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A REVIEW ON SUCCESSIVE GENERATIONS OF NATTOKINASE BASED FIBRINOLYTIC AGENT

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ABSTRACT

Nattokinase (NK) is one such fibrinolytic 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. NK, a potent blood-clot dissolving protein used for the treatment of cardiovascular diseases, is produced by the bacterium *Bacillus subtilis* during the fermentation of soybeans to produce Natto. In this review we focused on screening of producing strains, Genetic Engineering and Fermentation Process optimization for microbial nattokinase production, extraction and purification of nattokinase.

KEYWORDS: Nattokinase, blood clot dissolving protein, *Bacillus substilis.*

INTRODUCTION

Cardiovascular diseases such as heart attack, high blood pressure and stroke are the leading causes of death worldwide. These diseases are caused by the accumulation of fibrin in the blood vessels, which results in thrombosis. Fibrin is the main component of blood clot, and it is normally formed from fibrinogen by the action of thrombin. Thrombolytic agents hydrolyse these insoluble fibrin fibres so as to lysis the thrombus and restore blood flow to the area of ischemia. In earlier days thrombosis was treated by the use of anticoagulants such as heparin and coumarin, which inhibit the fibrin clot formation. The *in vivo* lysis of fibrin that involves the conversion of inactive plasminogen into active plasmin led to an alternative approach based on enzyme.[1]

Proteases, also known as peptidyl-peptide hydrolases are industrially useful enzymes which catalyze the hydrolysis of a peptide bond in a protein molecule.[2] Nattokinase is a serine protease from subtilisin family. It is particularly potent treatment, and it enhances the body's natural ability to fight blood clots in several different ways; because it so closely resembles plasmin, it dissolves fibrin directly. In addition, it also enhances the body's production of both plasmin and other clot dissolving agents, including urokinase. It digests fibrin both directly and indirectly. Indirectly, it activates pro-urokinase and tissue plasminogen activator(t-PA), supporting the fibrinolytic activity of plasmin.[3] These combined action 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.[4] The main interest about this enzyme is due to its direct fibrinolytic activity, being stable enough in the gastrointestinal tract makes this enzyme a useful agent for the oral thrombolytic therapy.

Thus, NK is regarded as a valuable dietary supplement or nutraceutical, proven safety and ease of mass production are other advantages of this enzyme. In addition to these valuable advantages, there are other applications attributed to NK including treatment of hypertension, Alzheimer's disease, and vitreoretinal disorders. Microbial fibrinolytic protease is considered as highly purified fibrinolytic enzymes from microbial sources is the most stable and economic way to obtain protein with fibrinolytic activity.[5]

Along with the development of biotechnology, great achievements have been made in the area of microbial production of nattokinase. The aim of this review is to detail the latest developments of the nattokinase producing strains, genetic engineering methods on nattokinase production, extraction and purification process, physiochemical properties of nattokinase from various microbes, comparison with other fibrinolytic agents, as well as its current status.

Properties of Natttokinase

Nattokinase (NK), named after the first producer strain *Bacillus subtilis* natto, is a serine protease from the subtilisin family (EC 3.4.21.62). The molecular mass ranges from 27.7-42 kDa and pI is ~8.7. Nattokinase exhibits considerable activity at pH 5.5 to 9.0. Strong Alkali destroys its functional and structure stabilities, and its activity decreases significantly when the temperature goes beyond 60°C. NK is composed of 275 amino acids and the gene sequence is homologous to those of other members of the subtilisin family (99.5% homology with subtilisin E, 86% with subtilisin BPN′, and 72% with subtilisin Carlsberg). The enzyme is a cysteine-free protease; thus, no disulfide bond is observed in its structure. Inhibition of NK by phenylmethylsulfonylfluoride (PMSF) indicates its membership to the serine protease family of enzymes. NK is encoded by the aprN gene. The protein is synthesized in a precursor form, in which a signal peptide and a propeptide are joined to the N-terminus of the mature polypeptide. The three-dimensional structure of NK at 1.74-Å resolution is resolved recently. The catalytic center of which contains three conserved residues, Asp-32, His-64, and Ser-221. Hydrogen bonds occurring in the catalytic triad and the oxyanion hole (Asn155) are very important to the catalysis of peptide bond.[5,6,7] It exhibits a 4-fold greater thrombus-dissolving activity than plasmin, can help the body to decompose the detrimental coagulation of blood, and potentiates endogenous fibrinolysis by inactivating plasminogen activator inhibitor type 1. Moreover, without raising the safety caution, the oral administration of nattokinase gives rise to a mild and frequent enhancement of the fibrinolytic activity in the plasma. These results indicate the potential use of nattokinase as a drug to treat embolism and as a dietary supplement to prevent cardiovascular diseases.[8] This enzyme has been found in many resources like Japanese natto, Chinese food douche, doufuru. It is also present in various microorganisms, the most important strain is the genus *Bacillus*. Many researchers have focused on the healthy food which was fermented by probiotics.[9]

Nattokinase as a Fibrinolytic Agent

It is noteworthy that fibrinolysis (nattokinase's main physiologic effect) is a natural physiologic process. Fibrin, a naturally occurring blood protein is broken up into fibrin-degradation products during fibrinolysis. There are several naturally occurring fibrin-degradation processes, all of which are well-documented in conventional literature. Nattokinase, therefore, undeniably promotes mechanisms of action that occur naturally. Once fibrin is degraded, clotting time is slowed. Nattokinase has been found to lyse (or break down) fibrin strands and plasmin substrates directly. In the process of clot-regulation, prourokinase is converted into urokinase, a process that is enhanced by nattokinase. Breaking fibrin down into its degradation products is also enhanced, converting plasminogen to plasmin.[6] Nattokinase increases tissue plasminogen activator also, enhancing fibrin breakdown and clotting reduction further.[10] By reducing thrombus formation, nattokinase decelerates the progression of plaque formation and reverses evolving atherosclerotic lesions. Moreover, nattokinase hydrolyzes active recombinant prokaryotic plasminogen activator inhibitor-1 (PAI-1), indicating that fibrin clot lysis by nattokinase also involves the cleavage and inactivation of PAI-1. These findings, along with the observation that nattokinase can be absorbed across the intestinal tract after oral administration make it a promising anti-clotting agent for the prevention and treatment of CVDs. Furthermore, a recent study has revealed that nattokinase can degrade amyloid which is believed to be associated with various neurodegenerative diseases.

Activity of free nattokinase decreases obviously in acidic pH conditions, entirely inactivated in simulated stomach environment (pH 1.2) after 1 h, which is a major barrier for oral administration effect. The pH stability of nattokinase can be improved by microencapsulation, while more than 95 % of activity is lost in simulated stomach environment. By compression coating of nattokinase tablets with methylacylic acid–methylmethacrylate copolymer, nattokinase were protected from acid inactivation in artificial gastric juice. The smelly taste of natto and nattokinase functional food is a special flavor for some consumers, while unpleasant for other consumers. Therefore, masking the smelly flavor is needed for extensive use. Compared to the tablet formulation, capsule shows better masking effect. Kollicoat MAE 100P (methacrylic acid–ethyl acrylate copolymer) is a pH-responsive material, which is resistant to the acidic environment and dissolves at neutral pH condition. This polymer has not been applied for coating of nattokinase capsule.[11] 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.[12]

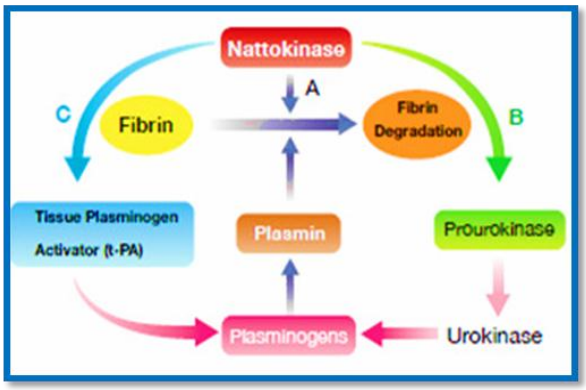

Figure 1: Physiological effects of Nattokinase on fibrin.[6]

Isolation and Identification of the Nattokinase

Most nattokinase-producing strains are isolated from Japanese natto food, and similar fibrinolytic enzymes are also isolated from other traditional fermented foods including Korean doen-jang and Chinese douchi. As a traditional fermented food, Chinese soybean paste is rich in food microorganisms, such as *Bacillus subtilis, Bacillus amyloliquefaciens, and Bacillus licheniformis.*[13] Casein plate method and fibrin degradation method were used for isolation of fibrinolytic enzyme productive strains.[14]

Enzyme production and purification

For the production of nattokinase, the bacterial isolate was grown in 100 ml of liquid medium in a conical flask containing containing 1 % SSP, 0.1 % K2HPO4, 0.05 % MgSO47H2O and incubated for 2 days at 30ºC.[15] For the separation of enzyme, the production media was centrifuged at 8,000 rpm for 20 min in a cooling centrifuge at 4^0C. Supernatant was collected, which served as the crude enzyme, and used for the enzyme assays and precipitation. The crude enzyme solution was concentrated by vacuum evaporation at 40ºC and filtered by 0.22 µm filter membrane to remove any bacterial cells. The concentrated crude enzyme solution was loaded onto a CM-Sepharose Fast Flow column (1.6 * 10 cm, bed volume 17 mL). The column was washed with 6 bed volumes of 10 mM Tris buffer (pH 7.4) and eluted with a linear gradient of 0 to 0.5 M NaCl in the same buffer at a flow rate of 0.2 mL/min. The fractions with fibrinolytic activity were pooled, concentrated and further loaded onto a Sephadex G-50 gel filtration column (2.6 * 90 cm). The column was eluted with 10 mM Tris buffer containing 0.5 M NaCl (pH 7.4) at a flow rate of 0.4 mL/min. The fractions containing the enzyme activity were combined, dialyzed against 50 mM Tris buffer (pH 7.8) and stored until further use. The protein concentration was determined by Lowry method using bovine serum albumin as the standard.[16]

Nattokinase activity measurement

The enzyme activity was quantified by cleavage of the synthetic substrate N-succinyl-Ala-Ala-Pro--Phe-p-nitroanilide, as determined by absorbance of product (p-nitroaniline) at 410 nm. The reaction mixture containing 0.5 mL of 1 mM synthetic substrate, 0.6 mL of PBS buffter, and 0.5 mL of the enzyme solution was incubated at 37 °C for 10 min. The reaction was then stopped by adding 0.5 mL of 0.2 M acetic acid. The absorbance of released p-nitroaniline can be measured at 410 nm using a UV spectrophotometer. One unit of amidolytic activity was expressed as nmol of p-nitroaniline released due to substrate hydrolysis/min/mL by the enzyme.[3]

Table 1: Physicochemical properties of Nattokinase from Various Microorganisms.

S. No	Organism	Molecular weight (KDa)	Optimum Temperature	Optimum pH	Reference
1	*Bacillus subtilis*	40-45	37ºc	9	[19]
2	*Bacillus subtilis* RJAS19	35	50ºc	9	[20]
3	*Bacillus subtilis*	28	37-55ºc	9	[21]
4	*Staphylococcus sciuri*	30	48ºc	8	[22]
5	*Pseudomonas aeruginosa* CMSS	21	25ºc	7	[10]
6	*Bacillus subtilis* REVS12	Not defined	40ºc	8	[23]
7	*Bacillus subtilis* YJ1	27.5	50ºc	8.5	[24]
8	*Bacillus subtilis* N1	46.5	55ºc	8	[16]
9	*Pseudomonas sp.* TKU015	21	50ºc	7	[25]
10	*Bacillus subtilis* TKU007	30	40ºc	8	[26]
11	*Bacillus subtilis*	Not defined	37ºc	9	[27]
12	*Bacillus subtilis*	Not defined	37ºc	7	[28]
13	*Bacillus subtilis*	Not defined	45ºc	5	[29]

Genetic engineering to enhance nattokinase production

With the development of molecular biotechnology, genetic engineering have been considered as the effective strategies to improve the nattokinase. The gene aprN coding for nattokinase has been cloned and expressed in many host strains, such as *B. subtilis, B. licheniformis, E. coli, L. Lactis, Spodoptera frugiperda*, and many genetic manipulation strategies have been utilized to improve the nattokinase production. Genetic modification of host strains was proved as one efficient strategy. Nattokinase yield was increased by 39% in the eight proteases deficient strain *B. licheniformis* BL10 (14.33 FU/mL), and by 2.6-fold in the multiple lytic genes deficient strain (5226 IU/mL). Also, an efficient autoinducible expression system was constructed for high-level production of nattokinase by promoter engineering, and the P23 promoter was confirmed as the optimal promoter for enhancing nattokinase production with the increased activity at 93.6 FU/ mL (Guan et al. 2016). The nattokinase yield (643 µg/ mL) was increased obviously by altering the −10 or −35 region of promoter PaprN. Meanwhile, the expression and transport components have also been engineered to enhance the nattokinase yield. By screening the signal peptides of extracellular proteins in *B. licheniformis*, the nattokinase yield was increased by 4.2-fold, which was attributed to the signal peptide of AprE, and overexpression of the signal peptidase I SipV also improved the nattokinase yield to 35.60 FU/mL in this optimized strain. Furthermore,

nattokinase was expressed as inclusion body in E. coli, and it was simply restored to the natural enzyme activity level by mixing protein with the refolding solution directly.[5]

Current status of Nattokinase based fibrinolytic drug

Natto contains a potent fibrinolytic enzyme that they named as nattokinase, and an oral form is now available to consumers worldwide as a supplement. The enzyme is currently considered in pharmaceutical industry as a promising drug for thrombolytic therapy. Now-a-days many pharmaceutical companies manufacture Nattokinase based fibrinolytic drugs. Currently using Nattokinase products are shown in below table.

Table: 2 Trade Name and Details of Some Nattokinase Based Products.

S. No	Trade name	Developed By	Product details	Reference
1	Clinical Grade Nattokinase	Dr. David Williams	Promotes healthy circulation and blood flow, helps in maintaining body's normal blood clotting mechanism.	https://www.drdavidwilliams.com/nattokinase-nt13
2	Nattovena	Arthur Andrew Medical	Circulatory and Heart Health, Superior Fibrin Degradation, Blood Cleansing and Purification, Normal Blood Viscosity, Healthy Plasmin Production	https://www.arthurandrew.com/products/nattovena
3	Natto-k	Enzymedica	Supports cardiovascular health, enhances circulation and promotes production of plasmin	https://www.a1supplements.com/enzymedica-natto-k
4	Nattokinase 2,000 FU's	Healthy origins	Supports Normal Circulatory Health	https://www.vitacost.com/doctors-best-nattokinase-2000-fu-90-vegetarian-capsules
5	Nattokinase with rutin	The vitamin shopee	Cardiovascular, Antioxidant	https://www.vitaminshoppe.com/p/nattokinase-rutin-100-mg-60-veggie-caps/vs-3448
6	Nattokinase	Puritans pride	Supports heart health	https://www.amazon.com/Puritans-Pride-Nattokinase-mg-60-Softgels/dp/B00B8XG052
7	Nattokinase complex	Solgar	Supports cardiovascular health	https://in.iherb.com/pr/solgar-nattokinase-complex-30-softgels/9375

Comparison of Nattokinase with other fibrinolytic agents

Nattokinase generates fibrin-degradation products for prolonged periods of time. Fibrin degradation products that result from nattokinase are generated for 8 hours and, in some cases, last up to 12 hours. However, unlike other conventional fibrinolytic agents that are administered orally, natto has been reported to prevent blood coagulation and encourage existing thrombi to dissolve. Thrombolytics (Tissue plasminogen activators-Alteplese/ Activase) is administered in a hospital setting during an acute myocardial infarction or thrombic stroke. t-PA is most effective within 3– 6 hours of onset. Acute hospital intervention, in many cases, is often too late and ineffective because the vessels of typical patients who sustain heart attacks or strokes contain hardened plaque beyond what a thrombolytic agent can affect. Urokinase is used to manage atrial heart chamber clots, clots in veins from IV catheters, and during thrombic heart attacks and thrombic strokes. It is indicated for dissolving thrombi in the heart, blood vessels, or lungs. Nattokinase maintains its activity for an 8–12 hour period; a longer half life than urokinase, which is effective for 4–20 minutes. Natto inhibits fibrin more effectively in-vitro than urokinase or plasmin and is relatively heat resistant. Streptokinase is indicated for treating an early myocardial infarction, especially an acute anterior myocardia l infarction within 6 hours prior to the onset of pain leading to hospital admission. Compared to t-PA, streptokinase has been found to be relatively ineffective when administered soon after a stroke. aspirin alone is used most often to prevent recurrent strokes and transient ischemic attacks. Aspirin acts by inhibiting blood clotting, which reduces platelet aggregation, and thereby prevents platelet plugs from forming. Aspirin inhibits cyclo-oxygenase irreversibly, which facilitates thromboxane A2 (TA2) production. TA2 is a potent inducer of platelet aggregation and vasoconstriction. Bromelain has been well documented for its ability to activate fibrinolysis via stimulating plasmin production. Because nattokinase inhibits fibrin more effectively in vitro than plasmin, nattokinase is a much more potent fibrinolytic agent than bromelain. Chinese ginseng-Panax ginseng has inhibited fibrinogen conversion to fibrin. The herb's mechanism of action appears to be via promoting urokinase's fibrinolytic activity. Natto and nattokinase inhibit fibrin invitro more significantly than urokinase does logic holds that

nattokinase is a more potent fibrinolytic agent than Panax ginseng or any other botanical or drug in vitro.[17]

Applications of Nattokinase

In recent years, nattokinase has drawn increasing attention of investigators because of its important physiological functions in heart and cerebral vessels. Nattokinase is a potent fibrinolytic enzyme that is considered to be a new promising agent for thrombolytic therapy[6], while the main drugs of urokinase (UK) and streptokinase (SK) are too expensive and their half life is short. Compared to the clinical thrombolytic drugs (urokinase and streptokinase), nattokinase has several advantages such as safe, low cost, and easy oral administration.[17] Nattokinase enhances our bodys natural ability to fight blood clots, and also has an advantage over blood thinners because it has a prolonged effect without side effects.[3] Nattokinase.

- Supports normal blood pressure
- Prevents blood clots from forming and dissolves existing blood clots
- Dissolves fibrin
- Enhances the body's production of plasmin and other clot dissolving agents, including urokinase (an enzyme produced by the kidneys and found in urine, which activates plasminogen).
- It has been shown to stabilize and assist the gastrointestinal tract
- May aid in the prevention of angina, varicose veins, muscle spams and pain
- Coronary artery disease — via heart attack prevention, morbidity, and recurrence reduction • Peripheral vascular disease —arterial atherosclerosis, venous thrombi
- Strokes—prevention, and morbidity and reduction recurrence

NK was reported to have an effect on both oxidative injury-mediated arterial thrombosis and inflammation-induced venal thrombosis. When ferric chloride (FeCl3) was administered to the injured arteries, it resulted in oxidative thrombosis and platelet adhesion. After treatment with NK, however, thrombus formation and platelet aggregation were inhibited. The effects of NK are similar to the well-known blood thinner, aspirin. Unlike aspirin, which often triggers bleeding or gastric ulcers, NK improves blood flow without any adverse effects. κ-Carrageenan-induced inflammatory thrombi formation in rat tails was used to examine the effect of NK. Twelve hours after gavage administration of NK, higher levels of fibrin degradation product (FDP) fragments and D-dimers were detected in blood samples. A greater than 50% decrease in thrombosis was observed in the blood vessels of the rat tail by biopsy analysis. At present, commercial NK products are widely used in Japan, China, Korea, European Union Countries, Canada, and the United States as a food supplement to thin blood, prevent blood clots, and improve blood circulation. Studies also indicate that NK can ameliorate other diseases such as hypertension, stroke, Alzheimer's disease, and atherosclerosis. The potential of using NK to decrease atherothrombotic risk and slow the progression of atherosclerosis as well as cognitive decline is currently being assessed in a Phase II clinical trial.[18]

CONCLUSION

Nattokinase is an exciting new compound with proven very potent fibrinolytic activity. Natto extracts with significant amounts of nattokinase are promising functional foods. All prior epidemiologic and clinical research points to nattokinase's effectiveness and safety for managing a wide range of diseases, including hypertension, atherosclerosis, coronary artery disease (such as angina), stroke, and peripheral vascular disease. Stressful era of modernization has led to high rates of cardiovascular diseases; thence it would then seem prudent to add this effective natural product to our heart health preventive arsenal as more recently, both clinical and non-clinical studies have demonstrated that Nattokinase supports heart health and promotes healthy circulation. Various animal and human trials have demonstrated that NK improves blood circulation and helps decrease the risk of a variety of cardiovascular diseases without producing any adverse side effects. Ongoing advances in genetic engineering are providing a promising future for the economically-viable, large-scale production of high-quality NK using recombinant gene technology. The current deal for nattokinase production is to decrease the cost of product by increasing the yield or integrated processes to reduce the processing cost and make the product available to a wide range of consumers.

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