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Restriction enzyme analysis of recombinant plasmids containing streptokinase gene

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Summary: The recombinant pTargeT.sk10 was digested with different restriction enzyme which showed that HindIII had 2 sites and produced two bands on agarose gel electrophoresis. The BamHI had only one site and therefore the recombinant plasmid was linearised and thus single band was observed. The PstI produced one band. The enzyme PvuI had two sites and thus produced two bands, the enzyme NheI had three sites but two sites were closely located producing one very small fragment and thus only two bands were observed on agarose gel electrophoresis. The enzyme SaII and NotI had one site each and therefore they linearised the recombinant plasmid producing only one band. The recombinant plasmid pSinCMV.sk11 digested with different RE showed that PstI and SpeI produced three bands while NheI, PmeI, NruI, SaII produced two bands each on 1% agarose gel electrophoresis

Key Words: Streptokinase gene, repicase vector, pTargeT vector, recombinant plasmid, restriction enzyme.

Introduction

Streptokinase is a single peptide, multi-domain secretory protein of 414 or 415 amino acid residues (47 kDa) produced by various strains of beta hemolytic Streptococcus (Malke et al., 1985; Jackson and Tang, 1982; Tillet and Garner, 1933; Nihalani et al., 1998). Many group A Streptococci bind plasminogen (Ullberg et al., 1989). Wong et al (1994) showed that streptokinase is one of the major blood clot dissolving agents used in many medical treatments.

Kapur et al (1995) studied the genetic analysis of SK gene of *Streptococcus pyogenes*. A 384 bp DNA fragment encoding two variable regions of the molecules was characterized in 47 isolates of *Streptococcus pyogenes*. The results revealed that alleles of the SK gene have a mosaic structure, and provide strong evidence for intragenic recombination. Moreover, organisms that are well differentiated in overall chromosomal character have identical SK alleles, which suggest that horizontal gene transfer and recombination have participated in the evolution of this locus. No simple relationship between SK allele and serum opacity factor production or specific disease was identified.

Materials and Methods

Recombinant Plasmids

The rplasmids pTargeT.sk10 and pSin.sk11 (Gangwar et al, 2010, 2012) constructed at IBIT Bareilly were used.

Agarose gel electrophoresis

Agarose gel electrophoresis was done as per the method of Sambrook & Russel (2001). Briefly appropriate quantity of agarose (analytical grade) was boiled with 1x TAE buffer in microwave oven to get the uniform molten agarose of desired gel percentage (0.7 or1.0 or1.5%) which was casted in appropriate gel casting tray fitted with acrylic comb and let for setting. Prior to casting, molten agarose was allowed to cool to 45°C and 0.5µg/ml ethidium bromide was added. The acrylic comb was carefully removed after the gel was perfectly set. The tray with the gel was submerged in an electrophoresis tank containing 1x TAE buffer, DNA to be analysed was mixed with appropriate volume of 6x DNA loading buffer making it 1x and charged into wells alongside DNA molecular weight marker. Electrophoresis was carried out at 70V or 90V until the tracking dye (Bromophenol blue) has just passed out of the gel. The DNA bands were then visualized under UV illuminator and photographed using UV Ultra Lum geldoc system (Germany).

Isolation of plasmid DNA

Miniprep

Small scale plasmid DNA isolation was done following the alkali lysis method (Birnboim and Dolly, 1979).

Restriction endonuclease analysis of recombinant plasmid

The presence of SK gene insert and its orientation in the recombinant plasmid was also confirmed by RE analysis using EcoRI, HpaI and AccI restriction enzymes. The reaction mixture consisted of recombinant plasmid DNA (100ng/ µl) 4µl, assay buffer 1.5µl, restriction enzyme 1µl, nuclease free water 8.5µl with total volume15µl. The digestion was done at 37°C for 3 hour and then the product was run on 1% agarose gel electrophoresis.

Effect of recombinant plasmid on blood clotting time in mouse

The recombinant plasmids were injected 50µg/mouse, in vector alone group vector DNA 50µg/mouse, intramuscularly in thigh of mice, 15 mice in each group, while 15 mice were kept as healthy control. At 24, 48 and 72 hour intervals, tip of the mouse tail was cut and blood clotting time was noted (fig 11).

Analysis of SK gene and recombinant plasmids using DNAstar

The SK gene as well as pTargeT.sk10 and pSinCMV.sk11 recombinant plasmids were analysed using DNAstar software.

Results and Discussion

The recombinant pTargeT.sk10 was digested with different restriction enzyme and the results obtained are shown in fig.1-2. It was observed that HindIII had 2 sites and produced two bands on agarose gel electrophoresis. The BamHI had only one site and therefore the recombinant plasmid was linearised and thus single band was observed. The PstI produced one band. The enzyme PvuI had two sites and thus produced two bands, the enzyme NheI had three sites but two sites were closely located producing one very small fragment and thus only two bands were observed on agarose gel electrophoresis. The enzyme SaII and NotI had one site each and therefore they linearised the recombinant plasmid producing only one band.

The recombinant plasmid was further analysed using DNAstar software which revealed that of 212 enzymes selected, 181 enzymes cut the DNA , it had 46 unique sites and 31 enzymes had no sites.

The recombinant plasmid pSinCMV.sk11 was digested with different RE and results are shown in fig.3. The enzymes PstI and SpeI produced three bands while NheI, PmeI, NruI, SaII produced two bands each on 1% agarose gel electrophoresis. The recombinant plasmid was also analysed using DNAstar software and it was found that 194 enzyme out of 212 enzyme selected cut the plasmid DNA, it had 24 unique sites and 18 enzymes had no site on the recombinant plasmid .

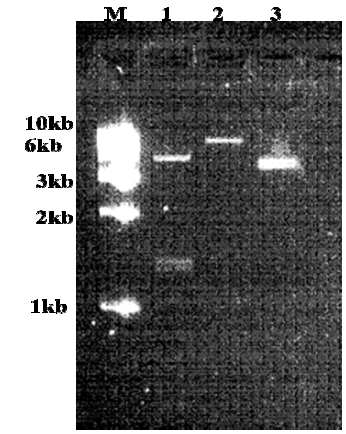

Fig. 1. pTargeT.sk10 digested with RE. Lane M-1kb DNA ladder (Fermentas), 1- HindIII, 2- BamHI, 3- PstI.

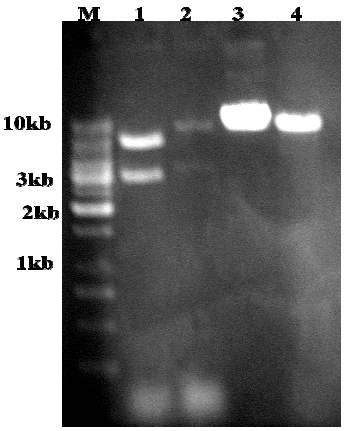

Fig.2. pTargeT.sk10 digested with RE. Lanes M: 1 kb DNA ladder (Fermentas); 1: PvuI; 2:NheI ; 3: SalI , 4: NotI,

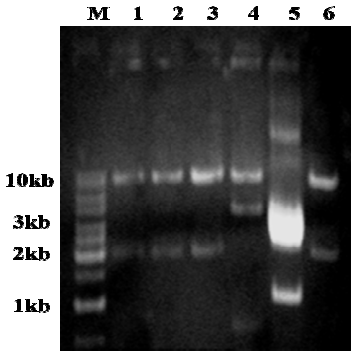

Fig. 3. Plasmid DNA pSinCMV.sk11 digested with RE. Lanes M: 1 kb DNA marker , 1: NheI, 2 :PmeI, 3: NruI, 4: PstI, 5: SpeI, 6: SalI,

Assay of recombinant plasmid on blood clotting time

The effect of recombinant plasmid on blood clotting time in mice was assayed and results are shown in table 1.

Table 1. Assay of blood clotting time in mice injected with recombinant plasmid**.**

S.No	Group	No of mice	DNA (µg)	Bleeding time (sec)		
				24 h	48h	72h
1	PTargeT.sk10	15	40	55.96±0.009	55.78±0.009	55.70±0.007
2	pSin.sk11	15	4	56.00+0.040	56.50±0.040	55.80±0.007
3	PTargeT vector	15	40	58.90±0.030	60.02±0.009	58.20±0.070

4	pSin vector	15	4	75.00+0.090	58.00±0.040	57.60±0.040
5	Healthy control	15	-	58.00+0.040	57.48±0.030	57.78±0.007

No significant difference in the blood clotting time in treated and control mice was seen.

The restriction enzyme analysis of the recombinant plasmid pTargeT.sk10 and pSinCMV.sk11 with different enzymes yielded valuable information on enzyme sites present on it. Presence of one site resulted in linearization of the plasmid and hence only one band was observed. The analysis of the recombinant plasmid using DNAstar software provided insight into the absence sites, number of sites present and it was found to correlate well with the observations of the digestion experiments. The DNAstar software analysis helped to select the enzyme for cutting the recombinant plasmids for checking the gene insert and its orientation as well.

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