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Characterization of an Extracellular Dipeptidase from *Streptococcus gordonii* FSS2

J. M. Goldstein,[1]† T. Kordula,[2] J. L. Moon,[1] J. A. Mayo,[1]* and J. Travis[1]

Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia,[1] and Department of Biochemistry, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, Virginia[2]

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PepV, a dipeptidase found in culture fluids of *Streptococcus gordonii* FSS2, was purified and characterized, and its gene was cloned. PepV is a monomeric metalloenzyme of approximately 55 kDa that preferentially degrades hydrophobic dipeptides. The gene encodes a polypeptide of 467 amino acids, with a theoretical molecular mass of 51,114 Da and a calculated pI of 4.8. The *S. gordonii* PepV gene is homologous to the PepV gene family from *Lactobacillus* and *Lactococcus* spp.

Streptococcus gordonii is an oral streptococcus that is important in both the formation of dental plaque and the production of infective endocarditis. Previous work with *S. gordonii* FSS2, a strain isolated from the blood of an endocarditis patient (10), has revealed a variety of cell-associated and extracellular glycosidolytic and proteolytic activities, thought to be important in the growth of this organism in the dental plaque and endocarditis vegetation environments (4, 5, 6, 11). In recent studies of extracellular proteinases made by this strain, an x-prolyl dipeptidyl peptidase (xPDPP) and an arginine aminopeptidase have been purified and characterized and the genes for both enzymes have been cloned and examined (4, 5). Here we report similar studies on a third such enzyme, *S. gordonii* PepV. *S. gordonii* PepV is a member of the PepV dipeptidase family, previously found in *Lactobacillus* and *Lactococcus* spp. (3, 7, 14, 15).

Materials and methods. *S. gordonii* FSS2 (previously *Streptococcus sanguinis* FSS2 [6]) was described earlier (6, 10, 11). Other oral (viridans group) streptococci (see Fig. 3) were provided by Vincent Fischetti, Rockefeller University. *S. gordonii* FSS2 was grown with pH control as previously reported (4, 5).

Enzyme assays measured both tripeptidase and dipeptidase activities of *S. gordonii* PepV (hereafter, PepV refers to *S. gordonii* PepV unless indicated otherwise; "*S. gordonii*" is added only when necessary to avoid ambiguity as to which version of PepV is meant). During purification (see Fig. 1) and most inhibitor studies (see Table 2), the tripeptidase activities of crude samples and purified PepV were measured in a two-step enzymatic reaction, with H-Ala-Phe-Pro-pNA (Sigma) as the substrate. PepV and the substrate (1 mM), both in assay buffer A (50 mM Tris, 1 mM $CaCl_2$ [pH 7.8]), were incubated; products of this reaction were NH_3^+-Ala-COO- and NH_3^+-Phe-Pro-pNA. Excess *S. gordonii* xPDPP (100 ng) then was added to release the pNA reporter group, and A_{405} was measured in a plate reader. Since *S. gordonii* xPDPP is a serine proteinase (4), potential inhibition of PepV by serine class inhibitors (diisopropylfluorophosphate, phenymethylsulfonyl fluoride, 3,4-dichloroisocoumarin, and TLCK [*N*α-*p*-tosyl-L-lysine chloromethyl ketone]; see Table 2) was examined in an independent dipeptidase assay that measured PepV-dependent release of ninhydrin-positive material using Leu-Gly as the substrate (2). Inhibitors were obtained from Sigma, Roche Molecular Biochemicals, or Calbiochem, except for Anstatin, which was a gift from Mirjana Grujic, Jozef Stefan Institute, Lubljana, Slovenia. For studies of cleavage specificity (see Table 1), hydrolysis of dipeptides, tripeptides, and higher peptides was measured in a high-performance liquid chromatography (HPLC) assay. The enzyme was incubated with the experimental peptide (enzyme-to-substrate ratio, 1:1,000) in 100 μl of 100 mM Tris, pH 7.8, for 2 h at 37°C, and the products were analyzed by HPLC.

PepV was purified from the culture fluid of a 15-liter stirred culture (pH was maintained at 7.5) in early stationary phase, at which time all glucose had been metabolized and approximately 25% of the total PepV activity was extracellular (data not shown). Cells were removed, and 80% ammonium sulfate was added to the culture fluid. The precipitate was collected, dialyzed, and chromatographed on DE-52. Active fractions were successively chromatographed on Superdex-75 HR 10/30, phenyl-Sepharose HP, Mono-Q HR 10/10, Cibacron Blue Sepharose CL-B6, Gly-Pro Sepharose, and Mono-P 5/5. Details of the purification scheme are available from the authors. Enzyme purity and molecular weight were assessed by Tris-HCl–Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12), silver staining (1), and gel filtration.

To clone the PepV gene, DNA from *S. gordonii* FSS2 was purified (Purgene; Gentra, Minneapolis, Minn.). The N-terminal peptide sequence of PepV (TIDFKAEVEKRREAL), obtained by Edman degradation, was used to search an *S. gordonii* database (ftp://ftp.tigr.org/pub/data/s_gordonii), resulting in identification of a 1,401-bp open reading frame encoding PepV. Subsequently, two PCR primers encoding the N and C termini were synthesized (5′-AGTGGATCCATGACAATTG ATTCTAAAGC-3′ and 5′-TTTGGATCCTTATTTGATTAG

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30602-7229. Phone: (706) 542-1713. Fax: (706) 542-3719. E-mail: jmayo@uga.edu.

† Present address: Elan Pharmaceuticals, South San Francisco, Calif.

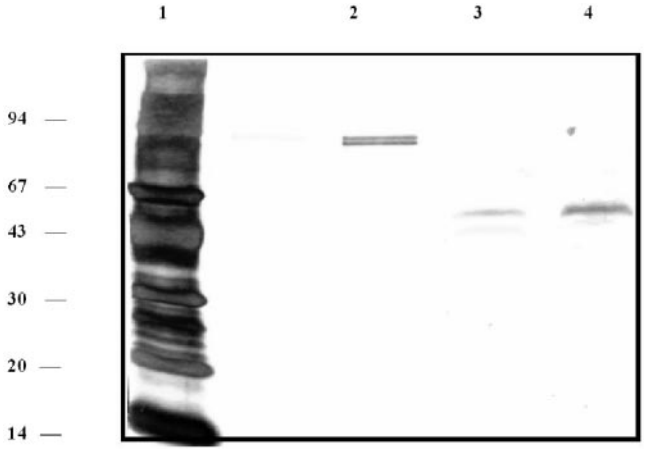

FIG. 1. Purification of PepV seen by SDS-PAGE (silver stained). Lane 1, 5 μg of molecular mass markers (phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa; cytochrome *c*, 14 kDa); lanes 2 to 4, boiled, reduced samples of pure protein from their respective final chromatography steps: 500 ng of purified *S. gordonii* xPDPP from Gly-Pro Sepharose (lane 2); 100 ng of partially purified PepV from Gly-Pro Sepharose (lane 3), and 200 ng of purified PepV from Mono-P (lane 4).

TTCGTAG-3′) and used in PCR to obtain the full-length DNA fragment encoding PepV. A single 1,410-bp PCR product was obtained, gel purified, subcloned, and sequenced. Details of the cloning procedure and Southern blot analysis are available from the authors.

Results. PepV copurified with the previously described *S. gordonii* xPDPP (4) through several steps and was isolated as a homogeneous protein by addition of a Mono-P chromatofocusing step, as shown in Fig. 1. The approximate molecular mass was 50 kDa by SDS-PAGE (Fig. 1). Gel filtration analysis (data not shown) indicated a molecular mass of 50 to 60 kDa, suggesting that native PepV is a monomer. Isoelectric focusing-PAGE and Mono-P standardization (not shown) indicated an approximate pI of 4.73. The cleavage specificity of PepV was examined with a variety of peptide substrates (Table 1).

TABLE 1. Cleavage specificity of *S. gordonii* PepV

Peptide[a]	% Cleavage[b]
Ala-↓-Ala	100
Ala-↓-Phe	100
Pro-↓-Phe	100
Leu-↓-Gly	100
Met-↓-Tyr	100
Gly-↓-Gly-↓-Ala	75
Gly-↓-Pro-Ala	20
Ala-↓-Phe-Pro	20
Ala-↓-Pro-Gly	6

[a] Cleavage sites are indicated by arrows. Cleavage sites for tripeptides were deduced from the appearance on HPLC of a dipeptide peak corresponding to the decrease in the tripeptide. No such dipeptide peaks were seen for Gly-Gly-Ala, indicating that both peptide bonds could be cleaved. The following peptides were not cleaved: Ala-His (carnosine), Ala-Pro, Gly-Pro, Ile-Pro, Leu- Pro, Lys-Pro, Ser-Pro, Gly-Gly, Gly-Pro-Arg-Pro (fibrin inhibitory peptide), and Trp-Ala-Gly- Gly-Asp-Ala-Ser-Gly-Glu (sleep-inducing peptide).

[b] The percent cleavage is defined as (1 − area of residual peptide peak after digestion/area of peptide peak in undigested control) × 100.

TABLE 2. Inhibition profile of *S. gordonii* PepV

Inhibitor[b]	Concn or time	Residual activity (%)[a]
DFP	5 mM	90
PMSF	2 mM	100
3,4-Dichloroisocoumarin	2 mM	97
TLCK	10 mM	100
Amastatin	350 μM	3
Anstatin	50 μM	100
Apstatin	500 μM	1
Diprotin A	500 μM	98
E-64	500 μM	157
Iodoacetamide	5 mM	100
EDTA	5 mM	2
EGTA	5 mM	6
1,10-*o*-Phenanthroline	5 mM	0
Phosphoramidon	1 mM	110
DTT	5 mM	215
β-Mercaptoethanol	5 mM	130
K^+	5 mM	76
Ca^{2+}	5 mM	35
Mg^{2+}	5 mM	136
Mn^{2+}	5 mM	143
Zn^{2+}	5 mM	9
Urea	1 mM	16
SDS	1%	1
100°C	10 min	0

[a] Residual activity was expressed as the percentage of the V_{max} of PepV with no inhibitor.

[b] DFP, diisopropylfluorophosphate; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol.

Several hydrophobic dipeptides were completely hydrolyzed, and a number of dipeptides with Pro in the P_1' position were not cleaved. Interestingly, tripeptide analogs (Gly-Pro-Ala and Ala-Pro-Gly) of two of such dipeptides (Gly-Pro and Ala-Pro) were partially degraded by PepV; the same was true for Gly-Gly and Gly-Gly-Ala. Larger peptides (fibrin inhibitory peptide and sleep-inducing peptide) were not cleaved even with prolonged incubation. Inhibitor studies (Table 2) indicate that PepV is inhibited by EDTA, EGTA, and 1,10-*o*-phenanthroline and thus is a metallopeptidase. The enzyme was sensitive to apstatin and amastatin (inhibitors of aminopeptidase P and glutamyl aminopeptidase/aminopeptidase A, respectively), but not to phosphoraminidon, an inhibitor of metalloendopeptidases.

Gene cloning technology provided an open reading frame encoding a 467-amino-acid polypeptide with a theoretical molecular mass of 51,114 Da and a calculated pI of 4.8. A homology search for this polypeptide, done with National Center for Biotechnology Information TBLASTN, against the EMBL, DDBJ, GenBank, and PDB databases, indicated that PepV is highly conserved within the dinuclear peptidase M20 family in the MH clan of metallopeptidases. Previous members of this family have been found in lactic acid bacteria, where they are know as the PepV family (3, 7, 14, 15). The *S. gordonii* PepV gene has closest homology to PepV genes cloned from *Lactococcus lactis* (60% identity), *Lactobacillus helveticus* (49%), and *Lactobacillus delbrueckii* (45%), as well as a gene encoding a hypothetical dipeptidase from *Bacillus subtilis* (49%). Sequence alignments of these enzymes are shown in Fig. 2. The crystal structure of *Lactobacillus delbrueckii* PepV has been solved (8), and the active site includes a zinc-coordinating

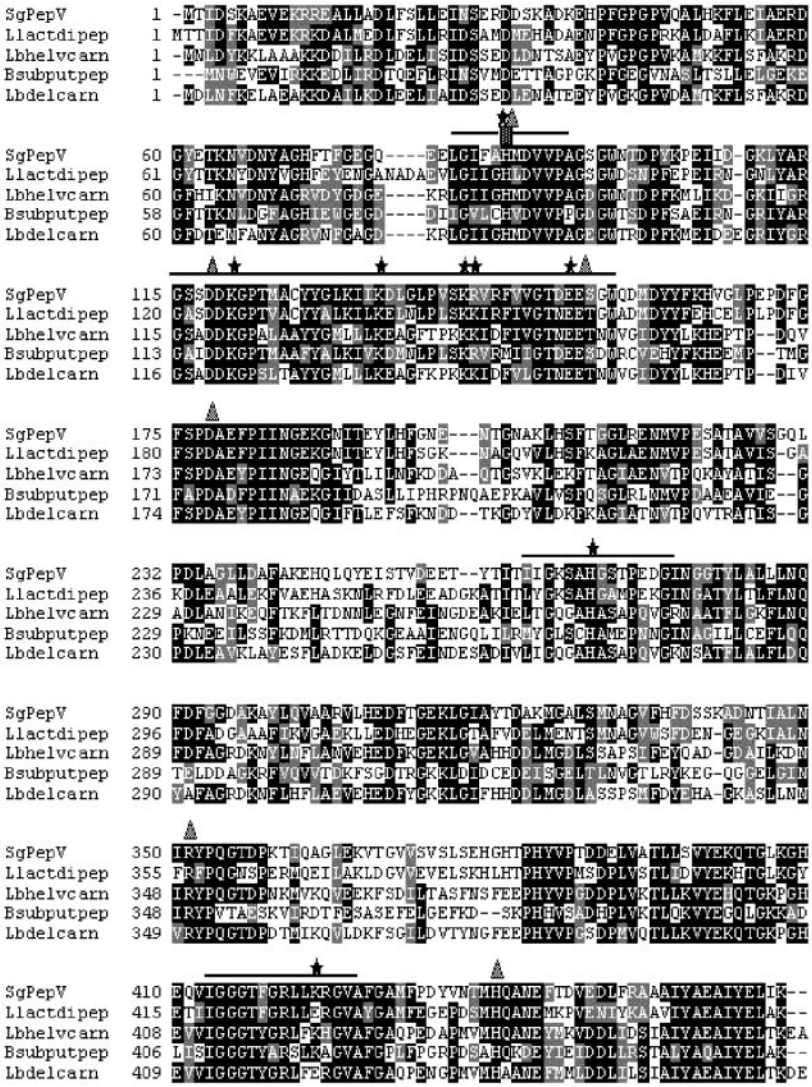

FIG. 2. Multiple sequence alignment of *S. gordonii* PepV (SgPepV) and bacterial homologues. Sequences of PepV from FSS2 and from *Lactococcus lactis* MG1363 (Llactdipep), *Lactobacillus helveticus* SBT2171 (Lbhelvcarn), *Bacillus subtilis* (Bsubputpep), and *Lactobacillus delbrueckii* subsp. *lactis* DSM7290 (Lbdelcarn) were aligned with the ClustalW multiple sequence alignment tool according to homology modeling (grey shading indicates similarity, and black shading indicates identity). Conserved box regions (boxes 1 to 4) are in sequential order and are denoted with single lines. Box, putative catalytic His residue within box 1; stars, conserved charged residues; triangles, residues implicated in the catalytic pocket from the crystal structure of *Lactobacillus delbrueckii* PepV.

histidine (His 88) within the motif LGIIGHMDVVP (Fig. 2, box 1). By analogy, His 87 of PepV is likely to be the active-site residue. Furthermore an additional five residues of *Lactobacillus delbrueckii* PepV (Asp 119, Glu 154, Asp 177, Arg 351, and His 439) have been implicated in critical metal coordination and nucleophile stabilization within the catalytic pocket (8). PepV maintains all of these essential residues, which are marked in Fig. 2. Finally, the PepV sequence includes three additional regions (boxes 2, 3, and 4) that are conserved within the M20 family of metallopeptidases. PepV shows high identity with these signature sequences, namely, 69% for box 2, 57% for box 3, and 93% for box 4. Furthermore, nine charged residues that are invariant in the M20 family are conserved in PepV.

The presence of the PepV gene in other oral streptococci is shown in Fig. 3. By Southern blot analysis using a PepV probe, a single copy of the gene was found in *S. gordonii* FSS2. Additionally, the gene was found in other *S. gordonii* strains (PK48, DL1, and PK2585) and in several species (*Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus parasanguinis*) that along with *S. gordonii* are members of the mitis group of viridans group streptococci (16). The PepV gene was not found in *Streptococcus salivarius*, which is not a member of the mitis group.

Discussion. The biochemical results (Fig. 1; Tables 1 and 2) are consistent with the interpretation that PepV is a metalloclass aminopeptidase with specificity for hydrophobic dipeptides. The enzyme has weaker tripeptidase activity but has no activity on tested peptides with four or more amino acids, suggesting a size limitation for the specificity pocket. A novel

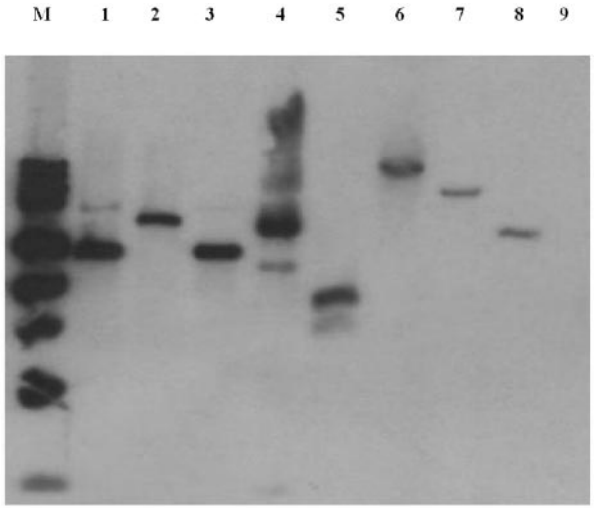

FIG. 3. Presence of the PepV gene in viridans group streptococci, as shown by Southern blot analysis. Four micrograms of bacterial DNA was digested with HindIII and hybridized with the PepV probe. Lanes: M, molecular mass markers; 1, *S. gordonii* PK48; 2, *S. gordonii* PK48; 3, *S. gordonii* DL1; 4, *S. gordonii* PK2585; 5, *S. parasanguinis* PK2584; 6, *S. mitis* J27; 7, *S. oralis* J21; 8, *S. oralis* PK34; 9, *S. salivarius* ATCC 27945.

feature is the resistance to cleavage of dipeptides with Pro in the P1′ position and the partial relief of that resistance when a third amino acid is added in the P2′ position (Table 1). Collectively, these results are comparable to those from PepV dipeptidases of *Lactococcus lactis*, *Lactobacillus helveticus*, and *Lactobacillus delbrueckii* (3, 7, 14, 15).

Genetic evidence (Fig. 3) indicates that in *S. gordonii* the PepV gene exists as a single copy and that this gene is conserved in the mitis group but not in at least one other group of the viridans group streptococci. The cloned gene for PepV (Fig. 2) reveals a protein sequence with significant homology (51% average identity) with PepV gene products of several lactic acid bacteria. The consensus sequence for the active site of PepV proteins from lactic acid bacteria (LGIIG HXDVVPAG) is present with slight modifications in *S. gordonii* PepV (Fig. 2, box 1). In *S. gordonii* PepV, the conserved IG in positions 4 and 5 of the consensus sequence is replaced by FA. Other notable features of PepV are the putative active-site His in box 1 and the strict conservation of charged residues in all four box motifs. The crystal structure of PepV from *Lactobacillus delbrueckii* has been elucidated (8), and there is substantial homology between PepVs of *Lactobacillus delbrueckii* and *S. gordonii* FSS2 (Fig. 3). Thus by analogy the active-site His and conserved charged residues in *S. gordonii* PepV also serve to stabilize zinc and the water nucleophile. These properties allow *S. gordonii* PepV to be included in the MH family of metallopeptidases.

This is the first report of the purification, characterization, and gene cloning of a PepV dipeptidase from the genus *Streptococcus*. The high degree of sequence conservation between *S. gordonii* PepV and PepV enzymes from *Lactococcus* and *Lactobacillus* spp. suggests the possibility of a common evolutionary gram-positive ancestor. The biological significance of the entire PepV subfamily is presently uncertain, particularly in view of the typically complex and redundant proteolytic systems of these bacterial groups (9, 13). Presumably *S. gordonii* PepV activity is useful for the growth of the mitis group of viridans group streptococci in dental plaque and heart valve vegetation environments. Future studies should focus on PepV expression and on the production of PepV knockouts in order to assess PepV's contributions to streptococcal growth and virulence in endocarditis.

Nucleotide sequence accession number. The sequence for the PepV gene was deposited in GenBank under accession number AY496433.

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