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Comparative Study of Two Tenecteplase Therapeutic Protein Purification Methods

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Abstract

Therapeutic Tenecteplase (TNKase) is a recombinant and site directed mutant version of human tissue plasminogen activator (TPA) with clinical advantages over TPA. Due to specific glycosylation, TNKase is preferred to over-express in mammalian cell lines such as Chinese Hamster Ovarian (CHO) Cells. The production and purification of this protein need huge efforts and costs, which directly increase the end product price and limits its medical applications in developing countries despite its benefits. In the current study, we compared two purification methods in order to minimize purification steps as well as purification costs. In the first method, DMEM medium containing CHO-C111 cells expressed recombinant TNKase was purified by a three columns protocol including Sephadex® G-10, HiPrepTM CM FF and L-lysine HyperD®. In the second method, because of its properties, only L-lysine HyperD® column was applied for purification of protein molecules with the lysine binding site, including TNKase. Our results showed that in the second method, higher purification fold and purification yield (1.14 and 1.25 times, respectively) have achieved compared to the first method. This finding in addition to reduction in purification steps, purification cost and time, make it possible to use this method for purification of TNKase. In addition, we suggest overexpressing this protein in serum-free cell lines such as CHO-DG44 in order to minimize impurities and make purification procedure easier.

Keywords: Human tissue plasminogen activator, Purification, Tenecteplase

1. Introduction

Tenecteplase (TNKase, DP00031) is a recombinant and site directed mutant version of human tissue plasminogen activator (tPA, EC 3.4.21.68) and is a member of the serine protease superfamily (1). Like tPA, TNKase contains 527 amino acid residues with 60-70 kDa molecular weight (2, 3). Human tPA is secreted by many cells e.g., endothelial, vascular smooth muscle and neural cells as well as monocytes and fibroblasts as a 562 amino acid residues native protein. The native protein undergoes processing via removing signal peptide and activation through binding to fibrin, thereupon dissolves clots via converting plasminogen (inactive proenzyme) to plasmin (active enzyme). The exact tPA activation process is yet to be known, however upon binding to fibrin, a molecular cleavage triggered by plasmin at R275-I276 of tPA molecule, which converts it into a two chain active molecule (Figure 1). The heavy chain (chain A) has 275 residues and approximately 38 kDa molecular weight and interferes in binding to fibrin, plasminogen activation and plasma clearance (4). This chain contains 4 domains: Fibronectin Finger Like Domain (Finger Like) starts at V4 and ends at S50 has a role in binding with fibrin. EGF like domain is conserved in many animal proteins and is homologous with epidermal growth factor, which contains three disulfide bonds. This

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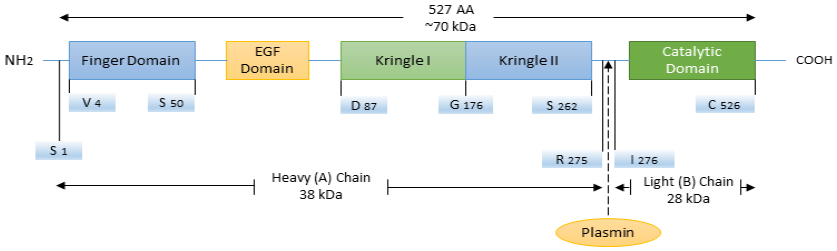

Figure 1. Schematic structure of TNKase molecule and its domains. Small pale blue boxes show the residues and their positions. Plasmin triggers cleavage at R275-I276 position and activates TNKase.

domain has a role in tPA uptake by hepatocytes and plasma clearance. Kringle Domain I (K1) starts at D87 and ends at E175. At TNKase K1 domain, three directed mutations have been introduced compared with native tPA: T103N, N115Q and N117Q. The T103N mutation decreases plasma clearance thus increasing the half-life of TNKase. The N115Q mutation has introduced in TNKase to compensate reduced fibrinolytic activity caused by T103N mutation. All these mutations can increase specify of TNKase compared with tPA to convert human plasminogen into plasmin with the outcome of dissolving blood clots (5).

The Kringle Domain II (K2) starts at G176 to S262. This domain also has a role in binding to fibrin. The binding of tPA to fibrin can be prohibited by its analogue, amino caproic acid. This is because of the presence of Plasminogen Like Lysine Binding Domains (PLLBD) on K2 (6, 7).

Light chain (chain B) starts at I276 and ends at P527 and has three key residues (S477, D371 and H322) of the serine protease family. This chain contains merely the catalytic domain. In TNKase, three direct mutations have introduced at L296, H297, R298 and R299 (4). All the residues have been substituted with alanine to make TNKase resistance to the inhibitor, Serpin E1.

Both intact and cleaved tPA are capable to split plasminogen at R561-V562 position, however, tPA activation increases in the presence of plasmin and cleavage of tPA molecule. tPA has one O-linked glycosylation and three N-linked glycosylation sites. The O-linked glycosylation site at T61 (EFG like domain) facilitate tPA uptake by hepatocytes. Up to now, two glycoform variants of tPA are found. In type I, glycosylation at N117 in K1 domain, N184 in K2 domain and N448 in the catalytic domain, while in glycoform II, glycosylation happens merely at N117 and N184 (6).

Due to its pharmaceutical properties and convenience use, the therapeutic TNKase is used as a thrombolytic treatment of acute ischemic stork (2). However, this drug is extremely expensive, as the time of writing this article in June 2021 the price of one intravenous kit of 50 mg TNKase is approximately USD 6,500 at https://www.drugs.com/price-guide/tnkase. This high price limits the usage of this invaluable medicine in many developing countries. Since a large portion of production cost relates to purification steps, therefore, minimizing purification steps can largely reduce the end product. In the current study, we compare two TNKase purification method with an eye on increasing the purity and decreasing the time and necessary funds for purification.

2. Material and methods

ÄKTA avant 150 FPLC system (GE Healthcare Bio-Science, Uppsala, Sweden) used in all chromatography steps.

2.1. Purification method I

2.1.1. Preparation of TNKase containing medium for purification

Two-chain TNKase was expressed in Chines Hamster Ovary Cell C-111 (CHO-C111) in DMEM medium containing 2.5 % FBS (8) and kindly provided by Royan Institute for Biotechnology, ACECR, Isfahan, Iran. One lit of fresh DMEM medium containing recombinant TNKase was centrifuged at 4000 g and 4 ºC for 20 min (Sigma 3-30 KS, Germany) to remove any debris. Then the medium was concentrated to 80 mL final volume using Amicon® Ultra centrifugal filter units Ultra-30, MWCO 30 kDa (Merck Millipore Ltd., Ireland), at the same conditions mentioned above. After centrifugation, TNKase

containing medium transferred to dialysis tubing (Sigma-Aldrich, USA) and dialyzed against total 10 lit sodium phosphate buffer 20 mM pH 6.5 at 4 ºC overnight. The buffer changed every 4 h and was magnet stirred continuously at 100 rpm. After buffer exchanged, TNKase containing medium was concentrated to 20 mL by centrifugal device as mention above.

2.1.2. Phenol red removal step

For this step, 50 mL Sephadex® G-10 (Pharmacia, UK) column was equilibrated with 3 bed volume sodium phosphate buffer 20 mM pH 6.5 at 0.5 mL/min flowrate. Concentrated sample was injected into column with the same flow rate. The eluted fractions were collected in 3 mL volumes. 250 µL aliquate of each fraction was added to 750 µL Tris-HCl 50 mM pH 8.5 and measured at 570 nm to detect fractions with phenol red and ensure the absence of phenol red in protein elutes fractions. In addition, each fraction was checked for TNKase activity and fractions containing TNKase were pooled and used for next step.

2.1.3. FBS removal step

In this step, the pooled fractions with TNKase activity from previous step, were loaded onto HiPrepTM CM FF 16/10 20 mL vlome which was pre-equilibrated with sodium phosphate buffer 20 mM pH 6.5 at 1 mL/min flow rate. The unbound proteins were washed with 3 bed volume of the same buffer at 2 mL/min flow rate. The elution of bound protein achieved by three-bed volume of linear ascending 0-1 M NaCl in sodium phosphate buffer 20 mM pH 6.5. Eluted proteins were collected in 3 mL fractions and each peak fraction checked with dot blotting and activity assay to confirm the presence of TNKase. The fractions containing TNKase were pooled and desalted with sodium phosphate buffer 20 mM 0.043% Polysorbate pH 7.2 and used in the next purification step.

2.1.4. TNKase purification with L-lysine HyperD®

Twenty-five mL of L-lysine HyperD® (Sartorius, Germany) was packed in XK16 column (GE Healthcare, USA) and was equilibrated with sodium phosphate buffer 20 mM 0.043% Polysorbate pH 7.2 at 2 mL/min flow rate. Pooled and desalted fractions from previous step was loaded into the column at 1 mL/min flow rate. Unbound proteins were washed out by 3 bed-volume of the same buffer. Six bed-volume of linear ascending gradient 0-1 M NaCl in above buffer was used to elute weakly bound proteins. In the next step for elution of TNKase, five bed-volume of linear ascending gradient of 0-200 mM amino caproic acid (ε-ACA) and 0-500 mM L-arginine in sodium phosphate buffer 20 mM 0.043% Polysorbate pH 7.2 was used. Fractions were collected in 3 mL volume and checked with dot blotting, activity assay and SDS analysis for the presence of TNKase.

2.2. Purification method II

2.2.1. Preparation of TNKase containing medium for purification

In this method, one lit of the DMEM medium containing recombinant TNKase was concentrated to 80 mL final volume as mentioned before. The concentrated medium then was dialyzed against 10 lit sodium phosphate buffer 20 mM pH 7.2 containing 0.043% Polysorbate with the same condition as mentioned before. The dialyzed sample then was diluted with 80 mL of the same buffer and pH was adjusted to 7.2.

2.2.2. TNKase purification with L-lysine HyperD®

Fifty mL of L-lysine HyperD® (Sartorius, Germany) was packed in two XK16 (GE Healthcare, USA) connected continuously and was equilibrated with sodium phosphate buffer 20 mM pH 7.2 containing 0.043% Polysorbate at 2 mL/min flow rate. The dialyzed and diluted sample from previous step then was injected into this column at 1 mL/min flow rate.

Unbound proteins and any residues of phenol red were washed out by four-bed volume of the same buffer. Then Six bed-volume of linear ascending gradient 0-1 M NaCl in above buffer was used to elute weakly bound proteins. In the next step for elution of TNKase, five bed-volume (250 mL) of linear ascending gradient of 0-200 mM ε-ACA and 0-500 mM L-arginine in sodium phosphate buffer 20 mM 0.043% Polysorbate pH 7.2 was used. Fractions were collected in 3 mL volumes and checked with dot blotting, activity assay and SDS analysis for TNKase.

2.3. SDS-PAGE analysis

Twenty µL of sample containing 30 µg protein was mixed with 5 µL non-reducing sample buffer and heated at 99 ºC for 5 min. After a brief spin down, sample electrophoresed on 12% poly acrylamide gel at 120 V and visualized with coomassie blue reagent.

2.4. Western and dot blotting

Anti-TPA Tissue Plasminogen Activator monoclonal antibody [2A153] ab21049 (abcam®, UK) and Anti-Mouse IgG A9044 (Sigma-Aldrich, USA) were used as primary and secondary antibodies, respectively, according to standard protocols as follow: separated proteins bands on SDS-PAGE gel were transferred to AmershamTM Hybond P 0.2 PVDF Western blotting membrane (GE Healthcare, Germany) followed by blocking in 5% skimmed milk in TBST buffer. The PVDF membrane was then incubated in TBST buffer containing 1 µg/mL primary antibody for 1 h. Then the secondary antibody was used at 1:300,000 concentration for 1 h. The AmershamTM ECLTM Prime Western Blotting Detection Reagent (GE Healthcare, Italy) was used for detection and AmershamTM HyperFilmTM ECL (GE Healthcare, UK) was used for recording. For dot blotting, 2 µL of the samples were directly transferred to PVDF membrane and after drying, the same procedure as above was carried out for detection.

2.5. The Enzyme assay

The TNKase assay was performed by monitoring p-Nitroaniline formation upon degradation of Chromogenix S-2288 (Chromogenix, USA) as a non-selective substrate in 180 s at 405 nm. Metalyse® 10,000 UI/ 50 mg (Behringer Ingelheim, Germany) was used as standard for enzyme assay. The standard solution of Metalyse® prepared with different concentrations containing 1, 2, 3 and 4 units of enzyme. The reaction buffer contains Tris-HCl 100 mM and 106 mM NaCl pH 8.4. The reaction mixture contained 200 µL of reaction buffer, 400 µL distilled water and 200 µL of standard Metalyse® solution and 200 µL substrate solution containing 1µM S-2288. All these ingredients were pre-heated at 37 ºC and the temperature was kept at 37 ºC during the incubation time for 180 s. Every 60 s, 50 µL of the reaction mixture was removed and added to 450 µL of 80 ºC preheated distilled water containing 40% acetic acid to simultaneously stop and dillute the rection. The production of p-Nitroaniline was measured at 405 nm and used for plotting the standard curve. The same reaction was carried out for each sample of cell culture and purified TNKase and then compared with the standard curve to determine the activity.

3. Results and discussion

3.1. Desalting and phenol red removal

Sephadex® G-10 (Pharmacia, UK) is well suited for desalting, buffer exchanging and removal of phenol red in cell culture media. In the current study, this size exclusion resin was used to remove phenol red as well as buffer exchanging and desalting, simultaneously, prior to the ion exchange chromatography column. The used buffer was sodium phosphate buffer 20 mM pH 6.5, which is the buffer to be used for equilibration of carboxymethyl resin in the next step. Figure 2 shows the chromatogram of phenol red removal using Sephadex® G-10. The first 7 fractions (about 21 mL)

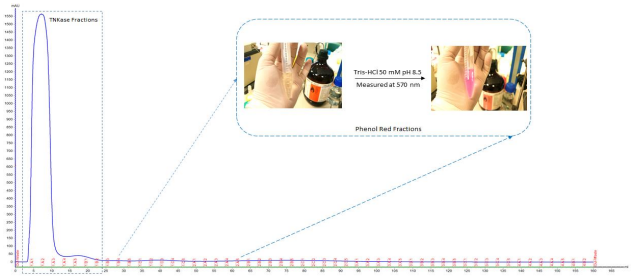

Figure 2. Sephadex® G-10 chromatogram. TNKase and phenol red fractions are labeled accordingly. The presence of TNKase in fractions was confirmed by enzyme activity assay and dot blotting. The presence of phenol red in fractions was confirmed by adding Tris-HCl pH 8.5 and measuring absorbance at 570nm.

were checked with SDS-PAGE and enzyme assay and confirmed the presence of TNKase. These fractions were pooled and used for the next step. The presence of Phenol red in each fractions was determined by adding Tris-HCl 50 mM pH 8.5 and absorbance measured at 570nm. Results showed the presence of phenol red in eluted fractions between 30 and 62 mL. Although CDC does not list the Phenol Red as a carcinogenic agent, its cytotoxic effects have reported before (9), thus using a phenol red free medium for production of therapeutic recombinant proteins is highly beneficial. In addition applying a phenol red free medium can reduce purification cost.

The used DMEM cell culture medium contained 2.5% FBS. Removing the FBS from the product is one of the most challenging steps in protein purification procedure. Albumin as the most abundant protein in FBS has 66.5 kDa molecular weight and overlaps with TNKase band on SDS-PAGE analysis. The best advantage that can be used to separate TNKase from albumin is the pI deference of these two proteins, as the pI of albumin is 4.7-5.5 and the pI of TNKase is 7.61, which is far above albumin.

In general, proteins in an environment with pH lower than their pI will be protonated and positively charged, but in an environment with pH higher than their pI will be deprotonated and negatively charged, it is the foundation of protein separation using an ion exchange chromatography method (10, 11).

In the current study, a carboxymethyl cation exchange chromatography resin, HiPrepTM CM FF 16/10 was used and equilibrated with phosphate buffer 20 mM pH 6.5. At this pH, TNKase is positively charged and will bind to carboxymethyl ligands, while albumin and other unbound proteins that are negatively charged were washed out. Subsequently, condition altered by ascending gradient of sodium chloride 0-1 M, in which TNKase molecules were eluted at about 150 mM sodium chloride gradient and were separated from other bound proteins (Figure 3).

In the next step, the fractions containing TNKase were pooled and desalted using sodium phosphate 20 mM pH 7.2 containing 0.043% poly sorbate and Amicon® Ultra centrifugal filter units Ultra-30, MWCO 30 kDa. The same buffer was used for equilibration of L-lysine HyperD® resin. This resin acts as an affinity chromatography as well as a cation exchange chromatography. L-lysine ligands were bonded to the Kringle II domain of TNKase molecules via its affinity properties and also to other positively charged proteins in the sample. Other unbound proteins were washed out.

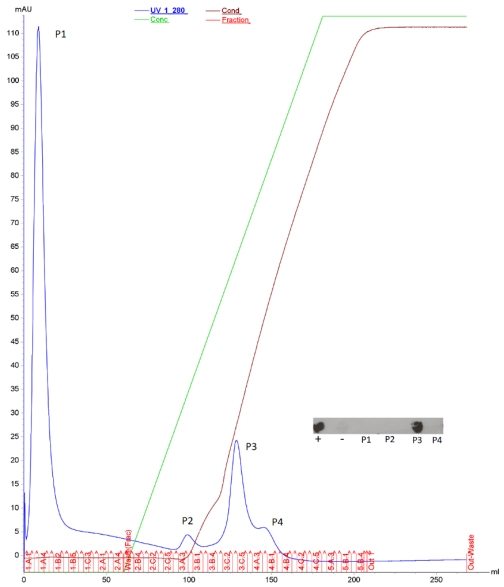

Figure 3. FBS removing by HiPrepTM CM FF 16/10. P1 shows unbound proteins including albumin washed out of column. P2, P3 and P4 are eluted proteins with NaCl ascending gradient. The Dot blotting analysis confirmed the presence of TNKase in P3. Symbols: P: chromatogram peak, +: positive control (Metalyse®), -: negative control (unexpressed CHO cell culture).

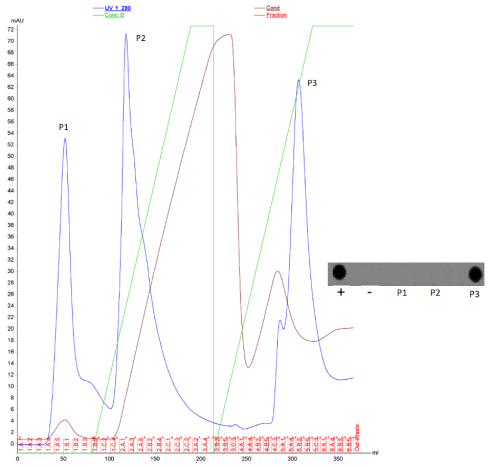

Figure 4. L-lysine HyperD® chromatogram. P1 shows unbound proteins to L-lysine HyperD® column and P2 shows eluted proteins by NaCl ascending gradient. For both P1 and P2 the absence of TNKase confirmed by dot blotting and activity assay. P3 shows eluted proteins by ε-ACA and L-arginine ascending gradient. The presence of TNKase in P3 confirmed by dot blotting and activity assay. Symbols: P: chromatogram peak, +: positive control (Metalyse®), -: negative control (unexpressed CHO cell culture).

In the first step of elution, an ascending gradient of NaCl (0-1 M) was used to elute and wash cationic and weakly bound proteins and any other residues of albumin. In the second step of elution, ascending gradients of ε-ACA and L-arginine were applied. In this step, TNKase molecules which were bound to L-lysine ligand via Kringle II domain L-lysine Binding Site (LBS) were eluted (Figure 4).

Ɛ-ACA is an analogue and derivative of L-lysine (12), which make it able to bind to specific residues in Kringle II domain LBS and in substitution with L-lysine at higher concentration (13, 14). Technically, L-lysine Hyper D® resin is useful in the purification of many biological molecules such as plasminogen and plasminogen activators from human and animal samples. This resin also is used in plasmin free serum production for further use in culture media. In addition, different subclasses of plasminogen and plasminogen activators could be separated by gradient of ε-ACA. This property of ε-ACA makes it invaluable in purification of TNKase and other tissue plasminogen activators.

In the second purification method, with an eye on reducing purification steps, increasing purification yield and reducing purification costs,

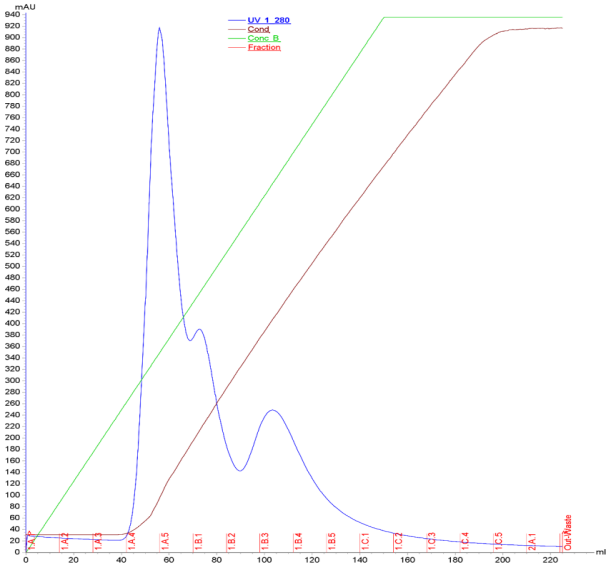

Figure 5. L-lysine HyperD® chromatogram elution with NaCl ascending gradient in 2nd purification method. The absence of TNKase in eluted peaks was confirmed by dot blotting and activity assay.

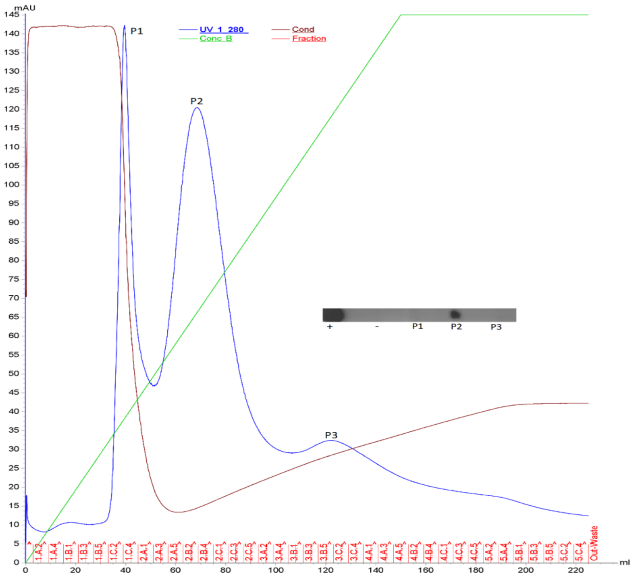

Figure 6. L-lysine HyperD® chromatogram elution with ε-ACA and L-arginine ascending gradient in 2nd purification method. The presence of TNKase in P2 confirmed by dot blotting and activity assay. Symbols: P: chromatogram peak, +: positive control (Metalyse®), -: negative control (unexpressed CHO cell culture).

a single purification step by using only L-lysine Hyper D® resin was performed.

In this step after loading samples onto the L-lysine Hyper D® column, phenol red and any other unbound proteins were washed out. Then, 6 bed-volume of ascending gradient of NaCl (0-1 M) was used to remove weakly bound proteins to this resin (Figure 5).

In the next step, in order to separate TNKase and other classes of proteins which bind to L-lysine ligands via LBS, an ascending gradient of ε-ACA (0-200 mM) was used (Figure 6).

Figure 7A shows the SDS-PAGE analysis of cell culture medium before and after expression of TNKase. Since the medium contains 2.5% FBS, in both analysis bands about 60 kDa is visible, while Figure 7B shows purified proteins from both purification methods. Figure 7C western blot

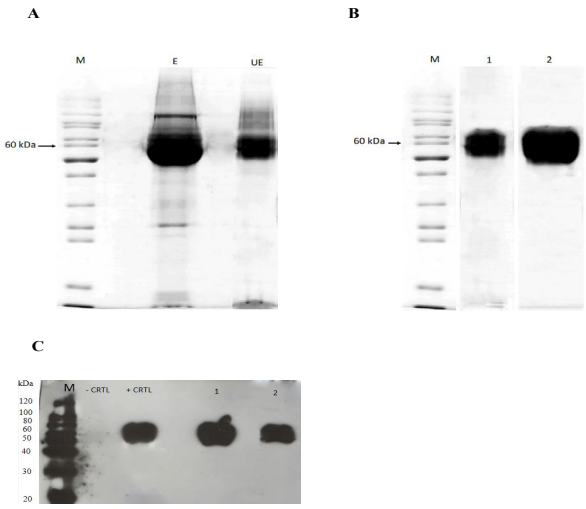

Figure 7. Figure 7A shows the SDS-PAGE analysis of TNKase expressed CHO-C111 cell line culture media supernatant (E) and CHO-C111 cell line culture media supernatant with no TNKase gene (UE) as negative control prior to the purification process. Figure 7B shows the SDS-PAGE analysis of the purified TNKase from the first purification method (1) and the second purification method (2). Figure 7C shows western blot analysis of negative control (-CRTL), positive control (+CRTL), purified TNKase from the first (1) and the second (2) purification methods.

Table 1. The purification table and summery of two purification methods.

Methods	Steps	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification Fold	Yield (%)
1st Method	Supernatant	490	2790	5.69	1	100
	HiPrepTM CM FF	23.64	1469	62.16	10.92	52.65
	L-lysine HyperD®	5.34	865.08	162	28.47	31
2nd Method	Supernatant	545.2	2986	5.47	1	100
	L-lysine HyperD®	6.3	1164.54	184.84	32.48	38.98

analysis of both purification methods.

Table 1 shows the summery of these two purification methods. As it is shown in this Table, in the second method, higher purification fold and purification yield (1.14 and 1.25 times, respectively) have achieved compared to the first method. This finding in addition to reduction in purification steps, purification cost and time, make it possible to use this method for purification of TNKase.

As it is shown in table 1, in the 2nd method, higher purification fold and purification yield (1.14 and 1.25 times, respectively) have achieved compared to the first method. This finding in addition to reduction in purification steps, purification cost and time, make it possible to use this method for purification of TNKase.

4. Conclusion

In the current study, purification of TNKase carried out by two modified methods in order to find the best method to minimize purification cost. All steps of the purification performed under cold chain. The recombinant CHO-C11cell line was cultured in DMEM medium containing phenol red and 2.5% FBS. Removing phenol red and FBS is a challenge in purification of therapeutic proteins (15) and performed successfully. We suggest the using of phenol red free DMEM medium and serum free CHO cell lines such as CHO-DG44 cell line, not only to reduce purification cost but also an easier and more effective purification strategy. One advantage of our current method is using Lysine HyperD® resin, which is excellent in binding to the Kringle domain of molecules such as plasminogen, plasminogen activator and TNKase. One of the other usage of this resin is producing plasmin free serum for further use in culture media (16). In addition, due to Lysine HyperD® resin properties (17) it is a good candidate for industrial purification process.

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Conflict of Interest

None declared.

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