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ISOLATION, PRODUCTION, ASSAY AND CHARACTERIZATION OF FIBRINOLYTIC ENZYMES (NATTOKINASE AND STREPTOKINASE) FROM BACTERIA

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ABSTRACT

Nattokinase, and Streptokinase are novel fibrinolytic enzymes which are isolated from Bacillus subtilis, β-haemolyti Streptococci and urine sample. The fibrinolytic enzyme Nattokinase, and Streptokinase 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 were 37-55°C and 9, 27-37°C and 9, respectively. The molecular weight of Nattokinase, Streptokinase was approximately 28 kDa, 47 kDa and 34 kDa, respectively, as determined by sodium dodecyl sulfatepolyacrylamide gel electrophoresis. The caseinolytic activity of Nattokinase, and Streptokinase 576.73 U, 467.73 U respectively, while fibrinolytic activity achieved by fibrin plate method were 10 U, 5 U respectively.

Key words: Anticoagulant activity, Protein fraction precipitation, casein, serum and plasminogen plate technique, enzyme thermodynamics, 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 ChungkookJang soy sauce and edible honey mushroom. Fibrinolytic enzymes have been purified from these foods and theirphysiochemical 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. Thrombolyticagents 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

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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 nonantigenic 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 asan 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% pyridoxineHCl, 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

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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 sulfatesaturation. 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, AgNO3, HgCl2 and SDS; residual activity was then determined. MgSO4, FeSO4, MnSO4, MnCl2 and CaCl2 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 1mm2 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 thesupernatant 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. **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 1mm2 at pH 7.7 and 35°C 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°C and showed 48.6, 38.5, 32.2 and 31.2% residual activity at 8, 27, 100 and -20°C, 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°C for 25 h. Above pH 11, enzymeactivity abruptly decreasedEffect 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), ethylenediaminetetraacetic acid (EDTA) and SDS, enzyme activity was completely inhibited (Figure 3). Enzyme activity was partially inhibited by HgCl2 and H2O2 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 MnCl2, CaCl2 and MgSO4 enzyme activity was activated but no activation was shown by the rest (Figure 4). SDS-PAGE The protein bands found on SDS PAGE for Nattokinasewere 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-SerPro-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

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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 MRVP, 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 ofenzyme solution that produced a clear zone of 1 mm2 at pH 7.7 and 35°C 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°C and showed 38.4, 34.5, 32.2 and 11.2% residual activity at 8, 55 and -20°C and 100°C, respectively. It was concluded that the enzyme was active in the range of 27 to 45°C (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°C 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 HgCl2, enzyme activity was inhibited.

DISCUSSION

It describes purification and characterization of Nattokinase, Streptokinase from B. subtilis, β-haemolytic streptococci and Pseudomonas sp. for assessment for its application as a thrombosis agent. 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- 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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