Extracellular Arginine Aminopeptidase from *Streptococcus gordonii* FSS2

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*Streptococcus gordonii* is a primary etiological agent in the development of subacute bacterial endocarditis (SBE), producing thrombus formation and tissue damage on the surfaces of heart valves. This is ironic, considering its normal role as a benign inhabitant of the oral microflora. However, strain FSS2 of *S. gordonii* has been found to produce several extracellular aminopeptidase- and fibrinogen-degrading activities during growth in a pH-controlled batch culture. In this report, we describe the purification, characterization, and partial cloning of a predicted serine class arginine aminopeptidase (RAP) with some cysteine class characteristics. Isolation of this enzyme by anion-exchange, gel filtration, and isoelectric focusing chromatography yielded a protein monomer of approximately 70 kDa, as shown by matrix-assisted laser desorption ionization, gel filtration, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis under denaturing conditions. Nested-PCR cloning enabled the isolation of a 324-bp-long DNA fragment encoding the 108-amino-acid N terminus of RAP. Culture activity profiles and N-terminal sequence analysis indicated the export of this protein from the cell surface. Homology was found with a putative dipeptidase from *Streptococcus pyogenes* and nonspecific dipeptidases from *Lactobacillus helveticus* and *Lactococcus lactis*. We believe that RAP may serve as a critical factor for arginine acquisition during nutrient stress in vivo and also in the proteolysis of host proteins and peptides during SBE pathology.

*Streptococcus gordonii* is a frequently researched member of the viridans family of oral streptococci (54). Viridans streptococci are primary colonizers of human dental plaque, where they serve a critical role in the establishment of microbial communities associated with good oral health. Although considered benign inhabitants of the oral microflora, members have been implicated in the systemic disease subacute bacterial endocarditis (SBE) (8, 33). The progression of this disease state requires: (i) trauma (congenital or inflammatory) to the endothelial valve surface such that it is predisposed to colonization, (ii) adhesion of organisms to the modified valve surface after their entry into the bloodstream via the oral cavity, and (iii) the propagation of infected vegetations consisting of bacteria in a fibrin-platelet meshwork (51). Despite the uniform susceptibility of these organisms to β-lactam antibiotics and their lack of classical streptococcal virulence factors, they can cause a chronic inflammation and/or life-threatening disease with periods of latency and recurrent infection (9, 18).

The ability of these organisms to form biofilm surfaces within two distinct microenvironments evoked studies of their patterns of gene expression and dynamic metabolism. *Streptococcus sanguinis*, studied as a model for viridans streptococcal pathogenesis, is known to express cell surface adhesins and a platelet aggregation-associated protein (PAAP) that facilitate both colonization and thrombosis (16, 19). Upon their entry into the bloodstream, bacteria undergo a shift from mildly acidic pH (6.0 to 6.5) in dental plaque to neutral pH (7.3) in the blood (43). This is significant, since among proteins extracted from *Streptococcus oralis* grown in batch culture and analyzed by two-dimensional electrophoresis, 39 showed altered expression at pH 5.2 versus 7.0 (55). Indeed, in vivo expression technology used on the *S. gordonii* rabbit model for infective endocarditis to detect genes activated in the new environment indicated an up-regulation of the *msrA* oxidative stress gene (53) and the induction of genes encoding carbohydrate metabolism enzymes, protein transporters, and cell surface proteins (26). The expression and secretion of glycogenidase and peptidase activities, as examined in pH-controlled batch cultures, was found to be down-regulated by acid growth conditions and up-regulated by growth in a neutral pH environment supplemented with serum (17).

Survival in vivo is dependent on the ability of the bacterium to remove sufficient quantities of nutrients from its environment. It is presumed that *S. gordonii* meets these needs by degrading salivary carbohydrates and glycoproteins in the oral cavity and utilizing plasma proteins when growing on heart valve surfaces. The free concentration of arginine in dental plaque has been measured in trace amounts (<1 μM) during periods of fasting, while meals present higher, variable levels (14). The amino acid constitutes 8% (by mass) of human fibrinogen, serving as a constant, plentiful reservoir. The vegetation biofilm is known to retard the diffusion of antibiotics (24), and solutes generally diffuse into the interior at lower rates than in water (50). Thus, access to carbohydrates and plasma proteins within the vegetation remains problematic. Under such conditions, where carbohydrates are scarce, oral streptococci can catabolize arginine via the arginine deiminase (ADI) pathway, resulting in the synthesis of both ATP and other essential metabolic precursors (7, 14). The utilization of
the amino acid is then prioritized, so the transport and metabolism of arginine-containing peptides may become particularly important. The amino acid and peptide transport systems of viridans streptococci have been described previously (2, 5, 10, 25, 31), and it has been shown that amino acids and small peptides are readily imported while those exceeding size limitations require hydrolysis by endo- and exopeptidases either present on the surface or secreted by these cells (6). One such activity that could meet the transport and metabolic challenges in vivo is an arginine aminopeptidase. Such an enzyme has been described in cell wall and cytoplasmic preparations of *Streptococcus mitis* and *S. sanguinis* (1, 12, 21) but has eluded detailed investigations. Here, we describe the purification, characterization, and partial cloning of the first reported extracellular arginine aminopeptidase (RAP) derived from *S. gordonii* FSS2, a strain previously isolated from the bloodstream of an SBE patient (37).

**MATERIALS AND METHODS**

culture media. Detection of this amidolytic activity in cell-free filtrate was observed during growth in culture supplemented with 50 mM glucose and 3.5 mM arginine and was dependent on a pH maintained in the range of 6.0 to 7.0. Growth and activity from a 1-liter pH-controlled (pH 7.0) batch culture were monitored at hourly intervals (Fig. 1). Detection of extracellular and cell-associated H-Arg-pNa activity from a single, defined peak of purified RAP (arginine aminopeptidase) from Mono-P.

**Physical properties.** SDS-PAGE analysis of the purified enzyme showed a single protein band, as judged by both Coomassie blue (Fig. 2) and silver staining, with an approximate mass of 70 kDa. Analysis of the protein by MALDI-TOF revealed a laser intensity peak that corresponded to a mass of 69,695.6 Da, while analytical Superdex

**TABLE 1. Purification of S. gordonii RAP**

<table>
<thead>
<tr>
<th>Step</th>
<th>Vol (ml)</th>
<th>Total activity (U)*</th>
<th>Total protein (mg)</th>
<th>Sp act (U/mg)</th>
<th>Fold purification</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>3,900</td>
<td>8,375</td>
<td>15,990</td>
<td>0.524</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation</td>
<td>43</td>
<td>1,707</td>
<td>433</td>
<td>3.94</td>
<td>7.52</td>
<td>20</td>
</tr>
<tr>
<td>DES52 anion exchange</td>
<td>10</td>
<td>3,284</td>
<td>176</td>
<td>18.7</td>
<td>36</td>
<td>40</td>
</tr>
<tr>
<td>Superdex 75</td>
<td>10</td>
<td>1,941</td>
<td>8</td>
<td>241.3</td>
<td>461</td>
<td>23</td>
</tr>
<tr>
<td>Mono-Q; FPLC</td>
<td>1</td>
<td>449</td>
<td>0.3</td>
<td>1,496</td>
<td>2,855</td>
<td>5.5</td>
</tr>
<tr>
<td>Mono-P; FPLC</td>
<td>2</td>
<td>232</td>
<td>0.12</td>
<td>1,835</td>
<td>3,506</td>
<td>2.8</td>
</tr>
</tbody>
</table>

*a* Based on enzymatic activity using H-Arg-pNa in which 1 U = 1 μmol of pNa released per s.
TABLE 2. Relative amidolytic activity of S. gordonii arginine aminopeptidase against various substrates

<table>
<thead>
<tr>
<th>Substrate (pNa)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-Arg</td>
<td>100</td>
</tr>
<tr>
<td>H-Lys</td>
<td>5</td>
</tr>
<tr>
<td>H-Ala</td>
<td>0</td>
</tr>
<tr>
<td>H-Glu</td>
<td>0</td>
</tr>
<tr>
<td>H-Ile</td>
<td>0</td>
</tr>
<tr>
<td>L-Leu</td>
<td>0</td>
</tr>
<tr>
<td>L-Pro</td>
<td>0</td>
</tr>
<tr>
<td>H-Val</td>
<td>0</td>
</tr>
<tr>
<td>H-Gly-Arg</td>
<td>0</td>
</tr>
<tr>
<td>H-Gly-Pro</td>
<td>0</td>
</tr>
<tr>
<td>Lys-Arg</td>
<td>0</td>
</tr>
<tr>
<td>Be-Arg</td>
<td>0</td>
</tr>
<tr>
<td>Ile-Pro-Arg</td>
<td>0</td>
</tr>
<tr>
<td>Sar-Pro-Arg</td>
<td>0</td>
</tr>
</tbody>
</table>

* Activity against H-Arg-pNa hydrolysis was taken as 100.

200 gel filtration indicated a molecular mass of 64 kDa and therefore a monomeric structure. Isoelectric focusing produced a pI of 5.6 for the native protein. The H-Arg-pNa activity was optimum at pH 6.5, with activity detected over a broad phosphate buffer range of 4.0 to 10.0. The enzyme in its pure form was unstable after a 24-h period at either 37 or 25°C, while it was minimally affected at 4°C. However, storage at −20°C over several weeks resulted in a major loss in enzyme, with complete inactivation after a 2-month period.

Enzyme specificity. Of the 14 chromogenic endo- and aminopeptidase substrates tested on the purified enzyme (Table 2), only H-Arg-pNa was rapidly hydrolyzed. Weaker activity (<10%) was detected against H-Lys-pNa and H-Leu-pNa. When Michaelis-Menten kinetics were measured using H-Arg-pNa as a substrate, a $V_{max}$ of 17.44 μmol min⁻¹ and a $K_m$ of 51 μM were determined.

To obtain further information on cleavage specificity, various peptides (2 to 13 amino acids) were tested as substrates for RAP (Table 3). Significantly, all proteolysis was restricted to peptides with an arginine residue in the N terminus, with cleavage detected only when a hydrophobic or an uncharged residue occupied the second site. There were no apparent additional restrictions for amino acids present downstream of the cleavage site. Extended incubation with peptides failed to yield additional peptide fragments, indicating the absence of endopeptidase activity or contaminating aminopeptidases. The inability of RAP to catalyze endo-specific proteolysis, as tested on whole protein substrates (azocasein, gelatin, collagen type IV, and fibrinogen), further supported the role of the purified enzyme as an exopeptidase.

Inhibition and activation studies. Studies with class-specific inhibitors (Table 4) supported the assignment of a serine class mechanism with some cysteine class characteristics. Moderate inhibition was observed using serine protease inhibitors (Pefabloc and, to a lesser extent, DFP and 3,4-dichloroisocoumarin). However, cysteine class inhibitors, iodoacetamide and E-64, had little or no effect on activity, while pCMB, a sulfhydrylsensitive reagent, was a potent inhibitor of RAP, indicating the possibility of a functional cysteine residue near the vicinity of the active site. Reducing agents (cysteine and β-mercaptoethanol) stimulated activity by approximately 50% and had a more pronounced effect at lower enzyme concentrations. With metallo class inhibitors, 1,10-orthophenanthroline provided no inhibition and EDTA actually stimulated RAP activity in a manner similar to that of the reducing agents. Activity was inhibited by the heavy-metal ions Zn²⁺ and Co²⁺ at 1 mM but was increased in the presence of lower-mass mono- and divalent cations. 1-Arginine served as a competitive inhibitor, while 1-lysine gave minimal inhibition, providing further evidence for strict specificity in the P₁ position. Compounds specific for eukaryotic arginine aminopeptidase/aminopeptidase B (EC 3.4.11.6) and leucine aminopeptidase (bestatin), as well as glutamyl aminopeptidase/aminopeptidase A (amastatin), were not inhibitory even at high concentrations. However, RAP was sensitive to the dipeptidyl-peptidase IV (DPP IV)-specific inhibitor H-Glu-pyrollidide and the aminopeptidase P-specific inhibitor apstatin, as evidenced by 98 and 50% inhibition, respectively, at 100 μM. Human plasma inhibitors, α-1-proteinase inhibitor and α-2-macroglobulin, had no effect on enzymatic activity.

These results are consistent with previous studies conducted

| Substrate Sequence |
|-------------------|------------------|
| Tyrosine kinase substrate Arg-Glu-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly |
| Anastatin-related peptide Arg-Cys-Arg-Val-His-Cys-Pro |
| Fibronectin-binding inhibitor Arg-Gly-Glu-Pro-Phc-Pro-Ile |
| Sexual agglutination peptide Arg-Gly-Pro-Phc-Pro-Arg |
| Protein kinase C substrate Arg-Phe-Leu-Arg-Gly-Glu-Pro-Asn-Val |
| Platelet aggregation inhibitor Arg-Gly-Glu-Ser |
| Peptide 7 Arg-Gly |
| Thymopentin fragment Arg-Lys-Val-Tyr |
| Peptide 9 Arg-Phe |
| Substance P Arg-Pro-Lys-Pro-Gly-Leu-Met |
| Bradykinin Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg |
| Dipetide A Arg-Pro |
| Angiotensin III Arg-Val-Tyr-Ile-His-Pro-Ile |
| Fibrin polymerization inhibitor (tetrapeptide) Gly-Pro-Arg-Pro |
| Kallidin Lys-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg |
| Lymphocyte activating pentapeptide Leu-Pro-Pro-Arg |

* †, cleavage site.
with cell-associated arginine aminopeptidases from *S. sanguinis* (12) and *S. mitis* (21).

**RAP sequence analysis.** The failure to generate a protein sequence from an apparently blocked N terminus required the sequencing of internal peptides after trypsin digestion. This partial structure resulted in the isolation of a peptide, DLTA DGSTLFGR, which was used to design a degenerate primer. In turn, the primer was employed to search the genomic clone of an *S. gordonii* strain in the Unfinished Microbial Genomes Database of TIGR. A matching contig (bvs 3948) was identified which comprised an incomplete open reading frame of 324 bp and included the internal sequence of RAP. The potential for errors in the electronic sequence necessitated the use of additional cloning methods. N- and C-terminal sequences facilitated the construction of degenerative primers, and PCR against an FSS2 library resulted in the isolation of the 324-bp product that constituted a partial-length DNA fragment. Southern blot analysis was performed with the clone and revealed RAP to be a single-copy gene. A 108-amino-acid polypeptide with a theoretical molecular mass of 11,934 Da and representing approximately 17% of the sequence of the intact protein was encoded by this open reading frame (Fig. 3).

The sequence was analyzed for the presence of gram-positive posttranslational modifications. Predictions indicated an intracellular, positively charged N terminus, a hydrophobic transmembrane domain (H region), and a putative site for signal peptide cleavage (42). This translocation mechanism is well conserved among *Bacillus* spp. (49) and has been identified in *Bacillus subtilis* alkaline protease (48) and bacillopeptidase F (56). In RAP, an export signal sequence of 14 hydrophobic residues is located downstream from the N terminus, followed by a polar region (denoted QACS) that provides a cleavage site after alanine.

The finding of an export signal supports the data for extracellular localization in culture experiments. A protein homology search was performed with the 108-amino-acid fragment using CMR BLAST against EMBL, DDBJ, GenBank, and PDB databases. The results indicated that RAP has 65% identity and 82% similarity with the unpublished sequence of a gene encoding an amylase binding protein B (AbpB) from *S. gordonii* (L. N. Li and F. A. Scannapieco, EMBL AF354648. www.embl.heidelberg.de). RAP and AbpB have predicted transmembrane domains, share nearly identical export signals, and have masses of approximately 70 kDa upon release from the cell. RAP maintains less homology with a putative dipeptidase (TIGR no. NTLO1SPL1617) from *Streptococcus pneumoniae* (44% identity), dipeptidase DA from *Lactococcus lactis* (34% identity), and nonspecific dipeptidase A from *L. helveticus* (31% identity).

**DISCUSSION**

This report describes the purification, characterization, partial cloning, and sequence analysis of an arginine aminopeptidase from *S. gordonii* FSS2. While this activity has been described in studies concerning arginine proteolysis by *S.
sanguinis and S. mitis (1, 12, 13, 20, 21, 36), the results presented here are the first biochemical evidence of an extracellular arginine aminopeptidase (RAP) as well as the only study of the molecular structure of a viridans streptococcal arginine aminopeptidase. The enzyme, which was obtained from a pH-controlled culture, was purified over 3,500-fold and was determined to be a homogenous monomeric protein with a molecular mass of 70 kDa. Specificity studies conducted using both p-nitroanilides and peptides smaller than 13 residues indicated only an exopeptidase activity upon incubation with either type of substrate. Although minor Lys and Leu cleavage occurred, efficient aminopeptidase activity required an Arg in the first position, with a nonpolar or uncharged residue preferred in the second site. Inactivation studies propose a serine class catalytic mechanism in which DPPIV- and aminopeptidase P-specific inhibitors appear to be the most effective.

Collectively, the biochemical studies of RAP are consistent with those of arginine aminopeptidases purified from S. mitis ATCC 9811 and S. sanguinis ATCC 903. These enzymes represented the first aminopeptidase B/arginyl-exopeptidase activities isolated from a bacterial source. Previous work had identified two activities, a broad-substrate aminopeptidase and an Arg-specific aminopeptidase, each capable of hydrolyzing arginine-2-naphthylamide when cytoplasmic, cell wall, or membrane fractions were used in cultures undergoing late exponential-early stationary-phase growth (12, 21). These molecular forms differ from each other with respect to their physical properties, inhibition studies, immunchemical properties, and cellular localization. RAP is most closely identified with the Arg-specific, lower-molecular-weight form that is associated with the cell wall and membrane, has been found to be sensitive to heavy metals, and is classified as a cysteine protease based upon inhibition studies. The inhibition of the S. mitis arginine aminopeptidase by pCMB and metals is suggested to be indicative of a sulfhydryl group located near the active site and essential for enzymatic activity (21). Additionally, it was reported that the stimulating effects of reducing agents were evidence for a cysteine residue(s) prone to oxidation (12). However, the sensitivity of RAP to serine class inhibitors, together with general unresponsiveness to cysteine class inhibitors, calls such a characterization into question.

The majority of bacterial aminopeptidases, including the broad-substrate Arg-aminopeptidase, are members of the metallo class, and with the exception of the the PepC family, bacterial cysteine class aminopeptidases are a rarity. The elucidation of crystal structures and site-directed mutagenesis experiments with bacterial prolyl-aminopeptidase (PIP) family members indicated that they are serine peptidases resistant to fluoride compounds, activated by reducing agents, and sensitive to mercurial and heavy-metal salts. These features have made characterization of the enzyme ambiguous and led to their misclassification as cysteine (27). Analysis of PIP genes from Bacillus coagulans (28) and Lactobacillus lactis (29) were modeled upon elucidation of the Xanthomonas campestris threedimensional structure, a model for the PIP family. The data obtained indicated that the active site was composed of the catalytic triad Asp58, Ser101, and His 267, with Cys62 located at the bottom of the active site about 5 Å from the nucleophilic serine, presumably accounting for its cysteine class behavior (40). However, in the case of RAP, the absence of both a complete sequence and an analogous reactive cysteine provide inconclusive evidence for serine classification.

The pattern of substrate cleavage exhibited by RAP indicates an aminopeptidase with a strict specificity for arginine in the first position and a restriction against a positively charged (Arg or Lys) or polar (Cys) residue in the second. However, proteolysis is favored when a nonpolar or hydrophobic amino acid is present in that position. There is constraint for neither residues downstream of the hydrophobic site nor peptide length, although longer peptides and proteins were not tested.

The unique specificity of RAP for Arg- and Pro-containing substrates may represent a new paradigm for aminopeptidase catalysis. The selectivity of RAP for arginine distinguishes it from eukaryotic aminopeptidase B, which cleaves arginine and lysine from the N terminus equally well (23). Proteolysis with proline in the second position is generally reserved solely for members of the aminopeptidase P family (4). Nevertheless, RAP functioned on three peptides containing Pro in the second position. Furthermore, inhibition was observed with both astatin and H-Glu-lyprollidide, two compounds that target those peptidases that can accept a Pro in their specificity pockets.

This report provides the initial sequence data for a viridans streptococcal arginine aminopeptidase. A homology search using the partial sequence of RAP revealed significant homology with a group of dipeptidases from gram-positive sources. An unclassified putative dipeptidase from S. pyogenes has the most identity (44% identical; 60% similar) and predicted a protein of about 498 amino acids. It was also suggested to be a cell surface protein with an export signal (VSYACT) similar to that of RAP (VAQACS). Both Lactococcus lactis and L. helveticus express biochemically identical dipeptidases, PepDA (459 amino acids) and cytosol nonspecific dipeptidase EC 3.4.13.18 (474 amino acids), which are approximately 30% identical and 47% similar to RAP (11). These enzymes are biochemically distinct from RAP, are specific for dipeptides of variable composition, and have been isolated from the cytoplasm of prokaryotic cells (34, 35).

Currently, all reported viridans streptococci arginine aminopeptidases have been associated with the cytoplasm, cell wall, or cell membrane (13, 20). The apparent export signal discovered in RAP represents the first extracellular protease from S. gordonii known to follow this secretion mechanism. Similar to other gram-positive exported proteins, RAP maintains N-terminal hydrophobic transmembrane and C-terminal regions in its unprocessed form. The consensus cleavage site (OACS), between residues 24 and 27, most closely resembles those sites in Bacillus sp. proteins. Extracellular proteases from B. subtilis and Lactobacillus lactis (PrsA and PrtM, respectively) maintain SACS sites that are processed via signal peptidase II (32). An oligopeptide-binding protein from S. gordonii also has an analogous AACS site (25).

Although arginine acquisition has been considered an important event inside the cell or in the space between the membrane and cell wall junction during peptide import, studies have not focused on extracellular peptidases capable of generating free arginine either on the cell surface or in the surrounding environment. As opposed to broad-substrate aminopeptidases, the specific arginine aminopeptidase has been implicated in the generation of a substrate for the ADI path-
way (22). Suitable concentrations of extracellular arginine are required for proper function of an antiporter that exchanges intracellular ornithine, a metabolite of the ADI pathway, for exogenous arginine (31, 45). The cell surface localization of the highly specific RAP is advantageous for the preservation of an energetically favorable concentration gradient. Its close proximity to the cellular membrane systems involved in amino acid and peptide transport could help meet nutritional requirements. The secretion of RAP would then regulate free arginine concentrations proximal to the cell and serve to scavenge proteins in the local environment, while retention of the broad-substrate aminopeptidase might be necessary for proteolysis of imported peptides and general protein turnover.

In *S. gordonii* G9B, extracellular protein profiles were altered by changes in pH, medium composition, and rate of growth (30). The secretion of two cytoplasmic proteins from *S. gordonii*, a GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (41) and an x-Pro DPP (15), was observed upon growth at a constant, neutral pH. These data are consistent with maximum expression of RAP activity at a controlled pH of 7.0 compared to growth at pH 6.0 and below (data not shown). Growth and activity curves revealed the peak of H-Arg-pNa activity to occur during early stationary phase, when excess glucose had been exhausted and a switch to arginine metabolism more potent than that of the intact peptide (57). The combined activation (3) or have been found to display biological activity and alter the balance between polymerization and fibrinolysis in favor of the growing vegetation. Future studies will focus on the expression and knockout of RAP in order to evaluate its relative contributions to streptococcal virulence and survival at the site of infection.

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**REFERENCES**


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