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Cloning and Characterization of an α-Enolase of the Oral Pathogen *Streptococcus mutans* that Binds Human Plasminogen

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Abstract

Streptococcus mutans is the etiologic agent of dental caries and is a causative agent of infective endocarditis. While the mechanisms by which *S. mutans* cells colonize heart tissue is not clear, it is thought that bacterial binding to extracellular matrix and blood conponents is crucial in the development of endocarditis. Previously, we have demonstrated that *S. mutans* cells have the capacity to bind and activate plasminogen to plasmin. Here we report the first cloning and characterization of an α-enolase of *S. mutans* that binds plasminogen. The functional identity of the purified recombinant α-enolase protein was confirmed by its ability to catalyze the conversion of 2-phosphoglycerate to phosphoenolpyruvate. The protein exhibited a K_m of 9.5 mM and a V_{max} of 31.0 mM/min/mg. The α-enolase protein was localized in the cytoplasmic, cell wall and extracellular fractions of *S. mutans*. Binding studies using an immunoblot analysis revealed that human plasminogen binds to the enolase enzyme of *S. mutans*. These findings identify *S. mutans* α-enolase as a binding molecule used by this oral pathogen to interact with the blood component, plasminogen. Further studies of this interaction may be critical to understand the pathogenesis of endocarditis caused by *S. mutans*.

Keywords

Streptococcus mutans; Enolase; Plasminogen receptor

Introduction

Streptococcus mutans is a gram-positive bacterium that is one of several oral streptococcal species that after trauma to the oral cavity can leave the oral cavity via the bloodstream. Once in the bloodstream, these organisms can colonize in the form of vegetations injured cardiovascular endothelium and cause chronic infective endocarditis [1]. Adherence of *S. mutans* cells to fibronectin and other components of the extracellular matrix has been suggested to play a role in the pathogenesis of endocarditis by this bacterium [2]. *S. mutans* cells have been shown to also have the capacity to bind and activate plasminogen to the serine protease, plasmin, as well as degrade fibronectin in the presence of plasminogen [3,4]. Plasminogen is a single chain glycoprotein with a molecular mass of 92 kDa that plays a critical role when

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activated to plasmin in fibrinolysis and tissue remodeling. Several gram-positive pathogens have been found to capture plasminogen on their cell surface, including *Streptococcus pyogenes* and *Streptococcus pneumoniae* [5,6]. This activity may facilitate the invasiveness of these pathogens in the host. *S. mutans* is not generally thought to be an invasive organism. However, Stinson and coworkers have shown that *S. mutans* has the ability to invade and kill human endothelial cells in culture [7].

Receptors for plasminogen are produced by a number of gram-positive bacteria [8]. Among streptococcal species, the glycolytic enzymes, glyceraldehydes-3-phosphate dehydrogenase and enolase have been identified as plasminogen-binding proteins. These generally cytosolic enzymes lack signal sequences and typical motifs for membrane anchoring, but the proteins have been shown to be surface-expressed on several streptococcal pathogens [6,9-12]. The association of glyceraldehydes-3-phosphate dehydrogenase and enolase with the cell surface may only occur at low pHs and expression of the proteins maybe enhanced during biofilm formation [13,14]. Surface-associated proteins, such as enolase, have the potential to be candidates for the development of new vaccines against many streptococcal infections.

In this study, we report the cloning of the gene and purification and characterization of an enzymatically active recombinant α-enolase protein of *S. mutans*. Analysis of cellular fractions of *S. mutans* demonstrated that the α-enolase protein was found in the cytoplasmic, cell wall, and extracellular protein fractions. The α-enolase was found to have the ability to bind human plasminogen that indicates that the protein can function as a plasminogen receptor.

Materials and methods

Cloning techniques

We obtained from the *S. mutans* genome sequence database a 1296 bp open reading frame (GenBank accession no. **AE014133**) that showed considerable homology to the α-enolase genes of *S. pyogenes*, *S. pneumoniae*, and the oral streptococcal species, *Streptococcus sobrinus* and *Streptococcus sanguinis* [15]. This sequence encoding the putative α-enolase of *S. mutans* was used to design oligonucleotide primers for PCR amplification of the sequence. The two oligonucleotide primers used to amplify the putative α-enolase gene using chromosomal DNA of *S. mutans* M51, prepared as described previously, were FE (5′- ATG TCA ATT ATT ACT GAT GTT TAC GCT CGC-3′), the forward primer and BE (5′- TTA TTT TTT CAA GTT GTA GAA TGA TTT AAG ACC ACG-3′), the backward primer [2, 16]. The forward primer began at base 1 and ended at base 30, while the backward primer began at base 1263 and ended at base 1296 of the sequence and includes a termination codon. The primer pair was used in PCR reactions with denaturing, annealing, and extension temperatures and times of 94 ° C for 1 minute, 50 ° C for 30 seconds, and 72 ° C for 3 minutes; respectively. The amplified PCR product generated was approximately 1.3 kb in size, which is the correct size based on the nucleotide sequence. The α-enolase PCR product was cloned into the TA expression vector pCR T7/NT TOPO-TA. The resulting plasmid DNA was transformed into *E. coli* TOP 10F' cells and spread on LB plates supplemented with 100 μg/ml of ampicillin. Plasmid DNA was isolated from several transformants and digested with restriction enzymes to verify the size of the insert DNA. The plasmid DNA was then sequenced for determination of the sequence of the insert DNA using Big Dye terminator reactions. The plasmid containing the α–enolase gene of *S. mutans* was designated pSmEno.

Expression and purification of S. mutans α-enolase

Purification of the *S. mutans* α-enolase under non-denaturing conditions was achieved using *E. coli* BL21 Star (DE3) cells that had been transformed with plasmid pSmEno. A 50 ml culture of BL21 Star (DE3)pLysS containing pSmEno grown at 37 ° C in LB broth supplemented with

ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) was inoculated into 1 liter of LB broth containing 100 μg/ml of ampicillin and 34 μg/ml of chloramphenicol and grown at 37° C until an OD_{600} of 0.6 was reached. IPTG was added to a final concentration of 1 mM and the culture was incubated for an additional 4 hrs. The cells (250 ml aliquots) were pelleted by centrifugation and the pellet was resuspended in 20 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10mM imidazole, pH 8.0). Lysozyme was added to a concentration of 1 mg/ml and the suspension was incubated on ice for 30 min, and the cells were lysed by sonication on ice for 1 min. The lysate was centrifuged and four ml of cleared lysate was added to 1 ml of 50% Ni^{+2}-nitrilotriacetic acid (Ni-NTA) slurry that was rotated gently at 4° C for 1 hr. The lysate-Ni-NTA mixture was loaded into a column, washed twice with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20mM imidazole, pH 8.0) and the his-tagged α-enolase protein was eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250mM imidazole, pH 8.0).

Measurement of α-enolase activity

Alpha enolase activity was determined by measuring the conversion of 2-phosphoglycerate (2-PGE) to phosphoenolpyruvate (PEP) [10]. Reactions were performed in 100 mM HEPES buffer pH 7.0, 10 mM $MgS0_4$, and 7.7 mM KCl and using different concentrations of 2-PGE in a final volume of 1.0 ml. Changes in absorbance per minute were monitored spectrophotometrically at 240 nm and were monitored at 5 second intervals for a period of 3 minutes. Values for K_m and V_{max} were calculated from plots of the resulting data using the Lineweaver-Burk equation and an extinction coefficient of 1.16×10^{-3} M^{-1} for PEP [10].

Preparation of α-enolase antiserum

Antisera against *S. mutans* α-enolase was prepared by immunizing subcutaneously New Zealand female rabbits with 200 μg of purified his-tagged α-enolase protein emulsified in Freund's incomplete adjuvant containing 10% muramyl peptide. Rabbits were boosted two-weeks later with the same amount of his-tagged α-enolase in Freund's incomplete adjuvant and after two additional weeks were bled. Antiserum to the α-enolase of *S. pyogenes* (anti-SEN) was a gift from Vijaykumar Pancholi [10].

Isolation of cellular protein fractions

Extracellular, cytoplasmic, and cell wall protein fractions of *S. mutans* M51 were prepared from 1-liter cultures grown at 37 ° C for 48 hours in Todd-Hewitt broth as described by Kuykindoll and Holt [17]. Total cell lysates of *S. mutans* M 51 were prepared from 20 ml of culture grown overnight at 37 ° C in Todd-Hewitt broth. Cells were collected by centrifugation and resuspended in 10 mM phosphate buffered saline, pH 7.0. The cell suspension was transferred to a 2 ml bead beater tube and 0.4 mg of 0.1 mm zirconia/silica beads per ml of cell suspension were added. The tube was placed in a Mini-Beadbeater 3110BX and run in one minute time periods with cooling on ice between time periods for a total of 3 minutes. The beads were allowed to settle and the supernatant fluid (total cell extract) was removed.

Immunoblot procedures

Protein samples were separated in 8% SDS polyacrylamide gels and electrophoretically transferred onto nitrocellulose membranes. The membrane was incubated 1 hr in 20mM Tris-HCl, pH 7.5, 150 mM NaCl (TBS) containing 5% non-fat dry milk at room temperature. The membrane was washed in 20mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween-20 (TTBS). For detection of α-enolase in *S. mutans* cellular fractions, membranes were incubated for 1 hr with antibodies to *S. mutans* or *S. pyogenes* enolase in TTBS containing 5% milk at room temperature. The membrane was washed three times in TTBS and then incubated for 1 hr with goat anti-rabbit immunoglobulin G alkaline phosphatase conjugate in blocking solution at room temperature. Detection of bound alkaline phosphatase conjugate was performed in a

substrate solution containing nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate. For detection of plasminogen binding to α-enolase of *S. mutans*, separated protein on a nitrocellulose membrane after blocking for 1 hr was incubated overnight with human plasminogen (10 μg/ml) in blocking solution. The membrane was washed followed by incubation for 1 hr with goat anti-human plasminogen. After washing in TTBS, the membrane was incubated 1 hr with rabbit anti-goat IgG horseradish peroxidase conjugate in blocking solution. Detection of plasminogen binding was achieved using the ECL enhanced chemiluminescent detection reagents and exposure of the membrane to Kodak BIOMAX Light film.

Results

Cloning and expression of putative α-enolase of S. mutans

A 1,296 bp open reading frame was identified in the genomic sequence of *S. mutans* UA159 and was used to design oligonucleotide primers for PCR amplification of the putative α-enolase gene. The open reading frame encoded a 432 amino acid protein with a molecular weight of 47 kDa. We found that the putative *S. mutans* enolase, designated SMU.1247 in the *S. mutans* genomic sequence, possessed more than 90% identity with the enolases of *S. sobrinus*, *S. sanguinis*, *S. pneumoniae*, and *S. pyogenes* when the translated amino acid sequence of the putative α-enolase gene of *S. mutans* was compared in a multiple sequence alignment (Fig. 1). The PCR-amplified DNA fragment containing the putative α-enolase gene of *S. mutans* was cloned into an expression vector to generate the 4.2 kb plasmid, pSmEno. After induction of expression in *E. coli* containing pSmEno of the putative α-enolase protein, the his-tagged recombinant protein was purified under non-denaturing conditions using nickel affinity chromatography. The recombinant protein will be referred to as rSmEno.

Alpha-enolase activity and enzyme kinetic properties of rSmEno

After purification of rSmEno, we determined that the recombinant protein possessed the functional capacity to catalyze the conversion of 2-phosphoglycerate to phosphoenolpyruvate, the enzymic reaction catalyzed by α-enolases. This assay was also used to determine the kinetic properties of rSmEno. Enzyme reaction kinetics using purified his-tagged protein with various concentrations of 2-phosphoglycerate was performed and values for K_m and V_{max} were calculated from reciprocal plots (Fig. 2). This analysis revealed a K_m value of 9.5 mM for 2-phosphoglycerate and a V_{max} of 31 mM phosphoenolpyruvate/min/mg for rSmEno.

Localization of SmEno in S. mutans

To determine the subcellular location of α-enolase in *S. mutans*, antiserum raised against rSmEno was used in an immunoblot analysis to probe extracellular, cell wall, and cytoplasmic protein fractions of *S. mutans* for the presence of the protein. This analysis using anti-SmEno serum revealed a strong reactivity to a protein of approximately 50 kDa found in the extracellular, cell wall, and extracellular protein fractions (Fig. 3B). A similar immunoblot analysis using antibodies against the surface enolase (SEN) of *S. pyogenes* revealed cross reactivity to a protein of similar in size to the protein recognized by the anti-rSmEno sera in the extracellular, cell wall, and cytoplasmic protein fractions of *S. mutans* cells (Fig. 3C). Both antibodies reacted with the purified rSmEno protein.

Plasminogen binding by α-enolase of S. mutans

To test the ability of the *S. mutans* α-enolase to bind plasminogen, protein in a total cell extract and an extracellular protein fraction of *S. mutans* along with purified rSmEno were separated by SDS-PAGE and transferred to nitrocellulose membranes. The blot was incubated with human plasminogen and then probed with antibodies against human plasminogen. The results

demonstrated that plasminogen bound to a protein of approximately 50 kDa in the total cell extract, extracellular protein fraction and with purified rSmEno that migrated with a mobility that was very similar to a protein in each of the preparations that reacted with anti-rSmEno sera (Fig. 4).

Discussion

This study provides evidence that oral streptococci, such as *S. mutans*, have the potential to use similar mechanisms as more virulent and invasive streptococci, such as *S. pyogenes* and *S. pneumoniae*, to facilitate the infectious process. *S. mutans* is the etiologic agent of dental caries but along with other viridans streptococci are responsible a substantial number of cases of chronic infective endocarditis [1,18]. In this study, the gene for the glycolytic enzyme, α-enolase, was cloned and the protein expressed and purified. Furthermore, the recombinant protein had the functional characteristics of an enolase and was found to bind plasminogen.

The *S. mutans* protein at the amino acid level had greater than 90% identity with enolases of a number of streptococcal species. The *S. mutans* enolase like several other streptococci had on its C-terminal end two lysine residues that have been reported to interact with the lysine binding sites of plasminogen [6,10]. Bergmann et al. [19] have demonstrated that the enolase of *S. pneumoniae*, designated Eno, has an additional internal plasminogen binding epitope and that this internal motif is crucial for the interaction of the Eno protein with human plasminogen. These investigators identified a motif of nine amino acids, FY**DKE**R**K**VY (amino acid residues crucial for plasminogen binding are bold and underlined), that bound human plasminogen (see Fig. 1). The acidic amino acids, aspartic acid (position 3) and glutamic acid (position 5), and the lysine residues at positions 4 and 7 in the motif were found to be important for plasminogen binding by the *S. pneumoniae* enolase. Furthermore, mutant strains of *S. pneumoniae* with substitutions in the critical amino acids of the internal plasminogen-binding motif were substantially less virulent than a wild-type strain of *S. pneumoniae*. The *S. mutans* α-enolase has the sequence, FY**D**NG**VY, that has five amino acids in common with the internal plasminogen-binding motif of *S. pneumoniae*. However of the amino acids required for plasminogen binding, only the aspartic acid at position 3 is present and two of the amino acids in the motif are not present at all. A similar sequence was found in the enolase of *S. sobrinus*, another caries causing oral streptococcus. This finding indicates that the *S. mutans* enolase does not have a functional internal plasminogen-binding site and suggests that enolase-mediated plasminogen binding by *S. mutans* occurs primarily through interaction of plasminogen with the C-terminal lysyl residues. Previously, we have shown that plasminogen activation by *S. mutans* is almost totally inhibited by lysine or the lysine analog, ε-aminocaproic acid that will support this idea [3].

Functional identity of purified rSmEno was confirmed by its ability to catalyze the conversion of 2-PGE to PEP. The value for the enzyme kinetic constant, V_{max}, was found to be similiar to those reported for α-enolases from *S. pyogenes* and *S. pneumoniae* [6,10]]. However, the value for K_m for rSmEno was found to be six-fold higher than the purified α-enolase from *S. pyogenes* and two-fold higher than the recombinant α-enolase from *S. pneumoniae*.

Localization studies utilizing polyclonal antibody against purified rSmEno and antibody against the enolase of *Streptococcus pyogenes* were used to determine the cellular location of SmEno in *S. mutans*. α enolase was found to be located in the cytoplasm, cell wall, and extracellular protein of *S. mutans*. This finding is similar to results reported by Ge et al. [20] that utilized matrix-assisted laser desorption ionization-time of flight mass spectrometry to identify α-enolase as one of the proteins in a surface protein preparation isolated from *S. mutans*. rSmEno was found to be cross-reactive with anti-SEN serum. Eno from *S. pneumoniae* was also found to be cross-reactive with anti-SEN serum [6]. Reaction of *S.*

mutans subcellular fractions with anti-SEN serum confirmed the results obtained with the rSmEno antibody that showed that α-enolase is located in the cytoplasm, cell wall, and extracellular protein of *S. mutans*. Neither rSmEno nor other streptococcal enolases have a signal sequence or a hexameric LPXTG motif. These domains have been shown to be critical for translocation through the cell wall and for anchoring surface proteins to the cell wall of Gram-positive bacteria [21]. Chhatwal [22] suggests that these anchorless proteins with no signal peptide or anchoring motif are secreted by a yet unknown mechanism and are then displayed on the bacterial surface by reassociation with receptors on the cell where they can execute a biological function. This could the explanation why enolase of *S. mutans* was found in the extracellular protein.

Pancholi and Fischetti [10] observed that α-enolase from group A streptococci was able to bind plasminogen, while Bergmann et al [6] and Whiting et al. [23] observed that α-enolase from *S. pneumoniae* was also able to bind plasminogen. We find using a blot overlap analysis that the *S. mutans* α-enolase could translocate through the cell wall of *S. mutans* cells and be found in the extracellular protein. The α-enolase in the extracellular protein had the ability to bind human plasminogen. This finding suggests that enolase is present in the microenvironment of *S. mutans* cells and can probably reassociate with the cell surface as has been suggested by Chhatwal [22] for anchorless proteins. Subsequently, the enolase can bind plasminogen and the plasminogen can be activated to plasmin by bacterial or host plasminogen activators. This surface localized plasmin activity on the surface of *S. mutans* cells can then degrade extracellular matrix constituents, such as fibronectin, as we have previously shown [3].

In conclusion, we have characterized the α-enolase of *S. mutans* and provided evidence that this typically cytoplasmic protein can translocate the cell wall of the oral bacterium, *S. mutans*, and function as a receptor for plasminogen. This finding suggests that this generally non-invasive oral organism may have virulence potential similar to that of more pathogenic streptococci.

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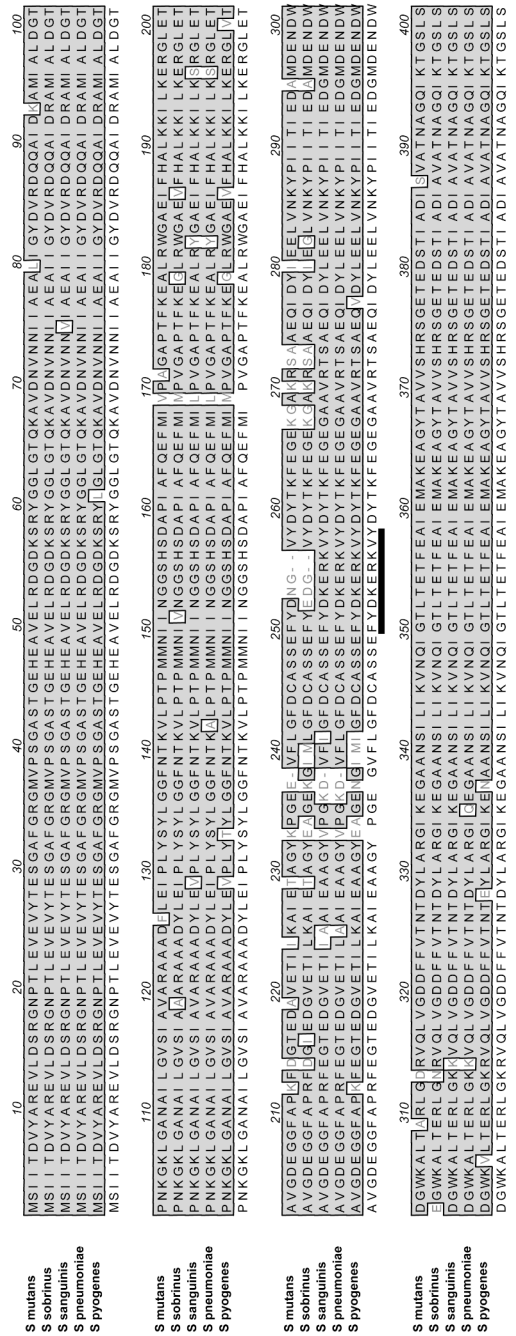

Fig. 1.

Clustal W sequence alignment of the translated protein sequences of *Streptococcus mutans* M51 α-enolase and enolases of *Streptococcus sobrinus*, *Streptococcus sanguinis*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes*. The internal plasminogen-binding motif of the *S. pneumoniae* enolase is denoted by the underlined amino acids.

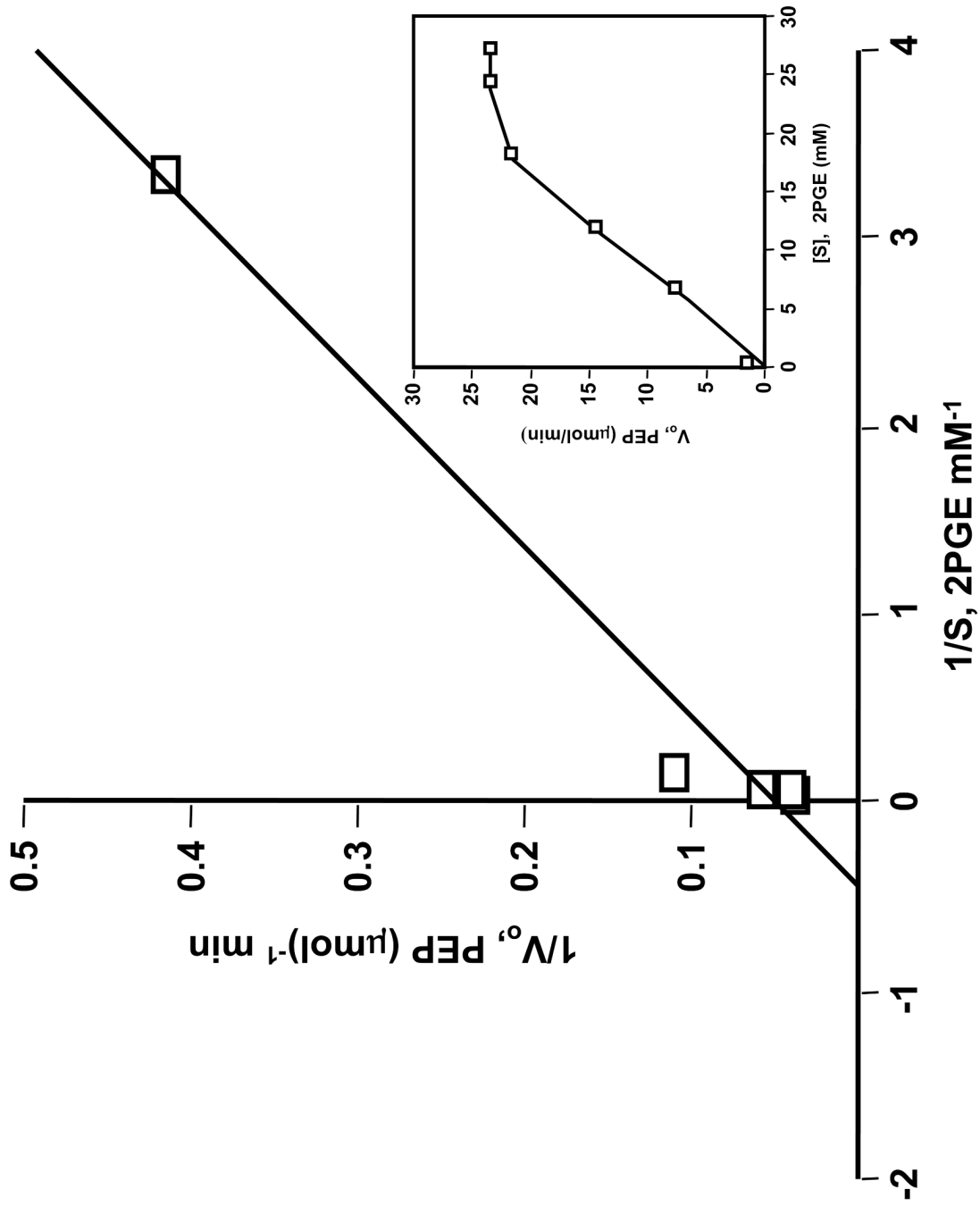

Fig. 2.

α-Enolase activity of purified rSmEno. Enzyme kinetics of rSmEno were determined by measuring the rate of conversion of 2-PGE to PEP in the presence of various concentrations of substrate (2-PGE; 0.3-27 mM) and 5 μg of the purified rSmEno as enzyme and monitoring the change in absorbance at 240 nm. Data was plotted by the method of Michaelis-Menten (inset) and V_{max} and K_m were calculated from double reciprocal plots.

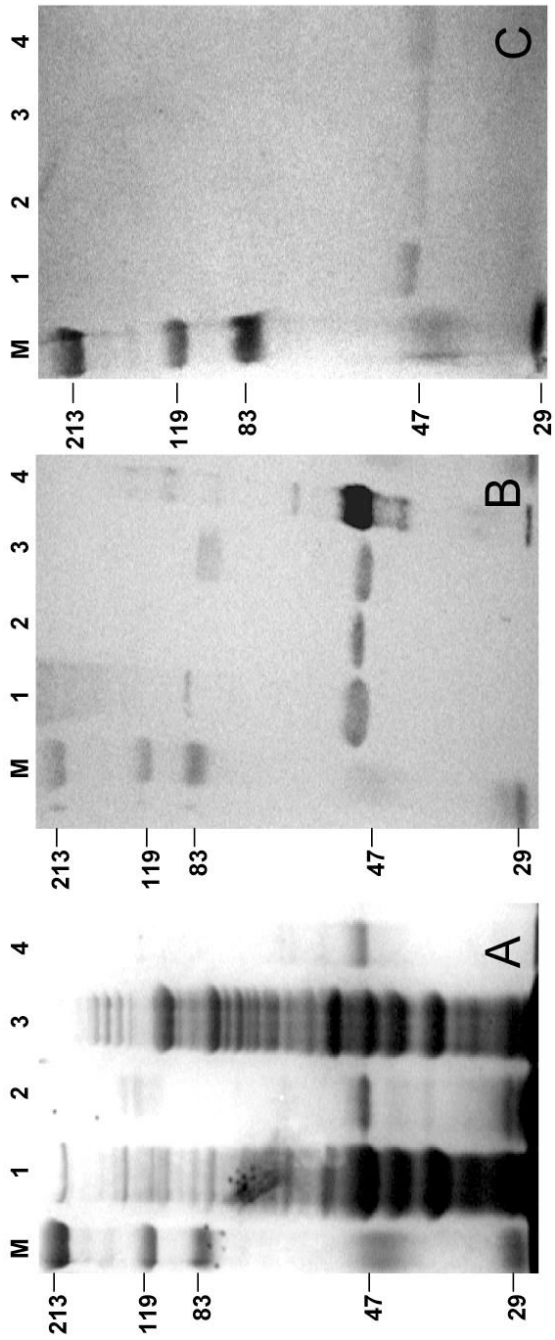

Fig. 3.

Localization of *S. mutans* enolase using anti-rSmEno and anti-SEN antibodies. Panel A; SDS-polyacrylamide gel of cellular fractions of *S. mutans*; Lane M, pre-stained molecular weight marker; Lane 1, extracellular protein fraction; Lane 2, cell wall fraction; Lane 3, cytoplasmic protein fraction; Lane 4, purified rSmEno. Panel B; immunoblot of gel probed with anti-rSmEno antibodies. Panel C; immunoblot of gel probed with anti-SEN antibodies.

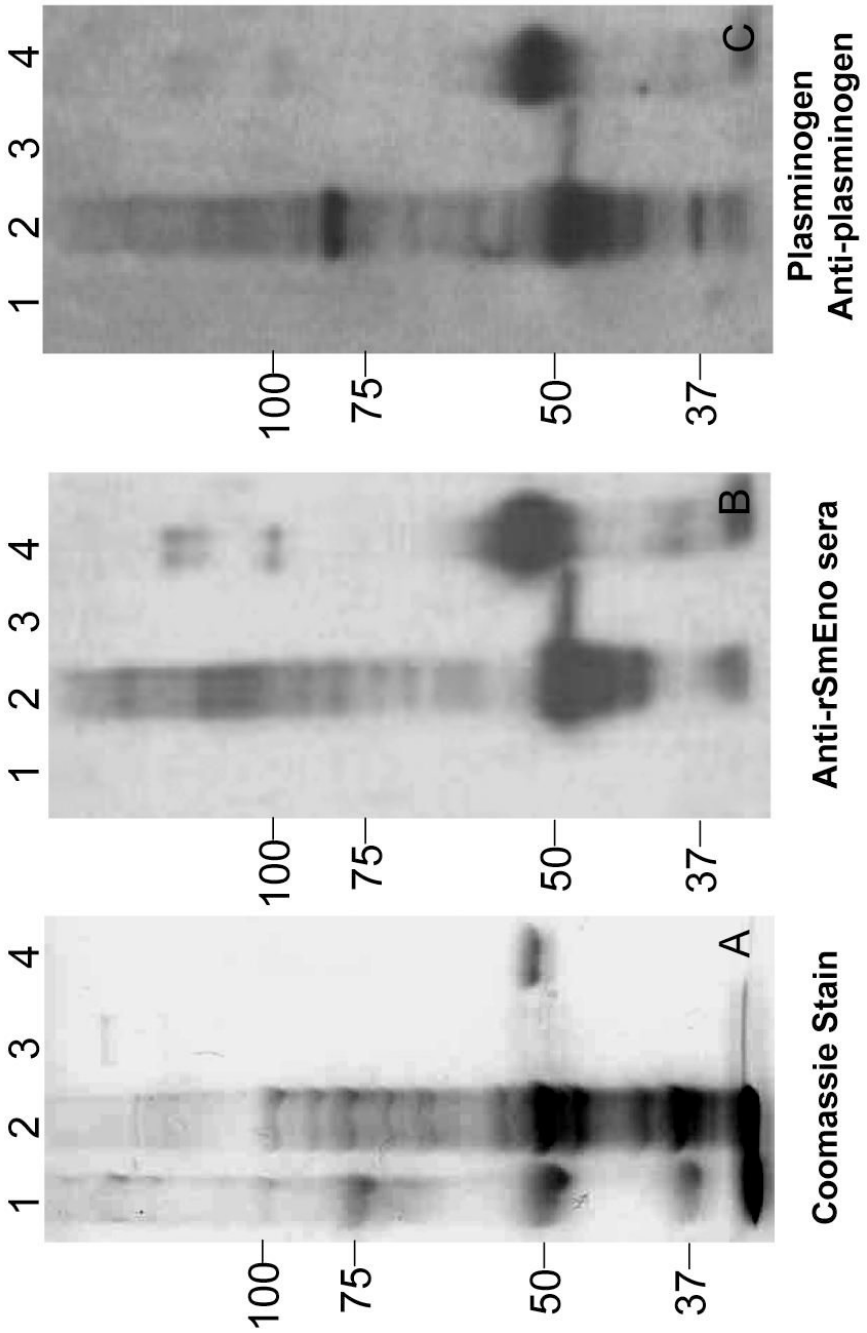

Fig. 4.

Plasminogen binding to the α-enolase of *S. mutans*. Panel A, SDS-polyacrylamide gel electrophoresis of a total protein extract and extracellular protein of *S. mutans* and purified rSmEno. Lane 1, molecular weight markers; Lane 2, total protein extract; Lane 3, extracellular protein; and Lane 4, purified rSmEno. Panel B, immunoblot of gel probed with anti-rSmEno antibodies. Panel C, Blot of gel incubated with human plasminogen and probed with anti-plasminogen antibodies.