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Research Article

PEGYLATION OF RECOMBINANT MUTEIN STREPTOKINASE FROM OVERPRODUCTION IN *ESCHERCHIA COLI* BL21 AND STUDY ON THE FIBRINOLITYC ACTIVITY *IN VITRO*

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ABSTRACT

Introduction: Streptokinase is a therapeutic protein with potent thrombolytic activity. However, its use as a therapy is limited in particular by short biological half-life due to plasmin degradation *in vivo*. Approaches that had been developed to overcome this problem were through structural modification both chemically and genetically (mutation). Chemical modification via PEGylation process has shown better stability as indicated by longer half life and better trombolitic activity. The aim of present study is to pegylate recombinant mutein streptokinase.

Methods: The recombinant mutein streptokinase was overproduced in *E. coli* BL21. PEGylation of protein consisted of two steps: activation of PEG with 1,1 'carbonyl diimidazol and conjugation of activated PEG to protein. Various factors influenced pegylation process were including incubation time and molar ratio of protein:activated PEG. Fibrinolytic activity of pegylated mutein streptokinase (PEG-streptokinase) was studied *in vitro* for blood clot lysis.

Result: Optimum condition for PEGylation process is incubation for 48 hours with molar ratio streptokinase:activated PEG 1:400. Study on the fibrinolytic activity in vitro showed that PEG-streptokinase has better fibrinolytic activity than non-pegylated form.

Conclusion: It can be concluded that recombinant mutein streptokinase was successfully pegylated with preserved and even prolonged *in vitro* fibrinolytic activity as compared to non-pegylated form.

Keyword: PEGylation, Streptokinase, Mutan K59Q-K386Q streptokinase, Fibrinolytic, Clot lysis assay.

INTRODUCTION

The lifestyle changing and food consumption in urban world nowadays have increased the risk of brain infarction (stroke) and myocardial infarction (heart attack) which are the leading cause of death world wide. The therapies for these two fatal infarctions are currently emphasized on how to minimaze tissue infarct in which severe cases can lead to death or permanent disability. Hitherto, preference of drugs opts to thrombolytic agent for its fast acting in dissolving thrombus. Optimum results from the therapy are only gained when the thrombolytic agents are administered as soon as the onset begins (Banerjee et al, 2003).

Some of commercial thrombolytic agents known are urokinase (uPA), tissue plasminogen activator (tPA), and streptokinase (sPA), which act as plasminogen activators. tPA and uPA are endogenous potent thrombolytic agents, and immunogically inert. On the other hand, streptokinase is exogenous thrombolytic agent in clinical practice, has comparative potency as endogenous agents, but no risk free. Administration of streptokinase to the circulatory system can illicit immune response and may cause hemorrhage due to its fibrin independent plasminogen activation mechanism (Reed et al, 1999; Reddy and Markus, 2000; Young et al, 1995 and 1998). In addition, its half time only about 23 minutes in circulation, which is not effective for therapy. Despite of its significant limitations, streptokinase remains the preferred therapeutic drug particularly for developing countries like Indonesia. This because of limitation of the available plasminogen activators, and streptokinase shows potent plasminogen activator with minimal side effects and lower price.

Due to short circulation half life, researchers in many countries and also in our laboratory have focused on structurally modifying streptokinase to extend its half life, to reduce its immunogenicity and to improve its plasminogen activation (Wu et al, 1998). Structurally modified streptokinase has been attained in several ways including genetic approach and chemical modification. The chemical modification of streptokinase is performed by complexion with polymers such as polyethylene glycol (PEG), and has been used and proved for extending its half life in circulation, and reducing immunogenicity.

PEGylation is a modification of protein, peptide or non-peptide molecule by the linking of one ore more polyethylene glycol (PEG) chains. The chemical attachment of PEG to therapeutic proteins produces several benefits, including enhanced plasma half life, lower toxicity, increased drug stability and solubility, and a decreased degradation by metabolic enzymes (Zeng et al, 2012). In certains instances, PEGylation of protein can increase its therapeutic efficacy by reducing the ability of the immune system to detect and mount an attack on the compound (Abuchowski et al, 1977; Harris et al, 2001,2003; Mehvar, 2000; Prajapati et al, 2008). PEGylation now plays an important role in drug delivery, enhancing the potentials of peptides and protein as therapeutic agents such as streptokinase.

PEG molecule specifically forms a covalent bond with free functional groups, like amines, thiols, or carboxylic acids on the surface of proteins. Therefore, one important requirement for pegylation is the presence of amino acid residue with an exposed amine or thiol group at the protein surface. Amino acid residues which can be act as conjugation targets are lysine, cysteine, arginine, N-terminal or hydroxyl group. As PEG is non reactive molecule, activation of this substance will initiate the conjugation reaction. The successful results only can be generated if activated PEG couple with a properly folded active side of the protein. But reactivity of target protein varies in response to local chemical environments created by neighboring amino acids at the surface of the folded polypeptide.

Rajagopalan *et al*., 1985, compared PEGs with average molecular weights of 2000, 4000, and 5000 conjugated to streptokinase. The results suggested that complex PEG 2000-streptokinase is the most resistant to plasmin cleavage, also the complex performed greatly reduced antigenicity and slower clearance rate than unmodified streptokinase. The current method of PEGyaltion in streptokinase utilizes CDI to activate PEG by synthesis an imidazole carbamate, derivative of PEG that can be subsequently react with lysine residues in substrate.

In present study we report PEGylation of recombinant mutein streptokinase obtained from overproduction in *E. coli* BL21 (Martius et al, in press). We studied on the effect of molar ratio of activated PEG:protein on degree of PEGylation. Fibrinolytic activity of pegylated streptokinase was then performed *in vitro* using blood clot lysis assay and compared with activity of non-pegylated form.

MATERIALS AND METHODS

Materials

Materials has Pro analysis and biology molecular quality: *Escherichia coli* BL21 contains pET-32b *ska* K59Q-K386Q plasmid, Luria Bertani medium for *E. coli* BL21 growth (yeast extract (Difco), tryptone (Difco), sodium chloride (Merck)), ampiciline (Sigma), isopropyl thio-β-D-galactoside (IPTG, Sigma), Sodium Dodesyl Sulphate (SDS, Merck), Sodium hydroxide (Merck), alcohol 70 %, dioxane, 1,1'-carbonyl diimidazole (CDI, Sigma), Polyethylene glycol 2000 (Sigma), Boric acid (Sigma), Phenylmethyl Sulfonyl Fluoride "PMSF" (Merck), protein marker (Amersham), Sterile aquadest, aquabidest, deionized water, coomasie brilliant blue, Dragendorff reagent (Bismuth subnitrate, nitrate acid dan kalium iodide) and materials for SDS-PAGE (acrylamide, bis-acrylamide, separating buffer, stacking buffer, SDS 10 %, ammonium polysulphate).

Recombinant ska K59Q-K386Q

E. coli BL21 containing pET-32b *ska* K59Q-K386Q was inoculated in 10 mL of liquid LB containing 100 µg/mL ampicilin for 18 jam, at 37 °C and at rotation of 150 rpm. One mL of inoculums then reinoculated in 250 ml LB medium with 100 µg/mL ampicilin, for 2 h until OD_{600} reached 0.7. Subsequently, 0.5 mM IPTG as an inducer was added and then incubated at 37°C for 3 hours. Cell harvesting was done by centrifugation at 8000 rpm for 20 minutes at 4°C. The pellet was resuspended using 5 mL binding buffer without imidazole, then PMSF was added to obtain final concentration of 1 mM. Cell lysis was performed with sonication at frequency of 4 kHz continuously. Debris cells were separated after centrifugation at 10.000 rpm, 4°C for 20 minutes. Recombinant streptokinase was purified using nickel column and analyzed using SDS-PAGE.

Activation of PEG

PEG was activated by the method of Beauchamp et al. Briefly, Polyethylene glycol (MW 2,000) was dissolved in dioxane at 37°C at a concentration of 50 mM. l,l'-carbonyldiimidazole was added to a final concentration of 0.5 M and the solution was incubated at 37°C for 2 h with stirring. The solutions then were dialyzed extensively against H_20 using Spectrapor membranes. Activated PEG preparations were lyophilized and stored desiccated at 4°C.

Characterization of activated PEG

Ultraviolet (UV) and Infra Red (IR) spectrophotometry

To identify the successful PEG activation i.e the presence of imidazole group at activated PEG 2000, spectrophotometry UV and FT-IR were performed. FT-IR spectroscopy was performed on fourier-transformed infrared spectrophotometer (8400S Shimadzu). The samples (PEG 2000, CDI and PEG-carbonylimidazole) were previously ground and mixed thoroughly with potassium bromide, an infrared transparent matrix, at 1:100 (sample:KBr) ratio, respectively. The KBr discs were prepared by compressing the powders at a pressure of 20 psi for 10 min on KBr-press and the spectras were scanned over wave number range of 4500-500 cm^{-1}.

Melting point test

The purity of PEG-carbonylimidazole was tested using melting point technique. Melting point of PEG 2000 alone was used as a comparison. It is indicated that a pure substance must show a melting point deviation not more than 2°C over its melting point.

Conjugation of activated PEG and recombinant mutein streptokinase

The influence of incubation time and variation of molar ratio between mutant streptokinase and activated PEG was observed.

Influence of incubation time on the PEG-streptokinase conjugation

PEGylation of mutant K59Q-K386Q streptokinase was performed using different concentration of streptokinase and PEG-carbonylimidazole but with same molar ratio (1:100). In this PEGylation process, 5.45 µg PEG-Carbonylimidazole was reacted with 7.5 µg and 30 µg mutant streptokinase in 1 ml boric buffer saline pH 8.5 and incubated for 72 hours at 4°C using digital incubator shaker. Sample was taken each 12 hours for characterization.

Influence of molar ratio of mutant streptokinase and activated PEG on the conjugation

Fixed amount of mutant K59Q-K386Q streptokinase used for this study was 30 µg. The PEG-carbonylimidazole was varied resulting in molar ratio of mutant streptokinase:PEG-carbonylimidazole as 1:100, 1:200, 1:400, 1:500, and 1:600. The reaction was allowed in 1.5 ml boric buffer saline pH 8.5 for 72 hours.

Product of pegylated mutant streptokinase was characterized with SDS-PAGE and protein molecular weight calculated using protein migration analysis by plotting on the semilog curve of molecular weight as function of ratio factor (Rf) of marker proteins.

Fibrinolytic activity test of pegylated mutant K59Q-K386Q streptokinase

Fibrinolytic activity of pegylated mutant K59Q-K386Q streptokinase (PEG-streptokinase) was tested *in vitro* using blood clot lysis assay according to Prasad, *et. al.* with small modification. Human blood (500 µL) was put in pre-weighed sterile microcentrifuge tube and incubated at 37°C for 45 minutes. After clot formation, serum was completely removed (aspirated without disturbing the clot formed) then weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone). Subsequently, 100 µl of solution containing 7.5 µg and 30 µg nonpegylated and pegylated mutant streptokinase were added. The reaction was then allowed at 37°C for 240 minutes. Clot lysis was furtheron recorded. This done by removing the supernatant obtained, then the residue was weighted. The weight different then expressed as percentage of clot lysis. Deionized water, *E. coli* BL 21 protein extracts and imidazole 200 mM served as negative controls.

RESULT AND DISCUSSION

Overproduction and purification of mutant streptokinase

The product of recombinant streptokinase cloning was presented in figure 1. As seen, the protein was shown as thick single band indicating

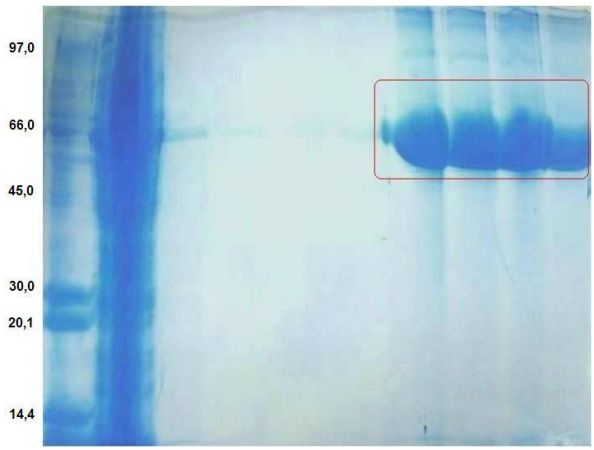

Fig. 1: SDS-PAGE analysis of recombinant mutant streptokinase after purified using nickel column.

Characterization of activated-PEG 2000

UV spectrophotometry analysis

As shown in figure 2, PEG alone does not absorb UV radiation. However, activated PEG exhibited spectra correspond to CDI spectrum with λ_{max} = 274 nm. This indicates that activation of PEG using CDI was successful.

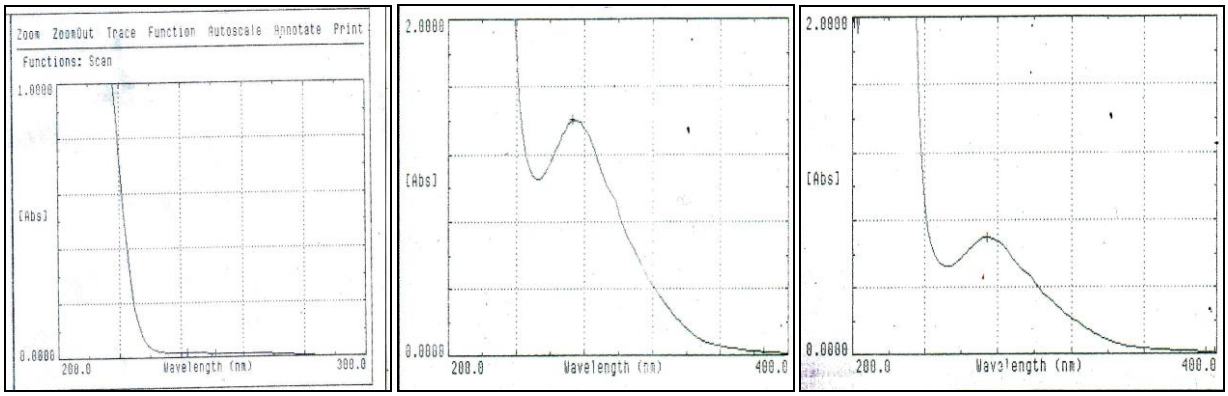

Fig. 2: UV spectra of PEG 2000 (a), Carbonyl diimidazole (CDI, b), PEG-carbonylimidazole (c).

FT-IR spectra analysis

To confirm UV analysis, FT-IR was performed (Fig. 3). As depicted in that figure, the FT-IR spectra between PEG-carbonylimidazole and PEG 2000 were clearly different. The presence of peaks in activated PEG at 2850-3220 cm^{-1} and 1500-1675 cm^{-1} are relevant to carbonyl group (C=O) and double bond group (C=C or aromatic C=N), respectively, the functional groups presence in CDI.

Purity of synthesized PEG-carbonylimidazole was indicated by the value of melting point recorded as 54-56°C (narrow range of 2°C). This means that PEG-carbonylimidazole was free of contaminant especially free CDI or free PEG.

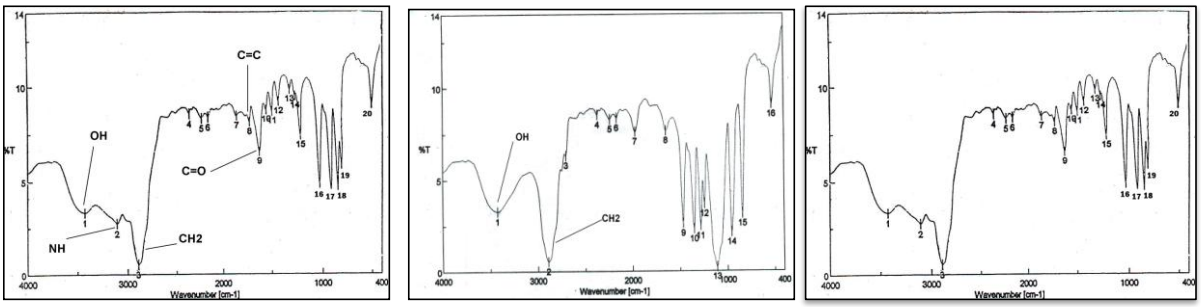

Fig. 3: FT-IR spectra of PEG 2000 (1), Carbonyl diimidazole (CDI, ii), and PEG-carbonylimidazole (iii).

PEG-Streptokinase conjugation

The PEG-streptokinase conjugation was presented in figure 4. As seen, not only the incubation time but also the molar ratio of PEG:streptokinase for conjugation process influenced the degree of pegylation. The conjugation was only occurred after 48 hours, indicated by the increase of molecular weight (figure 4, lane 7). However, prolonging the incubation time up to 60 and 72 hours did not improve the amount of PEG attached to the protein (figure 4, lane 8 and 9). This means, 48 hours is the optimum time for PEG-streptokinase conjugation.

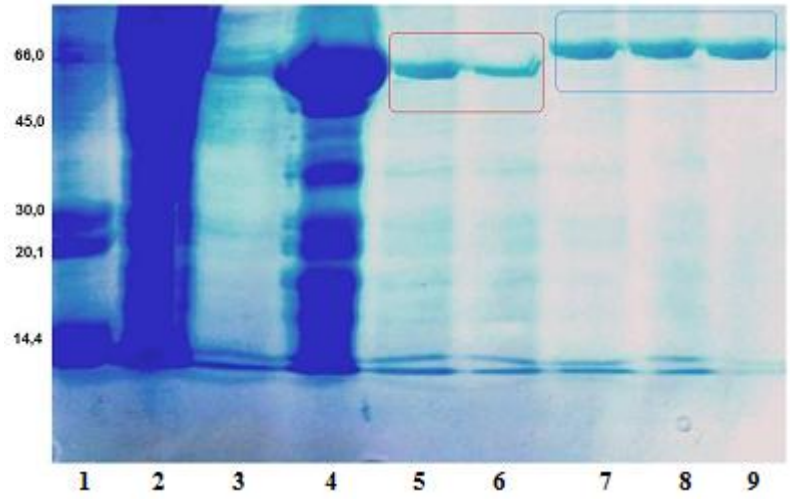

Fig. 4: SDS-PAGE of the samples produced at optimized incubation time optimation: protein marker (1), protein crude (2), elution fraction (3-4), after 12 hours incubation (5), after 36 hours incubation (6), after 48 hours incubation, after 60 hours incubation (8), and after 72 hours (9).

The influence of molar ratio of PEG and protein on the degree of pegylation was shown in figure 5. The concept was the more PEG, the more chances to interact with the protein. However, we observed that any limitation amount of PEG until the maximum attachment of PEG to the protein was reached. The linier correlation between the number of PEG and the degree of conjugation was recorded when the molar ration was 1:100, 1:200, and 1:400 (figure 5, lane 5-7). The higher molar ratio of 1:500 and 1:600, in fact, did not improve the PEG attachment (figure 4, lane 8 and 9). PEG-carbonylimidazole is one of the activated PEG which its targets are amine group of lysine residues or N-terminus of protein with covalent attachment (Rajagopalan, *et al.,* 1985). Lysine residue amounts of mutant K59Q-K386Q streptokinase can influence the degree of PEGylation. Result of amino acid sequencing in our previous work (data not published) showed that mutant K59Q-K386Q streptokinase has 31 lysine residues providing of 31 PEG molecules to interact.

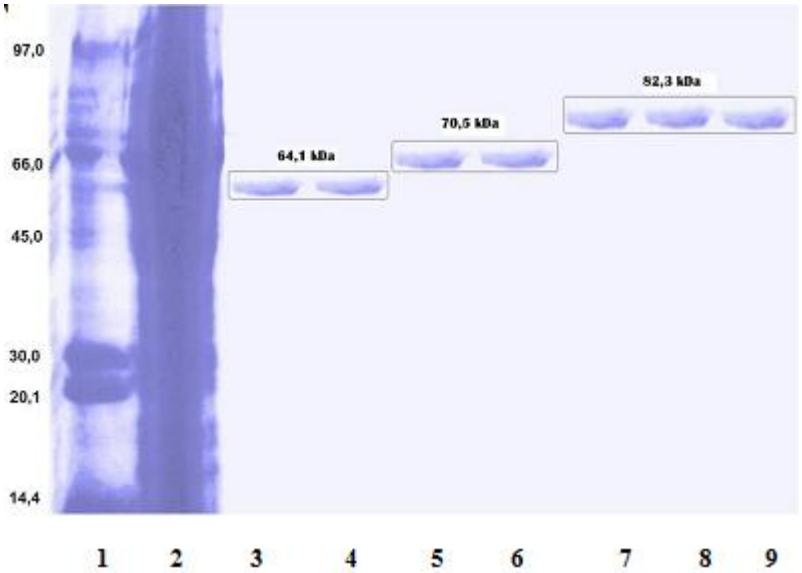

Fig. 5: SDS-PAGE of samples produced at optimized molar ratio of PEG:protein: protein marker (1), crude protein (2), non-pegylated streptokinase (3-4), PEG-protein 1:100 (5), 1:200 (6), 1:400 (7), 1:500 (8), and 1:600 (9).

The amount of conjugated PEG 2000 to streptokinase was determined by calculating the difference of protein MWs between pegylated and non-pegylated streptokinase. Protein MWs was obtained using semilog curve correlation between log MW and ratio factor (Rf) of marker protein. The linier regression equation resulted from the curve was y = -1.006x + 2.079 (r^2 = 0,996). The molecular weight of non-pegylated streptokinase was calculated as 64.117 kDa, pegylated streptokinase (1:100 and 1:200) was 70.477 kDa or corresponds with 3 PEG molecules, and pegylated streptokinase 1:400, 1:500 and 1:600 were 82.184 kDa or corresponds with 9 PEG molecules. Overall, 1:400 was preference molar ratio for PEG-streptokinase conjugation.

As reported by Rajagopalan, *et al.,* 1985, PEG 2000-streptokinase is the most resistant to plasmin cleavage, also the complex performed greatly reduced antigenicity and slower clearance rate than unmodified streptokinase. They did not observe other important factor which influences the degree of PEGylation process. We observed that incubation time and PEG-protein molar ratio are clearly influenced the degree of pegylation.

In vitro fibrinolytic activity of PEG-streptokinase

The issue of protein modification like pegylation is lost or reducing protein activity due to possibly active sites blockade (Veronese, 2001). Thus to evaluate whether our study to attach PEG on target amino acid on streptokinase will influence the streptokinase activity, the *in vitro* fibrinolytic activity was performed. Blood clot lysis assay was used for this purpose (Prasad, 2006). Blood clot lysis is an *in vitro* test method for fibrinolytic or thrombolytic drugs, simple and more economically and usually used. Fibrinolytic activity of pegylated mutant streptokinase was compared with non-pegylated form with concentration of 7.5 and 30 µg. Negative control was provided i.e deionyzed water, *E. coli* BL21 proteins, and imidazole 200 mM.

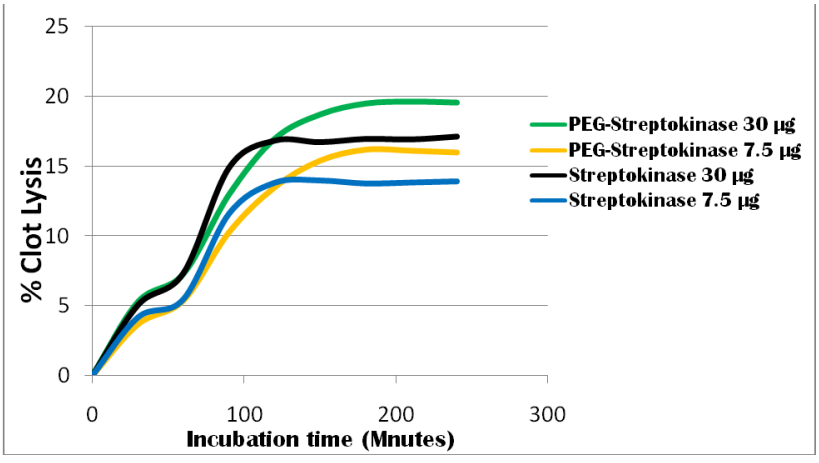

Fig. 6: Fibrinolytic activity of pegylated and non-pegylated mutant K59Q-K386Q streptokinase at molar ratio 1:400.

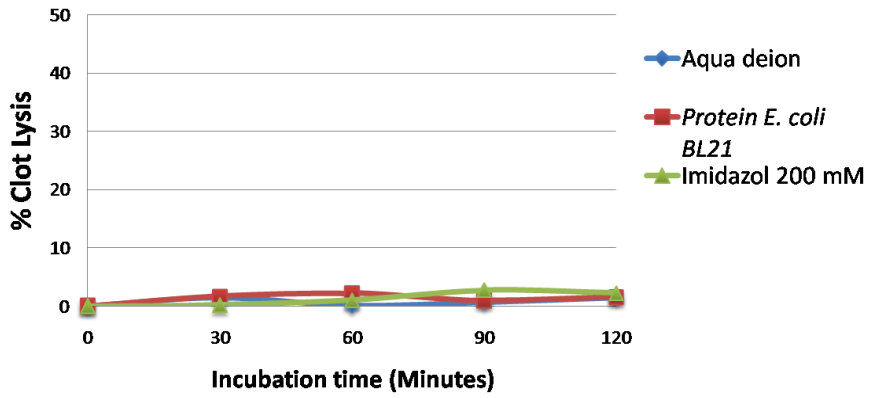

Fig. 7: Fibrinolytic activity of negative control.

Figure 6 shows the curve of clot lysis percentage as a function of time, type of protein and the concentration. While, figure 7 presents the clot lysis activity of negative controls. As indicated, the fibrinolytic activity was dose-dependent. The attachment of PEG on streptokinase contributed to the activity improvement. But, this was only significantly recorded after 120 minutes. This was relevant with the proteolytic activity of plasmin presence in serum sample. During this time, streptokinase in medium activated free plaminogen into plasmin and occurred continuously. It caused amount of plasmin in medium was accumulated and sufficient to inactivate streptokinase. Plasmin degraded peptide bond of streptokinase at other lysine residues next to pegylated sites. Mutation of cleavage site and PEGylation of lysine residues therefore protected streptokinase from plasmin attact, increasing the fibrinolytic activity of the streptokinase. This indicated that pegylation preserved the activity of streptokinase and even prolonged the fibrinolytic activity as 60 min.

CONCLUSIONS

Mutant K59Q-K386Q streptokinase overproducted in *E. coli* BL21 was pegylated successfully. Several factors were influenced the pegylation process such as incubation time, molar ratio of protein:activated PEG, and the number of available amino acid of protein. The more PEG-carbonylimidazole added the more PEG was attached to the protein. Attachment of PEGs on protein surface did not block or decreased the activity of streptokinase, even prolonged the fibronolytic activity of mutant K59Q-K386Q streptokinase.

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