Characterization of PepB, a Group B Streptococcal Oligopeptidase

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Group B streptococci were recently reported to possess a cell-associated collagenase. Although the enzyme hydrolyzed the synthetic collagen-like substrate *N*-(3-[2-furyl]acryloyl)-Leu-Gly-Pro-Ala, we found that neither the highly purified enzyme nor crude group B streptococcal cell lysate solubilized a film of reconstituted rat tail collagen, an activity regarded as obligatory for a true collagenase. We cloned and sequenced the gene for the enzyme (*pepB*). The deduced amino acid sequence showed 66.4% identity to the *PepF* oligopeptidase from *Lactococcus lactis*, a member of the M3 or thimet family of zinc metallopeptidases. The group B streptococcal enzyme also showed oligopeptidase activity and degraded a variety of small bioactive peptides, including bradykinin, neurotensin, and peptide fragments of substance P and adrenocorticotropin.

Group B streptococci (GBS) are the most frequent cause of serious, often fatal, bacterial infections of neonates in the United States and, in addition, are a common cause of peripartum maternal sepsis (19). Although GBS are harmless commensals most of the time, the organisms clearly possess the ability to systemically invade a susceptible human host and cause life-threatening infections. This ability appears to be due, at least in part, to the production of a variety of degradative enzymes that facilitate systemic invasion. We have extensively studied one such enzyme of GBS, a secreted hyaluronidase (9, 12) that is capable of breaking down hyaluronic acid, a major component of the extracellular matrix of many tissues.

The recent report by Jackson et al. (8) of a collagenase produced by GBS appeared to be yet another example of the production of an enzyme of GBS capable of degrading a component of the extracellular matrix and, possibly, facilitating invasion. The investigators speculated that the enzyme might contribute to the premature rupture of membranes frequently associated with neonatal infections. Jackson et al. (8) assayed the collagenase activity of GBS by using the synthetic substrate *N*-(3-[2-furyl]acryloyl)-Leu-Gly-Pro-Ala (FALGPA), which is hydrolyzed by all known collagenases and is thought to mimic the primary structure of collagen. We decided to isolate and purify the enzyme and further characterize it.

Enzyme purification. Intact cells of GBS were found to exhibit weak FALGPA-hydrolyzing activity, and in agreement with the results reported by Jackson et al. (8), much higher activity was found in cell lysates. Enzyme was therefore purified from the lysate of a cell pellet obtained from 60 liters of a log-phase culture ($A_{650} = 0.8$) of strain 3502 of GBS, a serotype III strain that had been isolated from the blood of an infected infant.

GBS were grown in a fermentor as described previously (12), by using the chemically defined medium described by van de Rijn and Kessler (17), which was purchased from Hazleton Research Products, Inc., Denver, Pa. Cells were harvested by centrifugation, washed in 0.1 M NaCl, and disrupted with a

continuous-flow cell breaker operated at 15,000 lb/in². The cell lysate was centrifuged at 11,000 × *g* for 1 h, and proteins in the supernatant were precipitated with 80% saturated ammonium sulfate overnight at 4°C. After centrifugation, the pellet was redissolved in 50 mM imidazole buffer, pH 7.0. Ammonium sulfate was removed by passing the mixture through a column (5 by 25 cm) of Bio-Gel P2 (Bio-Rad, Richmond, Calif.). Material in the void volume was applied to a column (2.5 by 22 cm) of DEAE-Sephacel (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.) equilibrated with 50 mM imidazole buffer, pH 7.0. The column was washed with the buffer, and adsorbed enzyme was eluted with a 0 to 1.0 M NaCl gradient in the same buffer. FALGPA-hydrolyzing activity of the fractions was measured by a modification of a procedure described by Van Wart and Steinbrink (18). A 10-μl sample was added to 300 μl of an enzyme assay mixture composed of 0.45 mg of FALGPA per ml, 0.24 M NaCl, 50 mM imidazole, 12 mM CaCl₂, and 2.4 μM ZnCl₂, pH 7.0. The rate of decrease in A_{345} was monitored with a recording spectrophotometer at room temperature.

Fractions containing enzyme activity were pooled, and the NaCl concentration was adjusted to 2.0 M. This mixture was loaded onto a phenyl Sepharose (Pharmacia) column (1.75 by 20 cm) that had been equilibrated with 2.0 M NaCl in 50 mM imidazole buffer. After washing with 120 ml of the same buffer, the enzyme was eluted with a linear decreasing gradient of 2.0 to 0 M NaCl in 50 mM imidazole buffer, pH 7.0. The active fractions were pooled and concentrated with a Centricon 30 ultrafiltration device (Amicon Inc., Beverly, Mass.). Final purification of the enzyme from GBS was done with a Bio-CAD Perfusion Chromatography Workstation (Perseptive Biosystems Inc., Cambridge, Mass.). Briefly, 0.5 ml of the concentrated, partially purified enzyme was adsorbed onto a Bio-CAD DEAE anion-exchange chromatography column and eluted by using a gradient from 0.4 to 1.0 M NaCl in 50 mM Tris-HCl (pH 7.4) buffer. Fractions containing the peak of enzyme activity were pooled and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (2).

Figure 1 illustrates the progressive purification of FALGPA-hydrolyzing activity by the various chromatographic procedures. The final product gave a single band with a molecular mass of approximately 70 kDa upon SDS-PAGE.

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FIG. 1. SDS-PAGE protein profiles of the enzyme preparation from GBS at different stages of purification. The gel was stained with Coomassie blue. Lanes: 1, cell lysate of GBS; 2, peak of enzyme activity on a DEAE-Sephacel column; 3, after phenyl-Sepharose column chromatography; 4, after purification on a BioCAD. The arrows indicate the migration positions of the molecular size standards, bovine serum albumin (66.2 kDa) and rabbit muscle phosphorylase *b* (97.4 kDa).

The pH optimum for FALGPA hydrolysis was 7.4. As reported by Jackson et al., enzyme activity was completely inhibited in the presence of the metal chelator EDTA or 1,10-phenanthroline (10 mM final concentration). A low concentration (2 μ M) of Zn^{2+} ions restored the activity of purified enzyme preparations and was routinely used in the standard assay mixture. A small increase in enzyme activity was observed in the presence of 10 μ M Mn^{2+} or Cu^{2+} ions, but the same concentration of Co^{2+} ions resulted in a large increase in enzyme activity (data not shown). Excess Zn^{2+} ions (10 mM) strongly inhibited the enzyme, whereas the same concentration of Co^{2+} ions activated the enzyme. We did not observe any increase in enzyme activity in the presence of dithiothreitol (0.2 to 5 mM).

Gelatinase activity was estimated by using an agarose plate assay. Samples (10 μ l) were placed in wells cut in a 1% agarose gel containing 0.1% gelatin, 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.5), and 10 mM $CaCl_2$ and incubated overnight at room temperature. The gel was then stained for 20 min with 0.1% Coomassie blue and destained in water on a rotary shaker. Gelatinase activity was revealed as a clear zone on a blue background. Surprisingly, neither the purified enzyme from GBS nor the original cell lysate exhibited any gelatinase activity in our agarose gel assay. This led us to test the ability of the enzyme from GBS to solubilize a film of reconstituted collagen fibrils.

Collagenase activity was assessed essentially as described by Birkedal-Hansen and Taylor (4). Acid-soluble rat tail tendon collagen (25 mg; Sigma) dissolved in 12 ml of 13 mM HCl was neutralized with 3 ml of 0.3 M HEPES buffer (pH 7.5), poured onto an agarose-coated polyester sheet (10 by 15 cm; Gelbond; FMC BioProducts, Rockland, Maine), and allowed to gel. The collagen gel was dried overnight at room temperature. It was then rinsed under running water for 1 min and again air dried. Test samples (10 μ l) were spotted onto the collagen fibril film and incubated for 2 to 16 h in a humid atmosphere. The film was then stained for 20 min in 0.1% Coomassie blue and rinsed

FIG. 2. Collagenase assay on a film of reconstituted rat tail tendon cartilage. A 10- μ l aliquot of *C. histolyticum* collagenase was placed at position 1. Position 2 received 10 μ l of the purified enzyme from GBS with equivalent FALGPA-hydrolyzing activity (measured in arbitrary units), and position 3 received 10 μ l of the enzyme from GBS containing 100 times as many units. The film was incubated at 37°C for 2 h in a moist environment and stained with Coomassie blue.

in water. Collagenase activity yielded a clear zone on a dark blue background.

Figure 2 shows the results obtained when collagenolytic activities of the purified enzyme from GBS and the collagenase from *Clostridium histolyticum* were compared. A 100-fold excess of the enzyme from GBS (based on FALGPA-hydrolyzing activity) compared to the clostridial collagenase showed no trace of activity on a reconstituted collagen film, even when the incubation time was extended to 16 h. This result indicated that the enzyme from GBS was not a collagenase.

Amino acid sequence determinations. N-terminal amino acid sequencing was carried out on protein bands electroblotted to polyvinylidene difluoride membranes following SDS-PAGE as described by Matsudaira (10). Tryptic peptides were obtained by digesting 120 μ g of the purified enzyme from GBS with 2 μ g of sequencing grade trypsin (Promega Corp., Madison, Wis.) in 75 mM NH_4HCO_3 buffer (pH 8.0). After 16 h of incubation at 37°C, peptides were separated by reversed-phase high-pressure liquid chromatography and sequenced as described previously (9).

The N-terminal amino acid sequence of the purified enzyme, as well as sequences of several tryptic peptides, were compared to sequences in the GenBank database. All sequences showed high similarity to amino acid sequences present in an oligopeptidase (PepF) isolated from *Lactococcus lactis* (11). On the basis of these sequences and a knowledge of their relative positions in the protein, degenerate oligonucleotide primers were designed for use in PCR amplification of internal fragments of the enzyme gene.

PCR amplification of internal gene fragments. The 100- μ l PCR mixture consisted of 200 μ M deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 50 pmol of each of the primers. PCR amplifications were done under conditions described previously (6). The amplified fragments were analyzed on 1% agarose gels and cloned into the pCR II vector by using a TA Cloning Kit (Invitrogen Corp., San Diego, Calif.). One pair of degenerate primers (1 and 2) permitted the amplification of a 384-bp fragment, which was then cloned and sequenced (PCR #4 in Fig. 3). It was capable of coding for a protein fragment that exhibited 58.6% amino acid identity (over 128 amino acid residues) with a sequence in the lactococcal oligopeptidase. The cloned gene fragment was excised from the plasmid by digestion with *Eco*RI. After separation on a low-melting-point agarose gel, the fragment was purified by phenol-chloroform extraction. It was then labeled with digoxigenin-11-dUTP by a random primer DNA labeling method (Genius system; Boehringer Mannheim Corp., Indianapolis, Ind.) and used as a probe to screen approximately 10^6 recombinants in a previously constructed λ phage library of

FIG. 3. Diagram of the 1.9-kb region containing the *pepB* gene sequenced in this study. The locations of PCR-amplified gene fragments 4 (used as a probe for library screening) and 12 (containing the 5' portion of the gene) are indicated. A full-length copy of the *pepB* gene was amplified by using primers 4 and 5.

chromosomal DNA fragments of strain 3502 of GBS (9). Positive phage clones were obtained and converted to recombinant pBK-CMV plasmids by in vivo excision. Clones with different restriction patterns were sequenced. One of them, clone 21, which contained a 1.475-kb enzyme gene fragment from GBS, was completely sequenced (Fig. 3). Sequence analysis showed that it contained a portion of a single open reading frame capable of coding for a 422-amino-acid segment.

The 5' portion of the gene was amplified by using the single-specific-primer PCR technique (14). Chromosomal DNA from

strain 3502 of GBS was digested with *EcoRI* and ligated into a similarly cut site in pUC19. The ligation mixture served as a template for PCR amplification with a gene-specific reverse primer (no. 3) and universal primer F-20 for pUC19 (Fig. 3). This permitted the PCR amplification of a 3.2-kb fragment, which was then cloned into the pCR II vector (Invitrogen) and sequenced. It overlapped with plasmid 21 by 660 bp. Further upstream was a nucleotide sequence capable of coding for an amino acid sequence identical to that obtained by protein sequencing of the N terminus of the purified enzyme.

FIG. 4. Features of the nucleotide and deduced amino acid sequences of the gene for oligopeptidase PepB from GBS. The N-terminal amino acid sequence of the purified enzyme and the sequences of six tryptic peptides are indicated by dotted underlining. A putative ribosome-binding region (SD) is indicated by bold double underlining. An inverted repeat 23 nucleotides after the stop codon (TAA) that may be a transcription termination sequence is indicated by opposing arrows. A typical Zn-binding motif, His-Glu-X-X-His, is boxed.

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1 MSDNRSHIEEKYQWDLTTVFATDELWETEVVELTQAIDDAKGF.SGHLDDSSQSLEITEVELELSRRLEKVVYVYASMKNDQDTTVAKYQEFQAKATALY PepB
1 MAKNRNEIPEKL TWDLTTIYKTDKEWEAELTRIKSEL SLVEETDPGHLDDSAESLLTITEKMLSISQQVEKLVYVYASMKNDQDTREAKYQEQSKATALY PepF
100 AKFSETFSFYEPPELLQLSESDYQSFLLEMPDLQKYDHFEEKIFANKPHVLSQNEEELLAGASEIFGAAGETFEILDNADMVFPVVKNAKGEEVELTHGNF PepB
101 VKFGEVYAFYEPEFLKISKEVYNKWLGLQKLKNYDHMFERLFAKKAHILSQKEEKLLAAAGEIFESPSETFEIFDNADIKLPMVKNESDEMIQLTHGNY PepF
200 ISLMESSDRTGRKEAYQAMYSTYEQFQHTYAKLTQTNVKSQNFKARVHHYQSARQSALSANFIPEEVYETLIKTVNHHLPLLHRYMKLRQKVLGLDDLKM PepB
201 SSLMESKNRGRKAAYKALYSNYEQYQHTYAKLTQTNVKVHNLNAQIRSYDSARQAALANFVPEKVYDVLMEAIHQHLLPLLHRYIELRKKILGITDLKM PepF
300 YDVTPLPQMDMSFTYDEALKKSEKVLAI FGEAYSERVHRAFTERWIDVHVNGKRSYGSGSYDTNAFMLLNWQDTLDNL YTLVHETGHSLSHTFTR PepB
301 YGIYTPLSNLGYKFNYEDGVKKAEEVLAIFGKEYKGKVKAAFEQRWIDVEENIGKRSYGSGSYDTNAFMLLNWQETLDDLF TLVHETGHSLSHSAFTRE PepF
400 NQPVYVGDYSIFLAEIASTTNNENILTETLLKEVKDDKNRFAILNHLYDGFKGTIFRQTQFAEFHAHVADQEGQVL TSEYLNLYAELEKYYGLTKED PepB
401 NQPVYVGNYPILFALAEIASTTNNENILTETLLKESKDDKERFALLNHWLD SFRGTVFRQSQFAEFQKIHADAAGEVLTSEYLNLYGEINEKYNLAVKG PepF
500 NHFIQYEWARIPHFYHNYVVFQYATGFAAANYLAERIVNGNPEDKEAYLNLYKAGNSDYPLNVIKAGVDMTSADYLDAAFRVFEERLVELENLVTKGVH PepB
501 NPEIQYEWARIPHFYHNYVVFQYATGFAAATFLAEKVHVGSTEDRQKYLEYLKAGSSAYPLEVIAKAGVDMESTYLDAAFELEFENRLESEKLVKGVH PepF
600 DN* PepB
601 L* PepF

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FIG. 5. Alignment of the amino acid sequence of oligopeptidase PepB from GBS with that of the oligopeptidase of *L. lactis* (PepF) illustrating regions of identity. Identical amino acid residues are joined by vertical lines. Double dots denote highly conserved amino acids, and single dots indicate moderately conserved amino acids.

As shown in Fig. 4, an open reading frame coding for a 69.6-kDa protein was identified (GenBank accession number U49821). The deduced amino acid sequence showed 66.4% identity to the oligopeptidase from *L. lactis* for the alignment depicted in Fig. 5. Adjacent to the N terminus was an ATG start codon preceded by a putative ribosomal-binding sequence, GGAGG (Fig. 5), 7 bp before the ATG codon. An inverted repeat, possibly a transcription termination sequence, was observed 23 bp after the TAA stop codon (Fig. 5). A typical Zn-binding motif, His-Glu-X-X-His, was present at amino acid positions 385 to 393. An isoelectric point of 4.90 for the enzyme from GBS was calculated by using the EditSeq program of DNASTAR (Madison, Wis.).

To express the gene for the enzyme from GBS in *Escherichia coli*, it was necessary to obtain a clone containing the entire gene. Primers (4 and 5 in Fig. 3) were therefore designed and used to amplify the gene from chromosomal DNA from GBS. A single 1.9-kb PCR product containing the Shine-Dalgarno sequence was obtained and cloned into the pCR II vector (plasmid 5). After transformation into INV α F'-competent cells (Invitrogen Corp., San Diego, Calif.), transformants were lysed by sonication and tested for enzyme activity to identify those possessing a functional gene. A transformant exhibiting very high enzyme activity was selected for further study. The recombinant enzyme was purified from cultures of this clone by using the same procedure used to isolate the enzyme from cultures of GBS.

Substrate specificity. Like the lactococcal enzyme, the enzyme from GBS showed no detectable proteolytic activity (data not shown). However, it did cleave a variety of small, biologically active peptides, including bradykinin, Ile-Ser-bradykinin, neurotensin, substance P fragment 2-11, and adrenocorticotropin fragment 1-10 (all obtained from Sigma). Each peptide (100 μ g) was incubated with 2 μ g of purified recombinant enzyme in 50 mM ammonium acetate–5.0 mM CaCl₂–5.0 μ M ZnCl₂ (pH 7.0) for 2 h at 37°C. Digestion was carried out on a smaller scale (10 μ g of peptide) for defensin HNP-2. Digestion mixtures were analyzed directly by using an API III triple quadrupole mass spectrometer (PE-Sciex, Thornhill, Ontario, Canada) operating in the electrospray mode. Aqueous solutions of the samples were injected into a 10- μ l/min flow of 50%

acetonitrile in water containing 0.1% formic acid by using a syringe pump to deliver the mixture to the electrospray interface.

The peptide cleavage results are summarized in Table 1. Bradykinin, neurotensin, adrenocorticotropin fragment 1-10, and substance P fragment 2-11 were all cleaved at the same positions reported for the lactococcal enzyme (11). However, in two cases there was mass spectroscopic evidence that limited cleavage of some additional peptide bonds had also taken place. In neurotensin, for example, the main cleavage occurred between the two Arg residues but a small amount of cleavage also took place between the Pro and Tyr residues. Similarly, adrenocorticotropin fragment 1-10 had a minor cleavage site between Met and Glu. The 29-amino-acid peptide defensin HNP-2 was not cleaved by the enzyme from GBS.

The PepF oligopeptidase from *L. lactis* has been placed in the M3 or thimet oligopeptidase family of metalloproteases (13), and the enzyme from GBS almost certainly belongs in that family. Members of this family exhibit almost identical substrate specificities and also possess highly conserved amino acid sequences around the zinc-binding active-site region. The original member of this enzyme family was called thimet oligopeptidase as an acronym for thiol-dependent metalloprotease (15). The enzyme from GBS, however, is not activated by thiols. This, of course, is not surprising since, like the PepF oligopeptidase, it contains no cysteine residues. Thimet oligopeptidase cleaves a number of different peptides, including bradykinin and neurotensin, at exactly the same sites that are cleaved by the enzyme from GBS and the lactococcal enzyme (3).

Camargo et al. (5) isolated and characterized endopeptidase 22.19 from rabbit brain, which also exhibits specificity similar to that of the enzyme from GBS. These investigators carried out a detailed study of the structural requirements of bioactive peptides for cleavage by the enzyme. They found that unlike that of other proteases and peptidases, substrate specificity was not determined by the amino acids flanking the sensitive bonds. Rather, it appeared that the length and flexibility of the peptide were the dominant factors. It was further suggested that the presence of a β -turn in the positively charged peptides may be an important factor for its interaction with the enzyme.

TABLE 1. Substrate specificity of the enzyme from GBS

Substrate	Cleavage site(s) ^a
FALGPA	Furanacryloyl-Leu-Gly-Pro-Ala
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Ile-Ser-bradykinin	Ile-Ser-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Neurotensin	Pyr-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu
Adrenocorticotropin fragment 1-10	Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly
Substance P fragment 2-11	Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH ₂
Defensin HNP-2	Cys-Tyr-Cys-Arg-Ile-Pro-Ala-Cys-Ile-Ala-Gly-Glu-Arg-Arg-Tyr-Gly-Tyr-Gly-The-Cys-Ile-Tyr-Gln-Gly-Arg-Leu-Trp-Ala-Phe-Cys-Cys

^a Large arrows indicate primary cleavage sites, and small arrows indicate minor cleavage sites.

Since defensins, abundant bactericidal peptides of neutrophils, are both positively charged and possess β -turns, we tested whether they were cleaved by the oligopeptidase from GBS. A highly sensitive mass spectrometric procedure, however, revealed no evidence of any cleavage of defensin HNP-2.

The oligopeptidase from GBS is completely inhibited by excess zinc (e.g., 10 mM), as are many other zinc oligopeptidases (1). Like some of these enzymes, the oligopeptidase from GBS is activated by low concentrations (10 μ M) of Co^{2+} and is not inhibited by high concentrations (e.g., 10 mM) of the ion.

The recent report of a collagenase from GBS (8) was based primarily on the ability of the enzyme from GBS to hydrolyze FALGPA. The continuous spectrophotometric assay for collagenase based upon the hydrolysis of FALGPA was introduced in 1981 (18) and has been widely used by numerous investigators. The method is convenient, rapid, sensitive, and quantitative. However, FALGPA hydrolysis is not completely specific for collagenases. In fact, numerous investigators have cautioned against assuming that activity on the synthetic peptide substrate proves that an enzyme is a collagenase (7). We found that neither the purified enzyme nor a crude cell lysate degraded collagen fibrils, an ability that is regarded as necessary for a true collagenase (7). The enzyme also did not exhibit any gelatinase activity. The data reported here indicate that the FALGPA-hydrolyzing enzyme from GBS is not a collagenase as previously reported, but rather is an endo-oligopeptidase (which we designate PepB oligopeptidase).

The biological function of PepB oligopeptidase is not known. If it is involved in the degradation of peptides in its surroundings, then the existence of a peptide transport system could be expected. Monnet et al. (11) recently suggested that a report of an oligopeptide transport system in lactococci (16) opens up the possibility that PepF oligopeptidase is involved in the degradation of peptides transported from outside the cell. No such system has been identified in GBS. Some researchers have speculated that the mammalian peptidases of the M3 family may be biologically important for the conversion and inactivation of bioactive peptides (5). The enzyme from GBS is certainly active on many of these peptides, but further studies are required to determine if this is important in pathogenesis.

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