A protease was purified from the culture filtrate of *Staphylococcus aureus*, strain V8, by a combination of procedures, such as precipitations with ammonium sulfate and acetone, chromatography on DEAE-cellulose, and preparative electrophoresis on polyacrylamide gel. The preparation is homogeneous by disc electrophoresis and sedimentation in the ultracentrifuge. Sedimentation velocity yields a value of $s_{20, w} = 2.9$ S. The molecular weight of the protease is estimated to be 12,000 by sedimentation equilibrium studies, and 11,400 by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The amino acid composition indicates 115 residues, and the absence of sulfhydryl groups. Valine is the NH$_2$-terminal amino acid of the enzyme.

The protease is active in the pH range of 3.5 to 9.5 and exhibits maximum proteolytic activity at pH 4.0 and 7.8. It is inhibited by diisopropyl fluorophosphate but not by EDTA. Studies on the substrate specificity with ribonuclease as the substrate revealed that the staphylococcal protease specifically cleaves peptide bonds on the COOH-terminal side of either aspartic acid or glutamic acid. In this report a procedure for the purification of an extracellular protease of *S. aureus*, strain V8, is described and some of the characteristics of the purified enzyme such as pH optima, molecular weight, amino acid composition, and substrate specificity are presented. This protease was shown to cleave specifically the peptide bonds at the carboxyl-terminal side of either aspartic acid or glutamic acid.

**EXPERIMENTAL PROCEDURE**

*Materials—* SDS, DFP, EDC, and β-glycerophosphate (Grade III, containing approximately 30% of the α isomer) were purchased from Sigma. N,N,N',N'-Tetramethylethylenediamine, N,N,N',N'-methylenebisacrylamide, and acrylamide were purchased from Eastman Organic Chemicals Co. DEAE-cellulose (DE-52) was obtained from Whatman. Proteins used as standards for molecular weight determination were cytochrome c, hemoglobin, subtilisin, and ribonuclease from Sigma, chymotrypsinogen from Nutritional Biochemicals, trypsin and chymotrypsin from Worthington, and myoglobin and lysozyme from Calbiochem.

Substrates such as casein (Hammersten) and denatured hemoglobin were obtained from Nutritional Biochemicals. N,N-Dimethylaseatin was prepared by the procedure of Lin et al. (4). Amidation of the carboxyl groups of N,N-dimethylaseatin with glycine ethyl ester was conducted by the method of Hoare and Koshland (5) with minor modifications. To a 1% solution of N,N-dimethylaseatin in 8 M urea at pH 4.3, containing 1 M glycine ethyl ester, solid EDC was added to 0.1 M. The mixture was maintained at 25°C, pH 4.75, and 1 hour later an identical amount of EDC was added. The reaction was allowed to continue for 2 additional hours. The protein solution was then dialyzed exhaustively against distilled H$_2$O and lyophilized.

*Organism and Culture Conditions—* *S. aureus*, strain V8, was obtained from Dr. T. Wadstrom. The bacteria were grown in a medium slightly modified from that described by Moravec et al. for the production of staphylococcal nuclease (6). It contains (amount per liter of solution in distilled water): K$_2$HPO$_4$, 2.4 g; NaH$_2$PO$_4$, 0.4 g; MgSO$_4$·7H$_2$O, 0.2 g; MnCl$_2$·4H$_2$O, 10 mg; FeSO$_4$·7H$_2$O, 6.3 mg; CaCl$_2$, 0.74 g; Difco Bacto-yeast extract, 5.0 g; β-glycerophosphate (sodium salt), 10.0 g; and casein, 15.0 g. CaCl$_2$ was sterilized separately and added before inoculation. Growth was carried out in 10-liter batches in a New Brunswick fermentor. The production of extracellular proteolytic enzymes in culture filtrates of *Staphylococcus aureus*, observed as early as 1916, has been the subject of a number of studies (1). However, it was only recently that attempts have been made to purify and characterize these proteases. Tirunarayanan and Lundblad (2) have demonstrated the presence of two electrophoretically distinct proteases in a partially purified enzyme preparation from *S. aureus*, strain Walker, and the two enzymes were shown to differ considerably from the concentrated culture filtrates of strain V8. Vesterberg et al. (3) separated three main peaks with proteolytic activity by isoelectric focusing. However, the properties of the proteolytic enzymes were not investigated.

In this report a procedure for the purification of an extracellular protease of *S. aureus*, strain V8, is described and some of the characteristics of the purified enzyme such as pH optima, molecular weight, amino acid composition, and substrate specificity are presented.

**SUMMARY**

A protease was purified from the culture filtrate of *Staphylococcus aureus*, strain V8, by a combination of procedures, such as precipitations with ammonium sulfate and acetone, chromatography on DEAE-cellulose, and preparative electrophoresis on polyacrylamide gel. The preparation is homogeneous by disc electrophoresis and sedimentation in the ultracentrifuge. Sedimentation velocity yields a value of $s_{20, w} = 2.9$ S. The molecular weight of the protease is estimated to be 12,000 by sedimentation equilibrium studies, and 11,400 by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The amino acid composition indicates 115 residues, and the absence of sulfhydryl groups. Valine is the NH$_2$-terminal amino acid of the enzyme. The protease is active in the pH range of 3.5 to 9.5 and exhibits maximum proteolytic activity at pH 4.0 and 7.8. It is inhibited by diisopropyl fluorophosphate but not by EDTA. Studies on the substrate specificity with ribonuclease as the substrate revealed that the staphylococcal protease specifically cleaves peptide bonds on the COOH-terminal side of either aspartic acid or glutamic acid. Casein, in which all carboxyl groups had been blocked with glycine ethyl ester in amide linkage, is not hydrolyzed by the protease.

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MF128 fermentor at 37° for 18 hours with moderate aeration and the agitation set at 250 rpm. After sterilization the fermentor was inoculated with 100 ml of inoculum well into log phase. At the completion of the cultivation, cetrimide was added to a final concentration of 1:10,000. After 1 hour, the suspension was then chilled to about 10° and centrifuged.

**Enzyme Assays**—Proteolytic activity was measured by the method of Kunitz (7). With casein as the substrate the reaction mixture contained in a final volume of 5 ml: 0.1 M Tris-HCl buffer pH 7.8, and 1% casein adjusted to pH 7.8 with NaOH. With hemoglobin as the substrate, the tube contained 0.8% hemoglobin in 0.1 M sodium acetate buffer, pH 4.0. Enzyme was added to start the reaction and the mixture incubated at 37° for 10 min. At the end of the incubation period, the reaction was stopped by the addition of 5 ml of 10% trichloroacetic acid. After standing for 15 min, the contents of the tubes were filtered through a Whatman No. 42 filter paper and the absorption at 280 nm was measured in a Cary model 16 spectrophotometer. All tests were carried out in duplicate and a blank was run with each assay. A linear rate of increase was obtained up to an absorbance of 0.200 with both substrates. A unit of activity was assigned as that amount which yielded 0.001 OD₅₅₀ unit of change per min under the conditions mentioned above. The specific activity is expressed as the number of enzyme units per mg of protein. Protein was measured by the method of Lowry et al. (8), with bovine serum albumin as a standard.

In some experiments proteolytic activity was determined colorimetrically according to the method of Lin et al. (4). In this case, proteolytic activity was expressed as the number of peptide bonds hydrolyzed calculated with the molar extension coefficient of 1.3 X 10⁴.

**Electrophoresis**—Electrophoresis in polyacrylamide gels was performed in 15% gels with Tris-glycine buffer, pH 8.9, according to the method of Davis (9) and at pH 4.3 as described by Reisfeld et al. (10). Electrophoresis was carried out at 4° and at 3.0 ma per tube. The gels were stained for protein with Amido black (0.55% in 7.5%/₄ acetic acid) for 1 hour and destained electrophoretically. Preparative polyacrylamide electrophoresis was done with the "PolyPrep" apparatus (Buchler Instruments Inc.). Fractionation was carried out on a 3.2-cm-high column of 7.5% gel in Tris buffer, pH 8.9, supporting a 1.0-cm layer of concentrating gel. The composition of the gels and the ratio of the various ingredients were the same as described in the procedure manual supplied by Buchler Instruments Inc.

SDS-polyacrylamide gel electrophoresis for estimation of the molecular weight of the protease was performed by the method of Shapiro et al. (11) as described by Weber and Osборn (12). The following reference proteins, with their molecular weights, were used: pepsin, 35,000; subtilisin, 27,600; chymotrypsinogen, 23,700; trypsin, 23,300; hemoglobin chains, 15,500; lysozyme, 14,300; ribonuclease, 13,700; cytochrome c, 12,400; and the two chains of chymotrypsin, 13,000 and 11,000.

**Ultracentrifugation**—Sedimentation velocity runs were performed in a Spinco model E ultracentrifuge at 20°. A photoelectric scanner operated at 280 nm was employed for the lower protein concentrations, while conventional schlieren optics were used for the higher concentrations. Low speed sedimentation equilibrium ultracentrifugation was conducted at 20° in a 12-mm double sector cell. The partial specific volume used for the determination of the molecular weight was calculated from the amino acid composition (13) (Table II).

**Amino Acid Composition**—Amino acid analyses were performed with a Beckman model 120C amino acid analyzer. Samples were hydrolyzed in 5.7 x HCl in sealed evacuated tubes at 110° for 24, 48, and 72 hours. Methionine and cysteine were determined on samples oxidized with performic acid according to the method of Hirs (14). Tryptophan was estimated by the procedure of Benze and Schmid (15) and by the colorimetric method of Gaitonde and Dovey (16) in formic acid-HCl (Reagent b). Lysozyme and trypsin which contain 6 and 4 residues of tryptophan, respectively, were used as standards in the latter method. The NH₂-terminal residue of the protease was identified and quantitatively estimated by the cyanate method of Stark and Smyth (17).

**Specificity of Protease**—The substrate specificity of the protease was elucidated by identification of the NH₂- and COOH-terminal amino acids of peptides produced by the hydrolysis of ribonuclease. The peptides were obtained by incubating oxidized ribonuclease (0.2 mg) at 37° with 5 μg of protease in 50 mM potassium phosphate buffer, pH 7.8, for 18 hours. The final volume was 0.5 ml. NH₂-terminal analyses were carried out by the 1-dimethylaminonaphthalene-5-sulfonfyl method (18). The 1-dimethylaminonaphthalene-5-sulfonfyl amino acids were identified by thin layer chromatography on silica gel-coated plates as described by Gros and Labouesse (19). The COOH-terminal amino acids were determined by hydrazinolysis. One milligram of hydrolysate was incubated at 80° for 14 hours with 0.6 ml of 90% hydrazine. After lyophilization, the COOH-terminal residues were identified by direct amino acid analysis. Blanks in which the substrate or the enzyme was omitted were run in parallel.

**RESULTS**

**Purification of S. aureus Protease**—The purification procedure is summarized in Table I. All operations were carried out at 4° unless otherwise noted. Dialysis of the enzyme preparations was always carried out in acetylated dialysis tubing (20) to minimize losses.

**Step 1: Ammonium Sulfate Precipitation**—To 5 liters of centrifuged culture filtrate were added, with stirring, 3000 g of solid ammonium sulfate. After further stirring for 1 hour, the suspension was centrifuged in a Sharples T-1 continuous flow centrifuge. The dark brown precipitate obtained was collected and dissolved in 10 mM Tris-HCl buffer, pH 7.5, containing 2 mM CaCl₂ to a final volume of 500 ml.

![Table I](http://www.jbc.org/)

**Table I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific Activity</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Culture filtrate</td>
<td>5,000</td>
<td>1,805</td>
<td>47,500</td>
<td>38</td>
<td>100</td>
</tr>
<tr>
<td>2. Ammonium sulfate precipitation</td>
<td>500</td>
<td>1,480</td>
<td>6,850</td>
<td>216</td>
<td>82</td>
</tr>
<tr>
<td>3. Acetone fractionation</td>
<td>250</td>
<td>1,220</td>
<td>1,075</td>
<td>1,134</td>
<td>67.5</td>
</tr>
<tr>
<td>4. DEAE-cellulose chromatography</td>
<td>180</td>
<td>525</td>
<td>450</td>
<td>1,168</td>
<td>29</td>
</tr>
<tr>
<td>5. Preparative electrophoresis</td>
<td>225</td>
<td>454</td>
<td>391</td>
<td>1,161</td>
<td>25.1</td>
</tr>
</tbody>
</table>

* Determined at pH 4.0 with hemoglobin as the substrate.

a Total of two preparations.

*Preliminary experiments indicated that calcium protected the enzyme at this stage of the purification, but this was not investigated further.*
tion could be interpreted as suggesting that the enzyme preparation was essentially free of extraneous proteins after chromatography on DEAE-cellulose. In less otherwise indicated, chromatography did not increase the specific activity of the protease this observation could be interpreted as suggesting that the enzyme preparation was essentially free of extraneous proteins after chromatography on DEAE-cellulose. Unless otherwise indicated, characterization of the protease was performed with the preparation obtained after preparative electrophoresis.

**Estimation of Purity**—The fractions obtained after the last three purification steps were examined by disc electrophoresis at pH 8.9. In Fig. 3, Gels 1 and 2 represent, respectively, the preparations obtained after precipitation with acetone and chromatography on DEAE-cellulose. The brown color in these gels represents the pigmented material associated with the enzyme preparations which was removed by preparative electrophoresis (Gel 3). The three preparations were also examined for the presence of cationic proteins by disc electrophoresis at pH 4.3. When high concentrations of enzyme (about 300 μg) were applied to the gels three very faint bands could be seen with the acetone-precipitated fraction. These contaminants, however, were removed by chromatography on DEAE-cellulose. The presence of a single major protein band in each of the gels was in relation to enzyme activity which did not increase significantly in the last purification steps (see Table I).

The fully purified staphylococcal protease was also examined using the ultra centrifuge. Sedimentation-velocity experiments performed at protein concentrations ranging from 0.22 to 10 mg per ml indicated that the enzyme sedimented as a single component with no perceptible evidence of heterogeneity (Fig. 4). The sedimentation rate of the enzyme showed some concentration dependence yielding an s_{20, w} value of 9.9 S.

**Molecular Weight**—The molecular weight of the protease was estimated by polyacrylamide gel electrophoresis in SDS. A plot of the logarithm of the molecular weights of the standard proteins against mobility gave a straight line (Fig. 5). By interpolation, the molecular weight of the protease was estimated to be 11,400.

The molecular weight of the protease was also estimated by the conventional sedimentation equilibrium method. A plot of the optical density at sedimentation equilibrium versus the square of the radial distance showed a straight line, indicating homogeneity (Fig. 6). The molecular weight estimated from the slope was 12,000 with a partial specific volume of 0.708 calculated from the amino acid composition. This molecular weight is in agreement with the value estimated by gel electrophoresis in SDS and suggests that the enzyme is composed of a single polypeptide chain.

**Amino Acid Composition**—The amino acid composition of purified staphylococcal protease, expressed as residues per 12,000 molecular weight, is summarized in Table II. Spectrophotometric analysis (15) revealed a tyrosine to tryptophan ratio of 3.0, a value which is in agreement with that obtained by colori-

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**Fig. 1.** Chromatography of *Staphylococcus aureus* protease on DEAE-cellulose. The preparation obtained after acetone precipitation was chromatographed on a DEAE-cellulose column and eluted with a linear potassium chloride gradient. Enzyme activity. The absorbance at 280 nm is shown as a continuous line.

**Fig. 2.** Electrophoretic elution of *Staphylococcus aureus* protease from polyacrylamide gel columns. Enzyme activity. The absorbance at 280 nm is shown as a continuous line. The horizontal line under the peak indicates the fractions pooled.
FIG. 3 (left). Polyacrylamide disc electrophoresis of the protease at different stages of purification. Gel 1, protease obtained after precipitation with acetone; 2, protease preparation after chromatography on DEAE-cellulose; 3, protease preparation after preparative disc electrophoresis.

FIG. 4 (right). Ultracentrifugation patterns of purified Staphylococcus aureus protease in 50 mM potassium phosphate buffer, pH 7.5, and 0.1 M NaCl. Ultracentrifugation was carried out at 59,780 rpm at a protein concentration of 8 mg per ml. The pictures shown were taken at 32-min intervals.

FIG. 5. Determination of the molecular weight of the staphylococcal protease by SDS polyacrylamide gel electrophoresis. The marker proteins are: 1, pepsin; 2, subtilisin; 3, chymotrypsinogen; 4, trypsin; 5, hemoglobin; 6, lysozyme; 7, ribonuclease; 8, chymotrypsin chain 1; 9, cytochrome c; 10, chymotrypsin chain 2.

FIG. 6. Sedimentation equilibrium of Staphylococcus aureus protease. Low speed sedimentation equilibrium was performed at a concentration of 0.4 mg per ml with the purified enzyme in 0.05 M potassium phosphate buffer, pH 7.5, at 20,000 rpm and 20°C. The abscissa is measured from the center of the rotor; the ordinate is the optical density of the solution measured at 280 nm.

Fig. 5. Determination of the molecular weight of the staphylococcal protease by SDS polyacrylamide gel electrophoresis. The marker proteins are: 1, pepsin; 2, subtilisin; 3, chymotrypsinogen; 4, trypsin; 5, hemoglobin; 6, lysozyme; 7, ribonuclease; 8, chymotrypsin chain 1; 9, cytochrome c; 10, chymotrypsin chain 2.

metric analysis (16). Valine was identified as the NH₂-terminal residue. One striking feature of the amino acid composition of S. aureus protease is the fact that proline accounts for about 10% of the total amino acid residues in the protein. The complete absence of half-cysteine and the high content of dicarboxylic residues or their amides are other noteworthy aspects of the composition. In contrast, only 1 residue of methionine, tryptophan, and arginine, respectively, is present.

Effect of pH on Proteolytic Activity—The effect of pH on proteolytic activity was studied with both hemoglobin and casein as substrates. As shown in Fig. 7, S. aureus protease exhibited two pH optima when assayed with hemoglobin, one at pH 4.0 and the other at pH 7.8. An optimum pH of 7.8 was also observed with casein. However, because casein tends to precipitate at pH values lower than 6.0 it was not possible to perform the assay at pH 4.0 with this substrate.

In view of the two pH optima, the specific activity of the enzyme during purification was also examined at pH 7.8 (Table III). Although the specific activity measured at pH 4.0 (see Table I) increased slightly after DEAE-cellulose chromatography, a 50% loss in specific activity was observed when the assay was carried out at pH 7.8 with casein. A decrease in activity was also noted when hemoglobin was used as the substrate at this pH. Unfortunately, under the latter conditions, the activity could not be measured precisely because the reaction showed a nonlinearity with time and enzyme concentration.

The activity of the protease preparations before and after
TABLE II

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Period of hydrolysis</th>
<th>Nearest integer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hrs</td>
<td>48 hrs</td>
</tr>
<tr>
<td>Lysine</td>
<td>5.6</td>
<td>5.6</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.9</td>
<td>2.8</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>20.2</td>
<td>28.3</td>
</tr>
<tr>
<td>Threonine</td>
<td>7.9</td>
<td>7.8</td>
</tr>
<tr>
<td>Serine</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>10.7</td>
<td>10.4</td>
</tr>
<tr>
<td>Proline</td>
<td>10.9</td>
<td>10.8</td>
</tr>
<tr>
<td>Glycine</td>
<td>8.6</td>
<td>8.8</td>
</tr>
<tr>
<td>Alanine</td>
<td>6.8</td>
<td>6.7</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Valine</td>
<td>6.0</td>
<td>6.9</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.3</td>
<td>5.8</td>
</tr>
<tr>
<td>Leucine</td>
<td>3.9</td>
<td>3.9</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.1</td>
<td>3.2</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Tryptophan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₂-terminal valine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Value obtained by extrapolation to zero hydrolysis time.
* Average of recoveries after 72 hours.
* Determined spectrophotometrically (12) and colorimetrically (13).
* Determined by the cyanate method (14).

Fig. 7. Effect of pH on the proteolytic activity. The substrate solutions were adjusted to various pH values with NaOH or HCl. The buffer systems employed were: citrate-phosphate, pH 3.0 to 7.0; phosphate, pH 6.0 to 7.4; and Tris-HCl, pH 7.0 to 9.0. O, hemoglobin; O, casein.

DEAE-cellulose chromatography was also examined by the method of Lin et al. (4). Fig. 8 shows that casein was rapidly hydrolyzed during the 1st hour of incubation by the two enzyme preparations. However, after 6 to 15 hours, when no additional new amino groups were detected, the total number of bonds cleaved by the enzyme preparation precipitated with acetone was about twice as high as that cleaved by the purified enzyme.

The reason for the decrease in specific activity at pH 7.8 but not at pH 4.0 is not clear and this problem is under further investigation.

Evidence for Presence of Single Protease—The presence of two closely related enzymes with pH optima at 4.0 and 7.8, respectively, was ruled out on the basis of the following observations. The proteolytic activity examined at pH 4.0 with hemoglobin and at pH 7.8 with casein, was equally resistant to heat denaturation over a wide range of temperatures (Fig. 9). The partial inactivation, starting at a temperature between 40 and 50⁰C, was found to be due to some autodigestion as revealed by an increase in unhydrophilic-reactive groups. Moreover, when the
The asparaginyl and glutaminyl bonds were mostly cleaved.

Substrate must be unsubstituted for hydrolysis to occur. This enzyme was substituted. These results therefore indicate that casin but had no effect on the substrate when its carboxy group coupled with glycinyl ethyl ester in amide linkage. As not expected to hydrolyze proteins which are devoid of free peptide bonds involving only dicarboxyl amino acids, it would not lose any activity after extensive dialysis against distilled H2O suggesting that cations were not required for enzymatic activity or for protection of the enzyme against autodigestion. The addition of Ca++, Mg++, Mn++, Zn++, EDTA, or citrate, at concentrations ranging from 1 to 10 mM in the assay mixture, did not have any effect on substrate hydrolysis.

**Substrate Specificity**—The specificity of the purified protease was elucidated by the identification of the NH2- and COOH-terminal residues of the fragments produced from oxidized ribonuclease by the action of the protease. Only arginine, alanine, serine, threonine, glycine, alanine, and valine were found as new NH2-terminal amino acids. Upon examination of the amino acid sequence of ribonuclease (21) it is evident that S. aureus protease has a narrow specificity and only the peptide bonds on the carboxyl-terminal side of aspartic acid and glutamic acid were cleaved with the exception of the aspartyl-cysteic acid bond. As new COOH-terminal residues, only aspartic acid and glutamic acid were detected by hydrazinolysis in the hydrolysates of ribonuclease and casein. An identical specificity was exhibited by the enzyme from the culture filtrate and the preparation obtained after acetone precipitation suggesting that a single major extracellular proteolytic enzyme is produced by the organism.

If the staphylococcal protease has a specific requirement for peptide bonds involving only dicarboxylic amino acids, it would not be expected to hydrolyze proteins which are devoid of free carboxyl groups. This has been shown by examining the action of the protease on N,N-dimethylcasein which had all carboxyl groups coupled with glycine ethyl ester in amide linkage. As shown in Fig. 11 the protease readily digested N,N-dimethylcasein but had no effect on the substrate when its carboxyl groups were substituted. These results therefore indicate that the β and γ-carboxy groups of the acidic amino acids of the substrate must be unsubstituted for hydrolysis to occur. This is also in support of the observation that in oxidized ribonuclease the asparaginyl and glutaminyl bonds were not cleaved.

**DISCUSSION**

Of some 50 strains of S. aureus, strain V8 was found to produce by far the highest levels of protease. The quantity of enzyme produced by this strain, however, was found to vary considerably from one culture batch to another, although the cultural conditions appeared to be identical at all points. At present, no reasons can be given to explain this variation.

The observation that the specificity exhibited by the crude enzyme in the culture filtrate was identical with that of the purified enzyme strongly suggests that only one protease is produced extracellularly by this organism. This is, however, at variance with the conclusion of Tirunarayanan and Lundblad (2) who claimed the presence of two distinct extracellular proteases. Vesterberg et al. (13), using isoelectric focusing, detected the presence of three peaks with proteolytic activity in culture filtrates. However, the possibility that more than one protease is produced by other strains under different cultural conditions or both cannot be excluded.

The decrease in activity of the protease at pH 7.8 with casein as the substrate was consistently observed after the chromatographic purification of the acetone-precipitated preparation on DEAE-cellulose. Since the purified enzyme cleaved fewer bonds in casein than the enzyme before chromatography (Fig. 8), this could possibly account for the decrease in activity. Since even the crude and the enzyme obtained after acetone precipitation cleaved only aspartyl and glutamyl bonds, the purified enzyme would be expected to be more restrictive, perhaps affected by the presence of certain neighboring residues. This assumption, however, was not shown in the present study on the specificity of the protease with ribonuclease as the substrate. In this case, only the Asp-Cys (cysteic acid) linkage was not hydrolyzed and all the other susceptible bonds were cleaved by the purified enzyme. It is realized, however, that a limited number of aspartyl and glutamyl bonds exists in ribonuclease. Ultimate proof will depend upon examination of additional aspartyl and glutamyl bonds in proteins or peptides of known amino acid sequences.

Recently another proteolytic enzyme, isolated from germinated sorghum, was found to have a specificity identical with that of S. aureus protease (22). This enzyme differed significantly from the staphylococcal protease in a number of properties. Thus, while the sorghum protease had a molecular weight of about 80,000 and an acidic pH optimum, S. aureus protease has a molecular weight of the order of 12,000 and is active in the pH range of about 3.5 to 9.5. Although its inhibition by DFP (Fig. 10) cannot be considered as conclusive evidence for its classification as a serine enzyme, the observation that it is not inhibited by EDTA, is active at alkaline pH, and is free of ions was elucidated by the identification of the K+ and Ca++.
sulphydryl groups, strongly suggests that it is not a neutral, acidic, or thiol protease.

The most striking difference between the staphylococcal protease and other extracellular proteolytic enzymes of microbial origin resides in its high degree of substrate specificity. In general, other bacterial and fungal proteases have wide spectra of peptide bond specificity. Furthermore, many proteolytic microorganisms are known to produce more than one protease. In contrast, S. aureus appears to produce only one extracellular proteolytic enzyme which is highly specific for peptide bonds involving dicarboxylic amino acids. Since only relatively large peptides would be liberated by the action of this protease from proteins in growth media, this enzyme is expected to be of limited importance in the nutrition of the organism unless there is an efficient transport system for such peptides.

The small size of the protein makes it ideally suited for a study of structure in relation to activity. Furthermore, a comparison of the amino acid sequence of the staphylococcal protease with those of other proteases of the same class may lead to useful information on the evolutionary variations of this group of enzymes.

Acknowledgments—We are indebted to Mrs. Alice R. Rae for skilled technical assistance, and to Dr. Wadstrom for providing us the S. aureus strain V8. We also wish to express our thanks to Doctors L. Brakier-Gingras and A. G. Borduas for assistance in the ultracentrifugation studies and our appreciation also goes to Dr. L. Gyenes for his critical reading of the manuscript.

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Gabriel R. Drapeau, Yves Boily and Jean Houmard


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