because, as has been demonstrated in the present studies, crystalline preparations of the enzyme and all of the chromatographically separable components possess detectable carboxypeptidase activity. Components formed in this way might differ from one another by only a few amino acid residues at the carboxyl terminus and thus be difficult to distinguish by determinations of molecular weight or amino acid composition. Further study will be required to learn whether more controlled methods of proteolysis, by trypsin, for example, can give a higher yield of a single molecular species possessing potential enzymic activity.

It can be seen in Fig. 2A that there is a peak in the activity curve at 90 ml which is not reflected by a similar rise in the concentration of protein. Since the protein concentration is determined by ultraviolet absorption and by ninhydrin analysis after alkaline hydrolysis, this result may signal the presence of a component possessing a high ratio of activity to total amino acid residues and with a lower molecular weight. This result has been obtained reproducibly and means for increasing the yield of this fraction are under study. Since the structure of the active site is one objective of the present investigations, characterization of the smallest active fragment is obviously advantageous.

**Activation of Enzyme**—After conversion from zymogen to enzyme, reduction is essential before full catalytic activity, either proteinase or carboxypeptidase, is manifested. Reduction may be brought about by thiol compounds such as thiocethanol, thioglyeolic acid, cysteine, or 2,3-dimercaptopropanol (all are equally effective), by cyanide, or less effectively, by sodium borohydride (an activity of 0.8 units per mg has been obtained in preliminary experiments). Since, as the experiment with sodium borohydride shows the proteinase is active in the absence of thiols, these activators cannot be functioning as coenzymes. In this respect, the proteinase resembles papain (22).

EDTA at a concentration 0.001 M does not activate the enzyme nor does the chelating agent enhance the activity in the presence of thiocethanol. Other chelators such as O-phenanthroline and 8-hydroxquinoline were similarly ineffective as activators. Spectroscopic analyses, generously performed by Dr. Bert L. Vallee, have demonstrated the absence of more than a few tenths of an atom of heavy metal per molecule of enzyme.

All the available evidence indicates that the streptococcal proteinase is a sulfhydryl enzyme. Study of the role of the —SH group in catalysis is facilitated by the finding that there is only one potential such group per molecule of either enzyme or zymogen. It has been reported that the enzyme is apparently cyst(e)ine-free (23), but the methods used were probably not sensitive enough to measure one half-cystine residue per molecule. The chemical properties of this half-cystine residue are unusual. The potential —SH group in the zymogen does not react with iodoacetic acid until after reduction in the presence of 8 M urea. Even after conversion of zymogen to enzyme, the potential sulfhydryl group remains masked and will not react with sulfhydryl reagents until after reduction, although the presence of 8 M urea is not required. There is no evidence that this masked potential —SH group is part of an —S—S— bond of a cystine residue, because the analytical data permit only one half-cystine residue per molecule of either zymogen or enzyme, and the enzyme does not show a change in molecular weight from dimer to monomer on reductive activation. Definition of the chemical structure in which this half-cystine residue participates is a subject of continuing investigation. These studies, in conjunction with those on papain (24), should provide helpful comparisons of the chemical structures of two enzymes of different origins but which possess several properties in common.
SUMMARY

The extracellular proteolytic enzyme elaborated by Group A streptococci has been subjected to chromatographic purification, amino acid analysis, NH2-end group analysis, and molecular weight determination. The crystalline zymogen produced in the culture medium has a molecular weight of 44,000, gives a single peak when chromatographed on sulfoethyl-Sephadex, and has an aspartic acid residue in an NH2-terminal position. The most active major component of the mixture formed by proteolytic digestion of the zymogen has a molecular weight of 32,000; the preparations studied so far contain several chromatographically distinct fractions which have similar chemical properties, and predominantly glutamic acid in the NH2-terminal position. Both the zymogen and the most active fraction of the enzyme contain one residue of half-cystine molecule. The single potential -SH group seems to be essential for the activity of the enzyme; the protein requires activation by sulfhydryl compounds or other reducing agents and the reduced enzyme is inactivated by iodoacetate with the formation of one residue of carboxymethylcysteine. There is no change in molecular weight on reduction of the enzyme. In the zymogen or in the unreduced enzyme, even in the presence of 8 M urea, the potential -SH group is not available for reaction with iodoacetate, iodoacetamide, or N-ethylmaleimide.

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