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Review of streptokinase isolation studies from various techniques

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Abstract

Vector that can replicate in Streptococcus spp. and Escherichia coli has been constructed by joining the E. coli plasmid. The resulting chimeric plasmid is designated pSA3 (chloramphenicol, erythromycin, and tetracycline resistance) and has seven unique restriction sites.pSA3 was transformed and was stable in Streptococcus sanguis and Streptococcus mutans in the presence of erythromycin. We have used pSA3 to construct a library of the S. mutans GS5 genome in E. coli, and expression of surface antigens in this heterologous host has been confirmed with S. mutans antiserum. previously cloned determinant that specifies streptokinase was subcloned into pSA3, and this recombinant plasmid was stable in the presence of a selective pressure and expressed streptokinase activity in E. coli.culture strategy is often used for increasing production of heterologous recombinant proteins in Escherichia coli. This study was initiated to investigate the effects of dissolved oxygen concentration (DOC), complex nitrogen sources and pH control agents on cell growth and intracellular expression of streptokinase (SK) in recombinant E. coli BL21(DE3) s increase in SK could be correlated with increase in plasmid segregational stability. Supplementation of production medium with yeast extract and tryptone and replacement of liquid ammonia with NaOH as pH control agent further enhanced SK expression without affecting cell growth. SK concentration of 1120 mg l1 representing 14-fold increase in SK production on process scale-up from flask to bioreactor scale fed-batch culture is the highest reported concentration of SK to date.
Plasminogen activator was purified from the culture supernatant of the bovine pathogen *Streptococcus uberis* NCTC 3858. After the final reverse-phase high-performance liquid chromatography step a single protein with a molecular mass of 32 kDa was detected in the active fraction. A partial peptide map was established, and degenerate primers were designed and used for amplification of fragments of the gene encoding the activator. Inverse PCR was subsequently used for obtaining the full-length gene.

Key words: Streptokinase, Escherichia coli, Culture Dissolved oxygen concentration Plasmid stability, vector

Introduction

Streptokinase is used to dissolve blood clots that have formed in the blood vessels. It is used immediately after symptoms of a heart attack occur to improve patient survival. SK is a medicine and also enzyme that used to breakdown clot in some cases of heart attack.The low SK production yields from natural host and its pathogenicity are the main reasons for exploration of recombinant DNA technology route for this important protein.

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Escherichia coli is the most commonly used host for heterologous protein production and the preferred method for increasing the concentration of heterologous recombinant proteins, which is proportional to both cell density (unit cell mass per unit volume) and specific cellular product yield. The development of recombinant strain, E. coli BL21. containing the complete gene encoding for streptokinase from Streptococcus equisimilis H46A have been reported before . Herein, the effects of process parameters like dissolved oxygen concentration (DOC) and nutritional parameters like addition of complex nitrogen sources and type of pH control agents on the overproduction of the lifesaver protein drug, streptokinase (SK) in E. coli are described. As Streptokinase is a bacterial product, the body has the ability to build up an immunity to it. Therefore, it is recommended that this medication

should not be used again after four days from the first administration, as it may not be as effective and can also cause an allergic reaction. it is usually given only for a person's first heart attack. Similarly the increase in recombinant protein expression levels with the addition of complex nitrogen sources has been reported in many instances in E. coli fermentations. Although a fair amount of general literature is available on the large-scale production of heterologous proteins in E. coli, it should be realized that each expression system is unique in terms of promoter system, host–vector interactions, sequence and characteristics of recombinant product and the effect of the expressed foreign protein on host cell physiology. Hence the optimum requirements for growth and product formation also vary from case to case. The importance of growth parameters can be deduced from the fact that although parameters like stable maintenance of recombinant plasmid, plasmid copy number, protease degradation of the recombinant product and inclusion body formation are primarily a function of genetic makeup of the host and vector system, these are also known to be greatly affected by the cultivation conditions and media composition.novel plasminogen activators have recently been described. They were derived from the bovine mastitis-inducing pathogens *Streptococcus uberis* . plasminogen activator secreted from *S. uberis* was different from that of *Streptococcus pyogenes* (Lancefield group A) and *Streptococcus equisimilis* (Lancefield group C) strains, as it activated bovine but not human plasminogen. It also differed from Lancefield group E activity by not activating porcine plasminogen (13). By activation of plasminogen to plasmin through the action of its plasminogen activator, *S. uberis* was also shown to be able to acquire surface-localized plasmin activity and plasmin binding to the bacterial surface was susceptible to increasing concentrations of NaCl and lysine. For a mastitis-inducing pathogen, the production of a plasminogen activator could be of importance in two ways. In addition to generation of plasmin activity needed for degradation of extracellular matrix proteins and subsequent colonization, the activation of endogenous plasminogen present in milk would lead to hydrolysis of milk proteins and, thereby, liberation of peptides from which *S. uberis* could obtain essential amino acids. study we have performed purification and partial amino acid sequencing of the plasminogen activator from *S. uberis* and have cloned and sequenced its gene. By sequence comparison, the plasminogen activator was shown to be related to the already-known streptokinases.**sk** may also be used to treat blood clots in the lungs (pulmonary embolism) and in the legs (deep venous thrombosis) Streptokinase belongs to a group of medications known as fibrinolytics. and complexes of streptokinase with human plasminogen can hydrolytically activate other unbound plasminogen by activating through bond cleaves to produce plasmin.

The strain used in this study was ampicillin-resistant recombinant E. coli BL21 (DE3) developed for over-expression of the gene encoding for SK protein of S. equisimilis H46A. The over-expression of cloned gene was under the regulation of T7 polymerase responsive promotor and a lac operator in a pET-series expression vector viz. pET-23d . Also, the recombinant plasmid contained the ampicillin resistance gene for selection of plasmid containing bacterial clones. It was maintained in 30% sterile glycerol at 72 C.*Taq* polymerase was from HT Biotechnology Ready to Go PCR beads were from Pharmacia (Uppsala, Sweden), oligonucleotides were from DNA Technology (Aarhus, Denmark) all other enzymes were from New England Biolabs (Hitchin, United Kingdom). PCR was performed in a Hybaid (Middlesex, United Kingdom) ABACUS thermal cycler. Sequencing was performed with a dye terminator cycle sequencing kit from PE Applied Biosystems .

Inoculum development--A loopful of frozen glycerol stock (kept at 70 C) was streaked on a LB plate containing ampicillin, 50 lg ml1 and incubated at 37 C for 14–16 h. A single isolated colony was then transferred to LB medium incubated on a rotary shaker at 37 C and 200 rpm for 6–8 h. This pre-inoculum was transferred at a rate of 5% (v/v) to the main inoculum medium and incubated for 12–14 h.

Shake flask cultivation-----

The cells were grown on a rotary shaker at 37 C and 300 rpm and induced with 1 mM IPTG (final concentration) when the cell density (OD500) reached 1.0–1.2 in 2 h. The culture were further incubated for 5 h and then harvested.

Batch cultivation------

The batch culture were induced when cell density (OD500) reached 3.8–4.2 (at 5 h) and then, further cultivated for 5 h before harvest. DO was maintained at 30% (unless otherwise stated) by cascading agitation rate (300–1000 rpm) with DO concentration at constant aeration rate (1.25 vvm).

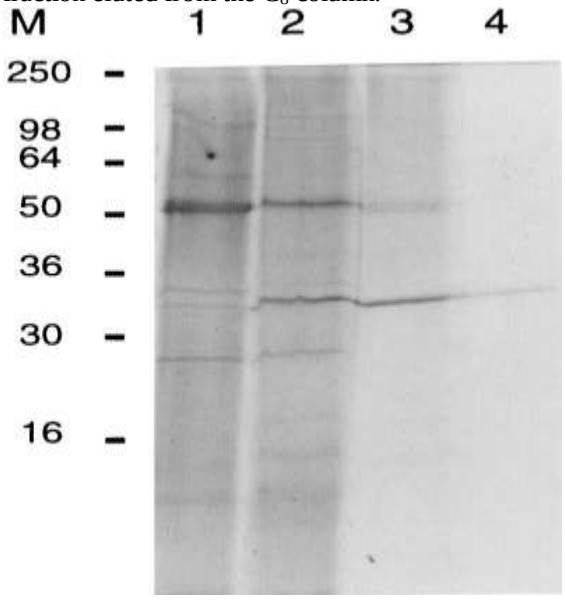
Bacterial strains and media. The E. coli K-12 strains used in this study were HB101 (7) and 294 (6). The

streptococcal strains included S. mutans GS5, S. sanguis (Challis), and the Challis strain harboring pGB305 (5). The basic media used were Luria Bertani for E. coli (14) and brain heart infusion for streptococci. When required, antibiotics were added at the following concentrations: tetracycline at 12.5 ,ug/ml, chloramphenicol at 100 ,ug/ml, and erythromycin at 50 ,ug/ml forE. coli 294 and 250 ,ug/ml for HB101; and erythromycin at 10 jxg/ml for S. sanguis and S. mutans. E. coli transformants containing S. mutans DNA inserts were inoculated into wells of 96-well microtiter plates containing 300 ,u1 of Luria Bertani medium with the appropriate antibiotic. After overnight incubation at 37°C, crude cell extracts were prepared by an adaptation of the method described by Clarke et al. (10). The plates were centrifuged at 2,000 rpm for 5 min, the supernatant fluid was decanted, and the cells were incubated at 30°C for 30 min with 300 p.1 of lysozyme at 0.25 mg/ml in 0.05 M Tris (pH 7.5). The cells were lysed by three cycles of freeze-thaw treatment and centrifuged to remove cell debris.

ampicillin was added at a final concentration of 100 mg l1 in all cases. The inducer, isopropyl-b-D-thiogalactopyranoside (IPTG) was also filter-sterilized and added to the culture when required.

E. coli-Streptococcus shuttle vector has been constructed and has proven useful for the molecular cloning of streptococcal genes. This vector, pSA3, consists of com plasmid pACYC184 may constitute an additional attribute of the pSA3 shuttle vector. Streptococcal plasmid pGB305 (4) (6.2 kilobases) and E. coli plasmid pACYC184 (8) (4 kilobases) were cleaved at their unique Aval sites, ligated, and transformed into E. coli HB101. Transformant clones with the chloramphenicol, erythromycin, and tetracycline resistance phenotypes were selected, and a plasmid designated pSA3 was isolated from one of these clones (Fig. 1). Digestion of the chimeric plasmid pSA3 with Aval gave two fragments corresponding to pACYC184 and pGB305 (Fig. 2). A physical map of pSA3 was derived from restriction analysis with HindIII (Fig. 1) and confirmed by double digestion with enzymes cleaving unique sites on the plasmid (data not shown). Plasmid pSA3 has seven single sites for cloning: EcoRI, EcoRV, XbaI, BamHI, SphI, SalI, and NruI. Insertion of DNA segments into the EcoRI and EcoRV sites would inactivate chloramphenicol resistance of E. coli, and cloning into the BamHI, Sall, and SphI sites would inactivate tetracycline resistance of E. coli.

The bovine plasminogen activator from *S. uberis* NCTC 3858 was purified from the culture supernatant by a combination of ammonium sulfate precipitation, DEAE–ion-exchange chromatography, denaturing Mono-S HPLC, and reverse-phase HPLC. Interestingly, the plasminogen activator appeared to be a very stable protein, as demonstrated by the fact that activity survived treatment with strong denaturing agents, such as 6 M urea, 8 M guanidinium hydrochloride, and 60% formic acid (data not shown), or passage over reverse-phase columns. The activator was identified as a 32-kDa protein, since only this band was present in the active fraction eluted from the C_8 column.

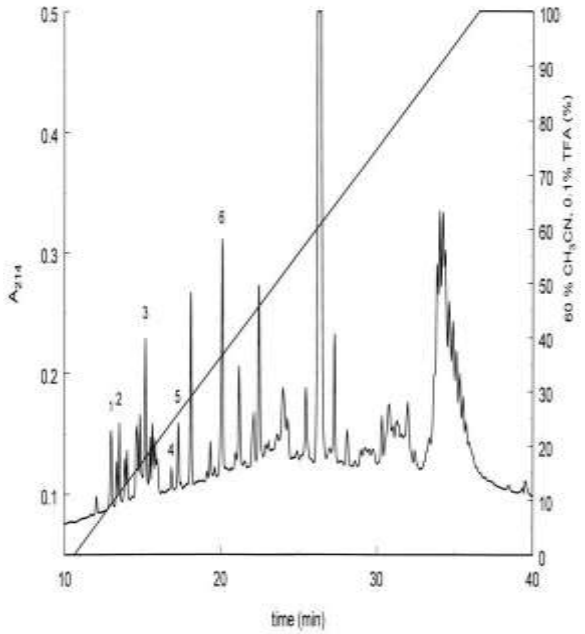


SDS-PAGE of material from different steps during purification of the plasminogen activator from *S. uberis*. Lanes: 1, ammonium sulfate precipitate; 2, pooled active fractions from DEAE–ion-exchange chromatography; 3, pooled active fractions .

E. coli BL21 (DE3) strain was initially evaluated in shake flasks and SK expression levels in LB and a formulated synthetic media (to be used for bioreactor cultivations) were compared. The expression levels as seen by running an SDS–PAGE of the whole cell lysates is shown . In LB medium, the concentration and specific yield of SK were 80 mg l1 and 74 mg g1 and the corresponding values for synthetic medium were 74 mg l1 and 68.5 mg g1 . The SDS–PAGE analysis of soluble and insoluble fractions of E. coli cells indicated SK.

Expression of streptokinase in LB and synthetic medium in shake flask culture. Recombinant E. coli cells were cultivated in LB and synthetic medium in separate flasks and induced with IPTG. Panel a: lane MW – standard molecular weight markers, lane 1 –

whole cell lysate from un-induced cells, lanes 2, 3 – whole cell lysate after 5 h of induction in case of LB and synthetic medium, respectively. Panel b: soluble (lane 1) and insoluble (lane 2) fractions of the induced sample. The arrow indicates the position of SK (47 kDa) band,



Tryptic peptides derived from degradation on the blot were separated by HPLC with a narrow-bore C_2/C_{18} reverse-phase column. The sequences of labeled peaks are shown in Table .TFA, trifluoroacetic acid.

The recovery of protein from the reverse-phase C_8 column was insufficient for generation of suitable amounts of peptides for use in amino acid sequencing, and therefore, tryptic degradation on the blot was carried out on material from the Mono-S column. These tryptic peptides were separated on a C_2/C_{18} reverse-phase HPLC column.

The identified amino acid sequences were used for design of degenerate oligonucleotides (Table) and these were subsequently used as primers for PCR on genomic DNA of *S. uberis* NCTC 3858, in order to isolate the gene encoding the plasminogen activator. The degenerate primer pair corresponding to the amino acid sequences. the degenerate oligonucleotide corresponding to the sequence was shown to hybridize with these amplicons, indicating that the PCR products were amplified from the plasminogen activator gene. The resulting sequences of the two fragments overlapped and comprised the codons for all the sequenced peptides, and they were subsequently used for design of primers for use in inverse PCR. The higher molecular mass of this streptokinase corresponds to the molecular masses of streptokinases from several other species and could indicate the presence of an extra domain .this streptokinase has a markedly different primary structure since no antibody cross-reactivity could be detected by Western blotting.

cultivations were carried out that involved initial cell cultivation in batch mode followed by subsequent addition of nutrients using different feeding strategies to build up high density of E. coli cells for induction. The entire fed-batch phase was carried out at 30% DOC set point under restricted glucose supply and the residual glucose level was maintained below 1.0 g l1 . An un-induced fed-batch experiment with glucose-feeding rate of 4.5 g l1 h1 resulted in a maximum cell density (OD500) of 42.0 F of SK. Further to this discussion, although the percentage of plasmid bearing cells only increased during initial 3–4 h of postinduction period by increasing DOC set point from 30% to 50%, the SK expression levels were significantly different (188 vs. 722 mg l1). Thus during bioreactor cultivations of the recombinant E. coli, not only the maintenance of DOC above a certain threshold level is important but the oscillations in the actual DOC profile should also be taken into account.

Overall, SK production increased from 74 mg l1 in shake flask to 1120 mg l1 in bioreactor scale fed-batch process, a 14-fold increase . . This yield of streptokinase is higher than previously reported streptokinase yields.

Conclusion

At the last we can conclude that cultivation parameters led to the attainment of streptokinase yield that is, to the best of our knowledge, highest recorded to date in the literature for this important therapeutic protein. Also, the investigations on the influence of DOC on expression level of this protein will help in better understanding of scale up of recombinant protein production process to large-scale bioreactors where the mixing time and DOC fluctuations are often large and have implications in formulating DOC control strategy for high cell density cultivation. In some instances, the streptokinase activity was lost in transformant clones; this was most likely due to damage occurring to the streptokinase gene during transformation by two-hit kinetics in streptococci. The shuttle capabilities of this vector were further demonstrated by inserting into pSA3 a cloned streptococcal determinant that specified the protein streptokinase.he potential of

streptokinase$_{S.}$ production in *Lactococcuslactis* (unpublished results) makes it attractive to investigate whether lactic acid bacteria could be used as vaccine carriers by mediating antigen presentation to the mucosal immune system. Future experiments employing domain swapping streptokinase$_{S.}$ might evaluate the species specificity of separate domains of the streptokinases.

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