African Journal of Biotechnology Vol. 10(8), pp. 1408-1420, 21 February, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1268
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Isolation, production, purification, assay and characterization of fibrinolytic enzymes (Nattokinase, Streptokinase and Urokinase) from bacterial sources

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Accepted 10 January, 2011

Nattokinase, Streptokinase and Urokinase are novel fibrinolytic enzymes which are isolated from *Bacillus subtilis,* β-haemolytic *Streptococci* and urine sample. The fibrinolytic enzyme Nattokinase, Streptokinase and Urokinase was purified from supernatant of *Bacillus subtilis,* β-haemolytic *Streptococci* and recombinant *E.coli* containing short fragment genomic DNA of *Pseudomonas* sp. Culture broth and showed thermophilic, hydrophilic, and strong fibrinolytic activity. The optimum temperature and pH of Nattokinase, Streptokinase and Urokinase were 37-55°C and 9, 27-37°C and 7 and 55°C and 9, respectively. The molecular weight of Nattokinase, Streptokinase and Urokinase was approximately 28 kDa, 47 kDa and 34 kDa, respectively, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The caseinolytic activity of **Nattokinase, Streptokinase and Urokinase were 576.73 U, 467.73 U and 785.73 U, respectively, while fibrinolytic activity achieved by fibrin plate method were 10 U, 5 U and 15 U, respectively.**

Key words: Anticoagulant activity, submerge fermentation, fibrinolytic enzyme activity, protein fraction precipitation, casein, serum and plasminogen plate technique, enzyme thermodynamics, haemolytic activity, enzyme screening, expression system, zymography, Edman degradation.

INTRODUCTION

Fibrinolytic enzymes were identified and studied among many organisms including snakes, earthworms, and bacteria: *Streptococcus pyogenes, Aeromonas hydrophila, Serratia* E15, *B. natto*, *Bacillus amyloliquefacens*, Actinomycetes and fungi: *Fusarium oxysporum*; *Mucor* sp, *Armillaria mellea* (Jian Sha et al, 2003).

Fibrinolytic enzymes can be found in a variety of foods, such as Japanese Natto, Tofuyo, Korean Chungkook-Jang soy sauce and edible honey mushroom. Fibrinolytic enzymes have been purified from these foods and their physiochemical properties have been characterized. Fermented shrimp paste, a popular Asian seasoning, was shown to have strong fibrinolytic activity. These novel fibrinolytic enzymes derived from traditional Asian foods are useful for thrombolytic therapy. They will provide an adjunct to the costly fibrinolytic enzymes that are currently used in managing heart disease, since large quantities of enzyme can be conveniently and efficiently produced. In addition, these enzymes have significant potential for food fortification and nutraceutical applications, such that their use could effectively prevent cardiovascular diseases (Yoshinori et al., 2005).

Accounts of cardiovascular diseases have become the leading cause of death in the Western world (Viles et al., 2004). Many blood clot-dissolving agents, such as urokinase, streptokinase, and tissue plasminogen activator (t-PA), have been utilized in clinical treatments for cardiovascular diseases. Hemostasis is a complex process obtained through an optimal balance between bleeding and blood clot formation. In an unbalanced state, fibrin clots may not be lysed resulting in thrombosis. Thrombolytic

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Abbreviations: t-PA, Tissue plasminogen activator; **SK,** streptokinase; **NAT,** nattokinase; **UK,** urokinase; **CU,** caseinolytic activity; **PTC,** phenyl thiocarbamoyl; **TLC,** thin layer chromatography; **PMSF,** phenyl methyl sulfonyl fluoride; **EDTA,** ethylene diamine tetraacetic acid; **SDS-PAGE,** sodium deodecyl sulphate polyacrylamide electrophoresis; **BSA,** bovine serum albumin; **PTH,** phenylthiohydantoins.

agents from various sources have been extensively investigated. Enzymes, such as urokinase, streptokinase and tissues plasminogen activators have been widely used in the treatment of thrombosis. However, these enzymes are often expensive, thermolabile and can produce undesirable side effects (Chitte and Dey, 2000).

Subtilisin nattokinase (NAT) (formerly designated Subtilisin BSP), produced by *Bacillus subtilis* natto, is a serine protease and is reported to have potent fibrinolytic activity (Nakamura, et al., 1992). Besides *in vitro* tests of fibrinolytic activity, many *in vivo* studies had been reported (Sumi et al. 1990). Fujita et al. (1995) treated dogs with nattokinase by oral administration, and the fibrinolytic activity in plasma increased and showed that subtilisin NAT could pass the rat intestinal tract and dissolve the chemically induced thrombosis. Suzuki et al. (1990) found that dietary supplementation of natto suppressed intimal thickening and modulated the lysis of mural thrombi after endothelial injury in rat femoral artery. Sumi et al. (1990) also reported a similar effect of dietary *Bacillus natto* productive protein on *in vivo* endogenous thrombolysis. Similar fibrinolytic enzyme-producing bacteria have also been isolated from Japanese shiokara, Korea chungkook-jang (Banerjee et al., 2004), and Chinese douchi. Nevertheless, it is still the most stable and economic way to obtain protein with fibrinolytic activity by *B. subtilis* natto. On the basis of its food origin, relatively strong fibrinolytic activity, stability in the gastrointestinal tract, and convenient oral administration, subtilisin NAT has advantages for commercially used medicine for preventative and prolonged effects (Uversky et al., 2004).

Streptokinase is an extra cellular protein, extracted from certain strains of beta hemolytic streptococcus. It is a non-protease plasminogen activator that activates plasminogen to plasmin, the enzyme that degrades fibrin cloth through its specific lysine binding site; it is used therefore as a drug in thrombolytic therapy (Mohammad et al., 2009). Streptokinase is currently used in clinical medicine as a therapeutic agent in the treatment of thromboembolic blockages, including coronary thrombosis (Banerjee et al., 2004; Endrogan et al., 2006).

Streptokinase (SK) a group of extracellular proteins produced by a variety of streptococci beta-hemolytic strains, and is a plasminogen activator composed of 414 amino acids with a molecular mass of 47 kDa. Unlike urokinase or tissue-type plasminogen activator that performs direct proteolysis, SK forms a high affinity equimolar complex with a plasminogen (Kim et al., 2000).

Urokinase (UK) is given to patients suffering from thrombolyticdisorders like deep vein thrombosis, thrombosis of the eye, pulmonary embolism, and myocardial infarction. This enzyme is a strong plasminogen activator which specifically cleaves the proenzyme/zymogen plasminogen to form the active enzyme plasmin (Kunamneni et al., 2008). UK is a serine protease, which specifically cleaves the proenzyme/zymogen plasminogen to form the active enzyme plasmin. It specifically catalyzes the cleavage of the Arg-Val bond in plasminogen. The active plasmin is then able to break down the fibrin polymers of blood clots. Clinically, UK is given to patients suffering from thrombolytic disorders. Among the plasminogen activators, UK provides a superior alternative for the simple reasons of its being more potent as compared to tissue-plasminogen activator and non-antigenic by virtue of its human origin unlike streptokinase. Based on these observations, UK is a strong plasminogen activator. Hence, UK, as one of the most potent plasminogen activators is attracting a great deal of attention. The mechanism of action, physico-chemical properties, *in vitro* production, cloning and expression, and clinical applications of UK are shown in the present study (Adinarayana et al., 2008).

MATERIALS AND METHODS

Isolation and Identification of microorganisms

B. subtilis

B. subtilis producing Nattokinase (NK) was isolated from soil sample collected from various regions in Kolkata, India and identified by colony morphology, Gram's staining, biochemical test and selective media. The identified microorganisms were stored at -20°C.

β-haemolytic streptococci

β-haemolytic streptococci with haemolytic activity was isolated from different samples of blood and biomass from infected throat. The bacteria was identified by blood agar selective media, Gram's staining and biochemical tests. The three isolates exhibited streptokinase activity (SK) and stored at -20°C.

Pseudomonas sp.

Pseudomonas sp producing Urokinase activity was isolated from human urine sample and identified by cetrimide agar selective media, Gram's staining and biochemical tests. The identified isolates were stored at -20°C.

Screening of enzymes

Screenings of fibrinolytic enzymes was done using nutrient agar medium containing 2% casein and 2 ml human serum.

Enzyme production

Nattokinase

B. subtilis was grown on basal medium containing (g/lit.) Soya Peptone, 10. K_2HPO_4, 2. $MgSO_4$, 1. Maltose, 20. Yeast extract, 10. Glucose, 2. and 1000 ml distilled water. The pH was adjusted to 7.2 with 2 M acetic acid and 2 M NaOH. Medium was sterilized by autoclaving at 121°C for 35 min and cooled to room temperature. One ml of uniformly prepared suspension of *B. subtilis* was used as

an inoculum; incubated at 37°C and 150 rpm in an orbital shaker. After 7 days of fermentation, cells were removed by centrifugation.

Streptokinase

β-haemolytic streptococci was grown on medium containing corn steep liquor 8% and 12% serelose, 7% KH_2PO_4, 0.33% K_2HPO_4, 0.2% cysteine, 0.01% Glycine 0.01% tryptone, 0.01% Uracil, 0.001% adenine sulfate, 0.001% nicotinic acid, 0.001% pyridoxine-HCl, 0.0018% calcium phosphate, 0.005% thiamine-HCl, 0.002% riboflavin, 0.001% and salt mixture 2 g/lit. The pH was adjusted to 7.0 with 1 M HCl and 1M NaOH. Medium was sterilized and cooled at room temperature. One ml of culture was used as inoculum; incubated at 37°C and 170 rpm in orbital shaker. After 75 h of fermentation, cells were removed by centrifugation.

Urokinase

The low molecular weight of DNA fragment isolated from *Pseudomonas sp* was ligated into Pet28a vector, transformed into *E. coli* BL21-RIL, and then induced to express under the control of T7 promoter. The transformed colony of *E. coli* was grown on medium containing (g/lit) casein enzyme hydrolysate, 10 potassium phos-phate, 2 calcium chloride, 1 sucrose, 20 peptone, 2 glucose, 2 yeast extract, 10 and distilled water 1000 ml. The pH was adjusted to 7 with 1 M acetic acid and 1 M NaOH. Medium was sterilized and cooled at room temperature. One ml of culture was used as inoculum; incubated at 37°C and 170 rpm in an orbital shaker. After 76 h of fermentation, cell were removed by centrifugation.

Enzyme purification

Cells were separated from culture broth by centrifugation (8,000 × g, 15 min) and the supernatant fluid was added to 3 volume of acetone. The mixture of supernatant and acetone was allowed to stand at 4°C for 1 day. After centrifugation (10,000 × g, 15 min) of the mixture, the resultant precipitate was purified by ion exchange column chromatography (DEAE Cellulose, MERK). The active fractions were added to 660 ml of acetone and allowed to stand at 4°C for 18 h. The precipitates were collected by centrifugation and then lyophilized. For further purification, gel filtration with Sephadex G200 (MERK) gel equilibrated with 10 mM glycine-NaOH buffer (pH, 9) was performed. The active fractions were precipitated with acetone and then lyophilized. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done by using 10 to 20% gradient polyacrylamide gel and a 4% stacking gel at 4°C.

Forward and backward extraction

Both forward and backward extraction was carried out with a volumetric phase ratio 6:6 (ml) in tightly stoppered 50 ml glass flask. In the forward extraction, 50 mM isooctane was used as the organic phase system and 1.0 mg/ml fibrinolytic enzyme in 20 mM/litre, pH 4.0 to 7.0 tris-buffer at the given salt species and concentration was used as aqueous phase system. The two phases were mixed on a orbital shaker with a speed of 240 rpm in water bath at 20°C. The mixtures were centrifuged at 4,000 rpm for 5 min. to reach a clear separation of two phases. During the investigation, fermentation broth was used as aqueous phase.

Ammonium salt precipitation

The fibrinolytic enzymes were also purified by ammonium sulfate saturation. The protein fraction was precipitated with 85% ammonium sulfate. Ammonium sulfate was found to activate the fibrinolytic activity after dialysis. Fibrinolytic enzymes were partially purified by using anion exchange column chromatography (DEAE Cellulose, MERK).

Enzyme assay and characterization

The relative activity and quantitative estimation of fibrinolytic enzymes were estimated by Lowry's method spectrophotometrically at 560 nm; L-arginine, casein, BSA, mixture of amino acids and phenylthiohydantoins (PTHs) was used as standard.

Effect of pH

Eight hundred (800) µl of serum was added to 100 µl of casein (3%) solution. The mixture was incubated at 37°C for 1 h then centrifuge. The precipitate was washed twice with 1 ml of phosphate buffer and vortexes. The purified enzymes were dissolved in sodium phosphate buffer (pH, 7.5). The enzyme solution (100 µl) was added to the serum casein solution and absorbance was taken by UV-spectrophotometer at 560 nm.

Effect of inhibitor/ activator

Purified enzyme was dissolved in 10 mM Glycine-NaOH buffer (Ph, 9.0) and mixed with each salt solution to give a final inhibitor and activator concentration of 0.5 mM. Enzyme samples were separately incubated at 37°C for 10 min with each of the following inhibitors: PMSF, EDTA, $AgNO_3$, $HgCl_2$ and SDS; residual activity was then determined. $MgSO_4$, $FeSO_4$, $MnSO_4$, $MnCl_2$ and $CaCl_2$ were used as activator.

Effect of temperature

Effect of temperature on the fibrinolytic activity was exa-mined at pH 8.5. Casein and serum was used as a substrate.

Enzyme assay

Fibrinolytic activity was determined by serum, casein and fibrin plate method. The Casein solution [2.5 ml of 2% (w/v) human fibrinogen (Merck) in 0.1 M Sodium Phosphate buffer, pH 7.4] was missed with 2 ml of human serum after sterilization of agarose solution in Petri dish (100 by 15 mm). After the dishes were allowed to stand for 30 min at room temperature, three holes were made on a fibrin plate by suction by using steel gel puncture (0.5 cm). 50 and 100 µl enzyme solution was dropped into each hole and incubated at 37°C for 18 h. After measuring the dimension of the clear zone, the number of units was determined. One unit of the enzyme activity was defined as the amount of enzyme in 25 µl of enzyme solution that produced a clear zone of $1mm^2$ at pH 7.7 and 35°C for 18 h.

Caseinolytic activity

Caseinolytic activity was assayed using the following procedure: A mixture (1 ml) containing 0.7 ml of 0.1 M sodium phosphate buffer(pH 7.5), 0.1 ml of 2% casein, and 0.1 ml of enzyme solution was incubated for 5 min at each temperature, mixed with 0.1 ml of 1.5 M trichloroacetic acid, allowed to stand at 4°C for 30 min and then centrifuged at RT. The absorbance at 560 nm for the

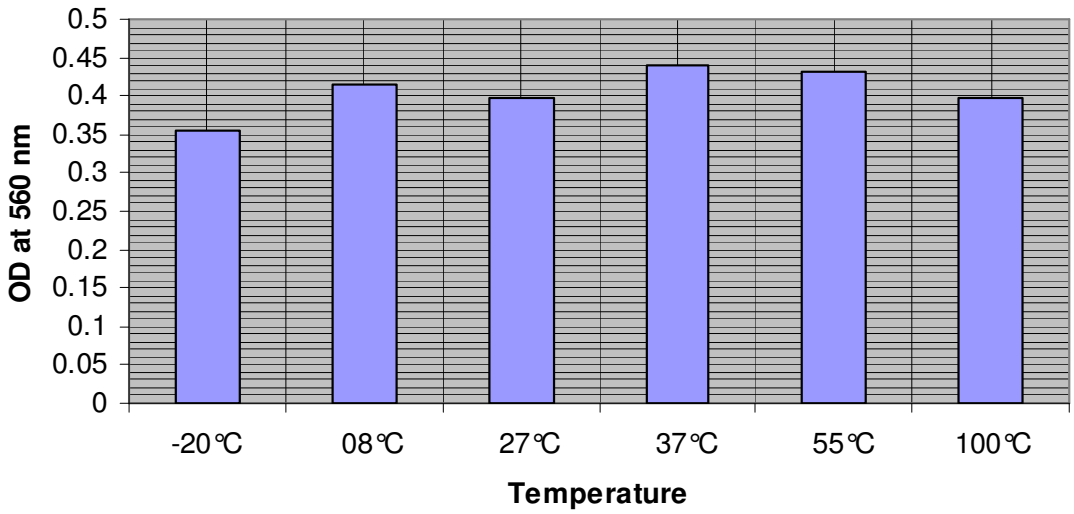

Figure 1. The relative activity of Nattokinase enzyme at different temperature.

supernatant was measured and converted to the amount of tyrosine equivalent. One unit of Caseinolytic activity (CU) was defined as the amount of enzyme releasing 1 µ mole of tyrosine equivalent/min.

Zymography

In Zymography, 20% acrylamide and 0.6% bis-acrylamide gradient was used. 0.1% of casein solution, 5 µl serum and 0.2% fibrin were used as a substrate in the gel. 4% stacking gel was used at 4℃.

Determination of the N-terminal amino acid sequence (Edman degradation)

After SDS-PAGE, purified enzyme/protein bands on polyacrylamide gel were cut and transferred inside the cellulose membrane containing phosphate buffer (pH 7.2). The 50 V electric current was supplied for 4 h. The protein bands were migrated to buffer from the gel by electroeleution method. The buffer containing protein was obtained by centrifugation at 10,000 rpm for 20 min; discarded supernatant and pellet was used for Edman process. The peptide was reacted with phenylisothiocyanate under mildly alkaline condition (30 mM phosphate buffer and 20 mM tris-HCl, pH 8.5), which converted the amino terminal of amino acid to a phenyl thiocarbamoyl (PTC) adduct. The peptide bonds next to the PTC adduct was then cleaved in a step carried out in anhydrous trifluroacetic acid (Merck), with removal of the amino terminal as an anilinothiajolinone derivative. The dramatized amino acid was extracted with isopropanol (95%) converted to the more stable phenylthiohydantoins derivatives by treatment with aqueous acid (45% acetic acid glacial or iodoacetate) and then identified. The Edman product was separated by thin layer chromatography (TLC), paper chromatography and electrophoresis. Mixture of amino acids was used as a marker. The retention factor (RF) value and bands of the N-terminal of amino acids were compared to the marker.

RESULTS

Nattokinase enzyme

B. subtilis organism was used for production of Nattokinase enzyme. These organisms are gram positive, rod shaped, aerobic and endospore forming bacteria. The biochemical test of *B. subtilis* showed positive result for MR-VP, starch hydrolysis, urease and casein hydrolysis test but negative result in TSIA, Gelatin, and indole production test.

Enzyme assay

The activity of Nattokinase enzyme was achieved was about 576.73U when compared to the BSA. The fibrinolytic activity of Nattokinase was also measured by casein, serum and fibrin plate technique. One unit of enzyme activity was defined as the amount of enzyme in 25 µl of enzyme solution that produced a clear zone of 1mm^2 at pH 7.7 and 35℃ for 18 h. The 7.5 U for 50 µl and 10 U of activity for 100 µl were achieved by Nattokinase.

Effect of Temperature

The effect of temperature on the fibrinolytic activity of Nattokinase was examined at pH 7. The temperature showing maximal enzyme activity was 37 to 60℃ and showed 48.6, 38.5, 32.2 and 31.2% residual activity at 8, 27, 100 and -20℃, respectively (Figure 1). It was concluded that the enzyme was thermostable metallo protease.

Effect of pH

The optimum pH for fibrinolytic activity of Nattokinase was around 9 and the enzyme activity decreased rapidly at level below pH 5. The enzyme was very stable in the range of 7 to 9 at 30℃ for 25 h. Above pH 11, enzyme

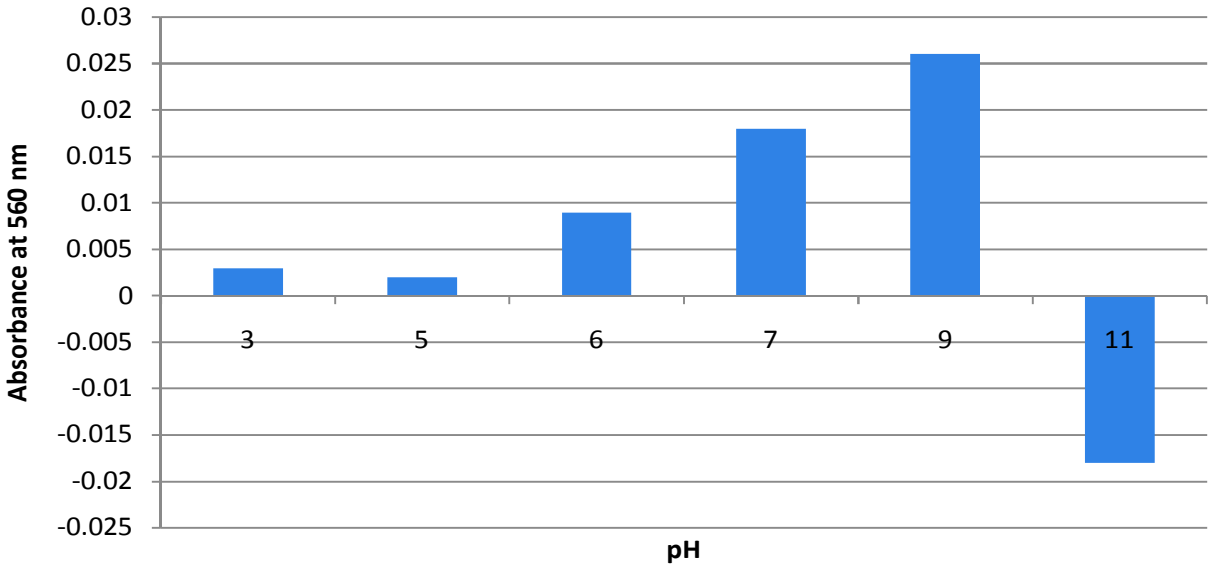

Figure 2. The relative activity of Nattokinase enzyme at different pH 3, 5, 6, 7, 9 and 11.

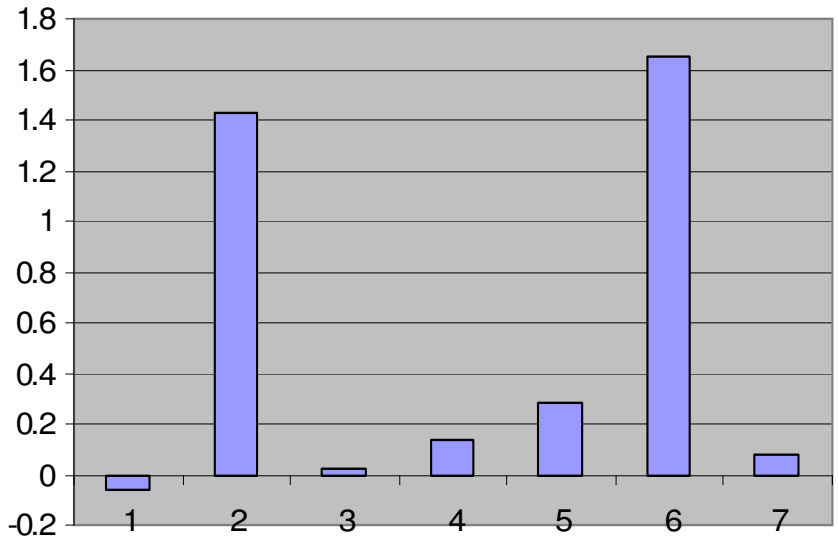

Figure 3. The relative activity of Nattokinase enzyme against different protease inhibitor: 1, PMSF; 2, $AgNO_3$;, 3, EDTA; 4, $HgCl_2$; 5, H_2O_2; 6, NaN_3; 7, SDS.

activity abruptly decreased (Figure 2).

Effect of inhibitor

When the enzyme (500 U/ml) was incubated at room temperature, for 10 min in phosphate buffer (1mM) with 1 mM phenylmethylsulfonyl fluorides (PMSF), ethylene-diaminetetraacetic acid (EDTA) and SDS, enzyme activity was completely inhibited (Figure 3). Enzyme activity was partially inhibited by $HgCl_2$ and H_2O_2 but no inhibition was shown by the others.

Effect of activator

When the enzyme (500 U/ml) was incubated at room temperature for 10 min in phosphate buffer (1 mM) with 1 mM $MnCl_2$, $CaCl_2$ and $MgSO_4$ enzyme activity was activated but no activation was shown by the rest (Figure 4).

SDS-PAGE

The protein bands found on SDS PAGE for Nattokinase

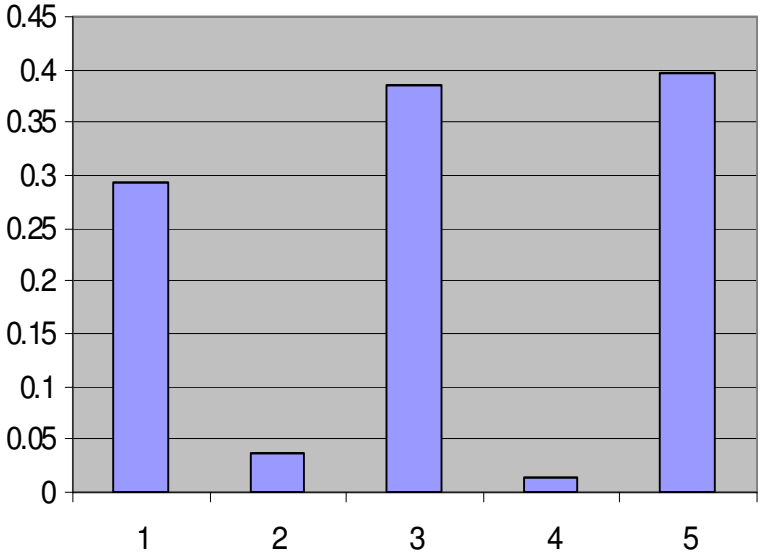

Figure 4. The relative activity of Nattokinase enzyme against different activator : 1, $MgSO_4$; 2, $FeSO_4$; 3, $CaCl_2$; 4, $MnSO_4$; 5, $MnCl_2$.

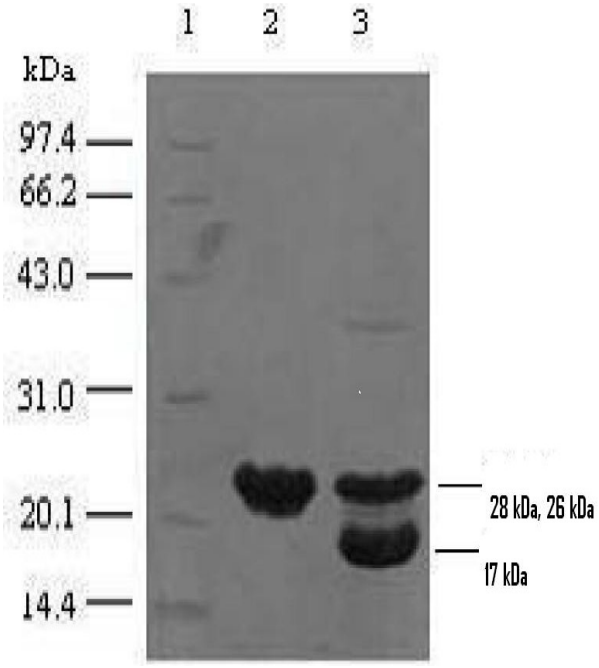

Figure 5. Molecular weight of Nattokinase. Lane 1, Marker; lane 2, active fraction II; and lane 3, crude Nattokinase.

were approximately 28 and 26 kDa (Figure 5). It was concluded that the molecular weight of purified protein was approximately to nattokinase 27.3 kDa.

Edman degradation

After the SDS PAGE purified enzyme on polyacrylamide gel was gone through Edman process, the Edman product was used for the N-terminal amino acid sequencing separated by thin layer chromatography. The N-terminal of amino acid sequence of the first 8 residues of purified enzymes was approximately Val-His-His-Pro-Arg-Ser-Pro-Tyr. Some sequence may be mismatch.

Recovery of nattokinase dissolved blood clotting

The recovery of enzyme was checked by dissolving human blood clotted. The experiment was done in laboratory in clean slide. About 100 mg of coagulated blood was dissolved by 200 µl enzyme within 2 h at 37°C temperature.

Result of streptokinase enzyme

Hemolytic streptococcus organism used for production of streptokinase enzyme was gram positive, cocci, aerobic bacteria. The biochemical test for *streptococcus* showed positive result in TSI, Gelatin, Nitrate, Citrate and Starch hydrolysis test while negative result was observed in MR-VP, urease and indole production test.

Enzyme assay

The activity of Streptokinase enzyme was achieved about 467.73U when compared to the casein. The fibrinolytic activity of Streptokinase was also measured by casein, serum and fibrin plate technique. One unit of enzyme activity was defined as the amount of enzyme in 25 µl of

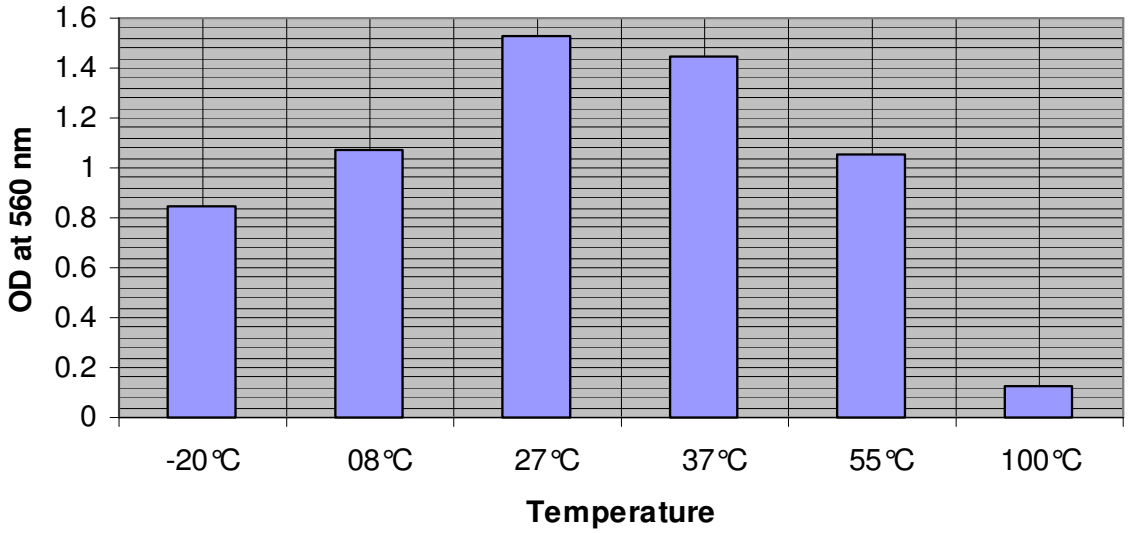

Figure 6. The relative activity of Streptokinase at different temperature.

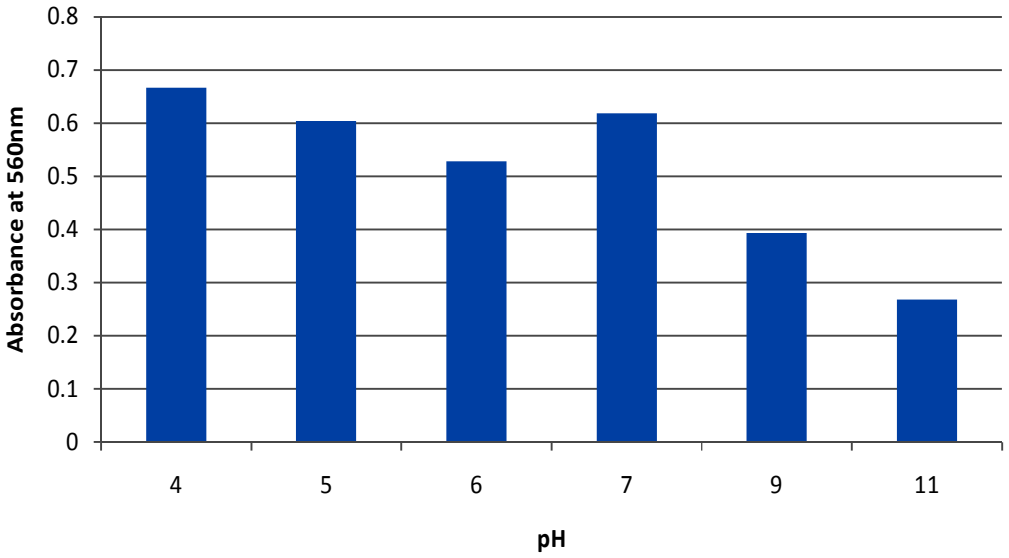

Figure 7. The relative activity of Streptokinase enzyme at different pH; 4, 5, 6, 7, 9 and 11, respectively.

enzyme solution that produced a clear zone of 1 mm^2 at pH 7.7 and 35℃ for 18 h. The 2.5 U for 50 µl and 5 U of activity for 100 µl were achieved by Streptokinase.

Effect of temperature

The effect of temperature on the fibrinolytic activity of Streptokinase was examined at pH 7. The temperature showing maximal enzyme activity was 27 to 37℃ and showed 38.4, 34.5, 32.2 and 11.2% residual activity at 8, 55 and -20℃ and 100℃, respectively. It was concluded that the enzyme was active in the range of 27 to 45℃ (Figure 6).

Effect of pH

The optimum pH for fibrinolytic activity of Streptokinase was around 4 to 7 and the enzyme activity decreased rapidly at level below pH 3. The enzyme was very stable in the range of 5 to 7 at 30℃ for 25 h. Above pH 11, enzyme activity abruptly decreased (Figure 7).

Effect of inhibitor

When the enzyme (500 U/ml) was incubated at room temperature for 10 min in phosphate buffer (1mM) with 1mM PMSF, EDTA and $HgCl_2$, enzyme activity was

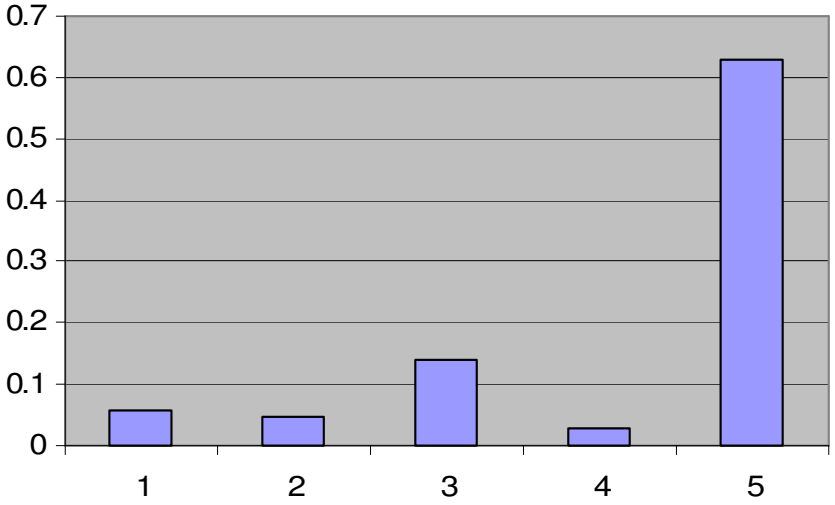

Figure 8. The relative activity of Streptokinase enzyme against different protease inhibitor: 1, PMSF; 2, EDTA; 3, SDS; 4, $HgCl_2$; 5, $AgNO_3$.

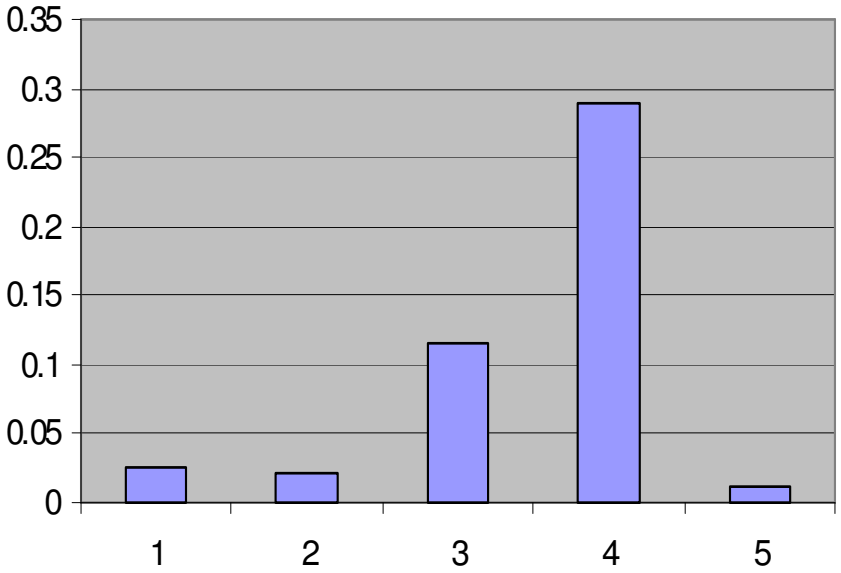

Figure 9. The relative activity of Streptokinase enzyme against different activator: 1, $MgSO_4$; 2, $FeSO_4$ Ca; 3, Cl_2; 4, $MnSO_4$; 5, $MnCl_2$.

completely inhibited (Figure 8). Enzyme activity was partially inhibited by SDS but no inhibition was shown by the others.

Effect of activator

When the enzyme (500 U/ml) was incubated at room temperature for 10 min in phosphate buffer (1 mM) with 1 mM $MnSO_4$, enzyme activity was activated and partially activated by $CaCl_2$ but no activation was showed by the rest (Figure 9).

SDS-PAGE

The protein bands found on SDS PAGE for Streptokinase were approximately 58, 47 and 40 kDa (Figure 10). It was concluded that the molecular weight of purified protein band 47 kDa was approximately to Streptokinase 47 kDa.

Edman degradation

After the SDS-PAGE purified enzyme on polyacrylamide gel has gone through the Edman process, the Edman

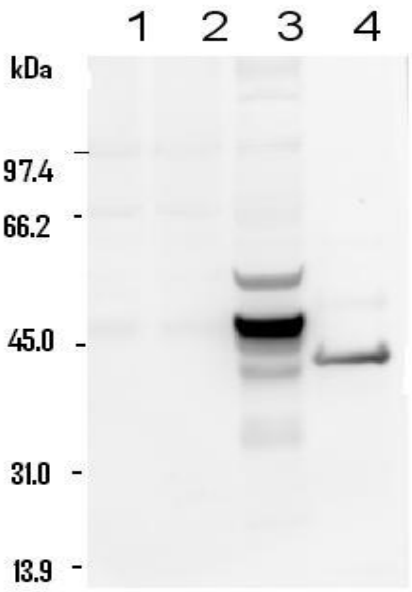

Figure 10. Molecular weight of streptokinase. Lane 1, marker; lane 2 active fraction II; lane 3, crude Streptokinase; and lane 4, active fraction III.

product was used for the N-terminal amino acid sequencing separated by thin layer chromatography (Figure 11). The N-terminal of amino acid sequence of the first 5 residues of purified enzymes was approximately His-Gly-Ser-Arg-Tyr. Some sequence may be mismatch**.**

Recovery of Streptokinase dissolved blood clotting

The recovery of enzyme was checked by dissolving human blood clotted. The experiment was done in laboratory in clean slide; about 100 mg of coagulated blood was dissolved by 200 µl enzyme within 2 h at 37°C temperature.

Urokinase enzyme

Pseudomonas sp. used for production of Urokinase enzyme was gram negative, rod shaped, and aerobic, unipolar motility bacteria. The biochemical test of *Pseudomonas.sp* showed positive result in indole, gelatin, nitrate and hydrogen sulfide and citrate test. Negative result was recorded in MR-VP, Urease and TSIA medium.

Enzyme screening

The zone showed by *Pseudomonas sp* was 1.5 cm on casein serum plate.

Enzyme assay

The activity of Urokinase enzyme was achieved about 785.73U comparing to the L-Arginine. The fibrinolytic activity of Urokinase was also measured by casein, serum and fibrin plate technique. One unit of enzyme activity was defined as the amount of enzyme in 25 µl of enzyme solution that produced a clear zone of 1 mm^2 at pH 7.7 and 35°C for 18 h. The 12.5 U for 50 µl and 15 U of activity for 100 µl were achieved by Urokinase.

Effect of temperature

The effect of temperature on the fibrinolytic activity of Urokinase was examined at pH 7. The temperature showing maximal enzyme activity was 55°C and showed 48.4, 34.5, 33.2 and 22.2% residual activity at 37, 27, 8, -20 and 100°C, respectively (Figure 12). It was concluded that the enzyme was active in the range of 37 to 58°C.

Effect of pH

The optimum pH for fibrinolytic activity of Streptokinase was around 7 to 9 and the enzyme activity decreased rapidly at level below pH 3. The enzyme was very stable in the range of 7 to 9 at 30°C for 25 h. Above pH 11, enzyme activity abruptly decreased (Figure 13).

Effect of Inhibitor

When the enzyme (500 U/ml) was incubated at room temperature for 10 min in phosphate buffer (1 mM) with 1 mM PMSF, enzyme activity was completely inhibited. Enzyme activity was partially inhibited by EDTA and SDS but no inhibition was showed by the rest (Figure 14).

Effect of activator

When the enzyme (500 U/ml) was incubated at room temperature for 10 min in phosphate buffer (1 mM) with 1 mM $MgSO_4$, enzyme activity was activated and partially activated by $FeSO_4$ but no activation was shown by the rest (Figure 15).

SDS-PAGE

The protein bands found on SDS PAGE for Urokinase were approximately 54, 34 and 17 kDa (not shown). It was concluded that the molecular weight of purified protein

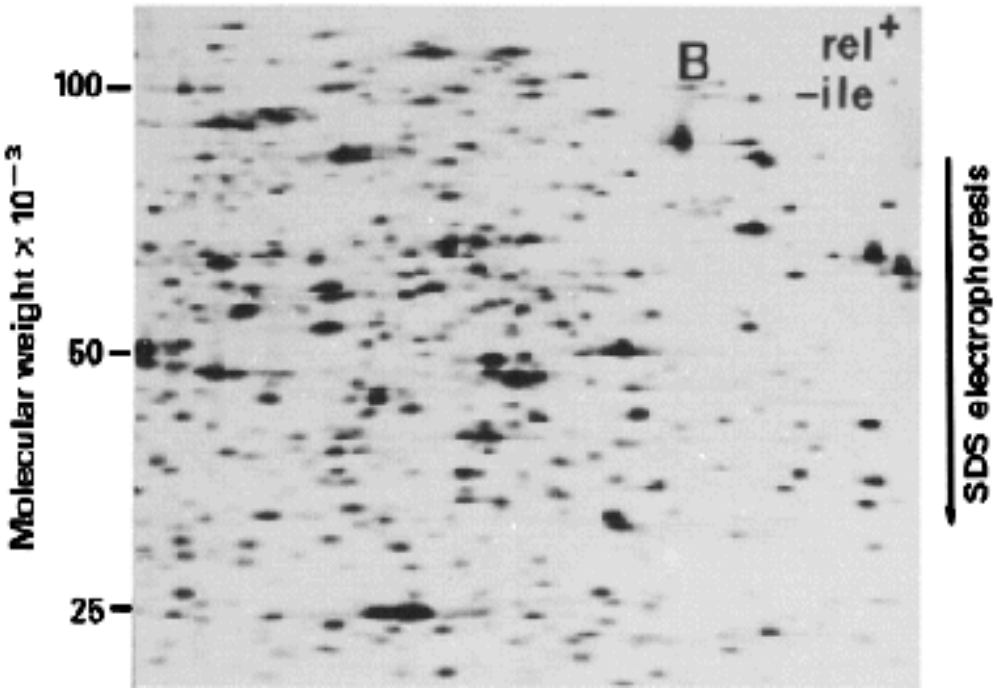

Figure 11. The product of Edman on SDS PAGE.

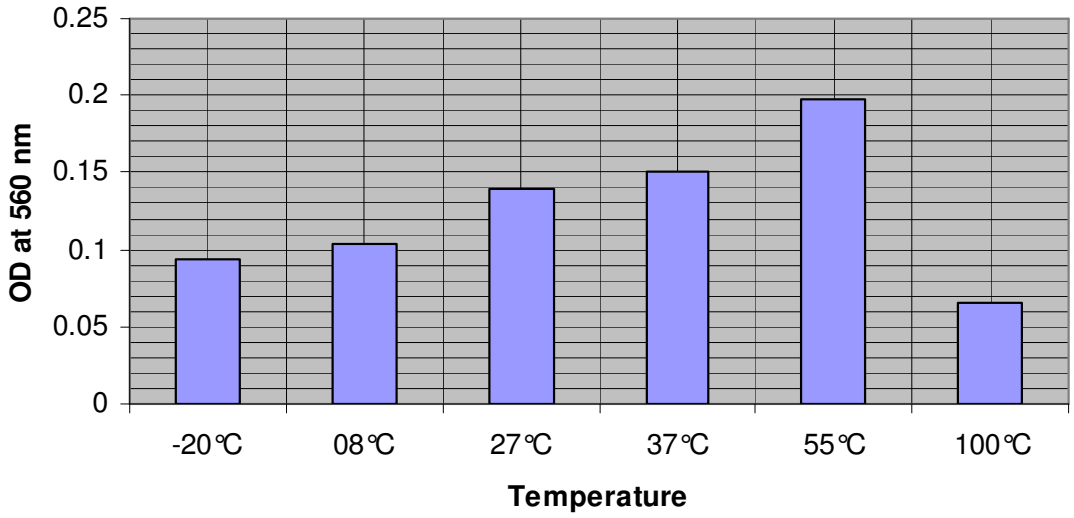

Figure 12. The activity of enzyme in different temperature.

band 54 kDa was similar to Prourokinase and 34 kDa Urokinase.

Estimation of Edman product by paper chromatography

After the SDS PAGE purified enzyme on polyacrylamide gel was done through Edman process, the Edman product was used for the N-terminal amino acid sequencing separated by thin layer chromatography. The N-terminal of amino acid sequence of the first 6 and 4 residues of purified enzymes was approximately Ala-Val-Thre-Tyr-Gln-Tyr and His-Asper-Ser-Pro. Some sequence may be mismatched.

Recovery of urokinase dissolved blood clotting

The recovery of enzyme was checked by dissolving

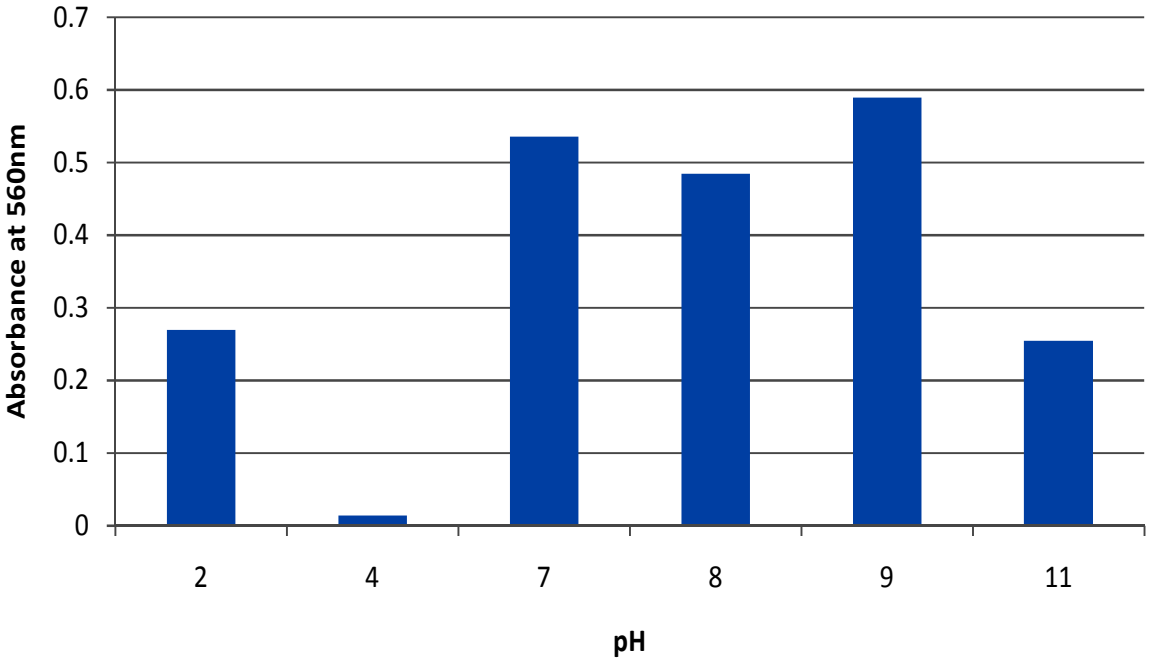

Figure 13. The relative activity of enzyme Urokinase as shown at different pH; 2, 4, 7, 8, 9 and 11, respectively.

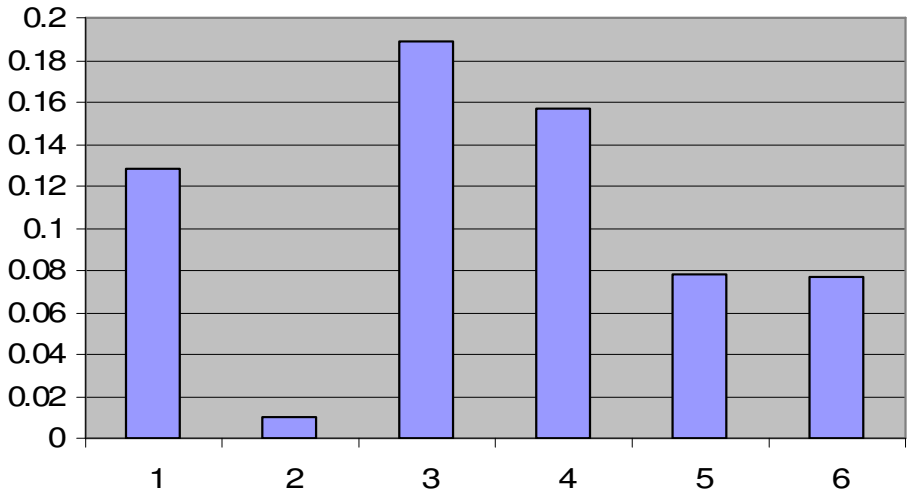

Figure 14. The relative activity of Urokinase enzyme as shown against different protease inhibitor: 1, $AgNO_3$; 2, PMSF; 3, $HgCl_2$; 4, SDS; 5, NaN_3; 6, EDTA.

human blood clotted. The experiment was done in laboratory in clean slide; about 100 mg of coagulated blood was dissolved by 200 µl enzyme within 2 h at 37°C temperature.

DISCUSSION

The article describes the purification and characterization of Nattokinase, Streptokinase and Urokinase from *B. subtilis, β-haemolytic streptococci* and *Pseudomonas sp.* for assessment for its application as a thrombosis agent. As mention above, intravenous administration of Urokinase and Streptokinase has been widely used for thrombosis therapy. Fibrinolytic therapy by oral drug administration has been recently investigated in animal models in which enteric-coated Urokinase capsule were given to normal and experimental dogs with saphenous vein thrombosis. Sumi et al (1990) reported that intravenous administration did not show any clear throm-

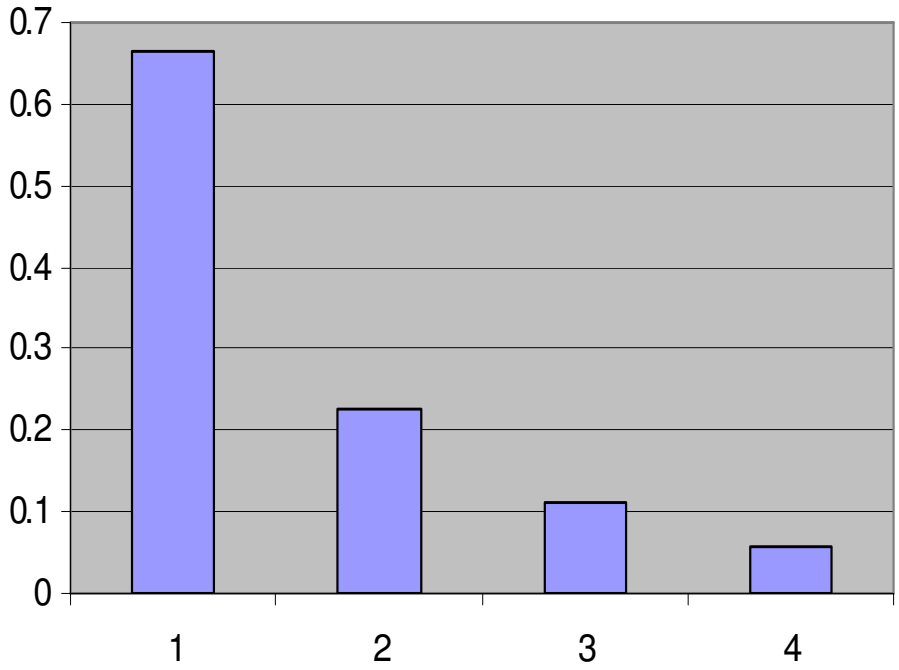

Figure 15. The relative activity of Urokinase enzyme as shown against different activator: 1, $MgSO_4$; 2, $FeSO_4$; 3, $CaCl_2$; 4, $MnSO_4$; 5, $MnCl_2$.

bolytic effect but oral administration enhanced fibrinolytic activity. In another study Sumi et al (1990) reported that when Nattokinase was given to human subject by oral administration, fibrinolytic activity and the amount of tPA and fibrin degradation product in plasma increased about two folds. On the basis of these reports, *B. subtilis* producing fibrinolytic enzyme were isolated from soil obtained from various regions of Kolkata. Among them *B. subtilis* showed strongest fibrinolytic, thermophilic and hydrophilic activity. According to Wonkeuk et al (1996) the fibrinolytic activity of CK, fibrinolytic enzyme obtained from *Bacillus* strain CK 11-4, was 0.33U-053U and 1 to 1.6 fold by fibrin plate method, but in contrast to our investigation, the enzyme activity was much better than CK, casein, Serum and fibrin plate method, for Natto-kinase 7.5U, 7.5 fold and 10 U, 10 fold. For Streptokinase 2.5U, 2.5 fold and 5U, 5 fold and for Urokinase 12.5U, 12.5 fold and 15 U and 15 fold were achieved. These several studies that have reported on the intestinal absorption of serum albumin. Bernik and Oller (1973) observed activation of a plasminogen through activator of the human kidney by trypsin treatment. In view of these reports, it can be suggested that Nattokinase from *B. subtilis,* Streptokinase from Streptococci and Urokinase from Pseudomonas sp can be given orally for use as a thrombolytic agent. All Streptokinases sequenced to date namely, those secreted by group C.S equisimilis H46 and group G *Streptococcus sp.* Strain G19909 was originally isolated from streptococci which had infected human host. These streptokinases have been shown to be remarkably similar to one another, more functionally and structurally, with greater than 85% homology at the amino acid level. In contrast, the streptokinase secreted by a streptococcus isolated from human host exhibited at the amino acid only 29.4% identity. The observation that streptokinases secreted by streptococci from different hosts were able to differentially activate only the plasminogen derived from the same host but were able to bind human, procine, equine, bovine and rabbit plas-minogen strongly suggests that these Streptokinases must share a common plasminogen binding domain. However, analysis of the primary sequence of these did not indicate any major contiguous regions of similarity. In addition, these three Streptokinases did not share the amino acids demonstrated to bind to the light chain of human plasminogen, as described by Wang et.al. (1998) Expression of human prourokinase in *E. coli* is very low, because there are several rare codons such as arginine (AGA), isoleucine (AUA), leucine (CUA) and Proline (CCC) in its coding sequence. The typical solutions for enhancing human prourokinase expression are time consuming and tedious. But in contrast to the present study, the gene of interest was moved to expression vector isolated from pseudomonas sp. and transferred the recombinant plasmid to a new bacteria *E. coli* BL21 and other host. It was less time consuming and more active than human prourokinase. In view of this report, it can be suggested that the fibrinolytic enzymes isolated from bacteria can be given orally for use as a throm-bolytic agent. *B. subtilis, Pseudomonas* sp. and some type *streptococci* have been recognized as being safe for humans. Further studies will test *Pseudomonas* sp.

obtained from human urine in vivo, and the cloning, sequencing and expression of the short fragment gene from chromosomal DNA of *Pseudomonas sp.* is proceeding.

Conclusions

Fibrinolytic enzymes such as Nattokinase, Streptokinase and Urokinase, used as thrombolytic agent but too costly and also used through intravenous instillation, needs large scale production by some alternative methods and high purity. So, isolation, production, purification, assay and characterization of fibrinolytic enzymes from bacterial sources are very effective and useful. In the future, the research will progress into the production of highly purified fibrinolytic enzymes from bacterial sources.

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