Purification and Substrate Specificity of a Strongly Hydrophobic Extracellular Metalloendopeptidase ("Gelatinase") from Streptococcus faecalis (Strain 0G1-10)*

(Received for publication, March 4, 1988)

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An extracellular Zn-endopeptidase was purified to homogeneity from the culture filtrates of Streptococcus faecalis (human oral strain OG1-10) by a procedure that comprised concentration in an Amicon Hollow Fiber System, ammonium sulfate precipitation, gel permeation chromatography, hydrophobic interaction chromatography (batch operation on phenyl-sepharose Cl-4B), followed by fast protein liquid chromatography (FPLC) on a phenyl-Superose HR 5/5 column, and finally FPLC on a Superose 12 HR 10/30 column. The enzyme is a 31.5-kDa strongly hydrophobic protein with an isoelectric point of 4.6 and a broad pH optimum of 6 to 8. The substrate specificity of the enzyme is similar to that of the mammalian membrane endopeptidase-24.11 and Streptococcus thermophilus thermolysin (EC 3.4.24.4) in hydrolyzing preferentially the Phe\(^{24}\)-Phe\(^{26}\) bond in insulin B-chain, followed by cleavage of the His\(^{5}\)-Leu\(^{16}\) bond. The enzyme was especially active on Azocoll and gelatin; soluble and insoluble collagens were hydrolyzed at a lower rate. S. faecalis sex pheromone-related peptides and several mammalian bioactive peptides were cleaved at sites involving pronounced hydrophobicity. The enzyme did not hydrolyze small synthetic peptide derivatives (phenylazobenzyloxy carbonyl L-Pro-L-Leu-Gly-L-Pro-D-Arg and 2-furylacryloyl-L-Leu-Gly-L-Ala) that are typically attacked by "true" bacterial collagenases. Chemical modification indicated the importance of histidine, carboxyl, and tyrosyl groups in enzyme activity, suggesting that this enzyme may thus be classified as a metalloproteinase II (EC 3.4.24.4). The enzyme is strongly inhibited by a 720-kDa factor present in rat inflammatory exudate. The pronounced ability of the enzyme to attack collagenous materials and certain bioactive peptides suggests its participation in inflammatory processes involving the presence of S. faecalis.

Streptococcus faecalis is frequently identified as the etiological agent of such opportunistic infections as soft tissue and urinary tract infections, intraabdominal abscesses, and root canal infections (1–5). Furthermore, group D streptococci have been found to be the causative agents in 10 to 20% of bacterial endocarditis cases (6), with the primary offending species being S. faecalis (7). Secondary bacteremia frequently results from S. faecalis soft tissue and urinary tract infection (8), and it is the most common Gram-positive organism identified in cases of polymicrobial bacteremia (9). The relationship of proteolytic enterococci to food poisoning and certain related disease conditions has also received attention in the past (10). Clinical isolates of S. faecalis are frequently resistant to antibiotics, a characteristic usually determined by plasmids or conjugative transposons (11). Finally, enterococci have been observed as prominent members of the flora in the advancing front of human dentine caries (12). A S. faecalis-like strain in combination with a proteolytic bacillus was cariogenic in rats (13). Gold et al. (14) found that the gelatin liquefying human strain 2SaR of S. faecalis induced caries, whereas non-proteolytic enterococci did not. These clinical findings suggest that the proteolytic strains of S. faecalis may display primary or secondary (proteolytic) aggressiveness in some clinical situations. Some strains of S. faecalis produce a hemolysin-bacteriocin (15), a trait which has been shown to contribute to virulence in a mouse model (16). Production of hemolysin has also recently been found to be common in a human isolate associated with parenteral infections (17).

The focusing of attention on hemolysin-bacteriocin production as contributing to aggressiveness of S. faecalis has perhaps ignored the possibility that extracellular proteinases produced by some of these organisms also contribute to virulence. Purification and properties of a 28–32-kDa metalloproteinase from S. faecalis var. liquefaciens were reported (18–21) 2 to 3 decades ago, but no further chemical information about this enzyme has since then been available. This enzyme has also been trivially called gelatinase, connoting that it liquefies gelatin. Previous studies show that this extracellular proteinase is newly formed, and no evidence for a proteinasezymogen was found (22). The enzyme was suggested to be a metalloenzyme with a metal specificity for zinc in reactivation after chelation (22). The purpose of the present paper is to describe a modern purification procedure for this enzyme and to report completely new substrate specificity data concerning proteins and bioactive peptides, including pheromone-related substances (11) produced by the organism. This new information, supplemented by results from chemical modification studies, was considered to facilitate the classification of the enzyme. This research effort is related to our aspiration toward a better understanding of the relationship between connective tissue and the extracellular proteinase profile of organisms pathogenic to humans (23).

EXPERIMENTAL PROCEDURES

Bacterial Strain and Cultivation—The strain used in this study was S. faecalis var. liquefaciens OG1-10 (24), a streptomycin-resistant mutant of the cariogenic human isolate 2SaR (14). Cells (1% inocula) were grown aerobically in a brain-heart infusion medium (Difco) in

* This study was supported by National Institute of Dental Research Grant DE02731 and the Finnsugar Research Foundation, Helsinki, Finland. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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enzyme was started by concentrating the 80-liter supernatant fluid in an Amicon Hollow-Fiber System DC2 (M, cut-off 10,000; cartridge H1P10) to 1604 ml.

Precipitation with Ammonium Sulfate—The enzyme present in the 1,604-ml concentrate was precipitated with ammonium sulfate at 50% saturation. After 2 h standing, the mixture was centrifuged at 27,500 x g for 15 min, and the pellets were suspended in 50 mM Tris-HCl, 1 mM Ca++, pH 7.8, to a final volume of 165 ml. The enzyme was completely precipitated at 50% ammonium sulfate saturation while most of the contaminating protein was excluded (Fig. 1A).

**Gradient Analysis**—The 165-ml sample was passed in seven successive lots through a Sephadex G-200 column (Fig. 1B). The active fractions from all separations were combined (2724 ml) and subjected to the next step.

**Hydrophobic Interaction Chromatography**—A pilot study (Fig. 2A) on a phenyl-Sepharose Cl-4B column showed that the enzyme was effectively purified with a high yield. This justified the use of batch operations in large-scale purification. Accordingly, suitable quantities of phenyl-Sepharose Cl-4B were washed with 50 mM Tris-HCl (1 mM Ca++, pH 7.8) and filtered through a Pyrex 40–50 glass filter. About 370-ml aliquots of the enzyme from the above Sephadex G-200 pool were mixed with 38 g of filter-dry gel. The mixtures were incubated with occasional stirring. Binding
was adsorbed in the presence of 35% ammonium sulfate. Elution was performed in three steps: 1) 35% ammonium sulfate in 0.1 M Tris-HCl (1 mM Ca\(^{2+}\), pH 7.0); 2) the same buffer without ammonium sulfate; 3) the same buffer with 50% ethylene glycol. Panel B, batch separation of the enzyme in the absence of ammonium sulfate. The amount of total protein in the starting material and in the ethylene glycol-desorbed enzyme is indicated. The protein concentrations show the total amount of protein present in the 100-ml aliquots of buffer used to desorb the enzyme. The results justified the use of batch technique in mass purification.

was almost complete within 1 h, but the reaction was continued overnight to ascertain complete binding. The mixtures were then filtered, and the gels were washed six times with 100-ml portions of the above buffer allowing the mixtures to stand each time for 30 min before filtration. No enzyme was washed out during this procedure. For the desorption of the enzyme, the gels were subsequently treated at 22 °C with eight successive 25- to 50-ml portions of 50% (v/v) ethylene glycol in 50 mM Tris-HCl (1.0 mM Ca\(^{2+}\), pH 7.8), 30 min at a time (Fig. 2B). The entire Sephadex G-200 pool was treated in this way after which all ethylene glycol-containing filtrates were combined, dialyzed, and concentrated with an Amicon stirred cell concentrator (Mr cut-off 10,000) under nitrogen to 43 ml. This concentrate was dialyzed for 50 h against 5,000 ml of 10 mM Tris-HCl (1 mM Ca\(^{2+}\), pH 7.0), changing the buffer three times. The concentration was thereafter continued on an Amicon Centricon-10 Microconcentrator to 4 ml. The enzyme was now dialyzed against 1,000 volumes of 5 mM phosphate buffer, pH 7.0, for 72 h (tubing Mr cut-off 12,000–14,000). The concentration of ethylene glycol in the enzyme was reduced below 0.001%.

**Hydrophobic Interaction Chromatography (Column)—**0.9-ml aliquots of the enzyme from the previous step were mixed with 0.1 ml of 2.5 M ammonium sulfate in 5 mM phosphate buffer, pH 7.0. The resulting samples were injected into a phenyl-Superose HR 5/5 column. The isocratic elution was performed with 5 mM phosphate buffer, pH 7.0, at a rate of 0.5 ml/min, collecting 0.5-ml fractions. At elution conditions indicated in Fig. 3A the proteinase was eluted at 10 to 20 min. The active fractions were combined from each separation, and the resulting pool was concentrated with Centri-10 membrane filters to 4 ml.

**Gel Permeation Chromatography by FPLC:** Purity of the

![Fig. 2](image-url)

**Fig. 2. Separation of the enzyme on phenyl-Sepharose Cl-4B gel. Panel A, column chromatography (0.8 × 10 cm). The enzyme was adsorbed in the presence of 35% ammonium sulfate. Elution was performed in three steps: 1) 35% ammonium sulfate in 0.1 M Tris-HCl (1 mM Ca\(^{2+}\), pH 7.0); 2) the same buffer without ammonium sulfate; 3) the same buffer with 50% ethylene glycol. Panel B, batch separation of the enzyme in the absence of ammonium sulfate. The amount of total protein in the starting material and in the ethylene glycol-desorbed enzyme is indicated. The protein concentrations show the total amount of protein present in the 100-ml aliquots of buffer used to desorb the enzyme. The results justified the use of batch technique in mass purification.**

![Fig. 3](image-url)

**Fig. 3. Final purification of the enzyme by successive FPLC procedures on a hydrophobic interaction and a gel permeation column. Panel A, the phenyl-Sepharose HR 5/5 column was equilibrated and eluted with 5 mM phosphate buffer, pH 7.0, at a flow rate of 0.5 ml/min. Panel B, the enzyme from the above step was chromatographed on a Superose 12 HR 10/30 gel column using 10 mM Tris-HCl (1 mM Ca\(^{2+}\), 100 mM NaCl, pH 7.0) as the elution buffer at a rate of 0.5 ml/min. Sample volume was 0.42 ml.**
**Enzyme**—Suitable aliquots of the enzyme from the previous step were injected into a Superose 12HR 10/30 gel column. The enzyme appeared as one homogeneous peak that coincided with the single protein peak observed (Fig. 3B). The purity and specific activity of the enzyme did not essentially improve in this chromatography (Table I), indicating that the enzyme was obviously already homogeneous before step 7 (Table I). The enzymes obtained after steps 6 and 7 were SDS-PAGE-pure (8-25% gradient, silver staining).

**Molecular Weight and Isoelectric Point**—The molecular weight of the enzyme was determined with the following results: SDS-PAGE, 33,000; Sephadex G-100, 32,000; Superose 12 (FPLC), 29,500 (mean 31,500). The isoelectric point of the enzyme was 4.6.

**Hydrophobicity of the Enzyme**—The S. faecalis proteinase was strongly hydrophobic: the enzyme was completely precipitated even from diluted protein solutions using 50% ammonium sulfate; precipitation started already at 20-30% saturation. The enzyme was also very strongly bound to phenyl-Superose and phenyl-Sepharose gels even in low ion concentrations. The strength and specificity of this binding which took place without added salt demonstrate the possibility that the hydrophobic phenyl residues of the gels directly interacted with a hydrophobic site of the active domain of the enzyme. The strong hydrophobicity of the active domain is supported by the ability of the enzyme to hydrolyze insoluble hydrophobic substrates and by the presence of hydrophobic amino acid residues adjacent to the scissile peptide bonds (see below). Table II gives the amino acid composition of the enzyme which shows, with regard to at least 11 amino acids, a rather close similarity with the amino acid composition of Staphylococcus aureus metalloendopeptidase (39). The portion of hydrophobic amino acids in the present enzyme was at least 43%, whereas in the S. aureus enzyme and the Bacillus cereus collagenase (23) the portion of these amino acid residues was about 40 and 37%, respectively.

**Substrate Specificity**—Azocoll proved to be a superior protein substrate for the S. faecalis proteinase; it was convenient to use and it was hydrolyzed at a high rate (Table III). None of the 24 commercially available (Sigma, Calbiochem) aminooacyl-2-naphthylamides (including Nα-benzoyl-DL-arginine-2-naphthylamide) was hydrolyzed by the enzyme. Nα-Benzoyl-DL-arginine-p-nitroanilide, phenylazobenzyloxy-carbonyl-L-prolyl-L-tyrosylglycyl-L-prolyl-D-arginine, and 2-furylacryloyl-L-tyrosylglycyl-L-prolyl-L-alanine were not hydrolyzed either. 3-(2-furylacryloyl)-glycyl-L-tyrosyl amide was hydrolyzed at a very low rate at the Gly-Leu bond. Elastin, Orcein and Hide Powder Azure were very poor substrates. Several bioactive peptides and S. faecalis pheromones were hydrolyzed at a higher rate. The specificity studies are summarized below.

**Proteins**—The enzyme was able to completely dissolve Azocoll in 50 mM Tris-HCl, pH 7.8 (containing 1 mM CaCl2); a 10-μg aliquot of the enzyme dissolved a 5-μg quantity of Azocoll in 3 h at 37 °C. When the completely dissolved material was subjected to FPLC on a ProRPC column, several hydrolysis products appeared (Fig. 4A); the products were eluted at a higher than 20% acetonitrile concentration. The molecular weight of the product was studied using Centricon membrane filters (M, cut-off 10,000 and 39,000) (Fig. 4A inset) and gel permeation chromatography on a Superose 12 column (Fig. 4B). Accordingly, products with a large molecular weight (>30,000) appeared very fast in the reaction mixture, accompanied or followed by a clearly slower liberation of products with molecular weights <30,000. Fig. 4C shows a similar separation of gelatin on Superose 12; the products were essentially the same size as in the Azocoll experiment.

**Peptides**—Table IV shows that four S. faecalis sex pheromone-related peptides (two pheromones and two inhibitors) were hydrolyzed at a site involving a Leu residue on the imino side of the scissile bond. The pheromone-related peptide CAM373 (Ala-ile-Phe-ile-Ala-Ser) was not hydrolyzed. Insulin B-chain was rapidly cleaved at Phe24-Phe25, followed by the hydrolysis at His2-Leu6. Prolonged incubation resulted in the hydrolysis of at least Ala14-Leu15 and His10-Leu11. All cleavage sites were characterized by the presence of a hydrophobic amino acid residue on the imino side of the peptide bond (Fig. 5). Insulin A-chain was hydrolyzed at a much lower rate than B-chain, the only scissile bonds (in 1-h incubation) being Gin10-Leu15 and Tyr14-Gln15, in this order of preference. The other peptides tested were also hydrolyzed at sites involving a hydrophobic amino acid residue on the imino side of the scissile bond. Leucine-enkephalin would theoretically be a good substrate, as judged by the presence of a Gly-Phe bond in the molecule. The very low rate of hydrolysis of this substrate indicates, however, that pentapeptides may represent the minimum substrate length that can trigger catalysis by this enzyme. Substance P liberated the following peptides: Arg2-Gln5, Arg1-Phe4, Arg1-Gly5, Phe3-Gly2, Phe1-Met4, and Leu10-Met11. The hydrolysis of most of these bonds progressed concretedly, but the formations of Arg2-Gly5 and Leu10-Met11 were the primary and the fastest hydrolytic

### Table 1

**Purification of the extracellular collagenolytic protease from S. faecalis var. liquefaciens (strain 0G1-10)**

| Step | Volume | Protein | Total protein | Specific activity | Yield | Purification factor |
|------|--------|---------|---------------|------------------|-------|-------------------|
| 1. Supernatant fluid of culture filtrate | 90,000 | 19.9 | 800 | 0.041 | 100 | 1 |
| 2. After Hollow-Fiber concentration | 1,604 | 19.5 | 31 | 0.966 | 91 | 23.5 |
| 3. After ammonium sulfate precipitation | 165 | 533 | 8.8 | 3.3 | 88 | 80.5 |
| 4. After gel permeation chromatography | 2,724 | 0.59 | 1.6 | 16.0 | 78 | 399 |
| 5. After hydrophobic interaction chromatography (batch operation) | 178 | 0.197 | 0.035 | 434.8 | 46.4 | 10,805 |
| 6. After hydrophobic interaction chromatography (FPLC column) | 4 | 1.17 | 0.0047 | 647 | 9.3 | 15,780 |
| 7. After gel permeation chromatography (FPLC column) | 4 | 1.10 | 0.0044 | 650 | 8.7 | 15,853 |

*In enzyme units min⁻¹ mg⁻¹ (1 unit of the enzyme produced an extinction coefficient of 0.001 in 1 min at 37 °C when tested in 50 mM Tris-HCl, pH 7.8, using Azocoll as substrate (cf. "Experimental Procedures")

*The enzyme was stored at -20 °C in 5 mM phosphate buffer, pH 7.0.
events, followed by the formation of Arg¹-Phe⁷ and Arg⁴-Gln⁶ (in this order). Of the products that contained the NH₂-terminal Arg⁴, only Arg⁴-Gly⁵ was hydrolyzed further. Table V compares the specific activities of the enzyme for the scissile bonds in insulin B-chain and substance P and shows the corresponding \( K_m \) values.

**Nature of the Amino Terminus of the Hydrolysis Products of Azocoll**—Azocoll was incubated with the enzyme for 24 h in 50 mM Tris-HCl, pH 7.8 (containing 1 mM Ca²⁺), and the NH₂-terminal amino acids of the hydrolysis products were identified by treating the peptides so liberated with aminopeptidase M (Pierce Chemical Co.). The gelatinase had hydrolyzed in Azocoll peptide bonds whose imino group belonged to one of the following hydrophobic amino acids: Ala, Leu, and Phe.

**Amino acid composition of the extracellular proteinase from S. faecalis (OG1-10)**

| Amino acid | S. faecalis enzyme | S. aureus metalloprotease | Thermolysin |
|------------|------------------|--------------------------|-------------|
| Aspartic acid | 44               | 58                       | 44          |
| Threonine | 22               | 23                       | 25          |
| Serine | 23               | 22                       | 26          |
| Glutamic acid | 32               | 41                       | 21          |
| Proline | 10               | 5                        | 8           |
| Glycine | 32               | 36                       | 36          |
| Alanine | 32               | 28                       | 28          |
| Cysteine | ND⁴              | 0                        | 0           |
| Valine | 18               | 18                       | 22          |
| Methionine | 4                | 5                        | 2           |
| Isoleucine | 13               | 12                       | 18          |
| Leucine | 19               | 19                       | 16          |
| Tyrosine | 22               | 22                       | 28          |
| Phenylalanine | 12              | 12                       | 10          |
| Histidine | 9                | 9                        | 8           |
| Lysine | 20               | 20                       | 11          |
| Tryptophan | ND⁴             | 2                        | 3           |
| Arginine | 10               | 7                        | 10          |

⁴The values cited were corrected for losses during hydrolysis. The hydrolysates were analyzed on a Beckman Model 6300 high performance automated amino acid analyzer using norleucine as internal standard. The samples were hydrolyzed for 4 h in 6 N HCl at 145 °C.

**Effect of S. faecalis proteinase on various peptides and proteins**

All assays were carried out at 30 °C in 50 mM Tris-HCl with 1 mM Ca²⁺, pH 7.8, for 3 h in a shaking water bath. The 1-ml reaction mixtures contained 0.2 μg of enzyme and 2 mg of substrate. No attempts were made to solubilize the protein.

**Effect of pH**—Azocoll was rapidly hydrolyzed by the enzyme over the pH range of 6 to 8 (Fig. 6A). The optimum pH of the hydrolysis of the Ser²-Leu⁴ bond in the pheromone cadI was about 7.2 (Fig. 6B). The sharper pH optimum observed in the hydrolysis of the pheromone indicates a different type of mechanism of hydrolysis at acidic pH values compared with Azocoll. The stability of the enzyme strongly decreased at acidic pH values: treating the enzyme for 17 h in 50 mM Mes, pH 4.8-7.5, showed that it was stable only above pH 5.6. These results are in line with the metalloenzyme nature of the enzyme; many metalloproteases are inactivated at low pH values because of loss of metal.
Metalloendopeptidase from *S. faecalis*

**TABLE IV**

Effect of *S. faecalis* proteinase on peptides

| S. faecalis pheromones-related peptides: | Amino acid sequence and sites of hydrolysis |
|----------------------------------------|------------------------------------------|
| iPDI*                                  | Ala-Leu-Ile-Leu-Thr-Leu-Val-Ser          |
| cADI*                                  | Leu-Phe-Ser-Leu-Val-Leu-Ala-Gly          |
| iADI*                                  | Leu-Phe-Val-Va1-Thr-Leu-Val-Gly          |
| cPDI*                                  | Phe-Leu-Val-Met-Phe-Leu-Ser-Gly          |
| Bradykinin                             | Arg-Pro-Pro-Lys-Phe-Ser-Pro-Phe-Arg      |
| [Lys+]Bradykinin                       | Lys-Pro-Gly-Phe-Ser-Pro-Phe-Arg          |
| Lys-Bradykinin                         | Lys-Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg      |
| Substance P                            | Arg-Pro-Lys-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH2 |
| Angiotensin I                          | Asp-Arg-Val-Tyr-Ile-His-Pro-His-Leu      |
| Angiotensin II                         | Asp-Arg-Val-Tyr-Ile-His-Pro-Phe          |
| Cholecystokinin'                       | Tyr-Met[S04H]-Gly-Trp-Met-Asp-Phe        |
| Glucagon'                              | Phe-Val-Gln-Trp-Leu-Met-Asn-Thr          |
| Leucine-enkephalin'                    | Tyr-Gly-Gly-Phe-Leu                      |
| β-Lipotropin'                          | Arg-Tyr-Gly-Gly-Phe-Met                  |
| Neurotensin'                           | Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu          |

* Competitive inhibitors of pheromones produced by plasmid-containing strains.
* Sex pheromones.
* Peninsula Laboratories, Inc. (San Carlos, CA); the pheromone-like peptides were from Milligen, and all other peptides were from Sigma.
* Only partial sequence is shown.

Identification of Active Site Groups—Previous studies have established that the zinc atom in this enzyme is essential for activity (20, 21). We confirmed this and additionally obtained new information from chemical modification studies which are summarized as follows. Phenylglyoxal and 2,3-butane-dione, studied under non-photooxidative conditions in borate buffer (38), were not inhibitory, indicating that the enzyme activity may not depend on active arginyl groups. One to 10 mM DEP, tested in 0.1 M phosphate buffer, pH 6.9, or in 90 mM Mes, pH 6.8, in the presence of 0.3 μM enzyme at 22 °C, caused a rapid and progressive loss of enzyme activity. Treatment of the inactivated enzyme with 0.1 M hydroxylamine for 17 h at 4 °C restored 50% of the activity. Five mM TNM, tested in 40 mM Tris-HCl, pH 7.5-11.2 at 22 °C, caused in 30 min a rapid, progressive, and irreversible inactivation of the enzyme that was more pronounced in the alkaline region: at pH 11 about 85% of the activity was destroyed (the enzyme was relatively stable at this pH). The pH profile of inactivation closely followed the nitration curve of free N-acetyltyrosine. o-Chloranil (3,4,5,6-tetrachloro-1,2-benzoquinone) was the most potent inactivator: 3 μM o-chloranil, tested in 45 mM Tris-HCl, pH 7.8 (containing 1 mM Ca²⁺), caused a 95% inactivation in 10 min. We have shown elsewhere (40) that this reagent is an almost "equimolar inhibitor" of *B. cereus, Clostridium histolyticum, and Achromobacter iphaghus* collagenases. Finally, 5 mM EEDQ, tested in 80 mM Tris-HCl (pH 7.0, at 25 °C; all EEDQ was dissolved), caused a 70% irreversible inactivation in 100 min.

Phenylmethanesulfonyl fluoride did not inhibit the enzyme in the normal assay mixture for Azo-coll hydrolysis. Ten mM L-leucine hydroxamate, a protease inhibitor (41), inhibited the enzyme by 60%. Phosphoramidon [N-(α-L-rhamnopyranosyl-hydroxyphosphinyl)-α-leucyl-α-tryptophan] inhibited thermolysin very effectively (42), but inhibits collagenases to a lesser extent. This reagent inhibited the *S. faecalis* enzyme less effectively than thermolysin (0.3 mM reagent inhibited by 50%). Straight-chain aliphatic alcohols (from methanol to butanol) caused a reversible inactivation, the degree of which was a linear function of the length of the carbon chain: 50 mM n-butyl alcohol inhibited by 100%, whereas 50 mM methanol caused only a 15% inhibition. The inhibitory potential of secondary and tertiary alcohols was weaker.

Inhibition of the Enzyme by a High Molecular Weight Factor Present in Rat Inflammatory Exudate—Samples of an inflammatory exudate were prepared under the dorsal skin in the rat (43). Aliquots of the exudate were subjected to FPLC on a Mono Q HR 5/5 anion exchange column (Fig. 7). The chromatographic fractions were then incubated with small
FIG. 5. Sequences of insulin A- and B-chain showing the peptide bonds primarily hydrolyzed by the S. faecalis protease. The arabic numerals above the vertical arrows indicate the order of preference of the hydrolysis sites of B-chain. The incubation times used with B-chain ranged from 15 min to 22 h. A-chain was incubated for 17 h.

TABLE V

Specific activity and the values of $K_m$ of the hydrolysis of insulin B-chain and substance P catalyzed by S. faecalis extracellular endopeptidase

| Scissile bond             | Specific activity* | $K_m$ (m) * |
|---------------------------|-------------------|------------|
| Phe$^6$-Phe$^7$ (insulin B-chain) | 75,573            | 7.25 $\times 10^{-5}$ |
| Substance P               |                    |            |
| Gly$^4$-Phe$^5$           | 9,417             | 4.9 $\times 10^{-5}$  |
| Phe$^5$-Phe$^6$           | 13,250            | 2.0 $\times 10^{-5}$  |
| Gly$^8$-Leu$^{10}$        | 120,760           | 1.2 $\times 10^{-4}$  |

* In nmol $\times$ min$^{-1}$ mg$^{-1}$. Determined at 37 °C with insulin B-chain and at 30 °C with substance P in 50 mM Tris-HCl, pH 7.8.
* Means of values determined using the Lineweaver-Burk, Hanes, and Hofstee methods.

amounts of the enzyme using Azocoll as the substrate under normal conditions (50 mM Tris-HCl, 1 mM Ca$^{2+}$, pH 7.8). The enzyme was strongly inactivated by a substance with the retention time of about 33 min. The molecular weight of this substance, determined by FPLC on a Superose 6 gel column (not shown), was 720,000–750,000 (human $\alpha_2$-macroglobulin has a $M_r$ of 725,000).

DISCUSSION

General—The present purification procedure of the S. faecalis extracellular protease allows structural studies. The enzyme appeared to be a strongly hydrophobic protein that interacted with hydrophobic and insoluble collagenous substrates. These studies confirmed the previous results (18, 19) concerning the pH optimum of the enzyme (i.e. 7.4–7.6) and the loss of enzyme stability below pH 6. The present studies provided new information about the pH optimum of the hydrolysis of Azocoll and certain S. faecalis peptides (i.e. pH 7.0–7.2). The pl value of 4.6 determined in this study corresponds well to the value of 4.7 reported earlier (21). Depending on the method used, the molecular weight of the enzyme ranged from 29,500 to 33,000. The consistency between the present and the previous (21) findings are further exemplified by the value of 32,000 obtained in this study with Sephadex G-100 gel and the same value reported previously using Sephadex G-75. Sedimentation equilibrium studies yielded a value of 28,000 (21). We also confirmed the previous findings (20)
on the metalloenzyme nature of the enzyme.

It is noted that the *S. faecalis* extracellular proteinase is a "neutral" proteinase, as evidenced by the present and earlier (18, 19) pH studies. However, the synthesis of the enzyme may reach its maximum in acidic environments. An optimal pH of 7.4 was reported by enteroococcal proteolytic activity (44), but an optimal pH of 6.3 was demonstrated for enzyme synthesis (45).

**Substrate Specificity**—Previous substrate specificity studies have shown only that the enzyme is capable of hydrolyzing casein, gelatin, hemoglobin, and a streptococcal lysin (46) and that the enzyme clots milk (18). The present study provides totally new information on the substrate specificity. Accordingly, insoluble Azocoll proved to be a superior protein substrate: it was totally solubilized by the enzyme producing high molecular weight protein fragments. Similar molecular weight distribution of the products was also observed when gelatin was used as substrate, suggesting the scission of similar peptide bonds in both substances. The *S. faecalis* proteinase can clearly be regarded as an endopeptidase with no aminopeptidase, dipeptidase, or tripeptidase activity. Because proteinases characteristically have an extended substrate binding site, the decrease in enzyme activity was caused by a substance with a retention time of 33 min.

**Active Site**—The DEP-caused inactivation and the 50% reactivation of the inactivated enzyme by hydroxylamine suggest that the modifier had perhaps reacted with an imidazole residue important to enzyme activity. In the pH range of 6 to 7 (used in the present experiments), DEP is highly specific for histidine (52), although certain other residues may also react with DEP. The failure to realize full recovery of enzyme activity with hydroxylamine treatment and complete discharge of the ethoxyformyl moiety with the enzyme may have resulted from the reaction of 2 eq of DEP per 1 eq of histidine followed by a Bamberger reaction to open the imidazole ring (53). It has been suggested that some dicarboxylation may occur when high molar excess of the modifier is used. Hydroxylamine readily removes the carboxy group from the monoderivatized residue but is ineffective at displacing the second carboxy function from the dicarboxyhistidyl residue formed by reaction with excess DEP (52). o-Chloranil, an extremely potent and fast irreversible inactivator of bacterial collagenases (49), was suggested to react with an active Tyr group of the enzymes. So far, only Cys and Tyr residues are known to react with TNM. Between pH 6 and 9 the rate of this reaction with Cys residues is independent of pH, permitting its differentiation from nitratin of Tyr residues (54). The rate of inactivation with the present proteinase was pH-dependent between pH 7 and 11, and the maximum inactivation was obtained at the vicinity of pH 10. The inactivation curve also closely resembled that of N-acetyltirosine. These data indicate that TNM...
may have reacted with an essential Tyr residue of the enzyme.

There are no conclusive data on the active site structure of the neutral endopeptidase-24.11, although evidence for an essential His (55, 56) and an Arg residue (55) was presented. These residues were also found to be present in the active site region of thermolysin (50, 57). His residues being most likely essential (57) and Arg residues most likely participating in substrate binding (58). Our experiments with diketones and ketone aldehydes suggested no direct involvement of an active Arg residue. EEDQ, a reagent highly specific for active carboxyl groups of enzymes (59), caused a sufficiently strong (irreversible) inactivation of the S. faecalis enzyme to indicate the importance of a carboxyl group in its activity.

Phosphoramidon which strongly inhibits thermolysin (42) and collagenase (60, 61), causing a 50% effect at low concentrations of 0.7 and 60 µM, respectively, required an about 300 µM inhibitor level for similar inhibition of the S. faecalis enzyme. Although the S. faecalis enzyme, thermolysin, and the mammalian endopeptidase-24.11 shared many properties related to substrate specificity and although these enzymes are inhibited by phosphoramidon, the accessibility of the active site groups of the S. faecalis enzyme to several common protease inhibitors was somewhat restricted compared with the reference enzymes. However, our data seem to be in agreement with previous ones in suggesting the importance of Tyr and His and carboxyl residues in the activity of this group of enzymes. Our studies thus suggest that the S. faecalis extracellular proteinase may be classified as a microbial metalloprotease II (EC 3.4.24.4).

Role of the Enzyme—Enzymes other than collagenases are biologically necessary for the process of physiological and pathological total collagen degradation. Degradation of collagen by specific collagenases results in the formation of collagen fragments that denature at body temperature into gelatin (62, 63). These fragments are then exposed to the action of other proteolytic enzymes, like bacterial "gelatinases" (similar to S. faecalis enzyme) or tissue proteinases (64). Because certain collagen fragments can be considered inflammatory mediators, the importance of microbial gelatinolysis to human health may be significant.

Although the present proteinase may be necessary for the nutrition or chemical defense of S. faecalis, this enzyme may also be involved in the decay of sex pheromones in culture filtrates (24, 65). Since an internal hydrophobic residue is a relatively common feature among biologically active peptides, the present proteinase, like endopeptidase-24.11, accordingly displays the required specificity to play a general role also in the hydrolysis of biologically active peptides (66). Such a role of the mammalian membrane endopeptidase-24.11 and a microbial endopeptidase with identical specificities in carrying out the termination of the physiological action of certain biologically active peptides indeed is a logical guess; the mammalian enzyme is strategically located at the cell surface, the S. faecalis enzyme being extracellular, both thus being capable of accomplishing similar reactions. Although many bioactive peptides are relatively small, the action of an endopeptidase is commonly required, especially because many such peptides have blocked NH₂ and COOH termini (67). It has been concluded that a limited number of cell surface peptides mediate a wide range of functions and that their cellular and subcellular localization, rather than peptide specificity, define their roles at different tissue sites (67). Because the S. faecalis extracellular proteinase exhibits strikingly similar substrate specificity to the 24.11-enzyme and because S. faecalis has been found associated with numerous inflammatory situations, it is possible that the chemical aggressiveness of this organism is partly attributed to the action of one of its major secretory products, the extracellular proteinase described in our study. This proteolytic action may in vivo be directed to collagenous substances and certain bioactive peptides, and it may be counteracted by specific tissue proteins (β2-macroglobulins?). Evolutionarily, the catalytic ability of this enzyme is not unique, but may represent a very old and widespread trait displayed by neutral proteinases from a variety of microorganisms, e.g. Bacillus, Pseudomonas, Aspergillus (68), and Streptococcus species.

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