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Fibrinolytic enzymes in Asian traditional fermented foods

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Abstract

Cardiovascular diseases, such as heart attack and stroke, are the leading causes of death in North America. According to data provided by the World Health Organization in 2000, heart diseases are responsible for 29% of the total mortality rate in the world. Since cardiovascular diseases have a major impact on an individual's quality of life, a tremendous amount of research has been done in the area of prevention and the treatment of the diseases. Fibrinolytic enzymes are agents that dissolve fibrin clots. Recently many food derived fibrinolytic enzymes have been found in various traditional Asian foods. Fibrinolytic enzymes can be found in a variety of foods, such as Japanese *Natto*, *Tofuyo*, Korean *Chungkook-Jang* soy sauce, and edible honey mushroom. Enzymes have been purified from these foods, and their physiochemical properties have been characterized. Fermented shrimp paste, a popular Asian seasoning, was shown to have strong fibrinolytic activity. These novel fibrinolytic enzymes derived from traditional Asian foods are useful for thrombolytic therapy. They will provide an adjunct to the costly fibrinolytic enzymes that are currently used in managing heart disease, since large quantities of enzyme can be conveniently and efficiently produced. In addition, these enzymes have significant potential for food fortification and nutraceutical applications, such that their use could effectively prevent cardiovascular diseases.

Keywords: Fibrinolytic enzyme; Asian fermented foods; Anticoagulant activity; Cardiovascular diseases; Plasmin; Nutraceutical; Functional foods

1. Cardiovascular diseases

Cardiovascular diseases, including acute myocardial infarction, ischemic heart disease, valvular heart disease, peripheral vascular disease, arrhythmias, high blood pressure and stroke, are the leading causes of death throughout the world. According to data provided by the World Health Organization (WHO) in 2000, heart diseases are responsible for 29% of the total mortality rate in the world (Fig. 1). These diseases, in particular, contribute to 38% of all deaths in Canada (Fig. 2). Indeed heart diseases not only affect the elderly, but are also the third leading cause of premature death in individuals under age 75 in Canada. Based on the mortality rates by different types of cardiovascular diseases, acute myocardial infarction and ischemic heart disease are the most important heart problems, starting at age 45 for men and 55 for women. Yet congestive heart failure and stroke affect older individuals over age 75, for both men and women (Health Canada, 2000).

Hemostasis is a tightly regulated process of keeping an optimal balance between coagulation and anticoagulation. Coagulation involves a series of enzymatic reactions, in which inactive plasma proteins are converted into active enzymes in each step of the pathway. As shown in Fig. 3, the cascade is initiated by the release of tissue factor or damaged collagen underneath the blood vessel endothelium. The final step involves the formation of a fibrin clot that stabilizes the platelet plug. The fibrin clot is formed from fibrinogen by thrombin.

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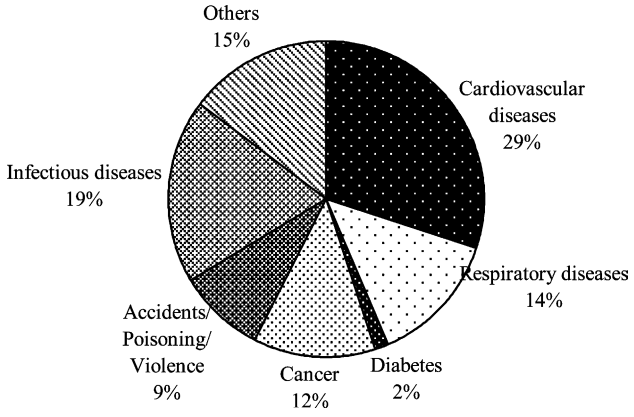

Fig. 1. The proportion of deaths by causes in WHO regions, estimates for 2000 (WHO, 2001).

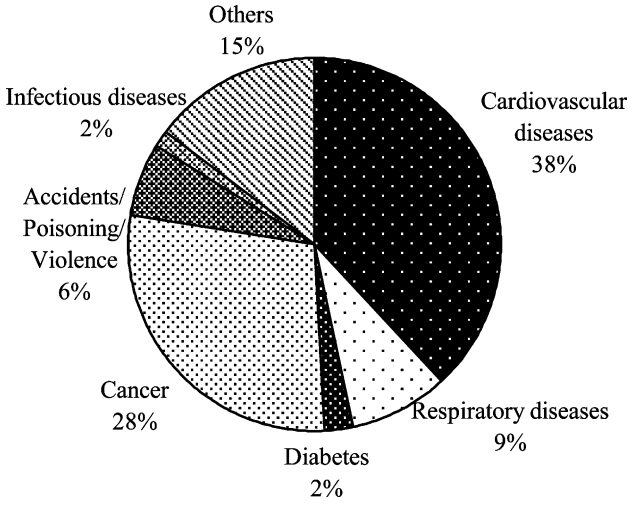

Fig. 2. The proportion of deaths by causes, in Canada, estimates for 2000 (WHO, 2001).

The dissolution of a blood clot is dependent on the action of endogenous plasmin, a serine protease that is activated by tissue plasminogen activator (Silverthorn, Ober, & Garrison, 1998).

An imbalance in hemostasis may result in excessive bleeding, or formation of a thrombus (an inappropriate blood clot) that adheres to the unbroken wall of the blood vessels. Accumulation of fibrin in the blood vessels can interfere with blood flow and lead to myocardial infarction and other serious cardiovascular diseases. Unless the blockage is removed promptly, the tissue that is normally supplied with oxygen by the vessel will die or be severely damaged. If the damaged region is large, the

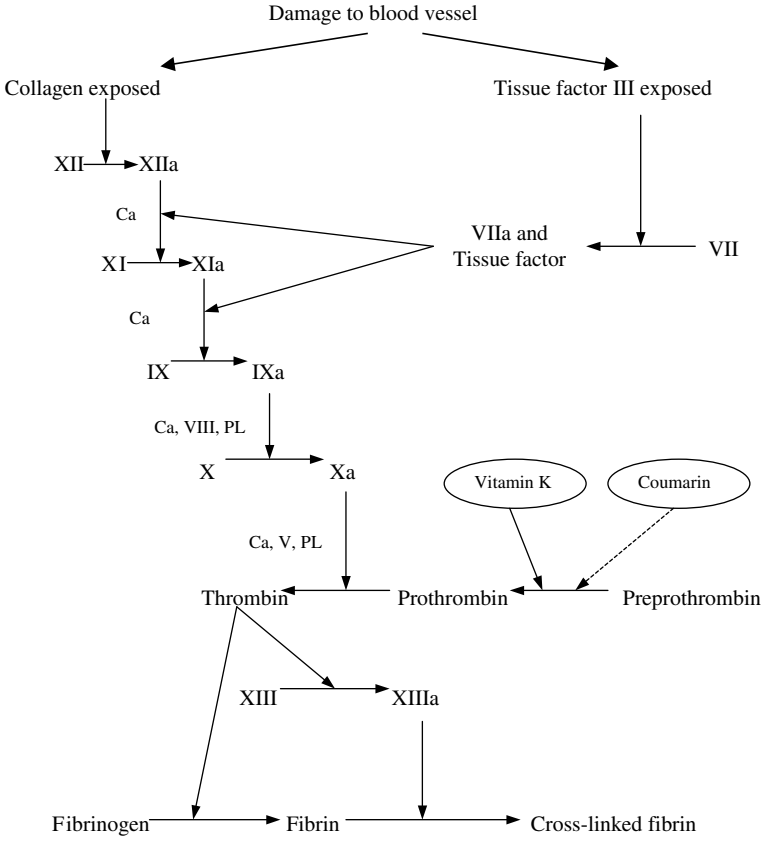

Fig. 3. A diagram of the blood-clotting cascade. The cascade involves a series of enzymatic reactions, in which inactive plasma proteins are converted into active enzymes in each step of the pathway. The final step is the formation of a fibrin mesh that stabilizes the platelet plug. The intrinsic pathway begins with collagen exposure and uses proteins already present in the plasma, while the extrinsic pathway starts when damaged tissues expose tissue factor to the plasma proteins. Solid and dotted arrows represent activation and inhibition of the components, respectively (Silverthorn et al., 1998).

normal conduction of electrical signals through the ventricle will be disrupted, leading to irregular heartbeat, cardiac arrest or death (Mihara et al., 1991).

Cardiovascular disease is a main contributing cause of death in the world, inflicting a devastating physical, emotional and major financial toll on its victims and their families. Thus, significant advances have been made during the past decade in the prevention and treatment of cardiovascular diseases. In general, there are four options for patients and they are summarized as follows.

Historically, management of heart disease and stroke has relied on the use of anticoagulant drugs and antiplatelet drugs. This is because the underlying pathophysiological process in myocardial infarction and stroke is the formation of a thrombus, which consists of fibrin and platelets. Therefore, an optimal antithrombotic prophylactic therapy can and should be directed towards both. Anticoagulants are chemicals that prevent coagulation from taking place. Most of them act by blocking one or more steps in the cascade that forms fibrin. Some drugs inhibit the synthesis of clotting factors, while others enhance the anticoagulant activity of the naturally occurring blood factors or prevent platelet plug formation. Indeed treatment with oral anticoagulants requires a constant balancing between under-treatment and over-treatment. In other words, the intensity of coagulation is monitored within narrow therapeutic margins, and the effect of the daily dose has to be checked regularly because of the influence of disease, food, and other drugs on coagulation (Oden & Fahlen, 2002). Warfarin is the most commonly used anticoagulant in Britain and the Western world. It inhibits coagulation by interfering with the incorporation of vitamin K into vitamin K-dependent clotting factors, including Factors II, VII, IX and X. There is a considerable variability in its effect on patients, and its effectiveness can be influenced by age, racial background, diet, and co-medications such as antibiotics. Another example of an anticoagulant is heparin, a glycosaminoglycan whose major anticoagulant effect is accounted for by its inhibition of thrombin, Factor IIa, and Factor Xa in the coagulation cascade. It has a short half-life and must be given parenterally, preferably by continuous intravenous infusion. The use of heparin is associated with haemorrhage, osteoporosis, alopecia, thrombocytopenia, and hypersensitivity (Fitzmaurice, Blann, & Lip, 2002).

Anti-platelet agents are used to prevent a clot from forming or from getting larger and occluding the entire vessel. Aspirin is the most widely used anti-platelet drug, and it inhibits platelet aggregation for the life of the platelets (7–10 days). It is prescribed in cases of acute myocardial infarction and prophylactically to prevent re-infarction. Antithrombotic doses used in clinical trials have varied widely from less than 50 mg/day to over 1200 mg/day. Adverse effects of aspirin are similar to that of warfarin. Other anti-platelet drugs, such as dipyridamole, clopidogrel and ticlopidine, work by inhibiting platelet-activating factor and collagen, and they are often prescribed for patients that have an aspirin allergy. These agents may be used in patients with atherosclerotic disease to prevent heart attacks, strokes, and coronary artery closure in patients undergoing angioplasty. Their usage, however, is associated with bone marrow suppression, in particular leucopenia (Blann, Landray, & Lip, 2002).

Unlike heparin and warfarin, which prevent extension and recurrences of thrombosis, thrombolytic agents (fibrinolytic enzymes) lyse pre-existing thrombus. These include urokinase (extracted from kidneys), streptokinase (extracted from bacteria), and genetically engineered tissue plasminogen activator (t-PA). Evidence has shown that patients with pulmonary embolism treated with streptokinase and urokinase are three times more likely to show clot resolution than patients taking heparin alone. These enzymes can also prevent some damage if the clot is removed soon after it occurs. Streptokinase, an effective thrombolytic agent for the treatment of acute myocardial infarction and pulmonary thromboembolism, is derived from streptococci. It can potentiate the body's own fibrinolytic pathways by converting plasminogen to plasmin. Being bacteria-derived, it is antigenic and repeated administration may result in the development of neutralizing antibodies and allergic reactions. On the other hand, t-PA is produced by recombinant DNA technology and it mimics an endogenous molecule that activates the fibrinolytic system. It does not elicit an allergic response and is considered to be more clot-specific. However, it has a short half-life and it needs continuous infusion to achieve its greatest efficacy. Because of the lack of site specificity for all of these fibrinolytic enzymes, adverse effects may include gastrointestinal haemorrhage, but severe anaphylaxis is rare (Turpie, Chin, & Lip, 2002).

Mechanical and surgical treatments are usually reserved for massive pulmonary embolism, where drug treatments have failed or are contraindicated. Several tests can be done prior to surgery. Heart catheterization and coronary angiography are usually performed to assess the function of the heart muscles, the valves within the heart and the small coronary arteries feeding the heart. Patients may also be asked to undergo an electrocardiogram, echocardiogram, exercise test, or holter monitoring before surgery (Wheatley, 2002).

Coronary bypass surgery has evolved to be one of the most common and the most successful operative procedures since the 1970s because of its symptomatic and survival benefits. Although surgical treatment is sometimes physically invasive and traumatic for the heart patients, technological advances in endovascular devices are making significant inroads into traditional coronary surgical practice. In fact, surgeons persistently develop new strat-

egies to maximize the effectiveness of coronary surgery and to minimize the injury associated with cardiopulmonary bypass. These new treatment modalities, however, require the use of an anticoagulant as an adjuvant therapy (Turpie et al., 2002).

2. Functional foods

The research areas of functional foods and nutraceuticals are rapidly expanding throughout the world. Scientists are actively working on the health benefits of foods by identifying the functional constituents, elucidating the biochemical structures, and determining the mechanisms behind the physiological roles. These research findings contribute to a new nutritional paradigm, in which food constituents go beyond their role as dietary essentials for sustaining life and growth, to one of preventing, managing, or delaying the premature onset of chronic disease later in life (Fitzpatrick, 1999, 2000; Hasler, 1998).

Research indicates that diets high in saturated fat, *trans* fat and sodium may increase the risk of cardiovascular diseases, whereas diets high in soluble fiber and antioxidants may help in preventing the diseases. For instance, the consumption of foods containing soy protein, such as soy beverages and tofu, in a diet low in saturated fat and cholesterol is associated with a reduced risk of coronary heart disease by lowering blood cholesterol levels. Scientific studies show that consumption of 25 g of soy proteins per day may lower the risk of heart diseases (Arliss & Biermann, 2002; Nestel, 2002; Puska et al., 2002). Plant sterols that are present in small quantities in many fruits, vegetables, nuts, seeds, cereals and legumes have also been shown to be beneficial in preventing heart diseases. It was shown that 1.3 g of plant sterol esters or 3.4 g of plant stanol esters per day in the diet is required to show a significant cholesterol-lowering effect in hypercholesterolemia patients (Meade, Ross, & Blackston, 2001). In addition, soluble fiber-containing foods, such as oats and psyllium, have been shown to be effective in lowering blood cholesterol levels. Clinical studies have proven that dietary fiber reduces blood cholesterol by decreasing the absorption of dietary cholesterol and increasing the excretion of bile acids in the gastrointestinal tract. Soluble fiber may also alter the serum concentration of hormones or short-chain fatty acids that affect lipid metabolism. As part of a low fat diet, 3 g of soluble fiber daily can help reduce blood cholesterol (Anderson et al., 2000; Bell & Goldman, 1999). Furthermore, omega-3 fatty acids from cold water fish like salmon, halibut and tuna, have been shown to reduce coronary heart disease mortality rates in patients. This is because long chain omega-3 fatty acids from fish oil can decrease triglyceride levels, favourably affect platelet function, and decrease blood pressure slightly in hypertensive individuals (Din, Newby, & Flapan, 2004). Ongoing research continues to explore the heart-health benefits of food ingredients such as conjugated linoleic acid and plant phytonutrients (Chagan, Ioselovich, Asherova, & Cheng, 2002; de Lorgeril et al., 1994; Hodgson, 1993).

3. Introduction to fibrinolytic enzymes

Fibrinolytic enzymes are agents that dissolve fibrin clots. The three enzymes that are currently being used for these purposes include urokinase, streptokinase, and genetically engineered tissue plasminogen activator. Yet fibrinolytic enzyme therapy, such as the intravenous administration of urokinase, is expensive, and patients may suffer from undesirable side effects such as resistance to reperfusion, occurrence of acute coronary reocclusion and bleeding complications (Bode, Runge, & Smalling, 1996). Consequently, several lines of investigation are currently being pursued to enhance the efficacy and specificity of fibrinolytic therapy. Recently, fibrinolytic enzymes have been discovered from both food and non-food sources. These enzymes have proven to be effective and they have been proposed as one of the potent fibrinolytic regimens.

3.1. The fibrinolysis system

The fibrinolytic system, as shown in Fig. 4, is composed of a proenzyme (plasminogen), enzymes that proteolytically activate plasminogen, and several inhibitors that regulate activation of plasminogen, activity of plasmin, and the stepwise degradation of fibrin. The structure-function relationships and the mechanisms of activation and inhibition of the main components of the system have been elucidated. Fibrin is a pathologic formation yet a structure that protects from bleeding at the site of vascular injury. Its removal is necessary for the restoration of normal blood flow, but this should occur only after the regeneration of the vessel wall. At the same time, t-PA, which is thought to be the primary initiator of fibrinolysis in the circulation, is the only protease of the hemostatic system that is continuously secreted by the endothelium in active form. Thrombotic occlusion at the site of vessel injury occurs when the growth rate of a thrombus exceeds that of its lysis. Impairments in these mechanisms may predispose patients to bleeding or thrombosis (Dobrovolsky & Titaeva, 2002).

3.2. Non-food sources

Fibrinolytic enzymes can be widely found in nature. They have been found in hemorrhagic toxin from snake venom (Nikai, Mori, Kishida, Sugihara, & Tu, 1984),

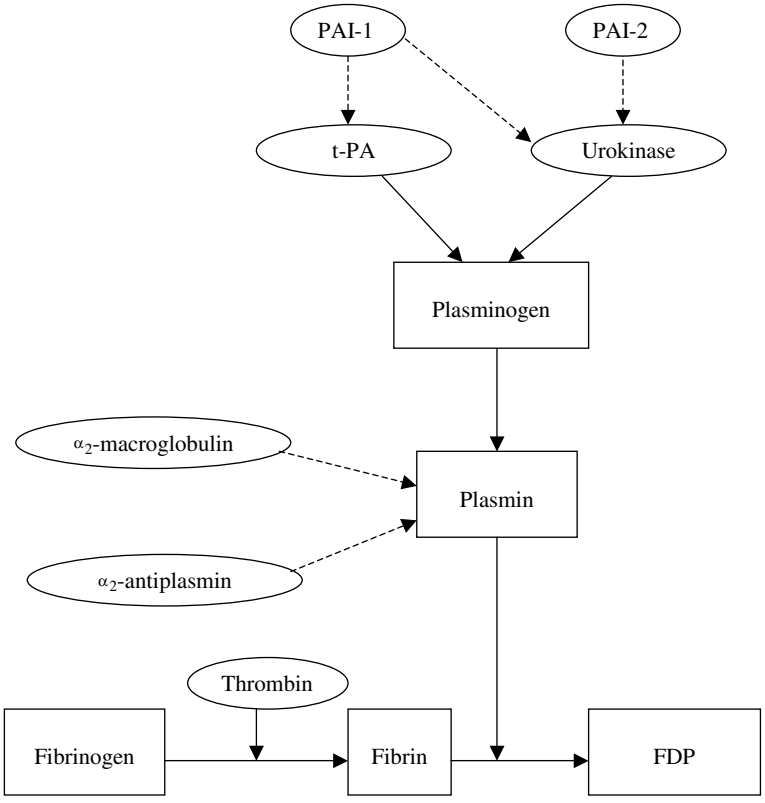

Fig. 4. A simplified diagram of the fibrinolytic system. Fibrin is derived from fibrinogen in response to thrombin. Meanwhile, fibrin is degraded into fibrinogen degradation product (FDP) by endogenous plasmin protease, which is an activated form of plasminogen. t-PA and urokinase are two physiological activators of plasminogen. Plasminogen activator inhibitor-1 (PAI-1) negatively regulates t-PA activity and plasminogen activator inhibitor-2 (PAI-2) serves as the primary regulator of urokinase in the extra-vascular compartment within the body. Alpha-2-antiplasmin and alpha-2-macroglobulin are plasmin inhibitors that terminate fibrinolysis. Solid and dotted arrows represent activation and inhibition of the components, respectively (Dobrovolsky & Titaeva, 2002).

earthworm secretions (Mihara et al., 1991), food-grade microorganisms (Chang, Fan, Kuo, & Sung, 2000; Jeong, Park, Baek, Park, & Kong, 2001), marine creatures (Sumi, Nakajima, & Mihara, 1992), and herbal medicines (Choi & Sa, 2000, 2001). In particular, a fibrinolytic protease has been isolated from *Spirodela polyrhiza*, an ingredient of traditional Oriental medicine that has been used for lowering blood pressure and detoxification of snake venom (Choi & Sa, 2001). Also, strong fibrinolytic enzymes are produced by *Bacillus* sp. strains that are used in food fermentation, invertebrates like *Stichopous japonicus*, as well as the seaweed *Codiales codium* (Jeon, Moon, & Kim, 1995).

3.3. Food sources

As shown in Table 1, fibrinolytic enzymes can be found in a variety of foods, such as Japanese *Natto* (Sumi, Hamada, Tsushima, Mihara, & Muraki, 1987), Korean *Chungkook-Jang* soy sauce (Kim, Choi, & Kim, 1996), and edible honey mushroom (Kim & Kim, 1999). Enzymes have been purified from these foods, and their physiochemical properties have been characterized. Among the food sources, fermented food products have been the focus of research. In particular, oral administration of the fibrinolytic enzyme extracted from Japanese *natto* can enhance fibrinolysis in dogs with experimentally induced thrombosis (Sumi, Hamada, Nakanishi, & Hiratani, 1990). Lysis of the thrombus can be observed by angiography (Kim et al., 1996). More importantly, fibrinolytic activity, the amounts of t-PA, and fibrin degradation by-product in the plasma are doubled when nattokinase is given to human subjects by oral administration. The underlying mechanism involves the absorption of the administered *natto* enzyme across the intestinal tract, and the release of endogenous plasminogen activator that induces fibrinolysis in the occluded blood vessel (Sumi et al., 1990).

4. A novel fibrinolytic enzyme in fermented shrimp paste.

A total of the thirteen traditional Asian fermented food samples, including fermented black beans, instant soybean paste, fermented shrimp paste, pickled gouramy fish, *tempeh*, light soy sauce, sweet bean paste sauce, yellow bean sauce, fermented bean curd (red), fer-

Table 1
Food sources of fibrinolytic enzymes

Food source	Origin	Description	Fibrinolytic enzyme	Reference
Natto	Japan	Bacillus fermented soybean	An extracellular serine protease (Nattokinase)	Sumi et al. (1987)
Tofuyo	Japan	Fermented bean curd	A soybean milk coagulating enzyme (SMCE)	Fujita et al. (1993)
Skipjack Shiokara	Japan	A salt-fermented fish product	An alkaline trypsin-like serine protease (Katsuwokinase)	Sumi et al. (1995)
Chungkook-Jang	Korea	Fermented soybean sauce	An alkaline serine protease (CK)	Kim et al. (1996)
Kimchi	Korea	Fermented vegetables	A *Bacillus protease*	Noh et al. (1999)
Armillariella mella	World-wide China	An edible honey mushroom	A neutral metalloprotease	Kim and Kim (1999)
Fermented shrimp paste	China	A salt fermented shrimp paste	Neutral Metalloprotease?	Wong and Mine (2004)

mented bean curd (white), fermented cow milk, glutinous rice in wine, and stinky bean curd powder, were screened for fibrinolytic enzyme activity. Fermented shrimp paste, a popular Asian seasoning, was shown to have a strong fibrinolytic activity (Wong & Mine, 2004). Its fibrinolytic enzyme was successfully purified by high performance liquid chromatography (HPLC). Fermented shrimp paste enzyme was determined to be a monomer with an apparent molecular mass of 18 kDa, composed primarily of β-sheet and random coils. The N-terminal amino acid sequence was determined to be DPYEEPGPCENLQVA. No inhibition was observed with PMSF, Pepstatin A, E64, and 1,10-phenanthroline, but the enzyme was slightly inhibited by EDTA and Cu^{2+}. N-terminal amino acid sequencing indicated that the fermented shrimp paste enzyme was novel with no homology to other existing fibrinolytic enzymes (Fig. 5). The enzyme was relatively specific to fibrin or fibrinogen as a protein substrate. It hydrolysed none of the plasma proteins, including BSA, IgG, thrombin, and hemoglobin. The enzyme was a neutral protease, showing broad pH stability around neutral, and an optimal activity from pH 3–7. Maximum enzyme activity was observed at 30–40 °C, and the enzyme was completely denatured at temperatures over 60 °C. It also had an anticoagulant activity measured by activated partial thrombin time (APTT), and prothrombin time (PT) tests. In addition, the shrimp paste enzyme was resistant to pepsin and trypsin digestion.

Fermented shrimp paste is traditionally made by fresh silver shrimp and salt (10–15% w/w). The mixture is crushed and kept for two days at room temperature. The partially fermented shrimp paste is then blended into a smooth paste and kept under the sun for another 2–30 days. The finished product is stored in a plastic or wooden bucket, or it is packed into glass bottles for retail sale. The fermentation process is due to bacteria from air, utensils or ingredients which are present in nature; they do not use any commercial cultures. It was concluded that the enzyme was produced during the natural fermentation stage (Wong & Mine, 2004). The source of this enzyme has yet to be determined. We have isolated four bacterial strains from this product. The identification and isolation of bacteria which produces this novel enzyme is under in progress in our laboratory. Kim et al. (1996) isolated a *Bacillus* sp. strain, which produces a strong fibrinolytic enzyme from *Chunkook-Jang*, a traditional Korean fermented soybean sauce. *Bacillus subtilis*, used for the production of domestic "*natto*" in Taiwan is another source of a potent fibrinolytic enzyme (Chang et al., 2000). A potent fibrinolytic enzyme (nattokinase; NK) was previously isolated from a traditional fermented food in Japan, *natto*, by Sumi et al. (1987). This enzyme is an extracellular serine protease produced from *Bacillus natto*. Sumi et al. (1990) further demonstrated that oral administration of *natto* or natto-

	1			5				10						15
Fermented shrimp paste	D	P	Y	E	E	P	G	P	C	E	N	L	Q	V	A
Natto (nattokinase)	A	Q	S	V	P	Y	G	I	S	Q	I	K	A	P	
Chungkook-Jang (CK)	A	Q	T	V	P	Y	G	I	P	L	I	K	A	D	
Bacillus subtilis BK-17	A	Q	S	V	P	Y	G	V	S	Q	I	K	A	P	A
Tofuyo (SMCE)	A	Q	T	V	P	Y	G	I	P	Q	I	K	A	D	
Skipjack "Shiokara" (katsuwokinase)	I	V	G	G	Y	E	Q	Z	A	H	S	Q	P	H	Q
Armillariella mellea	X	X	Y	N	G	X	T	X	S	R	Q	T	T	L	V

Fig. 5. Comparison of N-terminal amino acid sequence of the enzyme with other fibrinolytic enzymes. The first 14–15 amino acid residues at the N-terminal of the enzymes are shown (Chang et al., 2000; Jeong et al., 2001; Kim et al., 1996; Kim & Kim, 1999; Sumi et al., 1995).

kinase NK capsules enhance fibrinolysis in canine plasma in an experimental thrombosis model. Recently, Jeong et al. (2001) also isolated a fibrinolytic enzyme from the *Bacillus subtilis* BK-17 strain.

Thus, the shrimp paste might contain *Bacillus* sp., but produces a novel fibrinolytic enzyme. To confirm the physiological functions of the enzyme, the next step will be to examine the intestinal absorption of the enzyme in vitro using human intestinal epithelial cells, and to measure fibrinolytic activity of the enzyme in the blood and the organs using animal model systems.

The novel fibrinolytic enzyme derived from a traditional Asian food is useful for thrombolytic therapy like other potent fibrinolytic enzymes, such as nattokinase and earthworm enzyme. It will provide an adjunct to the costly fibrinolytic enzymes that are currently used in managing heart disease, since large quantities of enzyme can be conveniently and efficiently produced. In addition, this enzyme has significant potential for food fortification and nutraceutical applications, such that their use could effectively prevent cardiovascular diseases.

5. Future research directions

Cardiovascular diseases, such as heart attack and stroke, are the leading causes of death in North America. These diseases not only affect the elderly but are also the third leading cause of premature death in individuals under age 75. Since cardiovascular diseases have a major impact on an individual's quality of life, a tremendous amount of research has been done in their prevention and treatment. Fibrinolytic therapy such as intravenous administration of urokinase is widely used but the enzyme is expensive, and patients may suffer from undesirable side effects such as internal hemorrhage within the intestinal tract. Recent research, therefore, has been pursued to enhance the efficacy and specificity of thrombolytic therapy. Oral administration of an enzyme from *natto*, a Japanese fermented soybean, has been proposed as one of the potent fibrinolytic regimens. Thus, it is desirable to explore new sources of fibrinolytic enzymes in addition to *natto*, and to use the extracted enzyme as a practical agent in managing heart diseases.

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