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Research review paper

# Streptokinase—a clinically useful thrombolytic agent

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## Abstract

A failure of hemostasis and consequent formation of blood clots in the circulatory system can produce severe outcomes such as stroke and myocardial infarction. Pathological development of blood clots requires clinical intervention with fibrinolytic agents such as urokinase, tissue plasminogen activator and streptokinase. This review deals with streptokinase as a clinically important and cost-effective plasminogen activator. The aspects discussed include: the mode of action; the structure and structure–function relationships; the structural modifications for improving functionality; recombinant streptokinase; microbial production; and recovery of this protein from crude broths.

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## 1. Introduction

A blood clot (thrombus) developed in the circulatory system can cause vascular blockage leading to serious consequences including death. A healthy hemostatic system suppresses the development of blood clots in normal circulation, but reacts extensively in the event of vascular injury to prevent blood loss. Outcomes of a failed hemostasis include stroke, pulmonary embolism, deep vein thrombosis and acute myocardial infarction.

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Pathologies involving a failure of hemostasis and the development of clot require clinical intervention consisting of intravenous administration of thrombolytic agents (Collen et al., 1988; Collen, 1990; Francis and Marder, 1991). Streptokinase is one such agent. Other thrombolytic or fibrinolytic agents include urokinase and the tissue type plasminogen activator (tPA). This review outlines the process of clot dissolution (i.e., thrombolysis or fibrinolysis) and the various thrombolytic agents available to achieve it. One clinically important thrombolytic agent, streptokinase, and its production from microorganisms are discussed in detail.

Numerous trials have been conducted to compare the clinical efficacy of recombinant tPA and streptokinase. Generally, these investigations have not revealed a clear preference for either drug. Streptokinase is as effective as recombinant tPA in treating acute myocardial infarction (Sherry and Marder, 1991), and it is certainly more cost-effective; however, its use is not risk free. In view of the relatively recent availability of the competing recombinant tPA, skepticism is being expressed about the continued viability of streptokinase therapy (Sane and Little, 1998; Werf, 1999). Despite this, research on streptokinase continues, and it remains a vital affordable therapy specially in the world's poorer healthcare systems.

## 2. Thrombolysis or fibrinolysis

The physiology of the fibrin–clot formation is relatively well understood (Paoletti and Sherry, 1977; Wu and Thiagarajan, 1996). A blood clot or thrombus consists of blood cells occluded in a matrix of the protein fibrin. Enzyme-mediated dissolution of the fibrin clot is known as thrombolysis or fibrinolysis. In mammalian circulation, the enzyme responsible for fibrinolysis is plasmin, a trypsin-like serine protease (Castellino, 1981; Rouf et al., 1996). The fibrinolytically active plasmin is produced from the inactive protein plasminogen which is present in circulation. Conversion of the inactive plasminogen to fibrinolytic plasmin involves a limited proteolytic cleavage which is mediated by the various plasminogen activators (Castellino, 1984). Two plasminogen activators that occur naturally in blood are the tissue type (tPA) and the urokinase type (uPA). The fibrinolytic activity in circulation is modulated by inhibitors of plasminogen activators (e.g., plasminogen activator inhibitor-1, PAI-1, a fast-acting inhibitor of tPA and uPA) and plasmin (e.g.,  $\alpha_1$ -antiplasmin,  $\alpha_2$  macroglobulin) (Francis and Marder, 1991). The cascade of reactions leading to dissolution of fibrin clot is schematically illustrated in Fig. 1.

Recombinant forms of normal human plasminogen activators tPA and uPA are used in clinical intervention. Another commonly used plasminogen activator is streptokinase (sPA), a bacterial protein that does not occur naturally in human circulation. Streptokinase, tPA and uPA do not have a direct fibrinolytic activity and their therapeutic action is via the activation of blood plasminogen to the clot dissolving plasmin (Fig. 1). Unlike tPA and uPA, which are proteases, streptokinase possesses no enzymatic activity of its own (Castellino, 1981). Streptokinase acquires its plasminogen activating property by complexing with circulatory plasminogen or plasmin. The resulting high-affinity 1:1 stoichiometric complex (i.e., the streptokinase–plasminogen activator complex) is a

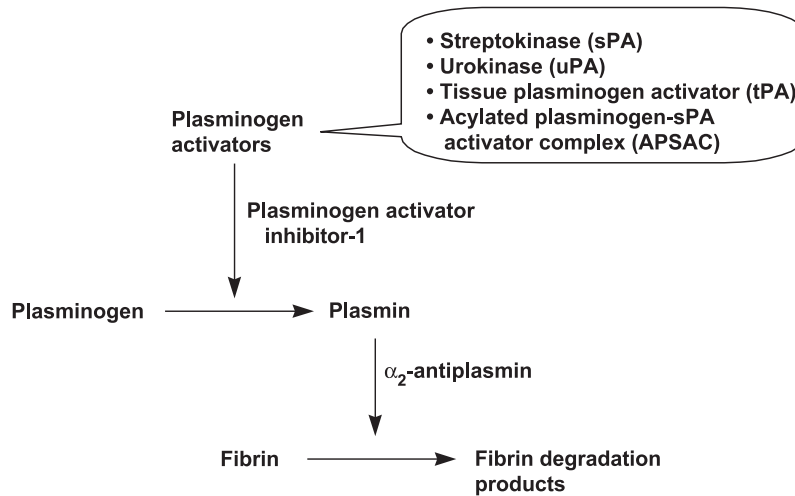


Fig. 1. Schematic representation of fibrinolysis.

high-specificity protease that proteolytically activates other plasminogen molecules to plasmin (Bajaj and Castellino, 1977; Castellino, 1981). Thus, the plasminogen activating action of streptokinase is fundamentally different from the proteolytic activation brought about by tPA and uPA.

### 3. Comparing plasminogen activators

Both tPA and uPA are trypsin-like serine proteases which activate plasminogen directly. They have the respective molecular weights of 70 and 55 kDa. Both tPA and uPA are glycoproteins. uPA is produced by the kidneys and secreted into the urine (Barlow, 1976; Paoletti and Sherry, 1977), whereas tPA is produced by the vascular endothelial cells (Camiolo et al., 1971; Strassburger et al., 1983).

tPA for therapeutic use is obtained mainly from cultures of recombinant animal cells (Rouf et al., 1996). Because of its high affinity for the fibrin clot, tPA activates the clot-bound plasminogen 100-fold more effectively than the circulatory plasminogen. Consequently, tPA is a poor activator of circulatory plasminogen relative to the other available plasminogen activators.

Of the three major available thrombolytic agents (i.e., tPA, uPA, sPA), the microbial sourced streptokinase is the least expensive, but immunogenic. Human tPA and uPA are immunogenically benign, but are short-lived in vivo and therefore of a limited therapeutic effectiveness. Comparative clinical trials and cost-effectiveness considerations suggest that streptokinase is the drug of choice for thrombolytic therapy (Mucklow, 1995; Gillis and Goa, 1996), but this is debatable (Rouf et al., 1996; Sane and Little, 1998; Werf, 1999). Streptokinase is a nonhuman protein, and its introduction into the circulatory systems can illicit severe anaphylactic response, including death (Lee, 1995; Jennings, 1996). The risk

of this immune response is dependent on the level of the anti-streptokinase antibodies present in circulation. This immunogenicity restricts multiple treatments with streptokinase (Bick, 1982; Collen, 1990; Lee, 1995; Jennings, 1996).

The plasmin produced through the streptokinase mediated activation of plasminogen breaks down streptokinase. This limits the in vivo half-life of streptokinase to about 30 min. Although streptokinase survives in circulation significantly longer than does tPA (a half-life of about 5 min), this is still short for efficient therapy (Wu et al., 1998).

Unlike microbial streptokinase, human urokinase is not antigenic or pyrogenic, but its recovery from urine is expensive and supply is limited for any extensive use in therapy (Rouf et al., 1996). Like streptokinase, urokinase activates both the circulating plasminogen and the clot-bound plasminogen. Because plasminogen activation by uPA and streptokinase is not specific to the clot-bound plasminogen, the use of these activators is associated with a serious risk of hemorrhage (Rouf et al., 1996).

Although streptokinase is the best known microbial plasminogen activator, it is not the only one. Staphylokinase (SAK) sourced from *Staphylococcus* sp. is a potential alternative plasminogen activator (Lack, 1948; Matsuo et al., 1990; Collen et al., 1992; Schlott et al., 1994; Okada et al., 2001); 1991a. The structure and mechanism of action of SAK are becoming better understood (Matsuo et al., 1990; Lijnen et al., 1991a; Okada et al., 2001). Recombinant staphylokinase has been produced in bacteria such as *Escherichia coli* (Sako, 1985; Collen et al., 1992; Schlott et al., 1994) and shown to induce fibrin specific clot lysis in human plasma milieu in vitro (Matsuo et al., 1990; Lijnen et al., 1991a). Attempts have been made to compare the fibrinolytic properties of staphylokinase and streptokinase using animal models of venous thrombosis (Lijnen et al., 1991b) and other methods (Collen et al., 1992).

Streptokinase and, to a lesser extent, staphylokinase are the best investigated fibrinolytic proteins of microbial origin, but there are others. Fibrinolytic proteins have been isolated from other species including microfungi (Bärwald et al., 1974) and anticoagulant proteins of potential therapeutic value occur in higher animals such as leeches, mosquitoes, snakes and bats.

Another activator in clinical use is the acylated plasminogen–streptokinase activator complex, APSAC, in which human plasminogen with an acylated active site has been complexed with bacterial streptokinase (Marder et al., 1983). The APSAC has an extended therapeutically effective half-life in circulation relative to streptokinase (Smith et al., 1981).

Because of the recognized shortcomings of the available plasminogen activators, attempts are underway to develop improved recombinant variants of these compounds (Nicolini et al., 1992; Adams et al., 1991; Lijnen et al., 1991a; Marder, 1993; Wu et al., 1998). Despite significant limitations, streptokinase remains the drug of choice particularly in the poorer economies because of its low relative cost (e.g., US\$200 per treatment).

#### 4. Streptokinase

The extracellular enzyme streptokinase (EC 3.4.99.22) is produced by various strains of  $\beta$ -hemolytic streptococci. The enzyme is a single-chain polypeptide that exerts its

fibrinolytic action indirectly by activating the circulatory plasminogen. The complete amino acid sequence of streptokinase was first established by Jackson and Tang (1982). Streptokinase has a molar mass of 47 kDa and is made up of 414 amino acid residues (Malke and Ferretti, 1984). The protein exhibits its maximum activity at a pH of approximately 7.5 and its isoelectric pH is 4.7 (De Renzo et al., 1967; Taylor and Botts, 1968; Brockway and Castellino, 1974). The protein does not contain cystine, cysteine, phosphorous, conjugated carbohydrates and lipids. Other physical and chemical data on streptokinase have been reported by De Renzo et al. (1967) and Taylor and Botts (1968). Streptokinases produced by different groups of streptococci differ considerably in structure (Huang et al., 1989; Malke, 1993).

#### *4.1. Structure of streptokinase and its mechanism of action*

A considerable degree of heterogeneity exists among the streptokinases produced by different groups of streptococci. Biophysical techniques such as circular dichroism, nuclear magnetic resonance (NMR) spectroscopy, Fourier transform infrared (FT-IR) spectroscopy and differential scanning calorimetry (DSC) have provided most of the available structural information on streptokinase (Radek and Castellino, 1989; Fabian et al., 1992; Misselwitz et al., 1992; Welfle et al., 1992; Teuten et al., 1993; Conejero-Lara et al., 1996; Parrado et al., 1996; Beldarrain et al., 2001). Studies on fragments of streptokinase have also yielded valuable information (Brockway and Castellino, 1974; Markus et al., 1976; Misselwitz et al., 1992; Siefring and Castellino, 1976; Rodriguez et al., 1994; Shi et al., 1994; Reed et al., 1995; Young et al., 1995; Kim et al., 1996).

Streptokinase consists of multiple structural domains (i.e.,  $\alpha$ -,  $\beta$ - and  $\gamma$ -domains) with different associated functional properties. Scanning calorimetric analysis suggests that the protein is composed of two distinct domains (Welfle et al., 1992). The N-terminal domain (i.e., residues 1–59) has been found to complement the low plasminogen activation ability of the 60–414 amino acid residue domain of the protein (Nihalani et al., 1998).

Recently, the crystal structure of streptokinase complexed with human plasmin light chain has been studied (Wang et al., 1998). Similarly, the crystal structure of streptokinase beta domain has been discussed (Wang et al., 1999b). Thermal denaturation of streptokinase has been discussed (Azuaga et al., 2002) and heat stability of specific domains of the enzyme has been studied (Conejero-Lara et al., 1996).

How streptokinase activates plasminogen has been the focus of extensive research (Bajaj and Castellino, 1977; Castellino, 1981; Nihalani and Sahni, 1995; Kim et al., 1996, 2000, 2002; Young et al., 1998; Reed et al., 1998; Robinson et al., 2000; Wu et al., 2001; Wakeham et al., 2002; Sundram et al., 2003; Zhai et al., 2003); nevertheless, mechanisms of activation of plasminogen by streptokinase are still being elucidated (Boxrud and Bock, 2000; Boxrud et al., 2001).

Streptokinase is known to activate plasminogen both by fibrin-dependent and fibrin-independent mechanisms (Reed et al., 1998). Streptokinase interacts with plasminogen through multiple domains. At least two independent plasminogen binding sites of streptokinase had been identified by 1995 (Nihalani and Sahni, 1995). The C-terminal domain of streptokinase is involved in plasminogen substrate recognition and activation

(Kim et al., 1996, 2002; Zhai et al., 2003). Similarly, the Asp41–His48 region of streptokinase is important in binding to the substrate plasminogen (Kim et al., 2000). The role of an adjacent region, i.e., residues 48–59, on plasminogen activation has been discussed (Wakeham et al., 2002). The coiled region of the streptokinase  $\gamma$ -domain is said to be essential for plasminogen activation (Wu et al., 2001). Similarly, the streptokinase  $\beta$ -domain is involved in forming the streptokinase–plasminogen complex that is responsible for activating the plasminogen (Robinson et al., 2000).

Streptokinase binds preferentially to the extended conformation of plasminogen through the lysine binding site to trigger conformational activation of plasminogen (Boxrud and Bock, 2000; Boxrud et al., 2001). Streptokinase–plasminogen activator complex interacts with plasminogen through long range protein–protein interactions to maximize catalytic turnover (Sundram et al., 2003). The first 59 amino acid residues seem to have multiple functional roles in streptokinase (Shi et al., 1994; Young et al., 1995). Without these N-terminal residues, streptokinase has an unstable secondary structure. Loss of residues 1–59 greatly reduces the activity of the remaining streptokinase fragment (i.e., residues 60–414) (Young et al., 1995). Species-specific plasminogen activation has been demonstrated using 56 isolates of the pathogenic group C streptococci (McCoy et al., 1991).

#### 4.2. *Enhancing streptokinase*

Immunogenicity of streptokinase and its relatively short half-life in circulation limit therapeutic potential of this protein. Streptokinase in circulation is proteolytically degraded by plasmin. Consequently, research has focussed on structurally modifying streptokinase to extend half-life, reduce or eliminate immunogenicity, and improve plasminogen activation. Any structural change needs to be informed by a thorough structure–function analysis of streptokinase domains and this has been the subject of extensive investigation (Reed et al., 1995; Fay and Bokka, 1995, 1998; Medved et al., 1996; Lee et al., 1997b; Wang et al., 1999a,c; Torrens et al., 1999a,b; Robinson et al., 2000; Kim et al., 2000, 2002; Wakeham et al., 2002; Sundram et al., 2003; Zhai et al., 2003). Structurally modified streptokinases have been produced in several ways including genetic mutation, recombinant DNA technology and chemical or enzymatic modification of the native streptokinase.

Mutant streptokinase with improved stability have been prepared (Shi et al., 1998). Two of the major sites of the proteolytic action of plasmin on streptokinase are Lys59 and Lys386 (Wu et al., 1998). This knowledge has been used to engineer variants of streptokinase that are resistant to plasmin (Wu et al., 1998) and, therefore, have a longer functional half-life. The plasmin-resistant forms of streptokinase have been found to be as active as the native streptokinase. Recombinant streptokinase produced in the yeast *Pichia pastoris* is glycosylated, and this appears to enhance its resistance to proteolysis (Pratap et al., 1996). Following a similar path, attempts have been made to extend the half-life of native nonglycosylated streptokinase by complexing it with polymers such as polyethylene glycol (PEG) (Koide et al., 1982; Rajagopalan et al., 1985). Plasmin-resistant, long-life variants of protein-engineered streptokinase have been produced in a protease-deficient recombinant *Bacillus subtilis* WB600 (Wu et al., 1998). It appears that the streptokinase

domains responsible for activity, stability and immunogenicity have considerable overlap. Therefore, a future therapeutically optimal streptokinase will not be necessarily the most active nor the longest lived.

Immunogenicity of streptokinase became known soon after its discovery in the 1930s (Tillett and Garner, 1933). The early studies showed that blood from patients with recent streptococcal infection inactivated streptokinase because of the presence neutralizing antibodies. Because streptococcal infections are common, a detectable level of antibodies against streptokinase occurs in most populations (Ojalvo et al., 1999a; Kazmi et al., 2002). Many different anti-streptokinase platelet-activating antibodies are known to exist and occur widely (Regnault et al., 2003).

The different domains of streptokinase differ in immunogenicity (Reed et al., 1993). Extensive work has been reported on the identification of antigenic regions of streptokinases (ParhamiSeren et al., 1996, 1997; Ojalvo et al., 1999b; Torrens et al., 1999a,b; Coffey et al., 2001). New antigenic domains were identified as recently as 2001 (Coffey et al., 2001).

Recombinant streptokinases with reduced immunogenicity have been produced (Ojalvo et al., 1999b). A mutant streptokinase that lacked the C-terminal 42 amino acids was found to be less immunogenic than the native molecule (Torrens et al., 1999b). One chemical modification has involved complexing streptokinase with PEG (Koide et al., 1982; Rajagopalan et al., 1985; Pautov et al., 1990), primarily for reducing immunogenicity.

Streptokinase variants with one or more of the normal amino acids residues replaced by others have been prepared in attempts to enhance plasminogen activation (Wu and Thiagarajan, 1996). Some of the modified variants displayed an enhanced stability. The preferred variant had Lys59 replaced with a glutamic acid residue. Streptokinase derivatives having platelet glycoprotein-binding domains have been reported (Galler, 2000). These derivatives produced higher local concentrations of plasmin in vivo when compared to unmodified streptokinase.

## 5. Production of streptokinase

### 5.1. Producing microorganisms

Streptokinase producing streptococci were first identified in 1874 by Billroth in exudates of infected wounds. Later, the blood of scarlet fever patients was shown to contain similar microorganisms. By 1919, *Streptococcus* sp. had been classified into alpha, beta and gamma variants based on the distinct types of hemolytic reactions the variants produced on blood agar plates.

In 1933, Lancefield used serologic distinctions to further differentiate the  $\beta$ -hemolytic streptococci into groups A to O (Lancefield, 1933). Most of the streptokinases are obtained from  $\beta$ -hemolytic streptococci of the Lancefield groups A, C and G. The group C are preferred for producing streptokinase as they lack erythrogenic toxins. The group C strain *Streptococcus equisimilis* H46A (ATCC 12449) isolated from a human source in 1945 has been widely used for producing streptokinase. The strain H46A was selected

from more than a hundred fibrinolytic isolates, because it yielded the most active streptokinase.

*S. equisimilis* H46A does not produce erythrogenic toxins and is less fastidious in its growth requirements than the majority of group A strains (Christensen, 1945). The H46A isolate can be grown on semisynthetic media to secrete large quantities of streptokinase (Christensen, 1945; Feldman, 1974). The H46A is also the main source of the streptokinase gene that has been expressed in various other microorganisms (Malke et al., 1985; Hagenson et al., 1989; Wong et al., 1994), as discussed elsewhere in this review.

#### 5.1.1. Recombinant producers

The streptokinase gene from *S. equisimilis* H46 A was sequenced by Malke et al. (1985). The transcriptional control of this gene has been studied (Gase et al., 1995) and the functional analysis of its complex promoter has been reported (Grafe et al., 1996). Considerable information exists, therefore, for effectively using this gene in producing streptokinase safely in nonpathogenic bacteria.

Studies of the streptokinase gene isolated from various sources suggest it to be polymorphic (Malke, 1993). The streptokinase gene (*skc*) cloned from *S. equisimilis* H46A has been expressed in several gram positive and gram negative bacteria including *B. subtilis* WB600 (Wong et al., 1994; Wu et al., 1998) and *E. coli* (Malke et al., 1984, 1985; Malke and Ferretti, 1984; Muller et al., 1989; Muller and Malke, 1990; Collen et al., 1992; Estrada et al., 1992; Ko et al., 1995; Narciandi et al., 1996; Avilan et al., 1997; Lee et al., 1997a; Perez et al., 1998; Pratap and Dikshit, 1998; Yazdani and Mukherjee, 1998; Zhang et al., 1999; Pupo et al., 1999; Yazdani and Mukherjee, 2002). Production in the yeast *Pichia* has been reported (Hagenson et al., 1989; Estrada et al., 1992; Pratap et al., 2000).

Plasmids bearing the *skc* and erythromycin resistance genes have been introduced into *S. equisimilis* H46A in attempts to select overproducer clones (Muller and Malke, 1990). Plasmids designed for high-level secretory expression of streptokinase have been successfully evaluated for producing it (Ko et al., 1995). An ability to produce recombinant streptokinase greatly enhances the possibilities for beneficial structural modifications of this protein and enhanced production of the desired recombinant streptokinase.

#### 5.2. Streptokinase fermentation

Group A hemolytic streptococci commonly require complex and rich media supplemented with various nutritional factors for growth (Bernheimer et al., 1942). A modification of the medium originally developed by Bernheimer et al. (1942) was used by Christensen (1945) for producing streptokinase from *S. equisimilis* H46A. This medium contained peptone, phosphate salts, glucose, biotin, riboflavin, tryptophan, glutamine and nucleic acids (thiamine, adenine and uracil). The glutamine content were only 25% of that in the original medium of Bernheimer et al. (1942). A low glucose concentration at inoculation allowed growth to become well established without excessive generation of acid. Further glucose was added after the initial overnight incubation. Cells proliferated to a high density. Alkali (5 M sodium hydroxide) was added at regular intervals to maintain a

neutral pH. In the absence of pH control, both the biomass growth and streptokinase production were adversely affected.

Batch cultures of streptococci have been characterized as having a rather extended lag phase followed by a relatively short period of exponential growth. Comparison with continuous culture has revealed a lower productivity of the equivalent batch fermentation. Studies on glucose and tryptophan limitation in continuous cultures of *Streptococcus faecalis* concluded that maximum biomass yield per unit energy source was attained when the energy source (glucose) was limiting (Rosenberger and Elsdén, 1960). In contrast, the production of streptokinase was maximized when glucose was in excess and the other nutrients were present in limiting amounts (Rosenberger and Elsdén, 1960).

Addition of polyoxyethylene sorbitan mono-oleate at a concentration of 0.01–0.10% to the medium prior to inoculation has been recommended for achieving high titers of streptokinase. Effect of amino acid feeding on steady-state continuous cultures of group A streptococci was reported by Davis et al. (1965). The culture had a dilution rate of  $0.5 \text{ h}^{-1}$  at a constant pH value of 7.5. Of the 14 amino acids tested, all except alanine could limit growth.

A study with continuous cultures of a group C  $\beta$ -hemolytic *Streptococcus* strain H46 assessed the biomass and streptokinase production at various dilution rates, pHs and temperatures in a complex medium with excess glucose (Holmstrom, 1965). At pH 7.0, the yield of biomass and streptokinase on glucose did not vary with the dilution rate; however, the biomass and streptokinase productivities increased with increasing dilution rate in the range of  $0.1$ – $0.5 \text{ h}^{-1}$ . Continuous culture had more than twice the streptokinase productivity of batch culture.

In a study of 19 strains of group E streptococci (SGE), 18 of the isolates were found to produce streptokinase in an enriched tryptose broth medium (Ellis and Armstrong, 1971). This medium contained tryptose, glucose, sodium chloride, uracil, adenine, glutamine, tryptophan, vitamins and salts. A medium containing corn steep liquor, cerelose,  $\text{KH}_2\text{PO}_4$  and  $\text{KHCO}_3$ , pH 7.0, was used by Feldman (1974) for producing streptokinase. Use of corn steep liquor instead of casein hydrolyzate enhanced streptokinase yield in cultures of the strain H46A. Baewald et al. (1975) used a simple and inexpensive medium to obtain high yields of streptokinase from *S. equisimilis*. The medium contained yeast autolyzate or corn steep liquor as the nitrogen source, glucose, and various salts. High titers of streptokinase were attained at  $28 \text{ }^\circ\text{C}$ , pH 7.2–7.4, within 24 h in agitated cultures.

A commonly used complex medium for producing streptokinase is the brain–heart infusion (BHI) medium. This medium is produced using bovine brain and heart tissue. Malke and Ferretti (1984) obtained good proliferation of *S. equisimilis* H46A on the BHI medium. The same medium was used by Suh et al. (1984) to produce streptokinase from a group C *Streptococcus* isolated from a human patient. The maximum activity of streptokinase was detected during exponential growth. A glucose and casein hydrolyzate medium supplemented with various salts was reported by Nemirovich-Danchenko et al. (1985) for producing streptokinase from *S. equisimilis*.

Chemically defined media (CDM) for growing group A streptococci have been developed to require only small inocula and without the need for a prior adaptation

regimen (McCoy et al., 1991). The doubling times of the streptococci in such media can be comparable to those in complex media. The group A streptococci grown in defined media can produce streptokinase and other secreted proteins (McCoy et al., 1991).

Influence of the cultivation temperature on growth and streptokinase production of *S. equisimilis* H46A has been investigated over the temperature range of 28–43 °C (Ozegowski et al., 1983). The H46A strain was capable of growth over the entire temperature range, but 28 °C was the optimal growth temperature. The amount of streptokinase produced per unit biomass concentration depended on the cultivation temperature.

Relatively recently, work has focused on elucidating the fermentation conditions for producing streptokinase from mutants of the wild-type streptococci and other genetically engineered microorganisms. Hyun et al. (1997) produced copious quantities of streptokinase using a mutant *Streptococcus*. The culture medium consisted of casein or serratio peptidase hydrolyzed casein, glutamine, cysteine, and yeast extract. The mutant was cultured at pH 6.8–7.2, 35–38 °C, in broth aerated at 0.1–1.0 vvm. The titer of streptokinase exceeded 8500 units per milliliter.

The *S. equisimilis* streptokinase gene expressed in *E. coli* has led to a 10-fold greater streptokinase titer than values obtained in cultures of group C streptococci such as *S. equisimilis* (Estrada et al., 1992). Work has been reported on localizing the core promoter region of the streptokinase gene, *skn* (Malke et al., 2000). The plasmid pSK100 has enabled high level secretory expression of streptokinase in *E. coli* cultured in ampicillin-containing LB medium. Expression titers of about 5000 units per milliliter have been attained (Ko et al., 1995). The LB medium consists of Bacto-tryptone, yeast extract, sodium chloride, and 50 µg·ml<sup>-1</sup> ampicillin at pH 7.3. Other media have been described for producing recombinant streptokinase in *E. coli* (Narciandi et al., 1996).

A recombinant streptokinase was produced in *E. coli* using the LB medium at 37 °C (Lee et al., 1997a). The plasmid used imparted ampicillin resistance to the bacterium and ampicillin provided the selection pressure for plasmid retention. The production of streptokinase was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the medium. The plasmid encoded a streptokinase that lacked the 13 N-terminal amino acid residues of the normal protein. This enhanced productivity of the recombinant protein and enabled secretion into the extracellular medium (Lee et al., 1997a,b). At least a part of the N-terminal domain is known to be functionally relevant in streptokinase (Young et al., 1995; Nihalani et al., 1998; Azuaga et al., 1999; Mundada et al., 2003), but this may not include the first 13 residues (Lee et al., 1997b). Other reports have also described IPTG-induced production of recombinant streptokinase (Yazdani and Mukherjee, 1998, 2002). The specific productivity of the recombinant protein was substantially enhanced by fed-batch cultivation compared to batch fermentations of IPTG-induced *E. coli* (Yazdani and Mukherjee, 1998). Some work on kinetic analysis and modeling of streptokinase fermentation has been reported (Stuebner et al., 1991).

### 5.3. Assaying streptokinase

Characterization of the streptokinase production by fermentation and assessment of the product require methods for assaying the streptokinase. Unlike for enzymes such as β-

galactosidase, no synthetic substrate has yet been identified for the streptokinase assay. Plasminogens of human, chimpanzee, monkey, cat, dog, and rabbit are the only known protein substrates for streptokinase (Castellino, 1981). Streptokinase assays rely on its ability to activate plasminogen to plasmin. Plasmin then hydrolyzes an indicator substrate and the extent of hydrolysis over a given period is related back to the concentration of streptokinase. The indicator substrates for plasmin include the fibrin clot, casein, other proteins and various synthetic esters (e.g., lysine methyl ester, lysine ethyl ester, *p*-toluenesulfonyl-L-arginine methyl ester).

In 1949, Christensen devised the first quantitative method for determining streptokinase (Christensen, 1949). This procedure required the determination of the smallest quantity of the streptokinase containing solution to cause lysis of a standard fibrin clot in 10 min at 37 °C, pH 7.4. Various modifications of this procedure have been used widely (Remmert and Cohen, 1949; Wasserman et al., 1953; Mullertz, 1955; Derechin, 1962; Wallen, 1962a,b; Tomar, 1968). Digestion of casein for estimating streptokinase had been established as early as 1947. A radial caseinolysis method with the agarose gel containing both casein and plasminogen is commonly used (Saksela, 1981). A quantitative enzymatic assay for human plasminogen and plasmin using azocasein as substrate was described by Hummel et al. (1965).

The fibrin plate method originally introduced for determining proteolytic activity in blood (Permin, 1947) has been widely used for measuring fibrinolysis. The method has been criticized for various shortcomings and numerous attempts have been made to improve it. In the fibrin plate method, a zone of lysis produced on a fibrin film in a petri dish is measured and related to the concentration of the fibrinolytic protein.

A heated fibrin plate method was advanced by Lassen in 1952. An agar diffusion assay for streptokinase was devised by Holmstrom (1965) as a modification of the Astrup and Mullertz method. A rapid fibrin plate assay was designed by Marsh and Gaffney (1977). This procedure reduced the required incubation time to 3 h from 16–20 h needed for the conventional fibrin plate assay. Incubation period was reduced by using fibrin films that had been enriched with plasminogen. Studies of fibrin clot lysis by streptokinase have identified the pH, the buffer concentration and the plasminogen concentration as the major variables influencing the lysis (Westlund and Andersson, 1991). The concentration of fibrinogen in the clot also affects the plate assay.

Synthetic amino acid esters were first introduced as sensitive substrates for assaying proteolytic enzymes (Mullertz, 1954) and soon afterwards were shown to be suitable for assaying plasmin (Troll et al., 1954; Kline and Fishman, 1961a,b; Sherry et al., 1964). Streptokinase–plasminogen/streptokinase–plasmin complex hydrolyzes methyl esters of lysine, acetyl-lysine and tosyl-arginine (Reddy et al., 1974). The hydrolysis of synthetic esters by plasmin generated by activating plasminogen with streptokinase can be followed by determining the carboxylic acid functional groups produced. Hestrin's photometric method established in 1949 can be used for this determination. This method relies on forming a ferric complex with hydroxamic acid (Mullertz, 1954). Alternatively, the free carboxylic acid groups produced by hydrolysis of synthetic esters can be determined by titrimetry (Davie and Neurath, 1953).

A modified Hestrin method has also been described (Roberts, 1958). Use of synthetic esters instead of fibrinogen has enabled a consistent and rigorous definition to be

established for the specific activity of streptokinase (Roberts, 1960; Taylor and Botts, 1968). The use of  $N^\alpha$ -CBZ-lysine-*p*-nitrophenyl ester (CLNE) as a substrate for plasmin has been reported (Silverstein, 1975). The assay based on this substrate is substantially more specific and convenient compared to assays that use  $N^\alpha$ -tosyl-L-arginine methyl ester (TAME) or  $N^\alpha$ -benzoyl-L-arginine ethyl ester (BAEE) (Silverstein, 1975). Compared to the TAME-based assay, the use of CLNE is 800-fold more specific for SK-PG/SK-PN complex. Similarly, the CLNE substrate is 40-fold more specific for SK-PG/SK-PN complex than is BAEE. A colorimetric plasmin assay based on TAME was developed by Castellino et al. (1976).

A solid-phase chromogenic assay for plasmin has been reported (Kulisek et al., 1989). In this assay, the plasmin generated by the action of nitrocellulose-bound streptokinase hydrolyzes the plasmin specific tripeptide *H*-D-valyl-leucyl-lysine-*p*-nitroaniline. Other similar spectrophotometric methods for assaying streptokinase have been described.

A highly sensitive and reproducible enzyme-linked immunosorbent assay (ELISA) for antibodies against streptokinase has been developed (Leonardi et al., 1983). An enzyme immunoassay for streptokinase was reported by Shemanova et al. (1995). This assay was 10 times more sensitive than the other methods used typically and required only micro-quantities of blood serum (cf.  $\geq 2$  ml of plasma is required for a typical plate or testtube assay). Eisenberg et al. (1990) reported the use of ELISA for measuring the cross-linked fibrin degradation products in plasma.

#### 5.4. Recovery and purification of streptokinase

Several schemes have been described for recovery and purification of streptokinase either from the commercially available crude preparations or the fermentation broths of various streptococci (De Renzo et al., 1967; Tomar, 1968; Taylor and Botts, 1968; Einarsson et al., 1979; Perez et al., 1998).

De Renzo et al. (1967) purified streptokinase from a relatively crude commercial preparation (Varidase; Lederle Laboratories, American Cyanamid, USA). Column chromatography on DEAE-cellulose was followed by column electrophoresis in sucrose density gradients to obtain a five- to sixfold increase in purity. Repeated chromatography was necessary to remove the last detectable traces of impurities. In a similar procedure, starting from crude Varidase, Taylor and Botts (1968) attained a final specific activity of 100,000 units of streptokinase per mg of protein. This required a combination of ion exchange (DEAE-Sephadex A-50) and gel permeation (Sephadex G-100) chromatography.

Tomar (1968) purified streptokinase from Varidase using a different procedure. Streptokinase was fractionated either by hydroxyapatite chromatography or ammonium sulfate fractionation. Precipitation of streptokinase with 40–50% ammonium sulfate resulted in a two- to threefold increase in specific activity. The precipitate was recovered by centrifugation and dialyzed against 0.09 M sodium chloride. The dialyzed solution was further purified by gradient elution from a DEAE-cellulose chromatography column. The major peak of eluted activity was concentrated 10-fold by ultrafiltration.

A highly pure streptokinase was recovered from the relatively crude commercial Kabikinase (Kabi Vitram, Sweden) by Einarsson et al. (1979). Ammonium sulfate

fractionation was first used to obtain a crude precipitate of streptokinase. This was redissolved and subjected to gel permeation chromatography. The eluted streptokinase fraction was further purified using column chromatography on DEAE-cellulose or DEAE-Sepharose.

Several affinity chromatography methods have been discussed for purifying streptokinase (Castellino et al., 1976; Andreas, 1990; Rodriguez et al., 1992, 1994; Liu et al., 1999). The earliest such procedure used insolubilized di-isopropyl fluorophosphate (DIP) plasmin as the affinity ligand (Castellino et al., 1976). This ligand was produced by the conversion of plasminogen to plasmin with urokinase and inhibition of the proteolytic activity of plasmin by DIP (Castellino et al., 1976). Purification through the affinity column caused a 30% decrease in the streptokinase activity, suggesting incomplete inhibition of the plasmin affinity ligand bound to the chromatography matrix. A different affinity purification used a monoclonal antibody ligand (Andreas, 1990).

Another affinity purification scheme used acylated plasminogen or plasmin as the affinity ligand (Rodriguez et al., 1992). The acylation of plasminogen or plasmin was carried out with *p*-nitrophenyl-*p*-guanidino benzoate (NPGB). Plasminogen acylation with NPGB allowed it to be used as an affinity ligand without requiring activation to plasmin. This probably reduced the plasmin associated proteolysis of streptokinase. Rodriguez et al. (1994) used a combination of two affinity matrices for chromatographic purification of recombinant streptokinase. The affinity ligands were human plasminogen and monoclonal antibody against streptokinase. Both ligands were bound to Sepharose as the chromatographic matrix. This purification method produced a preparation with about 50,000 units of activity per mg of protein and a purity of >93%.

Use of immobilized NPGB acylated plasminogen for affinity purification was further reported by Liu et al. (1999). A solution of urea was the eluent. Approximately ninefold purification was achieved with a yield of >90%. The specific activity of the purified material was 74,000 units/mg. Hernandez-Pinzon et al. (1997) recovered streptokinase by cross-flow ultrafiltration. Approximately 14% of the initial activity was lost as the protein solution was concentrated by eightfold. This loss was associated with denaturation of the streptokinase through unfolding and aggregation.

Streptokinase has been purified from the filtrate of a streptococcal fermentation broth using hydrophobic interaction chromatography on phenyl- or octyl-Sepharose column. A gradient elution with 21% ammonium sulfate was used to recover the streptokinase. Further purification involved gel permeation and ion exchange chromatography steps.

Perez et al. (1998) purified a recombinant streptokinase produced by fermentation in *E. coli* K12. To isolate the streptokinase, the biomass was first recovered by centrifugation and then washed, and the cells were disrupted. The streptokinase inclusion bodies were then dissolved and renatured. Hydrophobic interaction chromatography was then used to obtain the protein at a purity of >99%. The overall recovery yield was 49%. A similar yield of about 45% and a purity of >97% were attained by Zhang et al. (1999) in recovering recombinant streptokinase from inclusion bodies produced in *E. coli*. Generalized schemes for the recovery and renaturation of inclusion body recombinant proteins have been published (Chisti, 1998a).

Reverse-phase HPLC has been used for purifying a bovine plasminogen activator from culture supernatants of the bovine pathogen *S. uberis* (Johnsen et al., 1999). A single protein with a molecular mass of 32 kDa was detected in the eluted active fraction. This plasminogen activator lacked the C-terminal domain that is characteristic of the *S. equisimilis* streptokinase (Johnsen et al., 1999). Other work on *S. uberis* streptokinase has been reported (Johnsen et al., 1999, 2000) and its mechanism of action has been subjected to closer scrutiny (Sazonova et al., 2000).

Commercial production of streptokinase requires special attention to biosafety considerations because the protein is potentially immunogenic to process workers. In addition, care is necessary if streptokinase is being produced using natural strains of streptococci because all streptokinase producing streptococci are potentially pathogenic. The various safety considerations relevant to production of biopharmaceutical proteins have been discussed by Chisti (1998b).

## 6. Concluding remarks

Native streptokinase is useful for cost-effective thrombolytic therapy in clinical practice, but its use is not risk free. Large quantities of streptokinase can be produced inexpensively via bacterial fermentation. Cloning of the streptokinase gene in nonpathogenic microorganisms has enabled production of recombinant streptokinase that eliminates any risk of inadvertent inoculation of patients and production personnel with potentially pathogenic streptococci.

Various chemical modifications of streptokinase have been used for extending its half-life in circulation, improving plasminogen activation, and reducing or eliminating immunogenicity. These objectives have been attained to various degrees also by producing mutated and engineered streptokinases. The streptokinase domains responsible for antigenicity, stability and plasminogen activation appear to overlap to some degree. Consequently, it may be impossible to produce a modified streptokinase that combines the every desired functional trait at the desired level.

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