0273-2289/98/6903—0147$10.50

Large-Scale Preparation of the Δ10 Form of Staphylokinase by In Vitro Processing of Recombinant Staphylokinase with Purified Human Plasminogen

DEBASISH CHATTOPADHYAY,* JERRY E. STEWART, AND LAWRENCE J. DELUCAS

Center for Macromolecular Crystallography, University of Alabama at Birmingham, 276 Basic Health Science Building, 1918 University Boulevard, Birmingham, AL 35294-0005

Received June 26, 1997; Accepted September 3, 1997

ABSTRACT

The authors have developed a rapid and convenient method for purification of a low molecular weight form (Δ10) of the bacterial plasminogen activator, staphylokinase. Recombinant staphylokinase is expressed in *Escherichia coli*, with an amino terminal extension that facilitated purification by immobilized metal-affinity chromatography. Purified staphylokinase is treated with human plasminogen, and the resulting truncated form is purified using a combination of immobilized metal affinity chromatography and hydrophobic interaction chromatography. Purified protein is characterized by amino terminal sequencing and in vitro plasminogen activation assay.

Index Entries: Plasminogen; plasminogen activator; staphylokinase; immobilized metal affinity chromatography; hydrophobic interaction chromatography; recombinant protein.

INTRODUCTION

Plasminogen activators are agents that initiate fibrinolysis by converting the inactive zymogen plasminogen to the active protease plasmin. Staphylokinase, a small protein produced by lysogenic *Staphylococcus aureus*, is a fibrin-specific plasminogen activator *(1)*. Streptokinase and tis-

sue-type plasminogen activator (t-PA) are currently used in thrombolytic therapy. The relative molar potency of staphylokinase in thrombolysis is about 2× higher than that of streptokinase and about half that of t-PA *(2)*.

Staphylokinase, like streptokinase, is not an enzyme, but it forms a complex with native plasminogen that subsequently activates other plasminogen molecules *(3,4)*. The mechanism proposed for the fibrin specificity of staphylokinase is as follows: In the absence of fibrin, the plasminogen–staphylokinase complex is rapidly neutralized by α_2-antiplasmin, and, as a result, the systemic plasminogen activation is reduced in a plasma milieu; and in the presence of fibrin, the lysine-binding sites of the plasminogen–staphylokinase complex are occupied, and inhibition by α_2-antiplasmin is delayed, thus allowing preferential plasminogen activation at the fibrin surface *(4)*.

The gene (sak) encoding staphylokinase was cloned from the *S. aureus* phages sak42D and sakϕC, as well as from the genomic DNA of a lysogenic strain of *S. aureus (5–7)*. The sak gene encodes for a polypeptide containing 163 amino acid residues. The signal peptide, composed of amino terminal 27 amino acid residues, is cleaved during the secretion of the mature protein, which consists of 136 amino acid residues *(8)*. Staphylokinase has been purified from a lysogenic *S. aureus* strain. It has been expressed in *Escherichia coli*, *S. aureus*, and *Bacillus subtilis (5–9)*. Mature form of staphylokinase has been overproduced in *E. coli* using recombinant DNA methods *(10)*.

During plasminogen activation, staphylokinase is converted to low molecular weight (LMW) forms. These forms, STA Δ6 and STA Δ10, are generated by removal of six and ten amino acid residues, respectively, from the amino terminus of mature staphylokinase. A schematic diagram of the different molecular forms of staphylokinase is shown in Fig. 1. Both truncated forms have the same fibrinolytic activity and similar plasminogen activating potential in vitro *(11)*. This paper describes a rapid and convenient method for purification of active STA Δ10 in milligram quantities.

MATERIALS AND METHODS

All reagents used were of analytical grade. Human plasminogen (Hpg) was purchased from Sigma (St. Louis, MO), Chelating Sepharose and butyl Sepharose were purchased from Pharmacia (Piscataway, NJ). DNA sequencing kit Sequenase was purchased from United States Biochemical (Cleveland, OH).

Expression of Full-Length Staphylokinase with Hexahistidine Tag

Coding sequence for mature staphylokinase was amplified using polymerase chain reaction (PCR) and the template pSAK1 plasmid, which

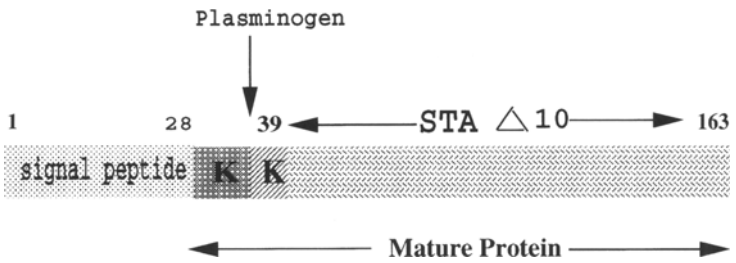

Fig. 1. A schematic diagram showing different molecular forms of staphylokinase.

contained the entire coding sequence. 5′ and 3′ PCR primers were designed to amplify the coding sequence for 136 amino acid residues, with an ATG start codon and an *Eco*RI and *Hind*III restriction site at the 5′ and 3′ ends of the amplified DNA, respectively. The amplified DNA after restriction digestion was cloned into the pKK223.3 vector (Pharmacia). The resulting plasmid pKST223.3 was transformed into *E. coli* JM109 for expression of a 137-amino-acid polypeptide that contains an extra methionine residue at the amino terminus. Since the level of expression was not satisfactory, the coding sequence for mature staphylokinase was subcloned into pQE-9 vector (Qiagen, Santa Clarita, CA). The resulting pQEST9 plasmid was transformed into competent *E. coli* DH5α(F′) cells, and transformants were selected on LB agar plates containing ampicillin (100 μg/mL). DNA from a positive clone was sequenced by dideoxy method using Sequenase DNA sequencing kit. The plasmid pQEST9 was transformed into competent *E. coli* M15[pREP4] cells, and transformants were selected on LB agar plates containing ampicillin (100 μg/mL) and kanamycin (25 μg/mL).

E. coli M15[pREP4] transformants containing pQST9 were grown in LB medium containing ampicillin (100 μg/mL) and kanamycin (25 μg/mL) at 37°C, until the OD (600 nm) of the culture reached 1.0. This culture was diluted 1:100 in fresh medium and grown at 37°C, with shaking at 300 rpm. When the OD (600 nm) of the culture reached 0.6, isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 1 m*M*, and the culture was grown for another 4 h post induction. Cells were harvested by centrifugation at 1860*g* for 15 min and stored at −80°C.

Purification of Recombinant Protein

E. coli cell pellet was suspended in 50 m*M* Tris-HCl buffer containing 100 m*M* NaCl, 1 m*M* phenylmethyl sulfonyl fluoride (PMSF), 1 m*M* benzamidine hydrochloride, and 1 μg/mL each of leupeptin and bestatin hydrochloride (pH 8.2). Cells were lysed with lysozyme (0.1 mg/mL final concentration) at 4°C for 30 min. The cell lysate was sonicated (2 cycles, 30 s each), followed by centrifugation at 104,630*g* for 30 min.

Recombinant protein was isolated from the soluble supernatant using immobilized metal affinity chromatography (IMAC). The supernatant was applied on the iminodiacetate column (2.5 × 10 cm) containing immobilized nickel, and equilibrated with lysis buffer. The column was washed with 50 m*M* Tris-HCl buffer (pH 8.2) containing 0.5 *M* NaCl, 1 m*M* PMSF, 1 m*M* benzamidine hydrochloride, and 35 m*M* imidazole. The recombinant protein (ST2) was eluted by the application of a 35–300 m*M* imidazole gradient (300 mL total volume; 50 m*M* Tris-HCl, 0.1 *M* NaCl, pH 8.2). Fractions of 5 mL were collected and analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Selected fractions were dialyzed against 0.1 *M* sodium phosphate buffer (pH 7.4).

In Vitro Plasminogen Activation Assay

The reaction mixtures containing varying concentrations of human plasminogen (0.09–1.84 μg), 0.1 *M* sodium phosphate (pH 7.4), and 24 μg of recombinant staphylokinase were incubated at 37°C for 1.5 h. The mixtures were boiled with 2X SDS sample denaturing buffer for 2 min and electrophoresed on 14% polyacrylamide gel containing 1% SDS.

In Vitro Treatment of Full-Length Staphylokinase with Human Plasminogen and Purification of the Truncated Form

Forty-three mg of recombinant mature form staphylokinase (ST2) in 0.1 *M* sodium phosphate buffer (pH 7.4) was incubated with 2.8 U human plasminogen (0.36 mg) for 2 h at 37°C. The mixture was cooled on ice, followed by the addition of equal volumes of 0.1 *M* sodium phosphate buffer (pH 8.0) and 0.2 *M* sodium chloride. The mixture was applied on an 8-mL nickel–iminodiacetate Sepharose column equilibrated with 0.1 *M* sodium phosphate and 0.1 *M* sodium chloride (pH 8.0). The column was washed with the same buffer until the absorbance (at 280 nm) of the flowthrough reached baseline. The column flowthrough and wash were pooled.

The collected protein was dialyzed against 0.1 *M* sodium phosphate buffer containing 1.5 *M* ammonium sulfate (pH 7.4). The dialyzate was chromatographed on a butyl Sepharose column (1.0 × 10 cm) equilibrated with the same buffer. After application of the sample, the column was washed with 10 column volumes of the equilibration buffer. The protein was then eluted with a descending gradient of ammonium sulfate from 1.5 to 0 *M* in 10 column volumes of equilibration buffer. Fractions of 5 mL were collected and analyzed by SDS-PAGE.

Amino Terminal Sequencing

The purified protein was subjected to amino terminal sequence analysis, using automated Edman degradation on a gas-phase microsequencing system Porton Model PI2090E (Beckman, Schaumburg, IL).

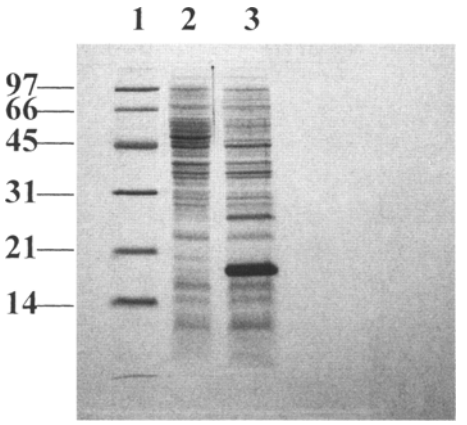

Fig. 2. Expression of ST2 in *E. coli*. Lane 1: Mol wt markers (molecular mass in kDa); lane 2: (−)ve control *E. coli* MI5[pREP4] cells with pQE-9; lane 3: *E. coli* MI5[pREP4] cells with pQEST9. Lanes 2 and 3 represent cells from 25 μL of bacterial culture boiled with 2× SDS sample denaturing buffer.

RESULTS AND DISCUSSION

Thrombotic complications of cardiovascular disease are one of the chief causes of death and disability. Thrombolytic therapy is becoming a routine treatment for patients with evolving myocardial infarction. Thrombolytic agents are plasminogen activators that convert the proenzyme, plasminogen, of the fibrinolytic system in blood to the active proteolytic enzyme plasmin. Plasmin dissolves the fibrin of a blood clot. At present, six thrombolytic agents are either approved for clinical use or under clinical trial *(12)*. Of these, the profibrinolytic agent, staphylokinase, forms a stoichiometric complex with plasminogen, which activates other plasminogen molecules *(4)*. Staphylokinase shows promise for fibrin-specific coronary arterial thrombolysis in patients with acute myocardial infarction *(12)*. Purification of staphylokinase in large quantity was not possible until recently *(10)*. In the present work, a construct of recombinant staphylokinase that was prepared can be easily purified using IMAC. The recombinant protein was expressed in *E. coli* as an intracellular protein with an amino terminal extension peptide MRGSHHHHHHSGSEP. Figure 2 shows an SDS-PAGE of the cells after induction with IPTG. When the soluble supernatent of the bacterial extract was applied to the chelating Sepharose column containing immobilized nickel, the recombinant ST2 bound to the column. Proteins that were weakly bound to the column were dissociated by washing with a buffer containing 35 m*M* imidazole. Recombinant ST2 was eluted from the column using a linear concentration gradient of imidazole. Fractions eluted between 0.1 and 0.2 *M* imidazole

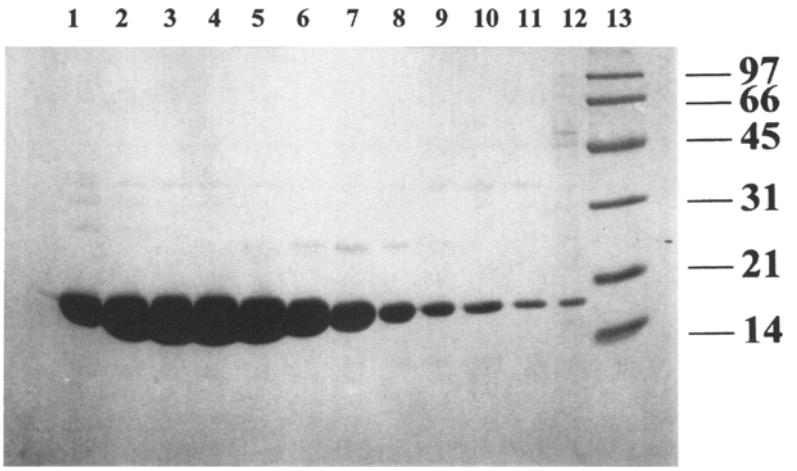

Fig. 3. SDS-PAGE of fractions from IMAC. Protein is eluted from the column using a linear concentration gradient of imidazole (35–300 m*M*). Lanes (from left) 1–11 (fractions 22–32): 10 μL of each fraction boiled with equal volumes of 2× sample denaturing buffer. Ten μL of resulting mixture was electrophoresed on 14% polyacrylamide gel containing 1% SDS. Lane 12: Soluble supernatent after cell lysis (1:5 dilution) prepared similarly. Lane 13: Mol wt marker (molecular mass in kDa).

showed the highest concentration of ST2 by SDS-PAGE (Fig. 3). Approximately 140 mg of nearly homogenous staphylokinase was obtained from 1 L of bacterial culture. Fractions were selected based on highest purity as judged by SDS-PAGE. The SDS-PAGE pattern of representative fractions (22–32) eluted with the imidazole gradient is shown in Fig. 3.

Useful conditions for plasminogen treatment were determined in the small scale. Experiments showed that 0.18 μg plasminogen was sufficient to cleave 25 μg purified staphylokinase at 37°C in 1.5 h (data not shown). The cleavage was scaled up. In a typical cleavage experiment, 25–40 mg of recombinant staphylokinase was converted to the LMW form in 2 h at 37°C. The resulting mixture, followed by adjustment of pH and ionic strength, was passed through a chelating Sepharose column charged with nickel. In this step, any uncleaved ST2 was bound to the column; the cleaved LMW form was obtained in the flowthrough and wash. The flowthrough and wash fractions were pooled, dialyzed against 0.1 *M* sodium phosphate (pH 7.4) containing 1.5 *M* ammonium sulfate, and subjected to hydrophobic interaction chromatography on butyl Sepharose column. LMW staphylokinase bound fairly strongly to the butyl Sepharose column, and was eluted with a descending gradient of ammonium sulfate, and is shown as a single protein peak in Fig. 4. Fractions from the butyl Sepharose column, when analyzed by SDS-PAGE, revealed that homoge-

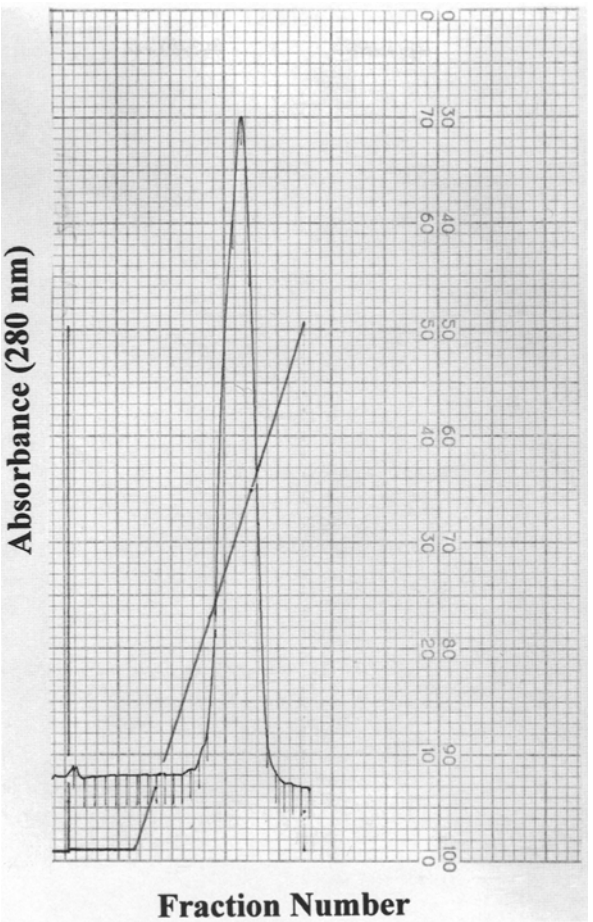

Fig. 4. Chromatograph showing elution of STAΔ10 from butyl Sepharose column. Protein from the column is eluted with a descending gradient of ammonium sulfate concentration (1.5–0 *M*).

nous LMW staphylokinase eluted at lower ionic strength. A sample of protein from the pooled fractions as analyzed by amino terminal sequence analysis. The amino terminal sequence was found to be KGDDASYFEP, which represents the STA Δ10 form of staphylokinase. Thus, plasminogen cleaved staphylokinase at the lysine (38)–lysine (39) peptide bond. Table 1 describes the relative yields of staphylokinase at various stages of purification. The purified Δ10 form of protein exhibited plasminogen activation in vitro. Figure 5 shows the SDS-PAGE of a sample of human plasminogen before and after treatment with the STA Δ10 form of staphylokinase (lanes 2 and 3, respectively). The untreated plasminogen is a single-chain polypeptide that is cleaved into two polypeptide chains, which dissociated under the reducing condition.

Table 1
Summary of Purification Steps for Recombinant STAΔ10:
Yield of Protein at Various Stages of Purification

Step	Starting with	Yield
IMAC	1 L bacterial culture	140 mg
Plasminogen activation		
Ni-chelating Sepharose	49 mg	23 mg
Butyl Sepharose	23 mg	22 mg

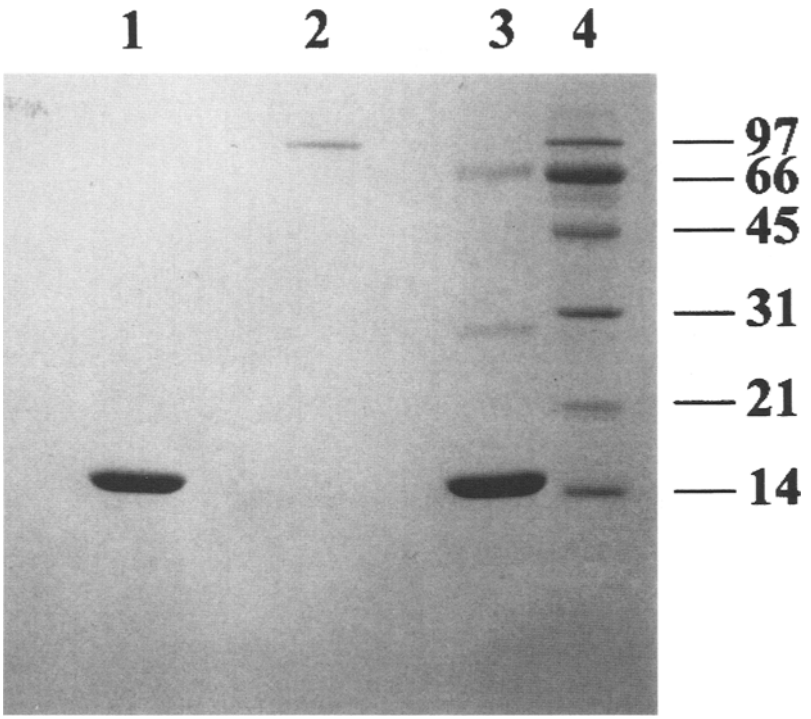

Fig. 5. Plasminogen activation assay using STAΔ10. Plasminogen is incubated with purified STAΔ10 at 37°C for 1.5 h. Five-μL aliquots from the reaction mixture were electrophoresed on 14% polyacrylamide gel containing 1% SDS. Lane 1: STAΔ10; lane 2: human plasminogen (Hpg); lane 3: Hpg treated with STAΔ10; lane 4: mol wt standard (molecular mass in kDa).

Profibrinolytic activity of staphylokinase has been known since the 1940s *(1)*. Staphylokinase possesses higher thrombolytic efficiency than the currently used bacterial plasminogen activator streptokinase *(2)*. However, proper evaluation of its thrombolytic potential has been elusive, because of its low expression levels and secretion of contaminant exotox-

ins. Methods for large-scale production of recombinant protein have been developed recently *(10)*, and recombinant staphylokinase variants have been studied *(13,14)*. An LMW form of staphylokinase which lacks 10 amino acid residues of the amino terminal of native protein, has been identified as a minimum unit with fibrinolytic activity in vivo *(12)*. Currently used bacterial plasminogen activator, streptokinase, suffers from two shortcomings; its limited efficacy for coronary recanalization; and its immunogenicity. The advantages of staphylokinase include a higher efficacy toward platelet-rich clots and a relatively low cost. Proper evaluation of the thrombolytic potential of staphylokinase will require the availability of large quantities of proteins of high purity, systematic structure–function analysis, and thorough clinical investigation. The method described in this paper is useful for preparing large quantities of the STAΔ10 form of staphylokinase in a highly purified form. STAΔ10 protein purified by this method possesses an authentic N-terminus created by the action of human plasminogen.

ACKNOWLEDGMENTS

Plasmid vector pSAK1, containing the entire coding sequence for staphylokinase, was purchased from ATCC. The authors thank Jill K. Moore for DNA sequencing. D. C. thanks the American Heart Association for a grant-in-aid (AL-G-960012). Part of this work was supported by NASA Cooperative Agreement NCC8-126.

REFERENCES

1. Lack, C. H. (1948), *Nature* **161,** 559, 560.
2. Robbins, K. (1995), *Thrombolytic Therapy for Peripheral Vascular Disease* Comerota, A. J. ed., J. B. Lippincott, Philadelphia, PA.
3. Collen, D., Van Hoef, B., Schlott, B., Hartman, M., Guhrs, K. S., and Lijnen, R. (1993), *Eur. J. Biochem.* **216,** 307–314.
4. Lijnen, H. R., Van Hoef, B., DeCock, F., Okada, K., Ueshima, S., Matsuo, O., and Collen, D. (1991), *J. Biol. Chem.* **266,** 11,826–11,832.
5. Sako, T., Sawaki, S., Sakurai, T., Ito, S., Yoshizawa, Y., and Kondo, I. (1983), *Mol. Gen. Genetics* **190,** 271–277.
6. Behnke, D. and Gerlach, D. (1987), *Mol. Gen. Genetics* **210,** 528–534.
7. Collen, D., Silence, K., Demarsin, E., De Mol, M., and Lijnen, H. R. (1992), *Fibrinolysis* **6,** 203–213.
8. Sako, T. and Tsuchida, N. (1983), *Nucleic Acid Res.* **11,** 7679–7693.
9. Sako, T. (1985), *Eur. J. Biochem.* **149,** 557–563.
10. Schlott, B., Hartmann, M., Guhrs, K., Birch-Hirschfeid, E., Pohl, H., Vanderschueren, S., Van de Werf, F., Michoel, A., Collen, D., and Behnke, D. (1994), *Bio/Technology* **12,** 185–189.

11. Ueshima, S., Silence, K., Collen, D., and Lijnen, H. R. (1993), *Thromb. Haemostasis* **70,** 495–499.
12. Collen, D. and Lignene, H. R. (1994), *Blood* **84,** 680–686.
13. Collen, D., Stockx, L., Lacroix, H., Suy, R., and Vanderschueren, S. (1997), *Circulation* **95,** 463–472.
14. Collen, D., Moreau, H., Stockx, L., and Vanderschueren, S. (1996), *Circulation* **94,** 207–216.