Chemical Methodologies

Original Research article

Optimization of Streptokinase Mutant Protein Purification Method using Affinity Chromatography Technique

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ARTICLE INFORMATION

ABSTRACT

Protein purification has always been one of the most critical and challenging stages of drug-protein production. Streptokinase as the most common cost-effective fibrinolytic drug is no exception. In this research study, the mutated streptokinase producing clone (SK263cyc) to which the histidine tag was grown in TY2x medium, and SDS-PAGE assessed protein expression after induction of protein expression. Three different methods did protein purification. In the first one, metal, ion affinity chromatography (IMAC) technique was used. In the second solution, first, by filtration with ammonium sulfate, the purification was carried out, and then by affinity purification, chromatography continued. In the third solution, hydrophobic chromatography was utilized to purify the streptokinase protein. The purity of the ophthalmic purity was 93.2%, and the purity of hydrophobic purity was found to be 90.4%, whereas the combination of pre-treatment with ammonium sulfate and the purity of the ophthalmic method did not achieve more than 88%. The results of this study revealed that, the IMAC method is more suitable as a final method at the process of streptokinase purification than the other two approaches.

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Graphical Abstract

The electrophoresis of purified products by HIC method
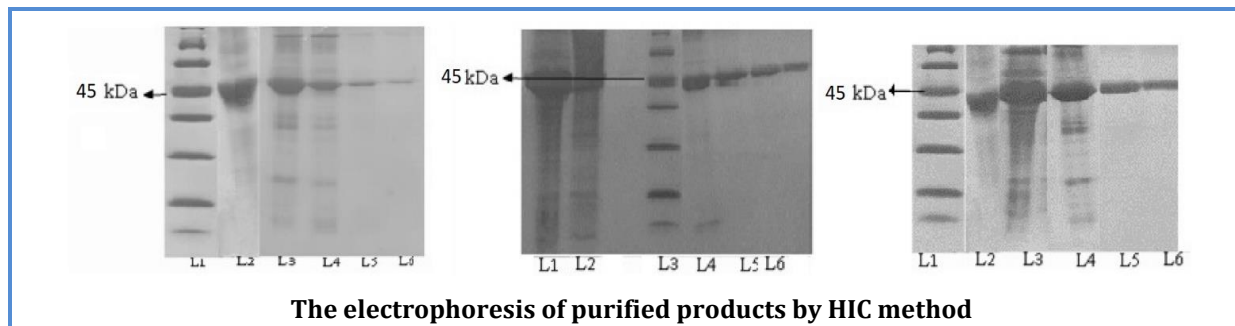
Introduction

When a blood clot forms in the circulatory system, it may cause blood clots, results in dangerous complications and, ultimately, and death. A healthy balance system prevents clots from forming in a natural circulation system [1-3]. The mechanism of blood clot formation is one of the most important and complex physiological systems in the body, and streptokinase is the most common and currently the most cost-effective fibrinolytic drug for this purpose. The most important part of designing a suitable "downstream process" for purification of a protein for analysis and treatment purposes is the separation of the desired protein from other available impurities and contaminants using various purification methods [4-6]. The highest increase in concentration is due to the separation stage of the product, however, the quality of the products dramatically increased during the initial purification stage. SK263cys is a mutant protein containing the cysteine amino acid at position 263 instead of glutamic acid and is designed to perform the specific pegylation on cysteine amino acid. High purity protein is required for this process. Generally, the HIC method is utilized to purify the streptokinase, as this process is costly and low yield [7].

The principle for protein adsorption to HIC media is complementary to ion exchange and size exclusion chromatography. Sample molecules containing hydrophobic and hydrophilic regions are applied to an HIC column in a high-salt buffer. The salt in the buffer reduces the solvation of sample solutes. As solvation decreases, hydrophobic regions that become exposed are adsorbed by the media. The more hydrophobic the molecule, the less salt is needed to promote binding. Usually a decreasing salt gradient is used to elute samples from the column in order of increasing hydrophobicity. Sample elution may also be assisted by the addition of mild organic modifiers or detergents to the elution buffer [8-10].

Escherichia coli is the most commonly used host for the production of recombinant proteins, both in research and industry. High-level expression of recombinant proteins in the form of a soluble intracellular product, secretory product, or as insoluble inclusion bodies depends on promoter system,

host-vector interactions, sequence, and characteristics of recombinant products and the effect of the expressed foreign protein on host cell physiology [11-14].

In this study, purification of streptokinase protein was performed by applying and optimizing the IMAC method and combining it with one step pre-purification by ammonium sulfate deposition method. We also tried to purify this protein with the highest purity and higher efficiency [15-19].

Experimental

Protein expression

The most commonly used host for expressing the *Escherichia coli*, the strain of *Escherichia coli* BL21 Rosetta (DE3), was used to express the mutant streptokinase protein. First, in a 5 cc TY2x medium containing kanamycin antibiotic 50 mL/μg, a unique culture was prepared overnight. After 24 h, after transferring the vertex tube, 100 μL of this medium was transferred to 50 mL of fresh and warm TY2x medium containing kanamycin antibiotic and stirred at 150 rpm at 37 °C until ambient light absorption at 600 nm (600 OD) (0.4 to 0.6).

To produce protein and induce the protein expression in large quantities, 50 μL of stock (M1 IPTG) was added to the culture medium to increase its concentration in 1 mM medium. The medium environment-friendly was returned to the incubator again and incubated for 3–4 h at 150 rpm at 37 °C. After incubation, the OD600 medium was re-recorded.

Protein purification

Cell failure was performed by cell lysis (8 mM urea + 10 mM tris + 100 mM sodium dihydrogen phosphate). To prepare the cell extracts from 50 mL TY2x culture medium, the culture medium was pre-divided into two falcons and centrifuged at 5000 rpm for 10 min. The supernatant was discarded, and the precipitate was kept. For precipitation, 5 mL of lubricating buffer was added. After one to two hours, the crystalline contents of the falcon were centrifuged at 5000 rpm for 10 min, and the supernatant was kept in the freezer after separation. The cell extract obtained was used in three strategies.

1: Hydrophobic interaction chromatography

2: Immobilized metal affinity chromatography

The first strategy is the use of affinity chromatography without pre-purification, and the second strategy is the use of ammonium sulfate precipitation as the pre-purification and the affinity chromatography as the final purification. Both of these solutions were compared with the primary approach used in the industry.

Purification by hydrophobic interaction method (HIC)

Gel filtration chromatography was performed using sephadex-25 G gel. By passing column equilibration buffer (20 mM tris, 0.2 mM sodium chloride and 8.5=pH), the sample was prepared for injection, and then 5 mL of sample was slowly loaded into the column by the sampler.

Purification by affinity chromatography

One of the important features of this purification system is its high capacity for binding to his-tag proteins (5-10 mL/mg) and insensitivity of the system to the precise 3D structure of the protein. For small-scale denaturing purification, after breaking the cell wall and extracting the cell contents, we centrifuge the liquid and load the supernatant after centrifugation on the chromatography column. After complete removal of buffer (pH 8), urea 8 mM + 10 mM tris + 100 mM sodium dihydrogen phosphate (5 mL sample was injected into the column by the sampler). When the sample was completely removed from the column, the washing buffer passed through the column. In the process of this study, pH of the buffer of the washing step was optimized in the range of 6.3 to 5.3 and a suitable pH of 5.7 was obtained. Figure 2b. demonstrates the effect of pH regulation on the efficiency of the IMAC method. At this point, proteins that are weakly bonded with nickel or have no bond at all with histidine are removed from the column. If the pH of the buffer used in the washout phase is not adequate, the target protein may also be removed from the column, resulting in a negative effect on the final purification. In the wash step, the buffer is introduced into the column at pH 4.5 to remove the target protein. The column outlet was collected in sterilized microtubes in a volume of 1 mL [4, 10].

Pre-Purification by ammonium sulfate deposition and purification by affinity chromatography

After preparation of the cell extract, the product was prepared with a pre-purification step by ammonium sulfate precipitation to enter the IMAC column. The amount of protein in the samples from each step of the process was determined by electrophoresis Figure 5. illustrates these results. The Table 3. also shows the analysis results of the gel through the software.

Protein measurement

Method: SDS-PAGE

To confirm the presence of the protein and to evaluate its value, the samples obtained from each step of the studied strategies were utilized by PAGE-SDS electrophoresis [7].

Optical method for calculating total protein

OD of each sample was measured using a spectrophotometer at 280 nm. The mean ODs were obtained and multiplied by the dilution and extinction coefficient of 0.925 for streptokinase, and the total protein content of the sample was obtained [9].

Calculate the process efficiency for the target protein and its purity

The gel electrophoresis image of each process was analyzed using the Lab image software, and the percentage of protein purity was determined at each step; the purification efficiency was then calculated using the degree of purity.

degree of purity = Total streptokinase/total protein concentration × 100

Yield = Purified protein concentration × degree of purity/total protein concentration

Results and discussion

The results of the electrophoresis demonstrated that the protein was produced properly (Figure 2). In the HIC purification strategy, the urea in the protein solution was first removed (Figure 1a). Then the isolated proteins were isolated from the streptokinase by HIC using the S-650 butyl gel (Figure 1b).
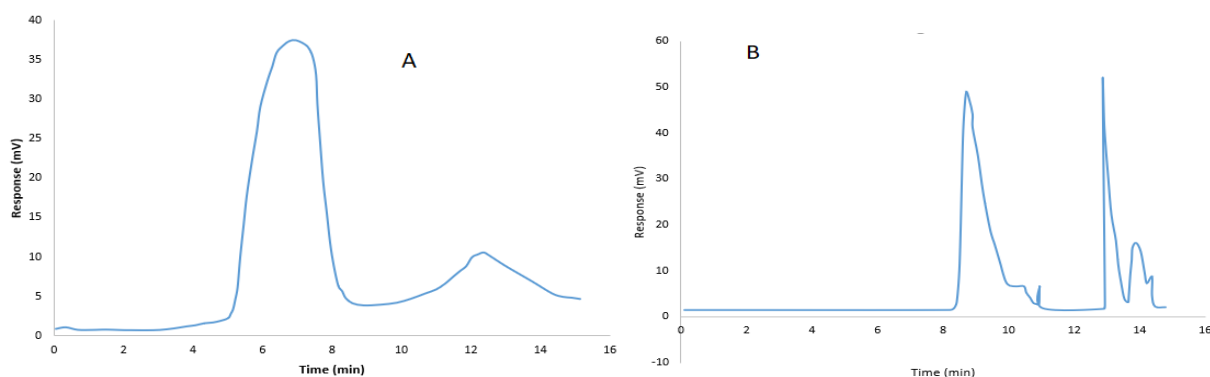

Figure 1. (a) Chromatogram obtained by gel filtration chromatography; **(b)** Chromatogram obtained by HIC

In vitro analysis of purified samples revealed that, all three purification strategies performed well. After optimizing the pH of the wash buffer, the amount of protein produced by IMAC at a concentration of 1.71 mg/mL was higher than the other two methods (Figure 2b). After careful examination using the Lab image software and determination of purity, it was found that the highest purity was related to IMAC 93% method. The results of the electrophoresis showed that, the protein was produced at the appropriate level at the beginning of the work.
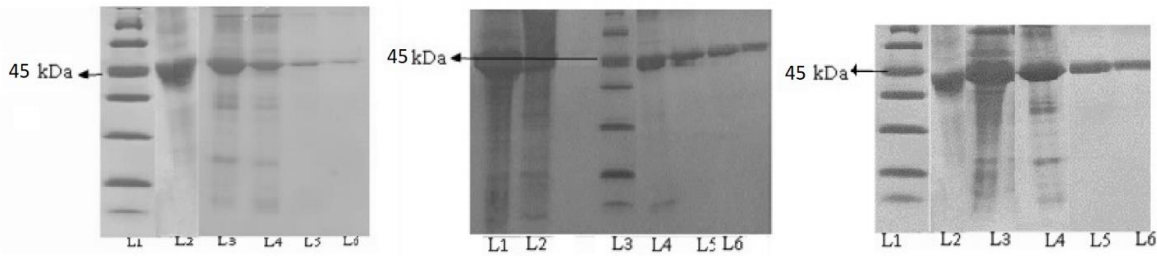
Figure 2. Electrophoresis of the products of the investigated methods. A shows the electrophoresis of purified products by HIC method. L3: protein before purification, L4: sample after gel filtration step, L5: Sample after ammonium sulfate deposition, L6: sample after the.HIC step b. The results of electrophoresis of the products show the method (IMAC method 1). L2: sample before purification, L4: sample (1) wash buffer purification (pH = 6.3), L5: sample (2) Purification by washing buffer   pH = 6, L6: Sample (3) Purification by washing buffer, L7: (pH = 5/7) sample (4) purification by washing buffer (pH = 5/3 C. Results of electrophoresis of the products of pre-purification by ammonium sulfate precipitation and purification by IMAC method) shows the two columns in the image, L3: protein before purification, L4: Sedimentation step protein, L5: First output, L6: IMAC second output IMAC

Measurement of protein concentration by the HIC method demonstrated that, the highest amount of protein and the highest yield was obtained by the IMAC method, while the lowest yield was obtained by combined IMAC method and deposition with ammonium sulfate (Figure 3a and b).
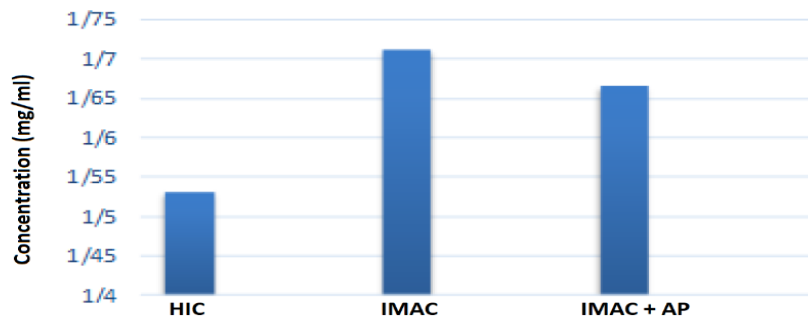
(a)
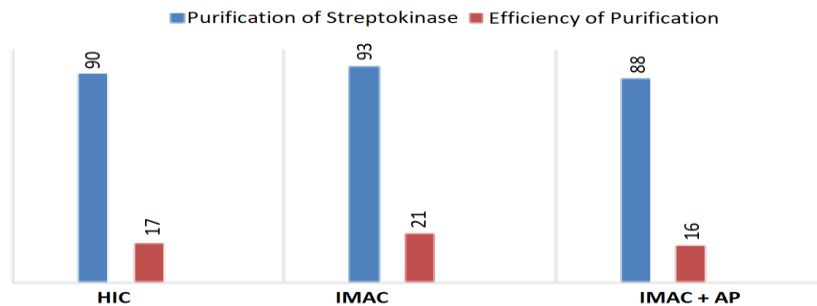
(b)

Figure 3. Comparison of Purification Results with Different Methods; **a):** Comparison of protein levels with three purification methods; **b):** Comparison chart and purity efficiency with three purification methods

Conclusion

The results of this study revealed that, the IMAC method had a higher efficiency than HIC in purification of streptokinase with the histidine tag. Also during the IMAC process based on the results, it was found that the pH of the wash buffer for the purification of streptokinase protein was 5.7.

Ethical clearance

This project did not need any ethical permission.

Source of funding

Conflict of Interest:

We have no conflicts of interest to disclose.

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