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RESEARCH ARTICLE

Isolation and Characterization of *Bacillus Subtillus* with Potential Production of Nattokinase

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Abstract

The aims of this study were to screened and characterized *Bacillus spp*. isolate that could produce a natural nattokinase with high activity. The study was carried out on 50 samples collected from different regions in Sudan. Primary screening and characterization of the microorganism showed that five samples (10%) were considered as *Bacillus subtilis* according to microscopic and biochemical characteristics. Selective medium was prepared for the extraction and production of nattokinase from these new isolates. The selected isolates *Bacillus subtilis* could produce active nattokinase with inhibition zone diameter ranged from 15-26 mm according to haemolysis and fibrinolytic activity.

INTRODUCTION

Nattokinase is a naturally-occurring proteolytic enzyme derived from natto , a traditional Japanese food produced from the fermentation of soybeans with *Bacillus subtilis* natto. Natto is made by fermenting cooked soybeans with a particular bacterium: *Bacillus subtilis* natto. The soybeans are fermented at 40°C (104 F) for 14 to 18 hours until the dark drown beans are covered with a sticky, viscous, string-like material. Natto has a slightly musty flavor and characteristic odor (Haritha et al., 2011). Indeed, 2011 WHO data shows nearly a tenfold increased incidence of death by Alzheimer's and dementia in the United States versus Japan, where natto is regularly consumed by a significant portion of the populace (Suzuki, 1994). Nattokinase is an enzyme that finds a wide range of applications in pharmaceutical industry, health care and Medicine, Its digests fibrin both directly and indirectly. Indirectly, it activates pro- urokinase and tissue plasminogen activator (t-PA), supporting the fibrinolytic activity of plasmin. In spite of its name, nattokinase is not a kinase enzyme, but a serine protease of the subtilisin family (Fujita et al., 1995). It exhibits a strong fibrinolytic activity. These combined actions promote healthy platelet function, contribute to the regular healthy function of the heart and cardiovascular system by maintaining proper blood flow, thinning the blood and preventing blood clots (Debajit et al., 2012).

The recommended amount of Nattokinase is more than 2,000 FU/ day, (FU which stands for Fibrin Degradation Unit). The Nattokinase activity level in natto commercially available varies from 1,400 FU/ pack (50g) to 2,000FU/ pack. It is recommended to take Nattokinase on a regular basis for those who are over 40 years old, stressed-out, have relatively high blood pressure, and have high blood viscosity due to hyperlipidemia or diabetes(Debajit et al., 2012)

In 1980, a researcher at the University of Chicago discovered the fibrinolytic benefits of natto when he tested 173 different foods for their effects on dissolving thrombi (Blood clots) associated with heart attack and stroke (JNKA, 2004).

Fibrinolytic enzymes were identified and studies among many organisms including snakes, earthworms and bacteria : *streptoccocus pyogens* , *Aeromonas hydrophlia B.natto*. Fibrolytic enzymes can found in a variety of foods such as Japanese natto, Tofuyo, Korean chungkook jang soy sauce, fibrolytic enzymes has been purified from these foods.

Subtilisin nattokinase (NAT) (formely designated Subtilisin BSP), produced by *Bacillus Subtilis natto,* is a serine protease and is reported to have potent fibrolytic activity (Sumi et al., 1987) besides in vitro tests of fibrolytic activity, many in vivo studies had been reported (Nakamura et al., 1992). One study treated dogs with nattokinase by oral administration and the fibrolytic acitivity in plama increased showed that subtilisin NAT could pass the rat intestinal tract and dissolve the chemically induced thrombosis (Sumi et al., 1990). Fujita et al., 1995 found that dietary supplementation of natto suppress intimal thickening and moulateded the lysis of mural thrombi after endothelial injury in rat femoral artery. Reports of a similar effect of dietary *Bacillus* natto productive protein on in vivo endogenous thrombolysis. (Debajit et al., 2012). Similar fibrinolytic enzyme-producing bacteria have also been isolated from Japanese shiokara , Korea chung kook-jang and Chinese douchi Nevertheless, it's still the most stable and economic way to obtain protein with fibrolytc activity by *B. subtilis* natto. On the basis of its food origin , relatively strong fibroltic activity , stability in the gastrointestinal tract , and convenient oral administration , subtilisin NAT has advantages for commercially used medicine for preventative and prolonged effects (Banerjee et al., 2004).

Nattokinase has been of considerable interest because of its capacity to digest fibrin in blood vessels (Suzuki, 1994; Debajit et al., 2012; JNKA. 2004). The enzyme is currently considered in pharmaceutical industry as a promising drug for thrombolytic therapy.

In addition, these enzymes have significant potential for food fortification and nutraceutical applications, such that their use could effectively prevent cardiovascular

disease (Yoshinori et al., 2005). In the future, the research will progress into the production of highly purified fibrinolytic enzymes from bacterial sources, it is still the most stable and economic way to obtain protein with fibrinolytic activity by *B. subtilis*.

The ultimate goal of this study is to isolate and characterize *Bacillus subtilis* from soil samples that could extract a promising nattokinase with potent activity.

Materials and Methods

This research was conducted between February to September 2014 at the Central Laboratory, Ministry of Science and Technology, Sudan. All the chemicals used in this study are of analytical grade unless otherwise stated.

Isolation of microorganisms

Fifty presumptive samples were recovered and collected into sterilized plastic bags from different locations in Sudan, and were given number prefixed with Is. Samples were taken from 15-20 cm depth after removing approximately 3 cm of earth surface. Isolation of the samples was performed by the soil dilution plate technique (Uversky and Fink, 2004).). In this technique; 1g of each soil sample was taken in 9 ml of sterilized distill water in pre- sterilized test tube. Serial aqueous dilutions were prepared by transferring 1ml of the soil suspension into 9 ml of sterilized distill water in sterilized test tubes. Different aqueous dilutions (10^{-7}) of the soil suspension were applied separately into sterilized petri-dishes containing sterilized nutrient and incubated for 24 hr at 37°C.

Screening and identification of the bacteria strain:

The isolated *Bacillus* strains were primarily screened for nattokinase synthesis after incubation of 100 mg of coagulated blood dissolved by 200 µl of the extract within 2 h at 37°C. Identification of the selected microorganism is determined according to the directions given by the Bergey's Manual of Systematic Bacteriology (You, 2004) and (Barrow and Feltham, 1993) and involved cultural microscopic and biochemical characteristics.

Extraction of the crude nattokinase

B. subtilis was grown on basal medium containing (g/L.) 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 inoculums; incubated at 37°C and 150 rpm in an orbital shaker. After 7 days of fermentation, cells were removed by centrifugation (Dubey et al., 2011).

Enzyme nattokinase assay

The enzyme was assayed using two different methods:

Blood haemolysis assay:

100 mg of coagulated blood was dissolved by 200 µl of the extract within 2 h at 37°C temperature (Collins and Lynee, 1995).

Fibrolytic activity assay:

Fibrolytic activity was determined by serum casein and fibrin plate method (Dubey et al., 2011). The casein solution (2.5 ml of 2 % (w/v) human fibrinogen in 0.1 M sodium phosphate buffer pH of 7.4 was missed with 2 ml of human serum after sterilization of agarose solution in petri-dish (100 by 15mm), the dishes were allowed to stand for 30 min at room temperature, three holes were made on a fibrin plate by suction using steel gel puncture (0.5cm). 50 ml of the extract solution was dropped into each hole and incubated at 37°C for 18 hours. Then measure 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 ml of enzyme solution that produced a clear zone of 1mm at pH of 7.4 and 37°C for 18 h.

Results and Discussion

Nattokinase is an enzyme considered to be a promising remedy for thrombosis healing due to its potent fibrinolytic activity (Suzuki, 1994). Due to its presence in food and relatively robust fibrinolytic activity, nattokinase has benefits over other available commercially used drugs in prophylactic and extended effects, particularly due to stability in the GI tract and comfortable oral administration (Sumi, 1987). Oral administration of nattokinase could diminish plasma levels of fibrinogen, factor VIII, and factor VII which may be useful as a nutraceutical for cardiovascular disease (Ruei-Lin et al., 2009).

Isolation and identification of microorganisms

Five out of 50 samples (10%) named Is (9), Is (13), Is (22), Is (28) and Is (37) were considered as *Bacillus subtilis* according to Microscopical and Biochemical characteristics table (1). These organisms showed gram positive, aerobic and endospore forming bacteria. *Bacillus subtilis* is a model organism for studying endospore formation in bacteria (Dubey et al., 2011) also showed positive result for casein hydrolysis, starch hydrolysis and even motility test was motile, but negative results for oxidase and indole production test . Nattokinase can now be produced by recombinant means and in batch culture, rather than relying on extraction from Nattō. (kwon, 2011). Nattokinase is traditionally produced by fermentation of various microorganisms, among which the genus *Bacillus Subtilis* natto is the preeminent nattokinase producer (Kim and Choi, 2000).

Table 1: Biochemical test results of Is (9), Is (13), Is (22), Is (28) and Is (37)

Primary screening of the microorganism

The isolates were screened for their abilities to give a clear zone around each hole. The primary screening of the microorganism revealed that the fibrinolytic activity of the enzyme was measured by casein and fibrin plate technique that give a clear zone around each hole which is indicator of nattokinase presence as showed in plate (1) below.

Table (1) Chemical and morphological characteristic of the isolate

Test	Gram Reaction	Sporforming	Starch	Casein	Motility	Oxidase	Indole	Catalase	Glucose	Aerobic Reaction
Result	+(ve)	Endo-spor forming	+	+	+	-	-	+	+	+

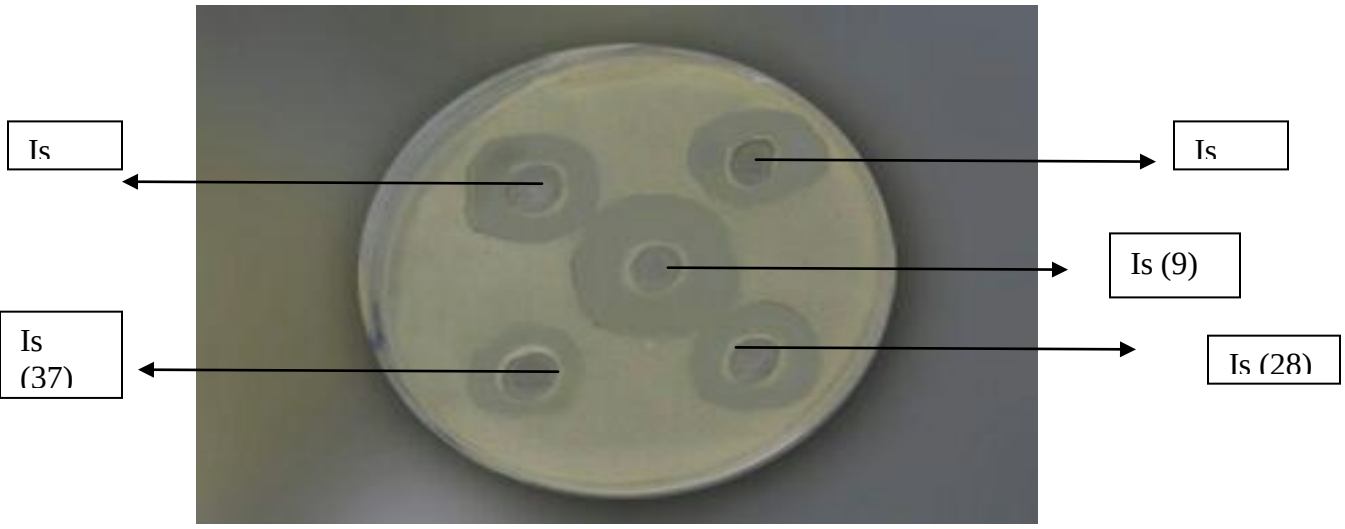

Plate (1) Primary screening of the microorganism using casien hydrolysis method

Extraction and recovery of crude nattokinase

After 7 days of the fermentation of the production medium, cells were removed by centrifugation at - 4°C and 12.000 rpm for 15 min. The supernatant was considered as extracellular crude nattokina according to Peng et al., (2005)

Kim and Choi 2000 concluded that nattokinase activity was significantly increased within the exponential cell growth and steadily approached to a maximum of 587 U/mL after 24 h. In addition, as it was anticipated while bacteria growth rate was increased steadily

Bacillu s subtilis secrete enzymes such as amylase, protease, pullulanase, chitinase, xylanase, lipase, among others. These enzymes are produced commercially but this enzyme production represents about 60 % of the commercially produced industrial enzymes (Schaechter et al., 2006).

Presence of nattokinase produced from *Bacillus substilis*

Fibrinolytic activity

The fibrinolytic activity of nattokinase was measured by casein and fibrin plate technique. It is an excellent quantitative performance according to (Suzuki, (1994). The measurement of the dimension of the clear zone around each hole which is the indicator of nattokinase activity U. Table (2) showed the clear zone of Is (9), Is (13), Is (22), Is (28) and Is (37) is 26 mm, 20 mm, 19 mm, 19 mm and 15mm respectively. One unit of the enzyme activity was defined as the amount of enzyme in 50 ml of enzyme solution that produced a clear zone of 1mm at pH of 7.4 and 37°C for 18 h. as showed in plate (1). Nattokinase degrades fibrin clots both directly and indirectly by affecting plasmingen activator inhibitor. Researchers suggest that nattokinase may promote normal blood pressure, reduce whole blood viscosity and increase circulation being an effective supplement to support cardiovascular health (J. B.S. L. 2004).). U could also be used when converting lysis area to knowing fibrin degrading enzyme activity. Sumi et al., (1990) reported that intravenous administration did not show any clear throm 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 Sudan. Among them *B. subtilis* showed strongest fibrinolytic, activity. In view of this report, it can be suggested that our fibrinolytic enzymes isolated from *B. subtilis* can be given orally for use as a thrombolytic agent.

Table (2) Illustrate the dimension of clear zone around casein hydrolysis

Sample no.	Dimension of the clear zone (mm)
Is (9)	**26**
Is (13)	**20**
Is (22)	**19**
Is (28)	**19**
Is (37)	**15**

Report showed that intravenous administration did not show any clear thrombolytic effect but oral administration enhanced fibrinolytic activity (Morikawa, 2006).

Haemolysis activity

The presence of nattokinase of the five selected bacteria was determined by dissolving human blood clotted according to (Atlas, 1997). Plate (2) (3) and (4). Nattokinase degrades fibrin directly in clot lysis assays with activity comparable to plasmin ((J. B.S. L. 2004). Thus it can be well concluded from the above experiments that different area in Sudan are indeed a rich source of microbial biodiversity holding within it immense novelty and potentiality of identifying new isolates for production of life saving drugs.

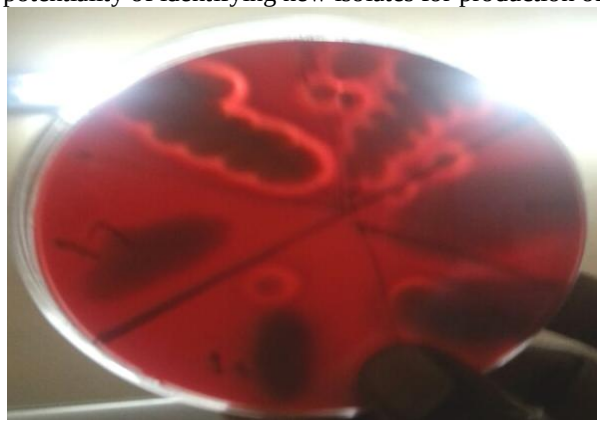

Plate (2) Nattokinase production using blood heamolysis

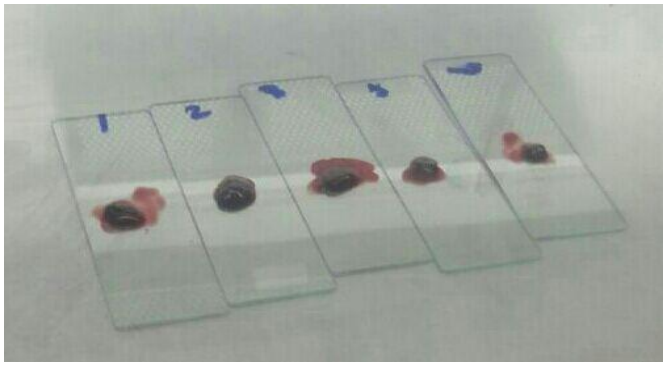

Plate (3) Human clotted blood before the addition of the crude nattokinase

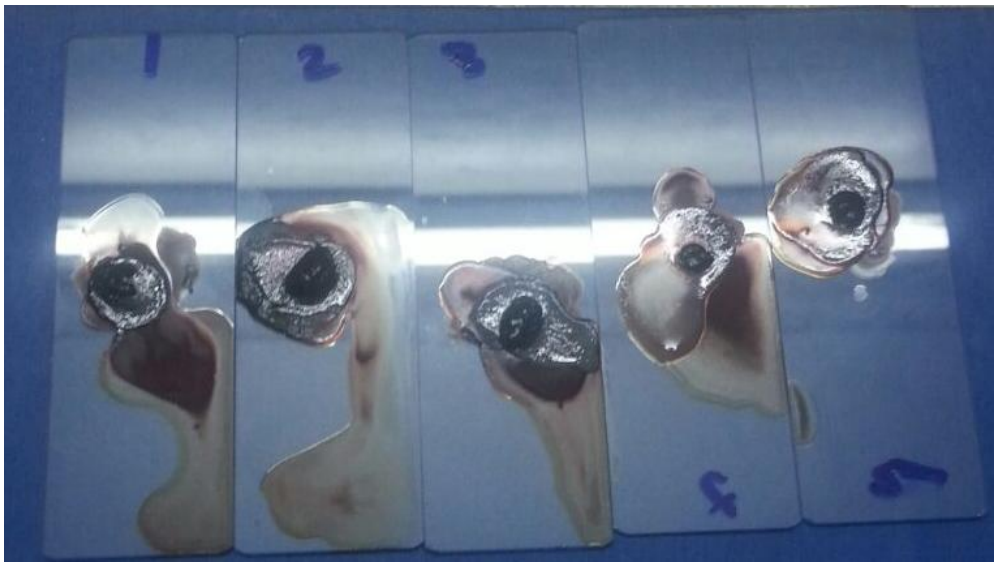

Plate (4). After 2 hours of the addition of the crude nattokinase

A 2009 study showed that nattokinase may be effective in catabolism of toxic amyloid fibrils associated with Alzheimers Disease as well as the insulin fibrils associated with diabetes and the prion peptide fibrils associated with Priom Disease (Ruei et al., 2009)

For a commercial use of nattokinase, it is, therefore, necessary to develop an efficient fermentation process that results in maximum production of the enzyme at high yield, high titer and high productivity

Conclusions

Fifty soil samples were brought from different locations in Sudan. Five isolates: Is (9), Is (13), Is (22) Is (28) and Is (37) out of 50 (10%) showed positive results of genus *Bacillus subtilis* according to conventional microscopic and biochemical analysis. The promising isolates also could produce an extracellular crude nattokinase indicating by heamolytic and fibrinolytic activity. In the future, the research will progress into the production of highly purified fibrinolytic enzymes from bacterial sources.

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References

Atlas, R.,M . 1997. Handbook of Microbiological Media; 2nd ed.,CRC press, Boca Raton, New York, London, Tokyo,1706 pp.

Banerjee, A., Chistic, Y. Banerjee, U. C. 2004. Streptokinase a clinically useful thrombolytic agent. Biotechnol. Adv. 22: 287-305.

Barrow, G. I. and Feltham, R. K .A. 1993. Cowan and Steel's manual for the identification of medical bacteria, 3rd ed. Cambridge University Press, Cambridge, p.352.

Collins, C. H. and Lynee, P. M. 1995. Microbiological methods. 7th edition. Butterworth- Heinemann Ltd.

Debajit, R. N .S. Yadavi, Ankus, Lubana and Kumar. 2012. Production, purification and characterization of nattokinase from *Bacillus* . Asian Journal of Pharmaceutical and Clinical Research. 5(3): 2012 .

Dubey, R. J. Kumar, D. Agrawala, T. Char and P. Pusp. 2011. Isolation, production, purification, assay and characterization of fibrinolytic enzymes (Nattokinase, Streptokinase and Urokinase) from bacterial sources. African Journal of Biotechnology. 10(8): 1408-1420.

Fujita, M. Hong, K. Ito, Y. Fujii, R. Kariya, K. Nishimuro, S. 1995. Thrombolytic effect of Nattokinase on a chemically induced thrombosis model in rat. Biol. Pharm. 18:1387-1391.

Haritha, M. Meena, V. Angalapati. 2011. Nattokinase: A review of fibrinolytic enzyme. Chemical, environmental and pharmaceutical research.2(1):61-66

Hsia, C. H. Shen, M. C. Lin, J. S. Wen, Y. K. Hwang, K. L. Cham, T. M. Yang, N .C. 2009. Nattokinase decrease plasma level of fibrinogen, factorV11, factor V111 in human subject. Nutr. Res. 29: 190-196.

JBSL Japan Bio-Science Laboratory Co, Ltd. 2004. Clinical Study report: an open clinical pilot study to evaluate the safety and efficacy of natural superkinase as an add - on oral fibrinolytic agents to low molecular weight heparin & anti-platelet in acute ischaemic stroke. DS- NK007.

(JNKA) Japanese Nattkinase Association 2004. http://www.j-nattokinase.org

Kim, S. H. Choi, N. S. 2000 Purification and characterization of subtilisin DJ-4 secreted by *Bacillus sp.* strain DJ-4 screened from Doen-Jang. Biosci. Biotechnol. Biochem. 64: 1722-1725

Kwon, E. Y. Kim, K. M. Kim, M. K. Lee, I. Y. Kim, B. S. 2011. Production of nattokinase by high cell density fed-batch culture of *Bacillus subtilis*". Bioprocess and Biosystems Engineering 34(7): 789–793.

Morikawa, M. 2006. Beneficial Biofilm Formation by Industrial Bacteria *Bacillus subtilis* and Related Species. Journal of Bioscience and Bioengineering. 101(1): 1-8.

Nakamura, T. Yamagata, Y. Lchishima, E. 1992. Nucleotide sequence of the subtilisin NAT gene, aprN, of *Bacillus subtilis* (natto). Biosci. Biotechnol. Biochem. 56: 1869-1871.

Peng, Y. Yang, X. Zhang, Y. 2005. Microbial fibrinolytic enzymes: an overview of source, production, properties, and thrombolytic activity in vivo. Appl. Microbiol. Biotechnol. 69:126–132.

Ruei-Lin Hsu, Kung-Ta Lee, Jung-Hao Wang , Lily, Y. L. Lee, and Rita, P. Y. Chen. 2009. Amyloid-Degrading Ability of Nattokinase from *Bacillus subtilis* Natto J. Agric. Food Chem. 57(2):503-508

Schaechter, M. J. L. Ingraham, F. C. Neidhardt. 2006. Microbial library (ASM Press, washington, DC) 99-102

Sumi, H. Hamada, H. Tsushima, H. Mihara, H. Muraki, H. 1987. A novel fibrinolytic enzyme (nattokinase) in the vegetable cheese Natto; a typical and popular soybean food in the Japanese diet. Experientia 43(10):1110-1.

Sumi, H. Hamada, H. Nakanishi, K. Hiratani, H. 1990. Enhancement of the fibrinolytic activity in plasma by oral administration of Nattokinase. Acta. Haematol. 84: 139–143.

Suzuki, K. 1994. Vitamin K-dependent proteins and their functions. In: Wanaga, S. Saito, H. Matsuda, M. (eds.) Vitamin K [in Japanese]. Tokyo: Medical Journal. pp. 109–253.

Uversky, V. N. Fink, AL. 2004. Conformational constraints for amyloid fibrillation: The importance of being unfolded. Biochem. Biophys. Acta, 1698: 131-153.

You, K. M. and Park, Y, K. 2004. A new method for the selective isolation of Actinomycetes from soil . Biotechnol. Techn.10:541-546.

Yoshinori, M. Ada, HKW. Jiang, B. 2005. Fibrinolytic enzymes in traditional fermented food. Food Res. In. 38: 243-250.