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MINI-REVIEW

Nattokinase: production and application

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Abstract Nattokinase (NK, also known as subtilisin NAT) (EC 3.4.21.62) is one of the most considerable extracellular enzymes produced by *Bacillus subtilis natto*. 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. This review tends to bring a brief description about this valuable enzyme and summarizes the various biotechnological approaches used in its production, recovery, and purification. Some of the most important applications of NK, as well as its future prospects, are also discussed.

Keywords *Bacillus subtilis* · Nattokinase · Medical applications · Subtilisin NAT · Supplement · Fibrinolytic activity

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Introduction

In 1987, Sumi et al. (1987) introduced a novel fibrinolytic enzyme from Natto (a typical and popular fermented soybean food in the Japanese diet) and named it nattokinase. Nattokinase (NK, also known as subtilisin NAT) (EC 3.4.21.62) is among the most considerable extracellular enzymes being produced by *Bacillus subtilis natto* (Hsieh et al. 2009; Liu et al. 2005; Unrean and Nguyen 2013). The present review aims to detail the current knowledge of the NK enzyme. This aim is followed by the critical review regarding its production, extraction, formulation, and applications. SCOPUS, Web of Science, and PubMed databases were reviewed until July 2014 using the "nattokinase" and "subtilisin NAT" keywords as the search terms. The lists of articles identified by this search strategy were studied and the relevant articles were selected.

Nattokinase structure

Natto is a traditional soybean food produced by *B. subtilis natto* fermentation. Natto is considered a rich source of valuable products including NK and menaquinone-7 (Berenjian et al. 2014b). We previously demonstrated this strain as the dominant microorganism for the industrial production of these health-promoting compounds (Berenjian et al. 2012, 2013).

NK is a serine protease composed of 275 amino acids (molecular weight 27.7 kDa and *p*I 8.6) which belongs to

the subtilisin family. The enzyme is a cysteine-free protease; thus, no disulfide bond is observed in its structure. Inhibition of NK by phenylmethylsulfonyl fluoride (PMSF) indicates its membership to the serine protease family of enzymes (Fujita et al. 1993; Yanagisawa et al. 2013; Zheng et al. 2005). 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 (Nakamura et al. 1992). The three-dimensional structure of NK at 1.74-Å resolution is resolved recently. The structure is almost identical with that of subtilisin E from *Bacillus subtilis* DB104. There are differences in residues at positions 85 and 192 between subtilisin E and NK. The finding was confirmed by the analysis of both the DNA sequence of subtilisin NAT gene (Nakamura et al. 1992) and the protein sequence of purified nattokinase (Fujita et al. 1993). Although NK is highly homologous to many subtilisins in serine protease family, only this enzyme shows high substrate specificity for fibrin (Peng et al. 2005). The enzyme shows considerable fibrinolytic activity at pH 6–12 but lacks functional and structural stability in alkaline environments. The enzyme activity also slows down at temperatures beyond 60 °C (Fujita et al. 1993; Maeda et al. 2001). NK retains more than 95 % of its activity after five rounds of freezing and thawing (Sumi et al. 1987). The NK characteristics are summarized in Table 1.

The catalytic triad of the NK enzyme is Ser-His-Asp (D^{32}, H^{64}, S^{221}) situated in a shallow groove on the surface of globular protein (Nguyen et al. 2013; Zheng et al. 2005). Hydrogen bonds occurring in the catalytic triad and the oxyanion hole (Asn^{155}) are very important to the catalysis of peptide bond. The four residues of Ser^{33}, Asp^{60}, Ser^{62}, and Thr^{220} form several hydrogen bonds which function to stabilize the transition state of the hydrolysis reaction (Zheng et al. 2006). A new nucleophilic catalytic mechanism for NK is proposed, and the action of hydroxyl in the catalytic environment as well as the action of S^{221} for locating the active peptide bond is clarified (Zheng et al. 2005). Residues of the S3 binding site of NK, namely Gly^{100}, Ser^{101}, and Leu^{126}, are critical for protease and fibrinolytic activities. Introducing amino acids with longer side chains at position Gly^{100} that project into the active site likely decreases substrate binding and catalytic activity of this enzyme. In opposition to the Gly^{100}, introduction of bulky side chains at position Ser^{101} increases protease activity. Leu^{126} is considered an essential structure component of the active cleft of NK which is buried in the active cleft and close to the catalytic triad. This residue is conserved and very important for the function among all subtilisins (Wu et al. 2007).

Fibrinolytic and antithrombotic activity

Several reports indicate the various fibrinolytic enzymes produced by *Bacillus* spp. (Chang et al. 2000, 2012; Ghasemi et al. 2012; Kim et al. 1996; Peng et al. 2003; Wang et al. 2009a; Yin et al. 2010). Among these enzymes, NK is assumed an interesting one. This considerable interest is due to the direct fibrinolytic activity of this enzyme, both *in vitro* and *in vivo* (Fujita et al. 1995a; Kamiya et al. 2010; Xu et al. 2014). It not only degrades fibrin directly but also increases the release of tissue plasminogen activator from cells to degrade fibrin (Yatagai et al. 2008). Moreover, it also enhances the body's production of clot-dissolving agents including plasmin and pro-urokinase. As compared to plasmin, NK is less sensitive on the cleavage of fibrinogen but more sensitive on the cleavage of cross-linked fibrin (Fujita et al. 1995c). Furthermore, NK inactivates plasminogen activator inhibitor type 1 and is capable of platelet aggregation inhibition by blocking thromboxane formation (Jang et al. 2013; Urano et al. 2001). The fibrinolytic activity of NK is retained in blood longer than 3 h (Tai and Sweet 2006). Decreasing the plasma levels of fibrinogen, factor VII, and factor VIII is another feature which makes NK a nutraceutical used in cardiovascular diseases (Hsia et al. 2009).

Thus, NK is currently considered an efficient, secure, and economic enzyme and also the focus of thrombolytic drug studies (Huang et al. 2013; Unrean and Nguyen 2013). Several strategies to minimize the side effects of NK to improve its stability and lengthen its circulation time for the treatment of thrombotic diseases hold great promise (Huang et al. 2013). As such, by means of site-directed mutagenesis experiments, the oxidative stability of NK was substantially increased by optimizing the amino acid residues Thr^{220} and Met^{222}, which were in the vicinity of the catalytic residue Ser^{221} of the enzyme (Weng et al. 2009). However, attempts for increasing thermostability of NK by introducing cysteine residues and therefore forming disulfide bonds were not successful. In this regard, Weng et al. (2014) constructed three double mutants of NK (G61C/S98C, T22C/S87C, and S24C/S87C) which contained two cysteines and determined their thermostability. The results demonstrated that disulfide bond was not formed within two cysteine residues and the three double mutants did not show increment in thermostability as compared to the wild-type NK.

Nattokinase production and formulation

Traditionally, most nattokinase-producing strains were isolated from the Japanese food Natto. Resembling fibrinolytic enzymes are also obtained from other traditional fermented foods such as Chinese douche, Korean doen-jang, Korean Chungkook-jang soy sauce, and Thua nao from northern Thailand (Inatsu et al. 2006; Kim et al. 1996; Wei et al. 2012). Besides *Bacillus* spp., alternative sources of obtaining this enzyme are *Pseudomonas* sp. and marine creatures (Mahajan et al. 2012; Sumi et al. 1992; Wang et al. 2009c). There are comprehensive studies regarding the optimal

Table 1 Nattokinase characteristics

Characteristics	Reference
Names and origin	Nakamura et al. (1992)
Gene name: *aprN*	
Open reading frame: 1143 nucleotide	
Initiation codon: GTG	
Protein name	Nakamura et al. (1992)
Recommended name: subtilisin NAT	
Alternative names: nattokinase; subtilisin BSP	
Sources	Mahajan et al. (2012), Wang et al. (2009c)
Major sources: *Bacillus subtilis natto*	
Other sources: *Pseudomonas* sp.; marine creatures	
Protein attributes	Fujita et al. (1993), Yanagisawa et al. (2010, 2013), Zheng et al. (2005)
Family: subtilisin serine protease	
Protein sequence length:	
Complete: 381 residues	
Signal peptide: 29 residues	
Propeptide: 77 residues	
Mature polypeptide: 275 residues	
Molecular weight of mature polypeptide: 27,700 Da (27,724 Da)	
Isoelectric point: 8.6	
3D-structure: PDB ID 4DWW (resolution of 1.74 Å)	
Commercial product examples	Peng et al. (2005)
NSK–SD™ (Pure encapsulations®)	
Capsules containing 100 mg (2000 fibrinolytic inits) NK	
NattoMax® (Jarrow Formulas®)	
Capsules containing 100 mg (2000 fibrinolytic units) NK	

fermentation conditions for NK production (Berenjian et al. 2014a; Chen and Chao 2006; Chen et al. 2007a; Cho et al. 2010; Deepak et al. 2008; Ku et al. 2009; Kwon et al. 2011; Liu et al. 2005; Rasagnya and Vangalapati 2013; Unrean et al. 2012; Wang et al. 2009b; Mahajan et al. 2010). Concentration adjustment and feeding strategy of essential nutrients are critical for enhancing NK production. As such, it has been observed that yeast extract, soy peptone, and glycerol are the most effective nutrients to enhance the NK production rate. Moreover, we recently demonstrated that compared to the batch fermentation, the fed-batch addition of glycerol during the cell growth phase increases NK production significantly (Berenjian et al. 2014a). Ku et al. (2009) optimized the cultivation medium composed of defatted soybean and glucose. Defatted soybean is a by-product formed in salad oil manufacturing, and glucose is regarded a very common and cheap carbon source. The most important advantage is the low cost of substrates used to produce valuable NK. Interestingly, *Pseudomonas* sp. TKU015 and *B. subtilis* TKU007 are able to produce NK using shrimp shell wastes as the sole carbon/nitrogen source. Shrimp shell powder is a fishery waste; thus, the production procedure using this substrate would be very cost effective and favors producing inexpensive fibrinolytic enzymes (Wang et al. 2009c, 2011). Fibrinogen and fibrin have also been used as substrates in culture media for NK production. Park et al. (2013) used fibrin- or fibrinogen-containing tryptic soy broth media to produce and purify NK from *B. subtilis* WRL101. It was observed that in spite of increased fibrinolytic activity obtained in the fibrinogen-containing medium, fibrin-containing medium decreased the activity.

NK gene from *B. subtilis natto* is also cloned and expressed in a variety of hosts including *B. subtilis*, *Escherichia coli*, *Lactococcus lactis*, and *Spodoptera frugiperda* insect cells (Chiang et al. 2005; Li et al. 2007; Liang et al. 2007a, b; Liu and Song 2002; Nguyen et al. 2013). Many efforts have been invested to increase the recombinant NK production. For instance, by altering sequences of the -10 or -35 elements of the NK gene promoter (P*aprN*), especially -10 element, the extracellular expression of recombinant NK was improved (Wu et al. 2011). Employing expression vectors with high structure stability also led to higher production of recombinant NK (Chen et al. 2007b). DNA family shuffling is another

mean to improve the fibrinolytic activity of NK. Using this method, it is feasible to generate a mutant library of NK to obtain a mutant with enhanced catalytic efficiency (Yongjun et al. 2011). Another promising procedure to enhance the extracellular production of NK in recombinant *B. subtilis* is assumed to be nutrient supplementation strategy. Using this technique, fourfold increase in recombinant NK production is reported with the medium supplemented with metal ions and glutamate (Chen and Chao 2006).

During downstream processing in the NK purification procedure, the traditional protein separation and purification methods, including organic solvent fractionation, salting out, and protein chromatography, have been utilized. However, some drawbacks such as lengthy separation and purification time, more operation units, and less activity recovery make these methods less useful (Chang et al. 2000; Deepak et al. 2009; Fujita et al. 1993; Rasagnya and Vangalapati 2013; Tonova and Lazarova 2008; Urano et al. 2001). Recently, novel methods for extraction and purification of NK are considered. For instance, reverse micelle extraction of this enzyme from fermentation broth using AOT/isooctane reverse micelles is reported for the first time, and the effects of temperature as well as phase volume ratio were examined (Liu et al. 2004, 2006). Three-phase partitioning (TPP), an efficient bioseparation technique, has been used to purify NK from fermentation broth of *Bacillus natto* NRRL-3666. In this technique, a combination of ammonium sulfate and *t*-butanol is exploited to precipitate protein between the lower aqueous layer and the upper organic layer (Garg and Thorat 2014). Alternative effective method of NK purification which is claimed to be faster compared to other methods is using magnetic poly(methyl methacrylate) (PMMA) beads immobilized with *p*-aminobenzamidine (Yang et al. 2006). In a study performed by Chiang et al. (2005) either NK or pro-NK could be overexpressed in *E. coli* as a recombinant protein fused to the C-terminus of olesin, a unique structural protein of seed oil bodies, by means of a linker polypeptide called intein. Active NK was released through self-splicing of intein, after reconstitution of artificial oil bodies. This action was induced by alteration in temperature and spontaneous cleavage of the propeptide.

In addition to the site-directed mutagenesis and protein engineering techniques for improvement of NK stability, other methods have been tried. NK immobilization is conducted with polyhydroxybutyrate (PHB) nanoparticles which increases the stability of the enzyme. Accordingly, activity was completely retained on storage at 4 °C for 25 days (Deepak et al. 2009). Microencapsulation of NK using γ-polyglutamic acid as a coating material can improve temperature and pH stability of microencapsulated NK compared to those of the free form (Hsieh et al. 2009).

In order to protect NK from being denatured in the gastric juice, a new formulation was designed to control its release rate when it passes through the human gastrointestinal tract. Hence, NK powder was first compacted into a tablet, which was then coated with a mixture of an enteric material, called Eudragit L100-55, and hydroxypropylcellulose by direct compression (Law and Zhang 2007).

Nattokinase as a dietary supplement and functional food

As a functional food, NK owns several advantages compared to the available clinical thrombolytic drugs, such as safety, low cost, confirmed efficacy, prolonged effects, preventative use, and easy oral administration (Peng et al. 2005; Zheng et al. 2005). NK is absorbed from the intestinal tract and has pH and temperature stability so that it can remain stable in the gastrointestinal system (Fujita et al. 1995b; Sumi et al. 1987). The effectiveness of *B. subtilis* NK capsules in dissolving thrombi in dogs is reported by Sumi et al. (1990). In a study performed by Suzuki et al. (2003b), dietary natto-extract supplementation caused suppression of intimal thickening produced by endothelial injury in rat femoral artery. Similarly, it has been observed that dietary supplementation with natto extract containing NK, suppresses intimal thickening after vascular injury (Suzuki et al. 2003a). As Ero et al. (2013) demonstrated, when administering oral single dose of NK supplement, peak serum levels were observed at approximately 13.3 h±2.5 h post-dose. In order to create *Lactococcus lactis* probiotic strain that is capable of producing or even secreting NK, Lee et al. (2010) combined the functions of *L. lactis* and the NK from *B. subtilis natto* and further assessed the safety and the risk of oral administration of this genetically modified microorganism. Murine models were also used to detect the allergenicity of this genetically modified lactic acid bacterium which showed to be regarded as safe to use (Chiang et al. 2011). Orally administrated NK combined with red yeast rice showed lipid-lowering effect. However, this effect was not associated with NK consumption alone, suggesting that combined NK and red yeast rice will be a better neutraceutical for patients with hyperlipidemia (Yang et al. 2009).

Effects on blood pressure

In a randomized double-blinded placebo-controlled trial, the effects of NK supplementation on blood pressure in prehypertension or stage 1 hypertension subjects were examined. In this regard, NK supplementation resulted in systolic and diastolic blood pressure reduction which is suggestive of its role in the prevention and treatment of hypertension (Kim et al. 2008). Inhibition of angiotensin I-converting enzyme (ACE) by NK and its degradation peptides may be attributed to suppression of blood pressure (Murakami et al. 2012). Therefore, consumption of functional foods containing NK

with antihypertensive activity is a successful strategy for the treatment of hypertension (Suwanmanon and Hsieh 2014). A research was conducted to elucidate the mechanism by which NK prevents hypertension and clarify whether the protease activity of this enzyme is needed or not. The results demonstrated that both intact form of NK and its fragments are absorbed from the intestine and reduce hypertension in spontaneously hypertensive rats. Nevertheless, depending on the form of oral administration, different mechanisms are suggested for antihypertensive effect. When intact NK (having protease activity) is administered, blood pressure may decrease through the reduction of blood viscosity by cleaving plasma fibrinogen. On the other hand, the fragments obtained from NK suppress hypertension via downregulation of plasma angiotensin II level (Fujita et al. 2011).

Further therapeutic applications of NK

When medical devices are in contact with blood, thrombogenesis is likely to occur immediately. After days to weeks without careful antithrombotic therapy, the thrombus complications may be evident. Therefore, considerations should be made to inhibit implant-associated thrombosis. To prevent this complication, NK is used as an innovative strategy for coating medical devices with antithrombotic activity. Local application of NK as novel composite particles has also been indicated for inhibition of implant-associated thrombosis (Wei et al. 2014). Oral administration of NK in the rat model of Alzheimer's disease has been demonstrated a remarkable effect in modulation of certain factors of this disease (Fadl et al. 2013). NK is also earning interest as a novel enzyme for pharmacologic vitreolysis in patients with proliferative vitreoretinal disorders. This action is due to its efficacy in inducing posterior vitreous detachment (PVD) (Takano et al. 2006). A significant, dose-dependent decrease of red blood cell aggregation and low shear viscosity of blood have been observed in NK administration. This fact indicates positive hemorheological effects of this enzyme which suggests its potential value as a therapeutic enzyme (Pais et al. 2006).

Deep venous thrombosis (DVT) and pulmonary embolism (PE) are matters of concern associated with prolonged air travel. Flite Tabs (Aidan, AZ, USA) displayed meaningful promotion of vein health and reduction of leg swelling in a randomized controlled study of subjects on international long-haul flights. This medication contains a new pharmacologic compound, pinokinase (a combination of pycnogenol and nattokinase). This mixture improves fibrinolysis and controls edema. Thus, administering NK can be considered an effective preventive strategy in subjects at high risk for DVT during long-haul flights (7–8 h) (Cesarone et al. 2003).

Miscellaneous applications of NK

Prion diseases are transmissible through contaminated surgical instruments. NK is able to tolerate a temperature of 50 °C and functions under basic conditions, such as pH 10, suggesting that it might be useful in instrument decontamination. Hsu et al. (2009) demonstrated that purified NK is capable of decreasing amyloid structure of recombinant human PrP fibrils (human prion peptide sequence 108–144) after 48 h of exposure (40 °C, pH 7). This method is also a valuable mean of enzymatic inactivation of prions in soil environments (Booth et al. 2013). An aqueous solution of NK provides lubricity of poly(etheretherketone), reducing the wear of the polymer. It can also be considered an effective lubricant in the presence of NaCl in the solution (Minn and Sinha 2012). Furthermore, NK is being used as the key ingredient of extraction method to recover encapsulated mesenchymal stem cells from 3D fibrin gels (Carrion et al. 2013). NK is gaining attention as a valuable enzyme in dairy processing too. During cheese ripening process, a variety of biochemical alterations occur. Proteolysis is one of the most significant of these biochemical changes which harden cheese varieties such as cheddar. Addition of NK as an exogenous proteolytic enzyme can accelerate this process. In addition, by using NK, higher levels of free amino acids is observed, which serve as substrates for many catabolic reactions and in turn improve the flavor of cheese (Upadhyay et al. 2006).

Conclusion and perspectives

Since NK is derived from food-grade microorganisms, it has the potential to be developed as functional food additive. NK also represents a promising drug candidate for use in both prevention and treatment of thrombotic disorders. Being stable enough in the gastrointestinal tract makes this enzyme a useful agent for oral thrombolytic therapy. Thus, NK is also regarded as a valuable food dietary supplement or nutraceutical. Proven safety and ease of mass production are among the advantageous factors. This enzyme has attracted more attention for its application in various industries. Besides nattokinase's effectiveness and safety for managing a wide range of thrombovascular diseases, it has a significant potential for the treatment of hypertension, Alzheimer's disease, and vitreoretinal disorders. The current retail prices for nattokinase in 100 mg quantities (equivalent to 2,000 fibrinolytic units) have a market price of US$50 to US$75. Therefore, 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.

References

Berenjian A, Mahanama R, Talbot A, Regtop H, Kavanagh J, Dehghani F (2012) Advances in menaquinone-7 production by *Bacillus subtilis natto*: fed-batch glycerol addition. Am J Biochem Biotechnol 8: 105–110. doi:10.3844/ajbbsp.2012.105.110

Berenjian A, Chan NL-C, Mahanama R, Talbot A, Regtop H, Kavanagh J, Dehghani F (2013) Effect of biofilm formation by *Bacillus subtilis natto* on menaquinone-7 biosynthesis. Mol Biotechnol 54:371–378. doi:10.1007/s12033-012-9576-x

Berenjian A, Mahanama R, Kavanagh J, Dehghani F, Ghasemi Y (2014a) Nattokinase production: medium components and feeding strategy studies. Chem Ind Chem Eng Q. doi:10.2298/CICEQ130928037B

Berenjian A, Mahanama R, Talbot A, Regtop H, Kavanagh J, Dehghani F (2014b) Designing of an intensification process for biosynthesis and recovery of menaquinone-7. Appl Biochem Biotechnol 172:1347–1357. doi:10.1007/s12010-013-0602-7

Booth CJ, Johnson CJ, Pedersen JA (2013) Microbial and enzymatic inactivation of prions in soil environments. Soil Biol Biochem 59:1–15. doi:10.1016/j.soilbio.2012.12.016

Carrion B, Janson IA, Kong YP, Putnam AJ (2013) A safe and efficient method to retrieve mesenchymal stem cells from three-dimensional fibrin gels. Tissue Eng Part C Methods 20(3):252–263. doi:10.1089/ten.tec.2013.0051

Cesarone MR, Belcaro G, Nicolaides AN, Ricci A, Geroulakos G, Ippolito E, Brandolini R, Vinciguerra G, Dugall M, Griffin M, Ruffini I, Acerbi G, Corsi M, Riordan NH, Stuard S, Bavera P, Di Renzo A, Kenyon J, Errichi BM (2003) Prevention of venous thrombosis in long-haul flights with Flite Tabs: the LONFLIT-FLITE randomized, controlled trial. Angiol 54:531–539. doi:10.1177/000331970305400502

Chang CT, Fan MH, Kuo FC, Sung HY (2000) Potent fibrinolytic enzyme from a mutant of *Bacillus subtilis* IMR-NK1. J Agric Food Chem 48:3210–3216. doi:10.1021/jf000020k

Chang C-T, Wang P-M, Hung Y-F, Chung Y-C (2012) Purification and biochemical properties of a fibrinolytic enzyme from *Bacillus subtilis*-fermented red bean. Food Chem 133:1611–1617. doi:10.1016/j.foodchem.2012.02.061

Chen PT, Chao YP (2006) Enhanced production of recombinant nattokinase in *Bacillus subtilis* by the elimination of limiting factors. Biotechnol Lett 28:1595–1600. doi:10.1007/s10529-006-9126-3

Chen PT, Chiang CJ, Chao YP (2007a) Medium optimization for the production of recombinant nattokinase by *Bacillus subtilis* using response surface methodology. Biotechnol Prog 23:1327–1332. doi:10.1021/bp070109b

Chen PT, Chiang CJ, Chao YP (2007b) Strategy to approach stable production of recombinant nattokinase in *Bacillus subtilis*. Biotechnol Prog 23:808–813. doi:10.1021/bp070108j

Chiang CJ, Chen HC, Chao YP, Tzen JT (2005) Efficient system of artificial oil bodies for functional expression and purification of recombinant nattokinase in *Escherichia coli*. J Agric Food Chem 53:4799–4804. doi:10.1021/jf050264a

Chiang SS, Liu CF, Ku TW, Mau JL, Lin HT, Pan TM (2011) Use of murine models to detect the allergenicity of genetically modified *Lactococcus lactis* NZ9000/pNZPNK. J Agric Food Chem 59: 3876–3883. doi:10.1021/jf104656m

Cho YH, Song JY, Kim KM, Kim MK, Lee IY, Kim SB, Kim HS, Han NS, Lee BH, Kim BS (2010) Production of nattokinase by batch and fed-batch culture of *Bacillus subtilis*. N Biotechnol 27:341–346. doi:10.1016/j.nbt.2010.06.003

Deepak V, Kalishwaralal K, Ramkumarpandian S, Babu SV, Senthilkumar SR, Sangiliyandi G (2008) Optimization of media composition for Nattokinase production by *Bacillus subtilis* using response surface methodology. Bioresour Technol 99:8170–8174. doi:10.1016/j.biortech.2008.03.018

Deepak V, Pandian SB, Kalishwaralal K, Gurunathan S (2009) Purification, immobilization, and characterization of nattokinase on PHB nanoparticles. Bioresour Technol 100:6644–6646. doi:10.1016/j.biortech.2009.06.057

Ero MP, Ng CM, Mihailovski T, Harvey NR, Lewis BH (2013) A pilot study on the serum pharmacokinetics of nattokinase in humans following a single, oral, daily dose. Altern Ther Health Med 19(3):16–19

Fadl NN, Ahmed HH, Booles HF, Sayed AH (2013) Serrapeptase and nattokinase intervention for relieving Alzheimer's disease pathophysiology in rat model. Hum Exp Toxicol 32(7):721–735. doi:10.1177/0960327112467040

Fujita M, Nomura K, Hong K, Ito Y, Asada A, Nishimuro S (1993) Purification and characterization of a strong fibrinolytic enzyme (nattokinase) in the vegetable cheese natto, a popular soybean fermented food in Japan. Biochem Biophys Res Commun 197(3): 1340–1347. doi:10.1006/bbrc.1993.2624

Fujita M, Hong K, Ito Y, Fujii R, Kariya K, Nishimuro S (1995a) Thrombolytic effect of nattokinase on a chemically induced thrombosis model in rat. Biol Pharm Bull 18(10):1387–1391

Fujita M, Hong K, Ito Y, Misawa S, Takeuchi N, Kariya K, Nishimuro S (1995b) Transport of nattokinase across the rat intestinal tract. Biol Pharm Bull 18(9):1194–1196

Fujita M, Ito Y, Hong K, Nishimuro S (1995c) Characterization of nattokinase-degraded products from human fibrinogen or cross-linked fibrin. Fibrinolysis 9(3):157–164. doi:10.1016/S0268-9499(95)80005-0

Fujita M, Ohnishi K, Takaoka S, Ogasawara K, Fukuyama R, Nakamuta H (2011) Antihypertensive effects of continuous oral administration of nattokinase and its fragments in spontaneously hypertensive rats. Biol Pharm Bull 34(11):1696–1701

Garg R, Thorat BN (2014) Nattokinase purification by three phase partitioning and impact of t-butanol on freeze drying. Sep Purif Technol 131:19–26. doi:10.1016/j.seppur.2014.04.011

Ghasemi Y, Dabbagh F, Ghasemian A (2012) Cloning of a fibrinolytic enzyme (subtilisin) gene from *Bacillus subtilis* in *Escherichia coli*. Mol Biotechnol 52:1–7. doi:10.1007/s12033-011-9467-6

Hsia CH, Shen MC, Lin JS, Wen YK, Hwang KL, Cham TM, Yang NC (2009) Nattokinase decreases plasma levels of fibrinogen, factor VII, and factor VIII in human subjects. Nutr Res 29(3):190–196. doi:10.1016/j.nutres.2009.01.009

Hsieh CW, Lu WC, Hsieh WC, Huang YP, Lai CH, Ko WC (2009) Improvement of the stability of nattokinase using γ-polyglutamic acid as a coating material for microencapsulation. LWT-Food Sci Technol 42:144–149. doi:10.1016/j.lwt.2008.05.025

Hsu RL, Lee KT, Wang JH, Lee LY, Chen RP (2009) Amyloid-degrading ability of nattokinase from *Bacillus subtilis natto*. J Agric Food Chem 57(2):503–508. doi:10.1021/jf803072r

Huang Y, Ding S, Liu M, Gao C, Yang J, Zhang X, Ding B (2013) Ultrasmall and anionic starch nanospheres: formation and vitro thrombolytic behavior study. Carbohyd Polym 96(2):426–434. doi:10.1016/j.carbpol.2013.04.013

Inatsu Y, Nakamura N, Yuriko Y, Fushimi T, Watanasiritum L, Kawamoto S (2006) Characterization of *Bacillus subtilis* strains in Thua nao, a traditional fermented soybean food in northern Thailand. Lett Appl Microbiol 43(3):237–242. doi:10.1111/j.1472-765X.2006.01966.x

Jang JY, Kim TS, Cai J, Kim J, Kim Y, Shin K, Kim KS, Park SK, Lee SP, Choi EK, Rhee MH, Kim YB (2013) Nattokinase improves blood flow by inhibiting platelet aggregation and thrombus formation. Lab Anim Res 29(4):221–225. doi:10.5625/lar.2013.29.4.221

Kamiya S, Hagimori M, Ogasawara M, Arakawa M (2010) In vivo evaluation method of the effect of nattokinase on carrageenan-induced tail thrombosis in a rat model. Acta Haematol 124(4): 218–224. doi:10.1159/000321518

Kim W, Choi K, Kim Y, Park H, Choi J, Lee Y, Oh H, Kwon I, Lee S (1996) Purification and characterization of a fibrinolytic enzyme

produced from *Bacillus* sp. strain CK 11-4 screened from Chungkook-Jang. Appl Environ Microbiol 62(7):2482–2488

Kim JY, Gum SN, Paik JK, Lim HH, Kim KC, Ogasawara K, Inoue K, Park S, Jang Y, Lee JH (2008) Effects of nattokinase on blood pressure: a randomized, controlled trial. Hypertens Res 31(8): 1583–1588. doi:10.1291/hypres.31.1583

Ku TW, Tsai RL, Pan TM (2009) A simple and cost-saving approach to optimize the production of subtilisin NAT by submerged cultivation of *Bacillus subtilis natto*. J Agric Food Chem 57(1):292–296. doi: 10.1021/jf8024198

Kwon EY, Kim KM, Kim MK, Lee IY, Kim BS (2011) Production of nattokinase by high cell density fed-batch culture of *Bacillus subtilis*. Bioprocess Biosyst Eng 34(7):789–793. doi:10.1007/s00449-011-0527-x

Law D, Zhang Z (2007) Stabilization and target delivery of Nattokinase using compression coating. Drug Dev Ind Pharm 33(5):495–503. doi:10.1080/03639040601050247

Lee KC, Liu CF, Lin TH, Pan TM (2010) Safety and risk assessment of the genetically modified Lactococci on rats intestinal bacterial flora. Int J Food Microbiol 142(1–2):164–169. doi:10.1016/j.ijfoodmicro.2010.06.018

Li X, Wang X, Xiong S, Zhang J, Cai L, Yang Y (2007) Expression and purification of recombinant nattokinase in *Spodoptera frugiperda* cells. Biotechnol Lett 29(10):1459–1464. doi:10.1007/s10529-007-9426-2

Liang X, Jia S, Sun Y, Chen M, Chen X, Zhong J, Huan L (2007a) Secretory expression of nattokinase from *Bacillus subtilis* YF38 in *Escherichia coli*. Mol Biotechnol 37(3):187–194. doi:10.1007/s12033-007-0060-y

Liang X, Zhang L, Zhong J, Huan L (2007b) Secretory expression of a heterologous nattokinase in *Lactococcus lactis*. Appl Microbiol Biotechnol 75(1):95–101. doi:10.1007/s00253-006-0809-4

Liu BY, Song HY (2002) Molecular cloning and expression of Nattokinase gene in *Bacillus subtilis*. Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao 34(3):338–340

Liu JG, Xing JM, Shen R, Yang CL, Liu HZ (2004) Reverse micelles extraction of nattokinase from fermentation broth. Biochem Eng J 21:273–278. doi:10.1016/j.bej.2004.07.008

Liu J, Xing J, Chang T, Ma Z, Liu H (2005) Optimization of nutritional conditions for nattokinase production by *Bacillus natto* NLSSE using statistical experimental methods. Process Biochem 40:2757–2762. doi:10.1016/j.procbio.2004.12.025

Liu JG, Xing JM, Chang TS, Liu HZ (2006) Purification of nattokinase by reverse micelles extraction from fermentation broth: effect of temperature and phase volume ratio. Bioprocess Biosyst Eng 28: 267–273. doi:10.1007/s00449-005-0032-1

Maeda H, Mizutani O, Yamagata Y, Ichishima E, Nakajima T (2001) Alkaline-resistance model of subtilisin ALP I, a novel alkaline subtilisin. J Biochem 129:675–682

Mahajan PM, Gokhale SV, Lele SS (2010) Production of nattokinase using *Bacillus natto* NRRL 3666: media optimization, scale up, and kinetic modeling. Food Sci Biotechnol 19(6):1593–1603. doi:10.1007/s10068-010-0226-4

Mahajan PM, Nayak S, Lele SS (2012) Fibrinolytic enzyme from newly isolated marine bacterium *Bacillus subtilis* ICTF-1: media optimization, purification and characterization. J Biosci Bioeng 113:307–314. doi:10.1016/j.jbiosc.2011.10.023

Minn M, Sinha SK (2012) The lubrication of poly(etheretherketone) by an aqueous solution of nattokinase. Wear 296:528–535. doi:10.1016/j.wear.2012.08.008

Murakami K, Yamanaka N, Ohnishi K, Fukayama M, Yoshino M (2012) Inhibition of angiotensin I converting enzyme by subtilisin NAT (nattokinase) in natto, a Japanese traditional fermented food. Food Funct 3(6):674–678. doi:10.1039/c2fo10245e

Nakamura T, Yamagata Y, Ichishima E (1992) Nucleotide sequence of the subtilisin NAT gene, *aprN*, of *Bacillus subtilis* (*natto*). Biosci Biotechnol Biochem 56(11):1869–1871

Nguyen TT, Quyen TD, Le HT (2013) Cloning and enhancing production of a detergent- and organic-solvent-resistant nattokinase from *Bacillus subtilis* VTCC-DVN-12-01 by using an eight-protease-gene-deficient *Bacillus subtilis* WB800. Microb Cell Fact 12:79. doi:10.1186/1475-2859-12-79

Pais E, Alexy T, Holsworth RE Jr, Meiselman HJ (2006) Effects of nattokinase, a pro-fibrinolytic enzyme, on red blood cell aggregation and whole blood viscosity. Clin Hemorheol Microcirc 35(1–2):139–142

Park CS, Kim DH, Lee WY, Kang DO, Song JJ, Choi NS (2013) Identification of fibrinogen-induced nattokinase WRL101 from *Bacillus subtilis* WRL101 isolated from Doenjang. Int J Adv Res Life Sci 1(3):66–74

Peng Y, Huang Q, Zhang RH, Zhang YZ (2003) Purification and characterization of a fibrinolytic enzyme produced by *Bacillus amyloliquefaciens* DC-4 screened from douchi, a traditional Chinese soybean food. Comp Biochem Physiol B Biochem Mol Biol 134(1):45–52. doi:10.1016/S1096-4959(02)00183-5

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(2):126–132. doi:10.1007/s00253-005-0159-7

Rasagnya PS, Vangalapati M (2013) Studies on optimization of process parameters for nattokinase production by *Bacillus subtilis* NCIM 2724 and purification by liquid-liquid extraction. Int J Innov Res Sci Eng Technol 2:4516–4521

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):1110–1111

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

Sumi H, Nakajima N, Mihara H (1992) Fibrinolysis relating substances in marine creatures. Comp Biochem Physiol B 102(1):163–167. doi: 10.1016/0305-0491(92)90290-8

Suwanmanon K, Hsieh PC (2014) Effect of γ-aminobutyric acid and nattokinase-enriched fermented beans on the blood pressure of spontaneously hypertensive and normotensive Wistar–Kyoto rats. J Food Drug Anal. doi:10.1016/j.jfda.2014.03.005

Suzuki Y, Kondo K, Ichise H, Tsukamoto Y, Urano T, Umemura K (2003a) Dietary supplementation with fermented soybeans suppresses intimal thickening. Nutrition 19(3):261–264. doi:10.1016/S0899-9007(02)00853-5

Suzuki Y, Kondo K, Matsumoto Y, Zhao BQ, Otsuguro K, Maeda T, Tsukamoto Y, Urano T, Umemura K (2003b) Dietary supplementation of fermented soybean, natto, suppresses intimal thickening and modulates the lysis of mural thrombi after endothelial injury in rat femoral artery. Life Sci 73(10):1289–1298. doi:10.1016/S0024-3205(03)00426-0

Tai MW, Sweet BV (2006) Nattokinase for prevention of thrombosis. Am J Health Syst Pharm 63(12):1121–1123. doi:10.2146/ajhp050509

Takano A, Hirata A, Ogasawara K, Sagara N, Inomata Y, Kawaji T, Tanihara H (2006) Posterior vitreous detachment induced by nattokinase (subtilisin NAT): a novel enzyme for pharmacologic vitreolysis. Invest Ophthalmol Vis Sci 47(5):2075–2079. doi:10.1167/iovs.05-0130

Tonova K, Lazarova Z (2008) Reversed micelle solvents as tools of enzyme purification and enzyme-catalyzed conversion. Biotechnol Adv 26:516–532. doi:10.1016/j.biotechadv.2008.06.002

Unrean P, Nguyen NH (2013) Metabolic pathway analysis and kinetic studies for production of nattokinase in *Bacillus subtilis*. Bioprocess Biosyst Eng 36:45–56. doi:10.1007/s00449-012-0760-y

Unrean P, Nguyen NHA, Visessanguan W, Kitsubun P (2012) Improvement of nattokinase production by *Bacillus subtilis* using an optimal feed strategy in fed-batch fermentation. KKU Res J 17: 769–777

Upadhyay VK, Kelly AL, Mcsweeney PLH (2006) Use of nattokinase, a subtilisin-like serine proteinase, to accelerate proteolysis in Cheddar cheese during ripening. Lait 86:227–240. doi:10.1051/lait:2006004

Urano T, Ihara H, Umemura K, Suzuki Y, Oike M, Akita S, Tsukamoto Y, Suzuki I, Takada A (2001) The profibrinolytic enzyme subtilisin NAT purified from *Bacillus subtilis* cleaves and inactivates plasminogen activator inhibitor type 1. J Biol Chem 276:24690–24696. doi:10.1074/jbc.M101751200

Wang C, Du M, Zheng D, Kong F, Zu G, Feng Y (2009a) Purification and characterization of nattokinase from *Bacillus subtilis natto* B-12. J Agric Food Chem 57:9722–9729. doi:10.1021/jf901861v

Wang JK, Chiu HH, Hsieh CS (2009b) Optimization of the medium components by statistical experimental methods to enhance nattokinase activity. Fooyin J Health Sci 1:21–27. doi:10.1016/S1877-8607(09)60004-7

Wang SL, Chen HJ, Liang TW, Lin YD (2009c) A novel nattokinase produced by *Pseudomonas* sp. TKU015 using shrimp shells as substrate. Process Biochem 44:70–76. doi:10.1016/j.procbio.2008.09.009

Wang SL, Wu YY, Liang TW (2011) Purification and biochemical characterization of a nattokinase by conversion of shrimp shell with *Bacillus subtilis* TKU007. N Biotechnol 28:196–202. doi:10.1016/j.nbt.2010.09.003

Wei X, Luo M, Xie Y, Yang L, Li H, Xu L, Liu H (2012) Strain screening, fermentation, separation, and encapsulation for production of nattokinase functional food. Appl Biochem Biotechnol 168:1753–1764. doi:10.1007/s12010-012-9894-2

Wei X, Luo M, Liu H (2014) Preparation of the antithrombotic and antimicrobial coating through layer-by-layer self-assembly of nattokinase-nanosilver complex and polyethylenimine. Colloids Surf B Biointerfaces 116C:418–423. doi:10.1016/j.colsurfb.2014.01.034

Weng M, Zheng Z, Bao W, Cai Y, Yin Y, Zou G (2009) Enhancement of oxidative stability of the subtilisin nattokinase by site-directed mutagenesis expressed in *Escherichia coli*. Biochim Biophys Acta 1794(11):1566–1572. doi:10.1016/j.bbapap.2009.07.007

Weng M, Deng X, Wu J, Zou G (2014) Thermostability of subtilisin nattokinase obtained by site-directed mutagenesis. Wuhan Univ J Nat Sci 19:229–234

Wu S, Feng C, Zhong J, Huan L (2007) Roles of s3 site residues of nattokinase on its activity and substrate specificity. J Biochem 142(3):357–364. doi:10.1093/jb/mvm142

Wu SM, Feng C, Zhong J, Huan LD (2011) Enhanced production of recombinant nattokinase in *Bacillus subtilis* by promoter optimization. World J Microbiol Biotechnol 27:99–106. doi:10.1007/s11274-010-0432-5

Xu J, Du M, Yang X, Chen Q, Chen H, Lin DH (2014) Thrombolytic effects in vivo of nattokinase in a carrageenan-induced rat model of thrombosis. Acta Haematol 132(2):247–253. doi:10.1159/000360360

Yanagisawa Y, Chatake T, Chiba-Kamoshida K, Naito S, Ohsugi T, Sumi H, Yasuda I, Morimoto Y (2010) Purification, crystallization and preliminary X-ray diffraction experiment of nattokinase from *Bacillus subtilis natto*. Acta Crystallogr Sect F Struct Biol Cryst Commun 66:1670–1673. doi:10.1107/s1744309110043137

Yanagisawa Y, Chatake T, Naito S, Ohsugi T, Yatagai C, Sumi H, Kawaguchi A, Chiba-Kamosida K, Ogawa M, Adachi T, Morimoto Y (2013) X-ray structure determination and deuteration of nattokinase. J Synchrotron Radiat 20:875–879. doi:10.1107/s0909049513020700

Yang C, Xing J, Guan Y, Liu H (2006) Superparamagnetic poly(methyl methacrylate) beads for nattokinase purification from fermentation broth. Appl Microbiol Biotechnol 72(3):616–622. doi:10.1007/s00253-006-0484-5

Yang NC, Chou CW, Chen CY, Hwang KL, Yang YC (2009) Combined nattokinase with red yeast rice but not nattokinase alone has potent effects on blood lipids in human subjects with hyperlipidemia. Asia Pac J Clin Nutr 18(3):310–317

Yatagai C, Maruyama M, Kawahara T, Sumi H (2008) Nattokinase-promoted tissue plasminogen activator release from human cells. Pathophysiol Haemost Thromb 36(5):227–232. doi:10.1159/000252817

Yin LJ, Lin HH, Jiang ST (2010) Bioproperties of potent nattokinase from *Bacillus subtilis* YJ1. J Agric Food Chem 58(9):5737–5742. doi:10.1021/jf100290h

Yongjun C, Wei B, Shujun J, Meizhi W, Yan J, Yan Y, Zhongliang Z, Goulin Z (2011) Directed evolution improves the fibrinolytic activity of nattokinase from *Bacillus natto*. FEMS Microbiol Lett 325(2):155–161. doi:10.1111/j.1574-6968.2011.02423.x

Zheng ZL, Zuo ZY, Liu ZG, Tsai KC, Liu AF, Zou GL (2005) Construction of a 3D model of nattokinase, a novel fibrinolytic enzyme from *Bacillus natto*: a novel nucleophilic catalytic mechanism for nattokinase. J Mol Graph Model 23(4):373–380. doi:10.1016/j.jmgm.2004.10.002

Zheng ZL, Ye MQ, Zuo ZY, Liu ZG, Tai KC, Zou GL (2006) Probing the importance of hydrogen bonds in the active site of the subtilisin nattokinase by site-directed mutagenesis and molecular dynamics simulation. Biochem J 395(3):509–515. doi:10.1042/bj20050772