Brazilian Journal of Medical and Biological Research (1997) 30: 1427-1430
ISSN 0100-879X Short Communication

Cloning, expression and purification of recombinant streptokinase: partial characterization of the protein expressed in *Escherichia coli*

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Abstract

We cloned the streptokinase (STK) gene of *Streptococcus equisimilis* in an expression vector of *Escherichia coli* to overexpress the profibrinolytic protein under the control of a *tac* promoter. Almost all the recombinant STK was exported to the periplasmic space and recovered after gentle lysozyme digestion of induced cells. The periplasmic fraction was chromatographed on DEAE Sepharose followed by chromatography on phenyl-agarose. Active proteins eluted between 4.5 and 0% ammonium sulfate, when a linear gradient was applied. Three major STK derivatives of 47.5 kDa, 45 kDa and 32 kDa were detected by Western blot analysis with a polyclonal antibody. The 32-kDa protein formed a complex with human plasminogen but did not exhibit Glu-plasminogen activator activity, as revealed by a zymographic assay, whereas the 45-kDa protein showed a K_m = 0.70 µM and k_{cat} = 0.82 s^{-1}, when assayed with a chromogen-coupled substrate. These results suggest that these proteins are putative fragments of STK, possibly derived from partial degradation during the export pathway or the purification steps. The 47.5-kDa band corresponded to the native STK, as revealed by peptide sequencing.

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Research supported by CDCHT/ULA (No. C-474-91).

Received March 21, 1997
Accepted September 16, 1997

Key words
- Recombinant streptokinase
- Plasminogen activators
- Protein purification

Streptokinase (STK), a protein of 415 amino acids that does not contain disulfide bridges, is produced and excreted by some strains of ß-hemolytic streptococci (1,2). Because of its ability to activate the fibrinolytic system, it is currently used as a thrombolytic agent for the treatment of patients with acute myocardial infarction (3,4). The production of STK by methods yielding a high benefit/cost or benefit/risk ratio is highly desirable. One of those methods is recombinant DNA technology that could be used to produce high quantities of recombinant STK (rSTK) avoiding the contamination risks that arise from the manipulation of pathogens such as those belonging to the streptococcus species. The first step in the development of such a method implied the cloning of the STK gene in the non-pathogen *Escherichia coli* JM105, and is the subject of the present study.

The following oligonucleotides were used

for gene amplification by the polymerase chain reaction technique: 5' TAG GAG GTT TCT ATG AAA AAT TAC TTA T 3' and 5' CCG TGG TTA TTT GTC GTT AGG 3'. The amplified STK gene of *Streptococcus equisimilis* was inserted into the unique *Sma*I site of pKK 223-3 (Pharmacia) to transform *E. coli* competent cells. The STK gene insertion, including a 26-amino acid residue signal sequence, was confirmed by multiple fragment nucleotide sequencing of the leading strand in the new plasmid isolated from one ampicillin-resistant/STK-positive clone. Furthermore, DNA sequencing data show that the cloned STK gene has the capacity to code for a 47.5-kDa mature protein. The recombinant plasmid, which contains a strong *tac* promoter, allowed the expression of at least 120 mg/l of the recombinant protein after induction with 0.5 mM isopropyl-ß-D-galactopyranoside (IPTG) during the exponential growth phase. Cells were harvested 3 h after induction and the periplasmic fraction extracted by treatment with 3.5 mM lysozyme in 50 mM Tris-HCl buffer, pH 8.0, and 20% sucrose for 30 min on ice (5). STK activity was determined by the casein-plasminogen plate assay (6). Briefly, 20 µl of the different fractions was used to fill wells cut into plates prepared from a medium containing 1% agarose, 1% skim milk, and 20 nM human plasminogen (Pg) in 50 mM Tris-HCl, pH 8.0. The area of the lysis zone surrounding the wells after 4-6 h of incubation at 37°C was correlated with dilutions of a standard native STK solution. Almost all the rSTK activity was obtained in the periplasmic fraction, confirming that the export pathway of *E. coli* is capable of recognizing the signal sequence of *S. equisimilis* STK (6). Three major rSTK derivatives of 47.5 kDa, 45 kDa and 32 kDa were detected by zymography and by Western blot analysis (7) (Figure 1).

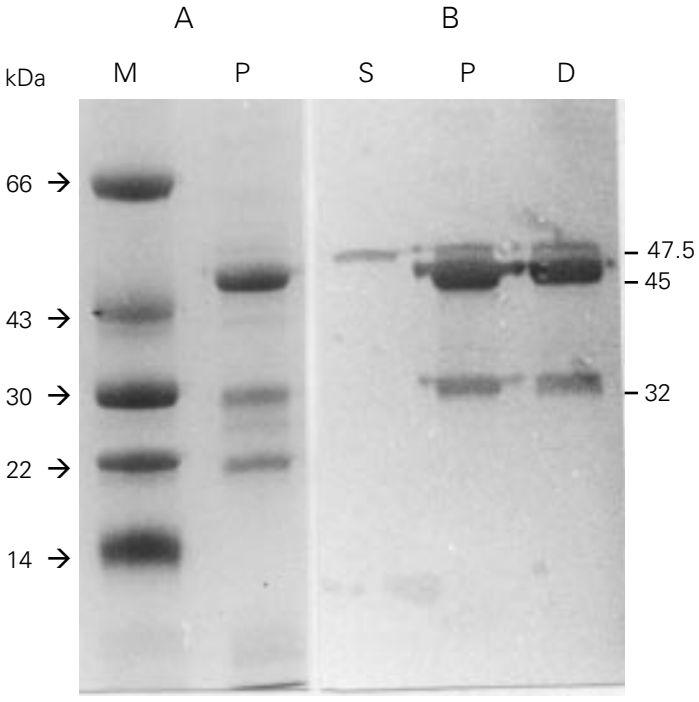

Figure 1 - SDS-PAGE (A) and Western blot analysis (B) of periplasmic streptokinase (STK). Periplasmic proteins were separated on a 0.1% SDS/15% polyacrylamide gel and stained with Coomassie blue. A replica was blotted onto a nitrocellulose membrane and analyzed with an anti-STK rabbit polyclonal antiserum developed against native STK. *A*, Lane M: molecular weight standards (serum albumin, 66 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 22 kDa; lysozyme, 14 kDa); lane P: periplasmic proteins. *B*, Lane S: native STK; lane P: periplasmic proteins; lane D: "pool" of periplasmic proteins eluted from the DEAE Sepharose column with STK activity. The major forms of rSTK are shown.

Periplasmic rSTK forms were purified by a two-step procedure including anion exchange chromatography on DEAE Sepharose and hydrophobic chromatography on a phenyl-agarose column. The rSTK species eluted between 4.5 and 0% ammonium sulfate when a linear gradient (10%-0%) was applied to the latter column (Figure 2). The 47.5-kDa band corresponded to the native form of STK, as confirmed by NH-terminal sequencing. The 45-kDa band with Pg-activating activity is similar to an active degradation product of the mature protein, lacking 31 or 32 C-terminal amino acids, as previously reported by Müller et al. (8). This fragment retained the biological activity to almost the same extent as native STK, when assayed with a chromogenic substrate for plasmin (K_m = 0.70 µM and k_{cat} = 0.82 s^{-1} *vs* K_m = 0.77 µM and k_{cat} = 0.80 s^{-1}, respectively) (9). The N-terminus of this particular band was chemi-

cally or sterically "compromised" and could not be sequenced.

It has been demonstrated that *E. coli* is capable of recognizing and processing Gram-positive signal peptides (10) and that heterologous proteins are secreted by a defective mechanism which could be implicated in some deleterious effects such as the reduction of cell growth, possibly due to a saturation of the export pathway (8). This also explains the physical basis of the structural alterations observed in rSTK and in recombinant staphylokinase, another bacterial exoprotein with profibrinolytic activity (11).

In the present study the expression of rSTK was tightly repressed until cells reached maximal growth; the use of a plasmid containing a *tac* promoter allowed us to overexpress the recombinant protein once the cells attained the end of the exponential growth phase. By using this strategy, the problems resulting from a defective excretion mechanism could be overcome.

As already stated, some putative fragments arise when heterologous proteins are excreted into the periplasmic space in *E. coli*. The 32-kDa species is possibly the result of nonspecific proteolytic cleavage of the gene product during the secretion pathway and/or during the purification steps (6), even though such low molecular form of rSTK was observed in the presence of 1 mM phenylmethylsulfonyl fluoride and 1 mM ethylenediaminetetraacetic acid. This fragment forms a complex with the zymogen (data not shown) but did not activate human Pg. Recently, Rodríguez et al. (12) demonstrated that different regions of the STK molecule mediate different aspects of the STK-Pg interactions including STK-Pg complex formation, active site exposure and impairment of the inhibition of plasmin by α-2-

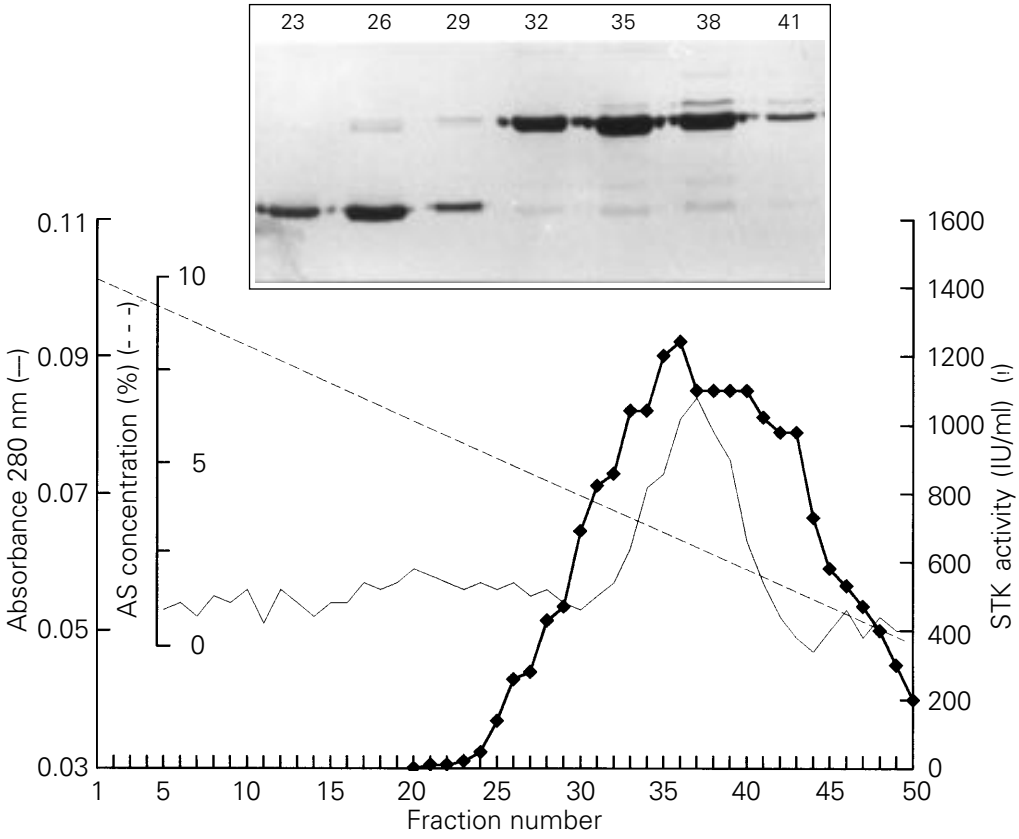

Figure 2 - Elution profile of rSTK produced in *E. coli* by phenyl-agarose column chromatography. The periplasmic protein fraction with streptokinase (STK) activity eluted from the DEAE Sepharose column was applied to a phenyl-agarose column equilibrated with 20 mM phosphate, and 10% ammonium sulfate, pH 7.0. Adsorbed proteins were eluted with a linear gradient of 10%-0% ammonium sulfate (AS) concentration in equilibration buffer. The fraction size was 1.3 ml per tube. *Full line,* Absorbance at 280 nm; *squares,* STK activity; *broken line,* ammonium sulfate concentration. Inset: Western blot analysis with an anti-STK polyclonal antiserum of selected fractions eluted from the phenyl-agarose column, showing the rSTK forms in each fraction.

antiplasmin. The results obtained in the present study add to those observations, showing that a putative fragment of 32 kDa is capable of binding to human plasminogen without activating it. Since structural modifications of proteins which participate in zymogen activation through the formation of an activation complex are of considerable interest for the understanding of structure-function relationships, it is clear that the biological significance of the 32-kDa fragment requires further investigation.

Acknowledgments

We thank D. Nieto, L. Romero and H. Arias for technical assistance, and Dr. M.H. Bueno da Costa and Dr. Marco Parada for helpful discussion.

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