IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Research Article
August 2015 Vol.:4, Issue:1
© All rights are reserved by Ashif Moidutty et al.

Production, Purification and Characterization of Fibrinolytic Enzyme Nattokinase from *Bacillus subtilis*

IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

***Ashif Moidutty[1], Balasubramanian T[2], Merit tardos[3], Fasalu Rahiman OM[4]**

1. *Department of Pharmacology, School of Pharmacy and Medical Sciences, Singhania University, Jhunjhunu, Rajasthan. India.*
2. *Department of Pharmacology, Al Shifa College of Pharmacy, Perintalmanna, Malappuram, Kerala, India.*
3. *Department of Pharmacy, Gulf Diagnostic Center Hospital, Abudhabi, United Arab Emirates.*
4. *Department of Pharmacology, MES Medical College, Perinthalmanna, Malappuram, Kerala, India.*

Submitted: 27 July 2015
Accepted: 3 August 2015
Published: 25 August 2015

Keywords: Nattokinase, *Bacillus subtilis*, Fibrinolytic activity, Enzyme Purification

ABSTRACT

Nattokinase (NK) is one such thrombolytic enzyme with a wide range of applications in Pharmaceutical industry, health care and medicine etc. It provides health benefits like cure of hemorrhoids, Diabetes, Muscle spasms, poor healing, chronic inflammation, helps to improve blood clotting mechanism, improves blood circulation, blood viscosity etc. In the present study, *Bacillus subtilis* was isolated from soil source and screened for the production of Nattokinase. Different source like cow fibrin and sheep fibrin were used in the production media. The maximum production was in the sheep fibrin containing media. The enzymes were purified to homogeneity by ammonium sulfate precipitation, dialysis and ion exchange chromatography. Characterization studies showed an optimum pH 7 and optimum temperature 37^0C. The enzyme was activated by $ZnCl_2$. The molecular weight of the enzyme as determined by SDS-PAGE was found to be 40-45 kDa. The anticoagulant property of the enzyme was also studied which gave delayed clotting time for different blood samples especially for human blood. From all these data it was concluded that, sheep fibrin was found to have increased production of Nattokinase which has anticoagulant property.

HUMAN JOURNALS

www.ijppr.humanjournals.com

INTRODUCTION

Nattokinase, a potent fibrinolytic enzyme, was primarily isolated from a traditional fermented food "Natto" in Japan[1]. The enzyme is a *subtilisin*-like serine protease composed of 275 amino acid residues and has a molecular weight of 27.7 kDa[2]. The enzyme, "nattokinase", has powerful antithrombic properties, which surpass those of even urokinase, giving it vast therapeutic potential. While this enzyme has been safely used in Japan for over 20 years, it was reported that nattokinase had potent fibrinolytic activity, which could be enhanced and prolonged in the plasma when it is taken orally[3]. Nattokinase is thought to be helpful to dissolve abnormal blood clots. Abnormal blood clots can cause heart attacks and strokes, as well as conditions such as phlebitis, pulmonary embolism, or deep vein thrombosis[4, 5].

It was further demonstrated that oral administration of nattokinase capsules enhanced fibrinolysis in canine plasma in an experimental thrombosis model. Moreover, fibrinolytic activity was retained in the blood for more than 3 h[6]. As to the fibrinolytic mechanism of nattokinase, the enzyme was reported not only to possess plasminogen activator activity, but also to directly digest fibrin by limited proteolysis[6]. Nattokinase could cleave plasminogen activator inhibitor-1 into low molecular weight fragments[7]. Studies suggest that nattokinase could be used as an agent for thrombosis therapy. *Bacillus natto* has properties that closely resemble plasmin and readily digests plasmin, fibrin, and similar synthetic substrates. It is not considered as human pathogen.

This work deals with production and purification of fibrinolytic enzyme nattokinase from *Bacillus subtilis* with its characterization. The produced enzyme was tested for its anticoagulant property for finding its pharmacological activity.

MATERIALS AND METHODS

Isolation and identification of organism producing Nattokinase: Different soil samples were collected from the Jayanagar 9th block garden and isolation of microorganisms was done by Serial dilution method. Pour plate method was used for quantifying microorganisms. Identification of organism was done by gram staining and biochemical tests. Screening for Bacteria was carried out by Skimmed Milk Agar and Gelatin Cup Zone Clearing Assay.

Production of Nattokinase: Production media was prepared and pH 7.5 was maintained. The media was then autoclaved at 121^{0}C for 15 mins. The substrate Fibrin (from Cow and Sheep)

was added after sterilization in the LAF chamber. The *B. subtilis* sp. was inoculated in both the media and the media was incubated in the shaker at 37^0C for two days for production of the enzyme.

Purification of crude enzyme: The enzyme sample was purified by Ammonium salt precipitation, dialysis and Ion-exchange chromatography.

Ammonium Sulphate Precipitation: The production media was centrifuged at 10,000 rpm for 12 mins to obtain the supernatant. And protein precipitation was done by using ammonium sulphate precipitation method (salting out method). The supernatant after centrifugation was collected and the volume of it was measured. And accordingly 70% ammonium sulphate was measured in order to conduct the salting out procedure. The supernatant was transferred into a conical flask and this flask was placed in ice cold condition on the magnetic stirrer, and the ammonium sulphate powder was added pinch by pinch until complete dissolving of ammonium sulphate takes place. After the addition of the salt and complete dissolving has been done, it was kept for overnight precipitation in the refrigerator. The pellet was collected and dissolved in 10 ml of 50 mm Tris hydrochloric acid solution. This solution contains the enzymes precipitated by ammonium sulphate. This sample was subjected to dialysis followed by ion-exchange chromatography.

Dialysis: Activation of dialysis membrane was done by standard method, and it was filled with the enzymes and sealed from the both sides without any air bubbles. The bag was kept in 500 ml of 50 mM Tris HCL pH 7.0 solutions on a magnetic stirrer in ice cold condition for 8 hrs. The buffer was changed frequently for every hour to avoid equilibration.

Ion Exchange Chromatography: Pre-processing of column was done by washing with methanol and drying it. The chromatography column packed with DEAE cellulose was washed using distilled water one to two times and kept for sonication for 15 mins. The matrix was activated using activation buffer A (25 mM Tris HCl+ 25 mM NaCl). The dialyzed enzyme sample was poured into the column followed by the elution buffer to elute the enzyme. The elutants were collected in the same test tube. The process of elution was carried out using solutions C, D, E & F which contains increasing concentration of NaCl.

Characterization of Enzyme

Effect of Temperature: The enzyme was assayed in the reaction mixture containing 2.0 ml of 0.5% casein solution in 0.1M carbonate buffer (pH 9.3) and 0.1 ml of enzyme solution in the total volume of 2.1 ml. After incubation at 4^0C, 37^0C, 55^0C, 80^0C, RT for 5 mins, the reaction was stopped by adding of 3.0 ml of 10% ice cold TCA and centrifuged at 10,000 rpm for 5 mins. The reading was taken at 280 nm in UV- Spectrophotometer.

Effect of pH: The enzyme was assayed in the reaction mixture containing 2.0 ml of 0.5% casein solution in 0.1M carbonate buffer of different pH (2, 4, 6, 8, and 10) and 0.1 ml of enzyme solution in the total volume of 2.1 ml. After incubation at 37^0C for 5 mins, the reaction was stopped by adding of 3.0 ml of 10% ice cold TCA and centrifuged at 10,000 rpm for 5mins. The reading was taken at 280 nm in UV- Spectrophotometer.

Effect of Substrate: The enzyme was assayed in the reaction mixture containing different concentration 0.5% casein solution in 0.1M carbonate buffer (pH 9) and 0.1 ml of enzyme solution in the total volume of 3.0 ml. After incubation at 37^0C for 5mins, the reaction was stopped by adding of 3.0 ml of 10% ice cold TCA and centrifuged at 10,000 rpm for 5 mins. The reading was taken at 280 nm in UV- Spectrophotometer.

Effect of Activator: The activator 10% zinc chloride was added to the enzyme and the enzyme assay was carried out to check the activation. The enzyme was assayed in the reaction mixture containing 2.0 ml 0.5% casein solution in 0.1M carbonate buffer pH 9 and 0.1 ml of enzyme solution in the total volume of 2.1 ml. After incubation at 37^0C for 5 mins, the reaction was stopped by adding of 3.0 ml of 10% ice cold TCA and centrifuged at 10,000 rpm for 5 mins. The reading was taken at 280 nm in UV- Spectrophotometer.

Effect of Inhibitor: The inhibitor 10% EDTA was added to the enzyme and the enzyme assay was carried out to check the activity of inhibitor. The enzyme was assayed in the reaction mixture containing 2.0 ml 0.5% casein solution in 0.1M carbonate buffer pH 9 and 0.1 ml of enzyme solution in the total volume of 2.1 ml. After incubation at 37^0C for 5 mins, the reaction was stopped by adding of 3.0 ml of 10% ice cold TCA and centrifuged at 10,000 rpm for 5 mins. The reading was taken at 280 nm in UV- Spectrophotometer.

SDS-PAGE: After the initial standardization the samples were loaded into different wells. The electrophoresis apparatus was attached to an electric power supply (20 mA). Run until

dye front reaches the bottom of the gel. Then switch off power supply, the gel was transferred to a clean or plastic container. Remove the stacking gel completely from separating gel. 5 gel volumes of 0.25% coomassie brilliant blue R-250, 50% methanol and 10% acetic acid were added and incubated 4 hrs to overnight at room temperature with shaking (Gel rocker). Stainer was removed and saved before replacing. The gel was de-stained by adding 20 ml of 5% ethanol, 7.5% acetic acid and allows it for 1 hour. The bands were viewed under the trans-illuminator. The gel was stored in the gel storing solution of 10% acetic acid.

Testing Anticoagulant property: Withdrawn 2 ml blood samples from rat, human, goat, cow, dog was placed in each test tube along with each control group. Hundred microlitre of purified enzyme was added and observed for clotting time.

RESULTS AND DISCUSSION

Cream colored bacterial colonies were produced as the result of pour plate method. The isolated organism was streaked on to the slants made with nutrient agar and again cream colored bacterial colonies (Fig. No.1) were formed when the slants were incubated at 37^0C overnight. Gram staining slides showed a violet color and rod shaped organisms (Fig. No.2), which indicated that the organisms were gram positive. From the various biochemical test results (Table 1) organism was found to be *Bacillus subtilis.*

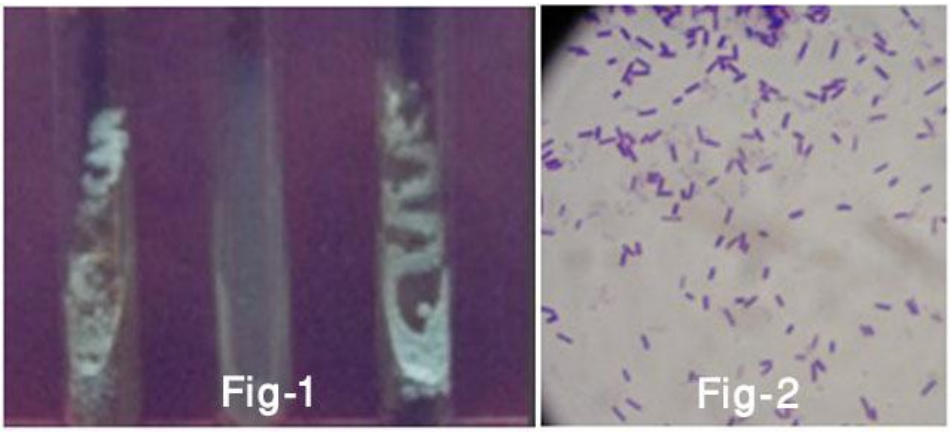


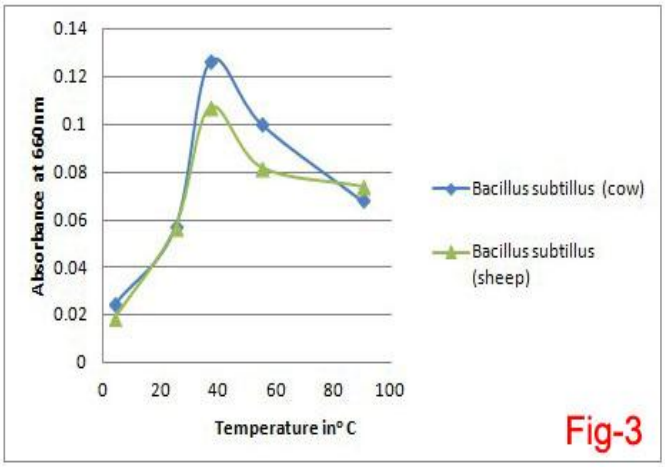
Figure No. 1-2: (1) *Bacillus subtilis* by Agar plating, (2) Microscopical view of *Bacillus subtillis*

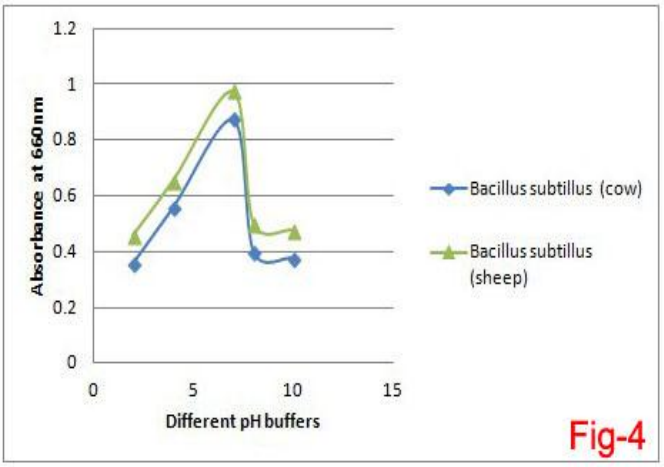
Table 1. Results for Biochemical tests

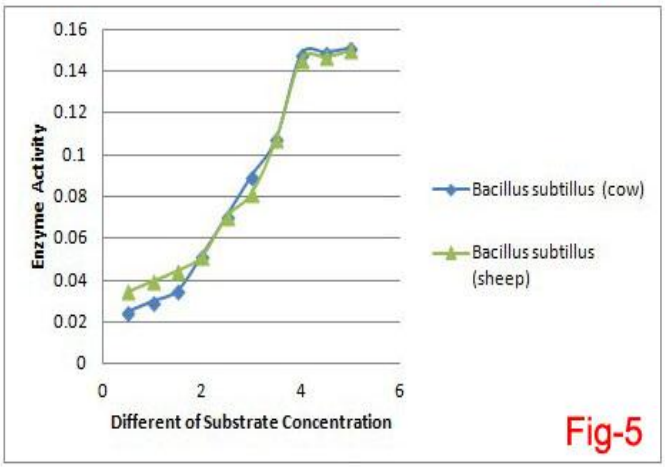
Biochemical test	Results
Indole	-ve
Methyl Red	-ve
Voges Proskauer	-ve
Citrate utilization	-ve
Urease	-ve
Catalase	+ve
Gelatin	-ve
Glucose fermentation	-ve
Lactose fermentation	-ve
H_2S	-ve

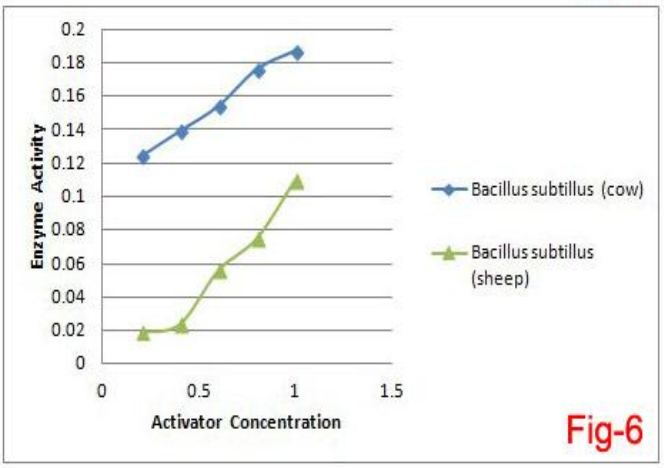
Different source like cow fibrin and sheep fibrin were used in the media for production of enzyme Nattokinase from the above isolated organisms. The production media containing 0.3% beef extract, 1% milk casein was used along with different source as mentioned above. The pH was adjusted to 7.0 with 1 M glacial acetic acid or 1 M NaOH and incubated at 37^0C in orbital shaker for 48 hrs at 86 rpm. Using different sources like cow fibrin and sheep fibrin enzyme activity was found to be 5 and 27.2 U/ml respectively.

The characterization results of the purified enzyme showed its optimum absorbance of 0.126 for cow fibrin and 0.107 for sheep fibrin at 37^0C (Fig 3), 0.875 for cow fibrin and 0.975 for sheep fibrin at pH 7 (Fig 4), 0.151 for cow fibrin and 0.15 for sheep fibrin at 5% substrate concentration (Fig 5), 0.187 for cow fibrin and 0.11 for sheep fibrin at 1 ml of 10% zinc chloride activator (Fig 6) and enzyme activity found to be decreasing for both cow and sheep fibrin gradually with the increase in the volume of the inhibitor (Fig 7). The molecular weight of the enzyme is found to be 40-45 kDa approximately by using different marker in SDS page electrophoresis (Fig 8).









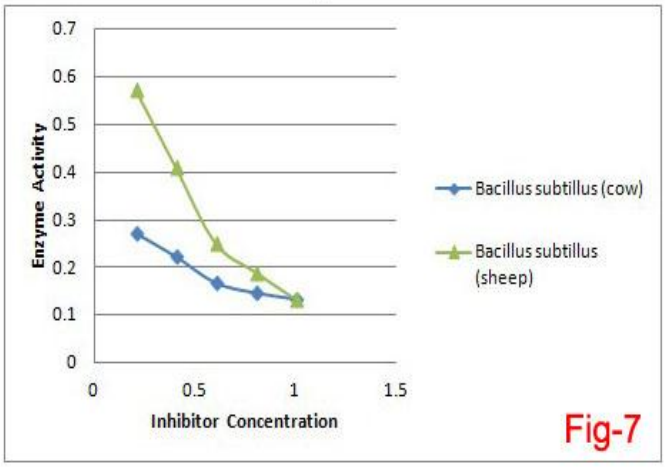


Figure No. 3-7: (3) Effect of temperature, (4) Effect of pH, (5) Effect of Substrate Concentration, (6) Effect of Activator, (7) Effect of Inhibitor

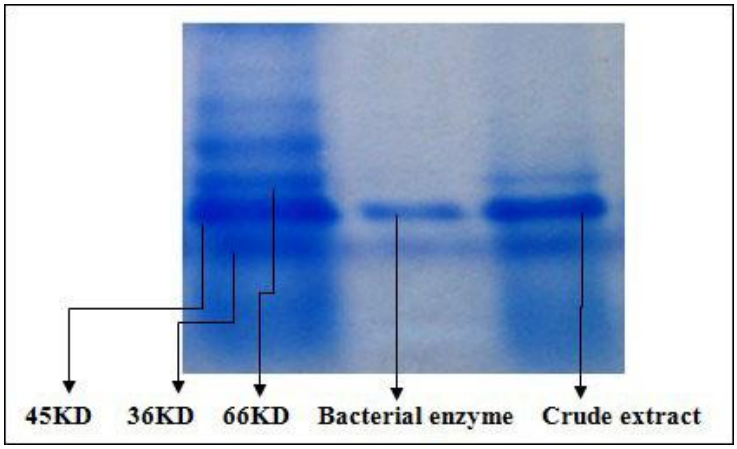


Figure No. 8. SDS-PAGE

Anticoagulant Property: Clotting time of different blood samples was found to be 420, 480, 540, 600, 660 Seconds for Rat, Goat, Cow, Dog and Human respectively (Table 2). By comparing with other species, Human test shows increased clotting time 660 seconds.

Table 2. Anticoagulant Property

Species	**Control** (in Seconds)	**Test** (in Seconds)
Rat	120-300	420
Human	300-480	660
Goat	240-420	480
Cow	300-420	540
Dog	360-480	600

CONCLUSION

The enzyme nattokinase has powerful antithrombic properties which surpasses even those of urokinase giving it vast therapeutic potential. Hence there is a need for cost effective, good quantity and large scale production of the enzyme. Isolation, production, purification, assay, characterization and fibrinolytic activity of nattokinase from bacterial sources are very effective and useful.

In the present study *Bacillus subtilis* was isolated from soil source and screened for the production of nattokinase. Different sources like cow fibrin and sheep fibrin were used in the media to achieve maximum production of enzyme using different sources as mentioned

above, maximum enzyme activity was achieved using sheep fibrin. The enzyme was purified to homogeneity by ammonium sulfate precipitation, dialysis and ion exchange chromatography. DEAE-Cellulose was used for purification of enzyme. The enzyme activity was checked at each and every step of purification. Characterization studies showed optimum pH 9 and the optimum temperature was found to be 37^0C at which enzyme showed higher activity. The molecular weight as determined by SDS-PAGE was found to be 40-45 kDa. The anticoagulant property of the enzyme was also studied which gave delayed clotting time for different blood samples. From all these data it was concluded that, sheep fibrin was found to have increased production of nattokinase which has anticoagulant property. The enzyme was more effective against human blood. This enzyme can be used as drug preparation effective in disease like cardiovascular disease, stroke, angina, venous stasis, thrombosis, emboli, atherosclerosis, fibromyalgia/chronic fatigue, claudication, retinal pathology, hemorrhoid, varicose veins, soft tissue rheumatisms, muscle spasm, poor healing, chronic inflammation and pain, peripheral vascular disease, hypertension, tissue oxygen deprivation, infertility, and other gynecology conditions.

REFERENCES

1. Sumi H, Hamada H, Tsushima H, Mihara H, Muriki H. A novel fibrinolytic enzyme (Nattokinase) in the vegetable cheese Natto: a typical and popular soybean food in the Japanese diet. Experientia. 1987; 43 (10): 1110-1111.
2. Chang CT, Fan MH, Kuo FC, Sung HY. Potent fibrinolytic enzyme from a mutant of Bacillus subtilis IMR-NK1. Journal of Agriculture and Food Chemistry. 2000; 48(8): 3210-3216.
3. Sumi H, Hamada H, Nakanishi K, Hiratani H. Enhancement of the fibrinolytic activity in plasma by oral administration of Nattokinase. Acta Haematologica.1990; 84 (3): 139-143.
4. Cesarone MR ,Belcaro G, Nicolaides AN, Ricci A, Geroulakos G, Ippolito E, Brandolini R, Vinciguerra G. Prevention of venous thrombosis in long- haul flights. Angiology. 2003; 54 (5):531-539.
5. Sumi H, Nakajima N, Mihara H. In vitro and in vivo fibrinolytic properties of nattokinase. Thromb haemostasis. 1992; 89:1267.
6. Fujita M, Hong K, Ito Y, Fuji R, Kariya K, Nishimuro S. Thrombolytic effect of Nattokinase on a chemically induced thrombosis model in rat. Biol. Pharm. Bul1.1995; 18:1387-1391.
7. Urano T, Ihara H, Umemura K, Suzuki Y, Oike M, Akita S, Tskamoto Y, Suzuki I, Takada A. The profibrinolytic enzyme subtilisin NAT purified from Bacillus subtilis Cleaves and inactivates plasminogen activator inhibitor Type 1. J. Biol. Chem.2001; 276:24690-696.