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Molecular Cloning of the Streptokinase Mutant Gene

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Abstract: Streptokinase, a bacterial protein produced by various strains of haemolytic streptococci, is widely used to treat humans with thrombolytic disease. This protein is antigenic and anti- streptokinase antibodies (Abs) cause allergic reactions and neutralize streptokinase therapeutic effects. To produce an engineered variant of streptokinase being functional and less antigenic than the native molecule. In this study the C-terminal immunodoinant epitope of native molecule was eliminated by Polymerase Chain Reaction (PCR) using specific internal primers. Then PCR-amplified sequence was cloned into pGEMEX-1 expression vector.

Key words: Streptokinase, mutant C-42, cloning

INTRODUCTION

Streptokinase is a streptococcal protein specially used for the treatment of thrombolytic disease and Acute Myocardial Infarction (AMI). This protein is a potent activator of the fibrinolytic enzyme system in humans streptokinase binds to plasminogen and generates a streptokinase-plasminogen activator complex. This complex catalyses the conversion of plasminogen to plasmin, an active enzyme that degrades the fibrin component of thrombi (Young *et al*., 1995).

Shortly after discovering the fibrinolytic effects of streptokinase, its immunogenicity was reported (Tillet *et al*., 1934). Antibodies to streptokinase are found in most individuals due to the high frequency of streptococcal infections (Lynch *et al*., 1991). Moreover, when patients receive streptokinase as therapy for thrombolytic disease, Abs titers to SK rise post-treatment. These neutralizing Abs reduce the efficiency of thrombolytic therapy and may cause a range of allergic reactions (Brugemann and Bom, 1993; McGrath and Patterson, 1984; Lew, 1984).

Previous reports have identified 5 antigenic regions in SK localized to fragments spanning amino acid residues 1-13, 14-127, 1-253, 120-352, 353-414 (Reed *et al*., 1993; Parhami-Seren *et al*., 1995). The present study is based on evidences about the presence of an immunodominant epitope in the C-terminal region of native molecule (Torrens *et al*., 1999b; Ojalvo *et al*., 1999). A deletion mutant (mut-C42), lacking the C-terminal 42 amino acids, showed to be less recognized and less neutralized by anti- streptokinase Abs from patients sera respect to the native protein (Torrens *et al*., 1999a; Torrens *et al*., 2000).

In this study, the SK mutant gene lacking the C-terminal 42 amino acids was cloned in expression vector, pGEMEX-1, to study the reactivity of anti-SK Abs with mut-C42 compared with native SK in Iranian patient sera.

MATERIALS AND METHODS

Genomic DNA from streptococci group A was isolated from 5 mL bacterial cultures grown in LB medium overnight at 37°C. The streptokinase mutant gene (1116 bp) was amplified by PCR (Sambrook *et al*., 1989) with two specific primers which designed based upon the previously published nucleotide sequence of the streptokinase gene from streptococcus equisimilis lancefield group C (Malke *et al*., 1985). The upstream primer (5 AGTCAATTGAGCTCAAATGA TTGCTGGACC 3) contained a SacI site and hybridized to the native SK nucleotide number 60 to 89 (N-terminal sequence encode leader peptide). Downstream primer (5 AGCATTCTGGATCCT AAGTCGCTTGCCC 3) contained a BamHI site and a stop codon and hybridized to the native SK nucleotide 1107-1116.

The amplified DNA sequence was digested with SacI and BamHI and cloned in the SacI- BamHI sites of pGEMEX-1 expression vector. Then this recombinant vector was transferred into DH5α cells as host. Transformants were plated on selective medium

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containing ampicillin. Colonies were screened and the integrity of cloned sequence was confirmed using restriction enzymes analysis.

RESULTS AND DISCUSSION

The PCR- amplified gene was electrophoresed on 1% agarose gel. Along with 100 bp DNA ladder marker, the length of the PCR product was confirmed (Fig. 1).

The PCR-amplified gene was cloned in the SacI- BamHI sites of pGEMEX-1 expression vector. Cloned gene was confirmed using restriction analysis with SacI and BamHI enzymes (Fig. 2).

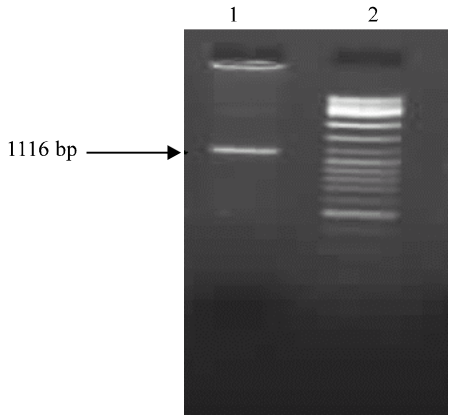

Fig. 1: 1% agarose gel electrophoresis.
Lane 1. Streptokinase PCR product. Lane 2. 100 bp DNA ladder marker 123

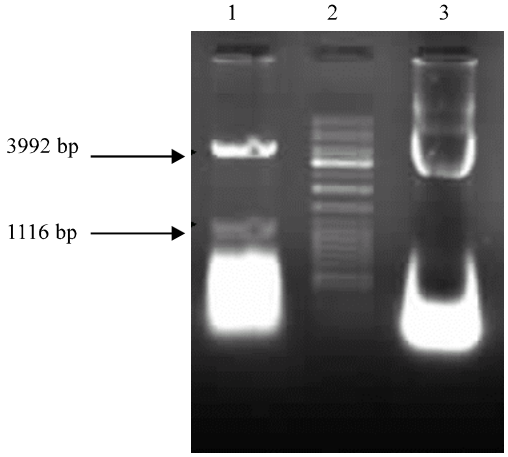

Fig. 2: Restriction analysis of recombinant plasmid.
Lane 1. Recombinant plasmid digested with SacI and *BamHI* enzymes.
Lane 2.100 bp DNA ladder marker.
Lane 3. Undigested recombinant plasmid.

Previous reports have shown 5 antigenic regions in streptokinase, mapped with soluble recombinant streptokinase fragments and anti-SK Abs from human sera from patients treated with streptokinase. These regions comprise amino acids 1-13,1-253,120-352 (containing two distinct, non overlapping epitopes) and 353-414. The data indicated that all patients raise Abs to two major discrete regions, these regions were 1-253,120-352. Two other epitopes in streptokinase constructed by amino acids 1-13 and 353-414 were not antigenic in all humans tested in this study (Parhami-Seren *et al*., 1995). Torrens *et al.* (1999a) mapped the antigenic regions (linear epitopes) located on streptokinase, using human total sera from patients treated with Heberkinase. As a result they obtained that C-terminal region 380-490 among other fragments is notably immunodominant. They also reported that around 30% from 1008 normal donors recognized a synthetic peptide resembling amino acids 373-414 from streptokinase C-terminal region (Ojalvo *et al*., 1999). Then the immunoreactivity of the synthetic 42-residue peptide with sera from AMI patients before and after Heberkinase therapy was tested. This peptide was recognized by 39% of patients before therapy and as it was expected, recognition increased to 64% after therapy. This is evidence about immune response towards C-terminal region of streptokinase (Torrens *et al*., 1999b). These studies were complemented by performing a competition experiment in which both native and mutant (lacking C-terminal 42 amino acids) proteins were tested in a neutralizing activity assay using patient's sera. For most of the individuals, mut-C42-neutralizing activity titer (NAT) significantly decreased with respect to native SK-NAT and mut-C42 was significantly less recognized by pre-existing anti- streptokinase Abs than the native streptokinase (Torrens *et al*., 1999a). Then, the immune response to mut-C42 and native molecule was tested in animals. 14 monkeys were subjected to treated with native molecule and mut-C42. All monkeys developed anti–streptokinase Abs, but in the case where treatment induced Abs directed against the C-terminus of SK, neutralizing activity against the native protein was significantly higher than that developed against mutant-C42 (Torrens *et al*., 2000). In conclusion, present data indicate that the immunodominant epitope in the C-terminal of native streptokinase can be eliminated without loss of specific activity. Based upon this result, at the present study the mutant gene, lacking the C-terminal 42 amino acids, was cloned into expression vector to compare the mutant protein with native protein regarding their antigenic properties in Iranian patient's sera.

If mut-C42 activity was less affected by neutralizing Abs compared with native streptokinase, this engineered variant could be a preferred alternative to native streptokinase for thrombolytic therapy.

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