A NOVEL FIBRINOLYTIC ENZYME FROM FERMENTED SHRIMP PASTE

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ABSTRACT

A NOVEL FIBRINOLYTIC ENZYME FROM FERMENTED SHRIMP PASTE

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The objectives of this research were to explore new sources of fibrinolytic enzymes from Asian fermented food products, and to elucidate the physiochemical properties of the enzymes. A novel fibrinolytic enzyme was purified from fermented shrimp paste, a popular seasoning used in Asian countries. Fermented shrimp paste enzyme was a monomer with an apparent molecular mass of 12.5 kDa, and it was composed primarily of β-sheet and random coils. Inhibition studies revealed that the enzyme was unique and it could not be classified into any classes of proteases. Its activity was optimized at neutral pH and at temperature from 30°C to 40°C. It was relatively specific to fibrin or fibrinogen as a protein substrate, yet it hydrolysed none of the plasma proteins in the studies. *In vitro*, the enzyme was resistant to pepsin and trypsin digestion. It also has an anticoagulant activity measured with activated partial thrombin time (APTT), and prothrombin time (PT) tests. These results suggested that fermented shrimp paste enzyme, which has a strong fibrinolytic activity, might be useful in clinical applications for preventing and treating heart attack. Also, it has a significant potential for food fortification and nutraceutical applications.

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1. BACKGROUND

1.1. Cardiovascular Diseases

1.1.1. Prevalence of CVD

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. In accordance with the data provided by World Health Organization in 2000, heart diseases are responsible for 29% of the total mortality rate in the world (Figure 1a). These diseases, in particular, contribute to 38% of all deaths in Canada (Figure 1b). Indeed heart diseases not only affect the elderly, but are also the third leading cause of premature death under age 75 in Canada. Based on the mortality rates by different types of cardiovascular diseases, as shown in Figure 2a and 2b, 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).

1.1.2. Pathophysiology of Heart Diseases

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 Figure 3, the cascade is initiated by the release of tissue factor or damaged collagen underneath the blood vessel endothelium. The final step involves the

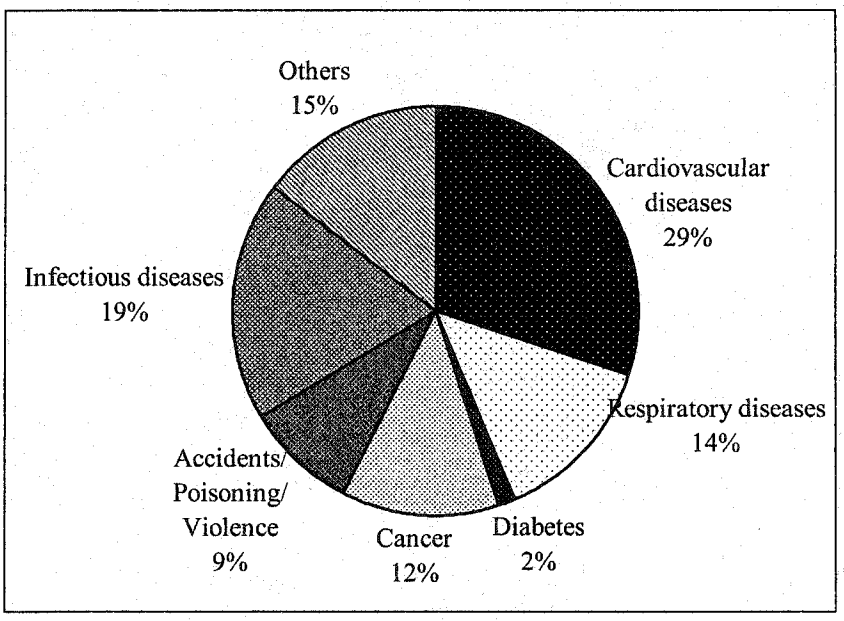

Figure 1a The proportion of deaths by causes in WHO regions, estimates for 2000 (WHO 2000).

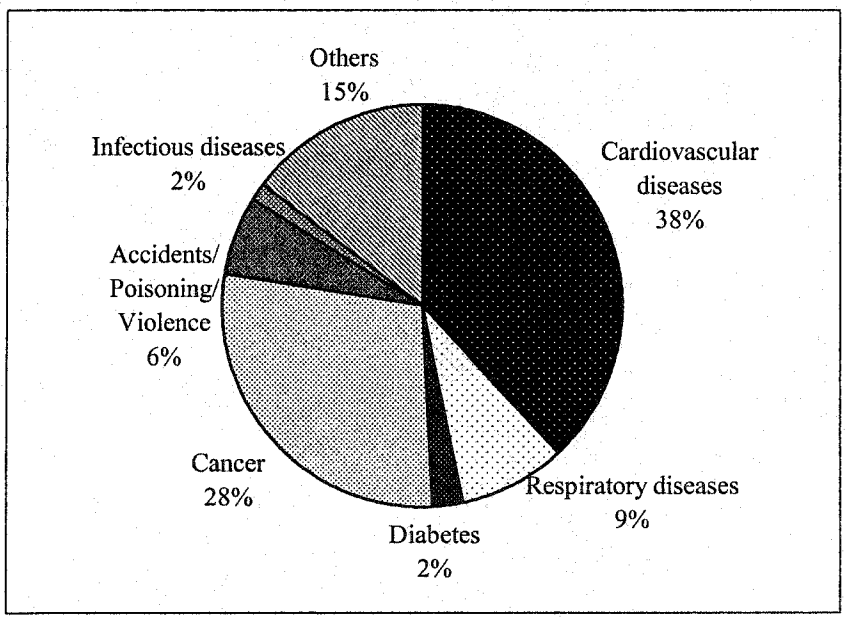

Figure 1b The proportion of deaths by causes, in Canada, estimates for 2000 (WHO 2000).

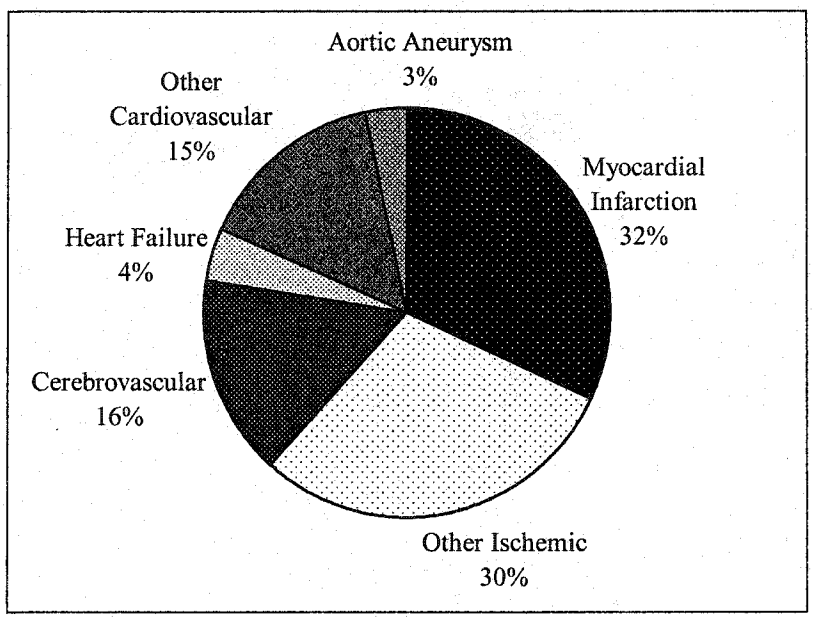

Figure 2a Prevalence of cardiovascular diseases by types in male Canadians (Health Canada 2000).

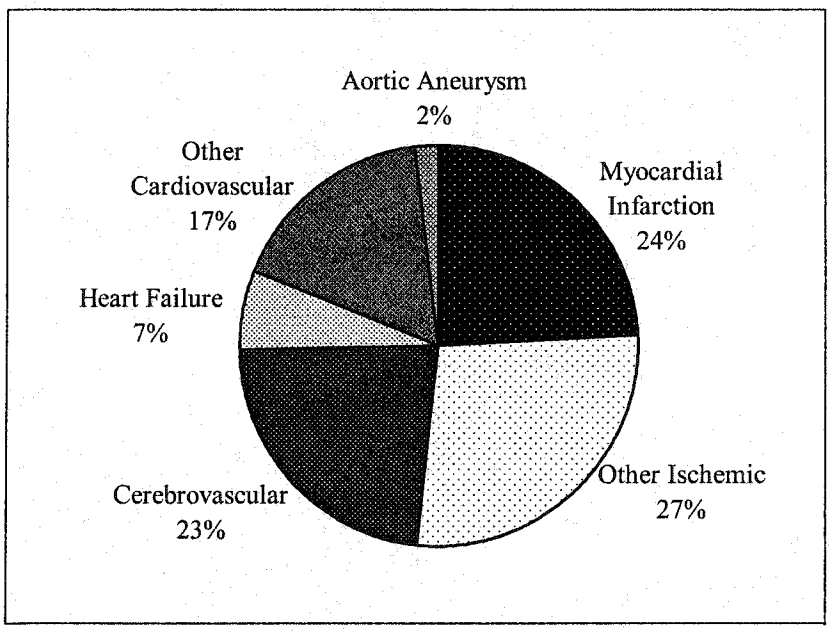

Figure 2b Prevalence of cardiovascular diseases by types in female Canadians (Health Canada 2000).

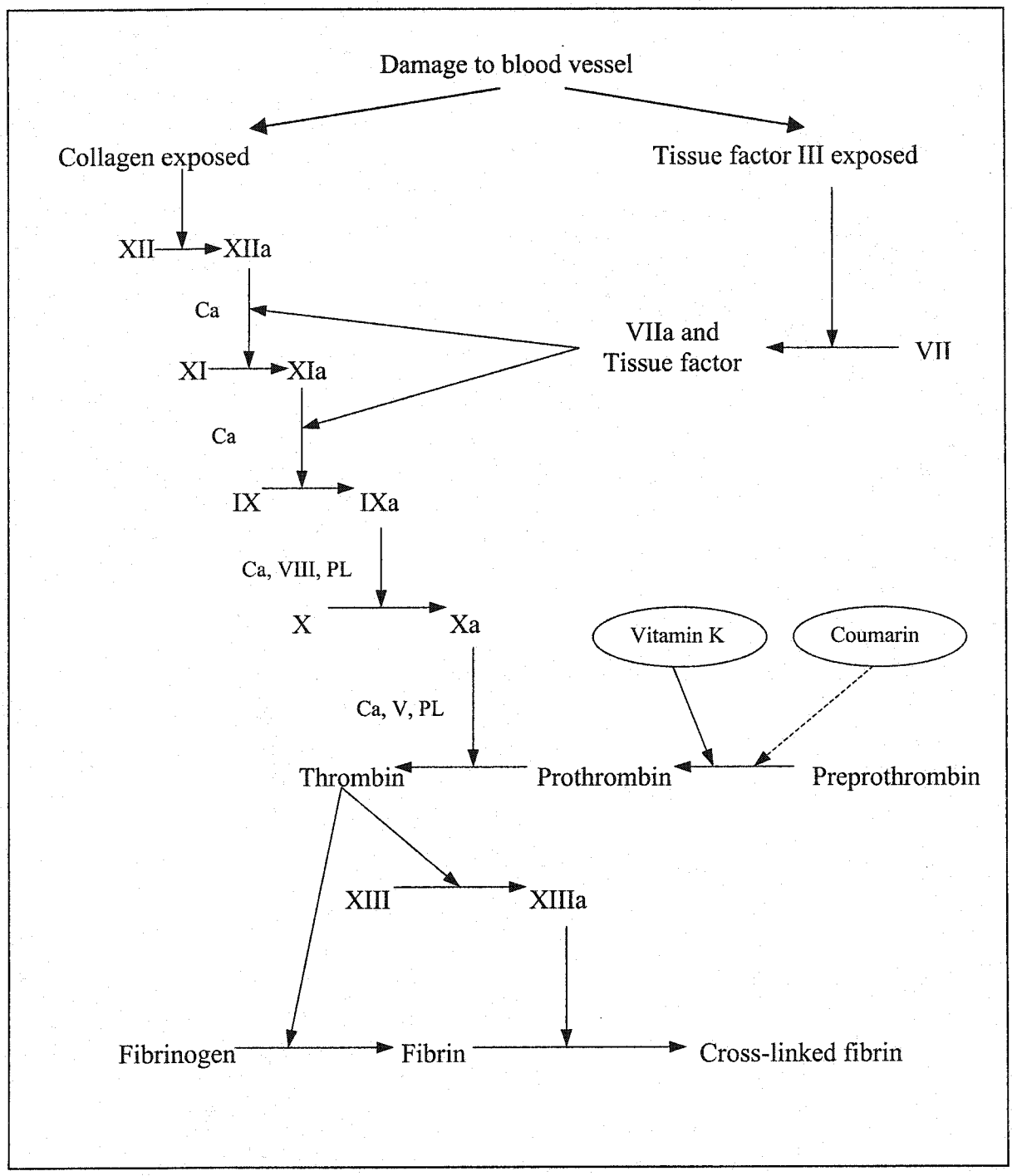

Figure 3 A diagram of the blood-clotting cascade. The cascade involves a series of enzymatic reactions, in which inactive plasma proteins are converted into active enzymes in each step of the pathway. The final step is the formation of a fibrin mesh that stabilizes the platelet plug. Intrinsic pathway begins with collagen exposure and uses proteins already present in the plasma; while 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).

formation of a fibrin clot that stabilizes the platelet plug. The fibrin clot is formed from fibrinogen by thrombin; whereas the dissolution of a blood clot is dependent on the action of an endogenous plasmin, a serine protease that is activated by tissue plasminogen activator (Silverthorn et al. 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 normal conduction of electrical signals through the ventricle will be disrupted, leading to irregular heartbeat, cardiac arrest or death (Mihara et al. 1991).

1.1.3. Current Treatments

Cardiovascular disease is the 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.

1.1.3.1. Pharmaceuticals

Historically, management of heart disease and stroke has relied on the use of anticoagulants 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. That is, an optimum antithrombotic prophylactic therapy can and should be directed towards both.

1.1.3.1.1. Anticoagulant drugs

Anticoagulants are chemicals that prevent coagulation from taking place. Most of them act by blocking one or more steps in the cascade that form 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 and 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. The drugs can be given orally or intravenously. Adverse effects of warfarin include haemorrhage, hypersensitivity, skin rashes, alopecia, and purpura. Another example of anticoagulant is heparin, a glycosaminoglycan whose major anticoagulant effect is accounted for by inhibiting thrombin, Factor IIa, and Factor Xa in the coagulation cascade. It has a short half-life and it must be given parenterally, preferably by continuous intravenous infusion. It is therefore inappropriate for home use. The dosage of this agent is based upon the

needs of the patients. Commercial heparin products, such as unfractionated heparins and low molecular weight heparins, come from beef lung or pork intestinal mucosa. The use of heparin is associated with haemorrhage, osteoporosis, alopecia, thrombocytopenia, and hypersensitivity (Fitzmaurice et al. 2002).

1.1.3.1.2. Anti-platelet agents

Anti-platelet agents are used to prevent a clot from forming or to prevent a clot from getting larger and occluding the entire vessel. Aspirin is the most widely used anti-platelet drug, which inhibits platelet aggregation for the life of the platelets (7 to 10 days). It is prescribed in the setting of acute myocardial infarction and prophylactically to prevent reinfarction. Antithrombotic doses used in clinical trials are 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, stokes, coronary artery closure in the patients undergoing angioplasty. Their usage, however, are associated with bone marrow suppression, in particular leucopenia (Blann et al. 2002).

1.1.3.2. Thrombolytic Agents

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). Evidences

show 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 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. Nevertheless, 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 et al. 2002).

1.1.3.3. Cardiac Surgery

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 conduct electrocardiogram, echocardiogram, exercise test, or holter monitoring before surgery (Wheatley 2002).

Coronary bypass surgery has evolved to be one of the commonest and the most successful operative procedures since the 1970s because of its symptomatic and survival benefits. It is an open-heart operation, in which arteries or veins are taken from another part of the body to channel the needed blood flow directly to the coronary arteries. The surgery is performed to improve patient's symptoms like chest pain or occasional difficulty in breathing, but also to protect the heart against a potential risk of a massive heart attack (Wheatley 2002). Another effective modality for acute coronary syndrome is called percutaneous transluminal coronary angioplasty (PTCA). It is a procedure used to dilate the narrowing in the coronary artery. A special catheter with a small balloon is positioned on the end within the narrowed section of the coronary artery. The balloon is then inflated and deflated several times to stretch the artery and to flatten the deposits against the walls of the artery (Turpie et al. 2002). In addition, inferior vena cava filters that are normally inserted via the internal jugular or femoral vein may be used in patients with recurrent symptomatic pulmonary embolism. It is considered as a primary prophylaxis of thromboembolism in patients at high risk of bleeding, such as patients with extensive trauma or visceral cancer (Harjai et al. 2002).

Some patients with coronary artery disease, however, may have symptoms refractory to percutaneous coronary intervention and coronary artery bypass surgery. Such patients are potential candidates for alternative forms of coronary revascularization, such as therapeutic angiogenesis. It is designed to promote the development of supplementary collateral blood vessels that will act as endogenous bypass conduits. Two major avenues for achieving therapeutic angiogenesis are currently under intense investigation, including gene therapy (the introduction of new genetic material into

somatic cells to synthesize proteins that are missing, defective, or desired for specific therapeutic purposes) and protein-based therapy (administration of the growth factors, instead of the genes encoding for the growth factors responsible for angiogenesis) (Harjai et al. 2002).

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 strategies to maximize 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).

1.1.3.4. 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; Hasler 1998).

Research indicates that diets high in saturated 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 beverage 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 grams of soy proteins per day may lower the risk of heart diseases (Arliss and Biermann 2002; Nestel 2002; Puska et al. 2002). Besides, plant sterols that are present in small quantities in many fruits, vegetables, nuts, seeds, cereals and legumes, are shown to be beneficial in preventing heart diseases. It is shown that 1.3 grams of plant sterol esters or 3.4 grams of plant stanol esters per day in the diet is needed to show a significant cholesterol-lowering effect in hypercholesterolemia patients (Meade et al. 2001). In addition, soluble fiber containing foods, such as oats and psyllium, are shown to be effective in lowering blood cholesterol levels. Clinical studies prove 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 grams of soluble fiber daily can help reduce blood cholesterol (Anderson et al. 2000; Bell et al. 1999). Furthermore, omega-3 fatty acids from cold water fish like salmon, halibut and tuna, have 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 (Daviglus et al. 1997). Ongoing research continues to explore heart-health benefits of food ingredients such as conjugated linoleic acid and plant phytonutrients (De Lorgeril et al. 1994; Hodgson 1993; Chagan et al. 2002).

1.2. 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 therapy such as 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 et al. 1996). Consequently, several lines of investigations are currently being pursed to enhance the efficacy and specificity of fibrinolytic therapy. Recently, fibrinolytic enzymes have been discovered from both food and non-food sources. These enzymes are proved to be effective and they have been proposed as one of the potent fibrinolytic regimens.

1.2.1. The Fibrinolysis system

The fibrinolytic system, as shown in Figure 4, comprises of a proenzyme (plasminogen), enzymes that proteolytically activate plasminogen, and several inhibitors that regulate activation of plasminogen, activity of plasmin, and 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 thrombus

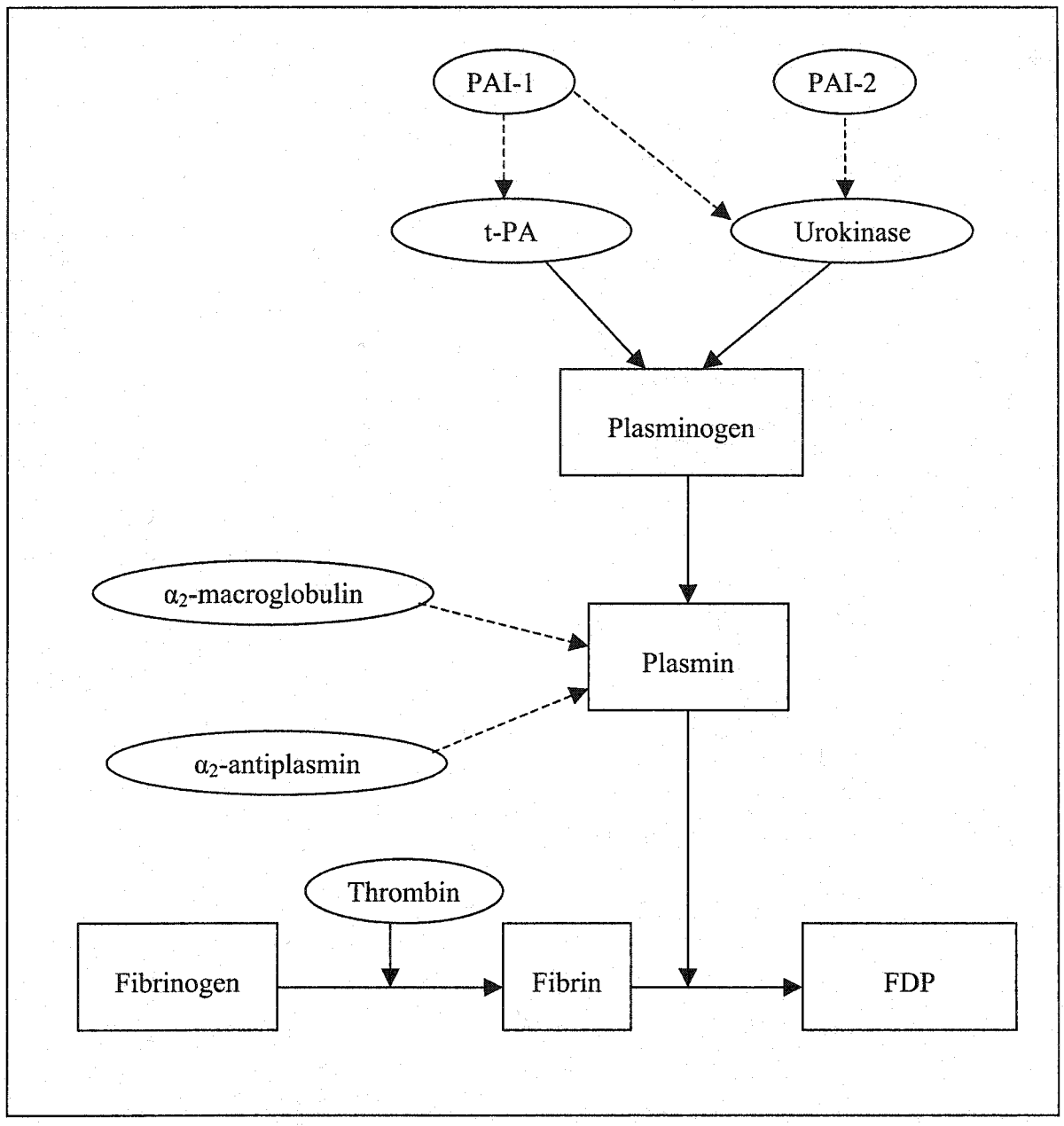

Figure 4 A simplified diagram of the fibrinolytic system. Fibrin is derived from fibrinogen in response to thrombin. Meanwhile, fibrin is degraded into fibrinogen degradation product (FDP) by endogenous plasmin protease, which is an activated form of plasminogen. t-PA and urokinase are two physiological activators of plasminogen; whereas 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 inhibitor that terminates fibrinolysis. Solid and dotted arrows represent activation and inhibition of the components, respectively (Dobrovolsky and Titaeva 2002).

exceeds that of its lysis. Impairments in these mechanisms may predispose to bleedings or thrombosis in patients (Dobrovolsky and Titaeva 2002).

1.2.2. Non-Food Sources

Fibrinolytic enzymes can be widely found in the nature. They have been found in hemorrhagic toxin from the snake venom (Nikai et al. 1984), earthworm secretions (Mihara et al. 1991), food-grade microorganisms (Chang 2000; Jeong et al. 2001), marine creatures (Sumi et al. 1992), and herbal medicines (Choi and Sa 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 and Sa 2001). Also, strong fibrinolytic enzymes are produced from Bacillus sp. strains that are used in food fermentation, invertebrates like Stichopous japonicus, as well as the seaweed Codiales codium (Jeon et al. 1995).

1.2.3. Food Sources

As shown in Table 1, fibrinolytic enzymes can be found in a variety of foods, such as Japanese natto (Sumi et al. 1986), Korean Chungkook-Jang soy sauce (Kim et al. 1996), and edible honey mushroom (Kim and 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. Lysis of thrombus can be

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., 1996
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	An edible honey mushroom	A neutral metalloprotease	Kim and Kim, 1999

observed by angiography (Kim et al. 1996). More important, 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).

1.3. Research Objectives

The main objectives of the current research project were to explore new sources of fibrinolytic enzymes from Asian fermented food products, to purify these novel enzymes by high performance liquid chromatography (HPLC), and to study the physiochemical properties of the enzymes.

2. SCREENING FOR FIBRINOLYTIC ENZYMES

2.1. Objectives

To screen for novel fibrinolytic enzymes from Asian fermented foods.

2.2. Introduction

As mentioned in section 1.2., fibrinolytic enzymes can be found in both non-food organisms and food products, especially fermented commodities. Fibrinolytic enzyme from Japanese fermented natto, for instance, has shown to be beneficial to prevent heart attack by enhancing the fibrinolytic system in animal models and human subjects. It is thus desirable to explore new sources of these enzymes from other Asian fermented foods since fermented foods, which are popular in Asian countries, are the focus of research for fibrinolytic enzymes. In this section, thirteen Asian fermented food products were screened for fibrinolytic activity by fibrin plate method.

2.3. Materials and Methods

2.3.1. Extraction of Fibrinolytic Enzymes

Each of the thirteen Asian fermented foods, 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), fermented bean curd (white), fermented cow milk, glutinous rice in wine, and stinky bean curd powder, was homogenized with a neutral extracting buffer, Tris Buffered Saline (TBS), at pH 8.0 to release enzyme from cells and organelle membranes of shrimp tissues. Insoluble material was removed by centrifugation at 12000xg for an hour at 4°C. The supernatant liquid was assayed for fibrinolytic activity by fibrin plate assay.

2.3.2. Fibrin Plate Assay

Fibrin plate assay according to Haverkate (1974) was used to measure fibrinolytic activity of the samples. In the assay, 0.35 Unit/mg of human plasmin (Sigma Chemical Co., St Louis, MO) was used as a standard fibrinolytic protease. Fibrin plate was prepared by dissolving 0.8% (w/v) of bovine fibrinogen in 0.8% (w/v) of agarose solution at 37 C. The fibrinogen solution was mixed with 0.2% (v/v) of 1Unit/μL of thrombin protease (Amersham Pharmacia Biotech, Uppsala, Sweden), and poured into a 5cm petri dish. The dish was left at room temperature for two hours to form a fibrin clot layer. A hole (2mm in diameter) was punched for sample application on the fibrin plate. After incubation of the plates for 17 hours at room temperature, the diameter of the clear zone was measured and fibrinolytic activity was expressed in plasmin units, which was determined from the plasmin standard curve.

2.4. Results and Discussion

The Asian fermented foods that have been screened for fibrinolytic activity are shown in Figure 5a. Most of them are fermented soybean products, and others include fermented seafood paste, fermented milk flavoring, and fermented rice produce. The fibrinolytic activities measured by fibrin plate method were shown in Figure 5b. All fermented soybean products except white fermented bean curd showed no fibrinolytic activity. Similarly, no activity was observed in pickled gouramy fish, fermented cow milk, and glutinous rice in wine. Fermented shrimp paste, however, had a strong fibrinolytic activity whereas fermented bean curd had a weak enzymatic activity. These enzymes directly hydrolysed the fibrin plate without plasminogen, and hence, they were regarded as a plasmin-like enzyme rather than a plasminogen activator.

Figure 5a Asian fermented food products that have been screened for fibrinolytic activities. Tempeh and stinky bean curd powder are not shown here.

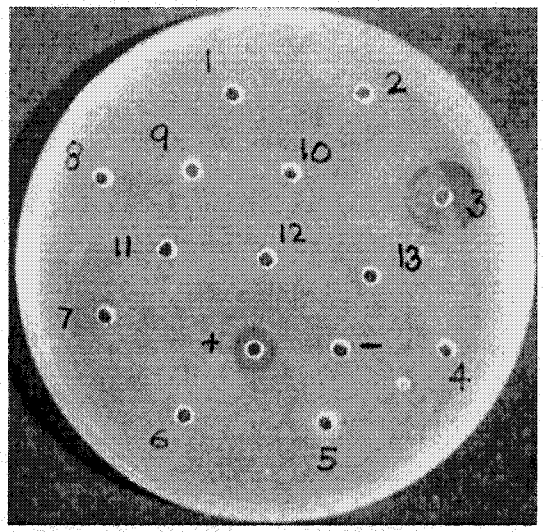

Figure 5b Fibrinolytic activities of thirteen Asian fermented food products. The crude enzyme from each of the samples was applied to the fibrin plate and incubated for 17 hours at room temperature. 1) Fermented black beans; 2) Soybean paste; 3) Fermented shrimp paste; 4) Pickled gouramy fish; 5) Tempeh; 6) Light soy sauce; 7) Sweet bean paste sauce; 8) Yellow bean sauce; 9) Fermented bean curd (white); 10) Fermented bean curd (red); 11) Fermented cow milk; 12) Glutinous rice in wine; 13) Stinky bean curd powder. Protease plasmin and sodium borate buffer were used as positive and negative control, respectively.

To determine whether this fibrinolytic enzyme is a natural component of shrimp or a derivative from the fermentation process of shrimp, shrimp muscle and intestine were both tested for fibrinolytic activity by fibrin plate method (data not shown). Neither of them showed fibrinolytic activity, and hence, it was concluded that the enzyme was produced during the fermentation stage. The source of fermented shrimp paste enzyme has yet to be explicated. It is possible that the enzyme is originated from intestinal bacteria of fresh shrimp, as fibrinolytic enzymes has been discovered from food-grade microorganisms in other salt-fermented seafood products. Since strong fibrinolytic activity was detected in the crude enzyme extract of fermented shrimp paste, this product was chosen for further studies in the current research project.

3. PURIFICATION OF FERMENTED SHRIMP PASTE ENZYME

3.1. Objectives

To purify a fibrinolytic enzyme from fermented shrimp paste.

3.2. Introduction

Fermented shrimp paste is a salt fermented shrimp product used widely in South-East Asia. It is mass-produced in summer time throughout Asian countries. The color of the fermented shrimp paste ranges from a pinkish to dark brown, and it has a very strong fishy flavor. It is a popular flavoring, which forms an integral part of curry pastes and marinades. It can also be used as seasonings for clay pot casseroles, fried rice, noodle dishes, or served as a dipping sauce. The components of fermented shrimp paste and the manufacturing process are shown in Table 2 and Figure 6, respectively. Fresh shrimps are mixed with salt in a big plastic bucket. The mixture is crushed and kept for two days. The partially fermented shrimp paste is then blended into a smooth paste and kept under the sun for another 20 to 30 days. The finished product is stored in plastic or wooden buckets, or it is packed into glass bottles for sale in the marketplace (Cheng 2002). The nutrition information for a commercial fermented shrimp paste is shown in Table 3. In this section, an enzyme from fermented shrimp paste was purified for characterization since a strong fibrinolytic activity was detected from the crude enzyme extract of fermented shrimp paste.

Table 2 Ingredients of fermented shrimp paste. The amount of salt used for fermentation is varied among different brands of fermented shrimp paste. No preservative is added to the product.

Ingredients	Percentage (%)
Silver Shrimp	85-90
Salt	10-15

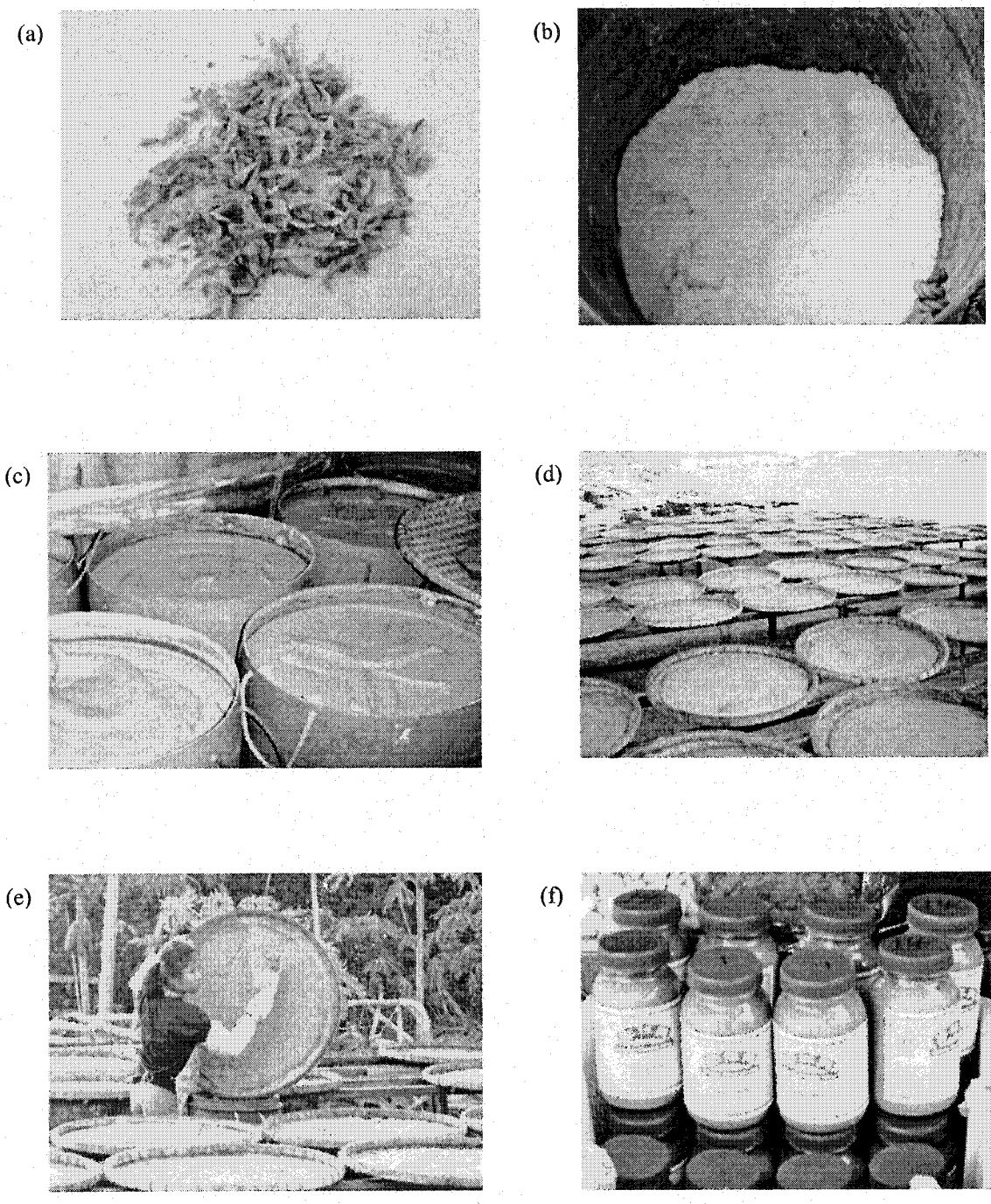

Figure 6 Production of fermented shrimp paste. The photos were taken in Cheng Cheung Hing Fermented Shrimp Paste Factory, Hong Kong. (a) and (b) silver shrimp and sea salt, respectively, for fermented shrimp paste production; (c) fermentation stage (2 days); (d) sun-drying stage (20-30 days); (e) harvesting stage; (f) fermented shrimp paste sold in bottles.

Table 3 Nutrition information for a commercial fermented shrimp paste. The information, calculated per 100g of serving, was provided by Cheng Cheung Hing Fermented Shrimp Paste Factory, 2002.

Energy	102kcal / 425kJ
Protein	20.6g
Fat	2.2g
Carbohydrates	1.0g
Sugar	0.2g
Starch	0.2g
Dietary fiber	0.6g
Sodium	8500mg
Calcium	40mg
Iron	5.5mg

3.3. Materials and Methods

3.3.1. Extraction of Crude Fermented Shrimp Paste Enzyme

The crude enzyme extract of fermented shrimp paste was prepared as described in section 2.3.1. In order to remove most of the impurities, the supernatant liquid was filtered with Whatman no.1 filter paper and dialyzed with 1 kDa cut-off cellulose membrane (Spectrum Laboratories Inc., Rancho Dominguez, CA) against deionized water for two days at 4 C. The dialysed protein sample was concentrated in a freeze-drier for two days.

3.3.2. Purification of Fermented Shrimp Paste Enzyme

3.3.2.1. Gel Filtration on Superdex 75HR Column

A 10mg/mL of crude fermented shrimp paste enzyme solution was prepared from freeze-dried sample in 10mM PBS at pH 7.0. About 5mg of crude enzyme was applied to the Superdex 75HR column (1.0x24cm) (Amersham Pharmacia Biotech, Sweden) in each run. The column was eluted with the same buffer at a flow rate of 0.5mL/min. Fraction containing fibrinolytic activity was collected and concentrated with a 10kDa ultrafiltration membrane (Millipore (Canada) Ltd., Nepean, ON).

3.3.3.2. Reversed Phase Chromatography

Reversed phase chromatography by using Sephasil Protein C4 5μm ST 4.6/250 column (0.46x25cm) (Amersham Pharmacia Biotech) was performed to separate the active enzyme fraction collected from gel filtration. The column was eluted with 10mM ammonium acetate (Buffer A) and 10mM ammonium acetate in 75% acetonitrile (Buffer

B) gradient at pH 7.0. Fraction containing fibrinolytic activity was collected and dialysed with 3.5kDa cut-off cellulose membrane (Spectrum Laboratories, Inc.) against deionized water for one day to remove acetonitrile. The dialyzed protein sample was concentrated with a 10kDa cut-off Centricon centrifugal filter (Millipore (Canada) Ltd.).

3.3.3. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a 15% polyacrylamide gel under constant pH at 150V for one hour (Laemmli 1970). The enzyme solution was treated with 5% (v/v) of β-mercaptoethanol at 96°C for 10 minutes to reduce disulfide bonds. Low molecular weight standard proteins (Sigma Chemical Co.) [bovine serum albumin (66kDa); ovalbumin (45kDa); glyceraldehydes-3-phosphate dehydrogenase (36kDa); carbonic anhydrase (29kDa); trypsinogen (24kDa); soybean trypsin inhibitor (20kDa); alpha-lactalbumin (14.2kDa); aprotinin (6.5kDa)] were used as molecular markers. Protein bands were stained with silver staining according to Heukeshoven and Dernick method (Westermeier 1993).

3.3.4. Protein Concentration Assay

The Bio-Rad DC assay kit was used to determine protein concentration in the experiment. The assay was similar to Lowry method, and the color development was primarily due to the amino acids tyrosine and tryptophan. Bovine serum albumin (Sigma Chemical Co.) was used as a protein standard. A 10μL of standards and samples were pipetted into a clean, dry microtiter plate. A 25μL of an alkaline copper tartrate solution (Reagent A) and a 200μL of a dilute Folin Reagent (Reagent B) were mixed with each of

the standards and samples. The mixtures were incubated at room temperature for 15 minutes and the absorbance was measured at 655nm.

3.3.5. N-terminal Amino Acid Sequence Analysis

Amino acid terminal sequence analysis of fermented shrimp paste enzyme was carried out with a PE Applied Biosystems Model 476A/494 Protein Sequencer (University of British Columbia, Canada).

3.3.6. Amino Acid Composition

Amino acid analysis of fermented shrimp paste enzyme was carried out with a Perkin Elmer Applied Biosystems Model 420A PTC derivatizer, with an in-line Perkin Elmer Applied Biosystems Model 130A PTC Amino Acid Analyzer (Iowa State University, U.S.A.).

3.3.7. Circular Dichroic Analysis

The far-UV spectrum of fermented shrimp paste enzyme was determined using a Jasco J-600 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan). Pure fermented shrimp paste enzyme (0.116mg/mL) was prepared in 10mM phosphate buffer at pH 7.0. Buffer and sample were filtered through a 0.22μm filter, and degassed before use. The enzyme spectrum was obtained by scanning the sample three times, from 190nm to 250nm, using a quartz cuvette with a path length of 0.1cm (Japan Spectroscopic Co., Ltd.). Phosphate buffer was used to establish a baseline, which was subtracted from the sample spectrum. The mean residue weight ellipticities were calculated, using a mean residue weight of 110, as described by Mulkerrin (1996), and

expressed in degrees centimetres squared per decimole. The percentages of the different secondary structures, including α–helix, β-sheet, β-turn and random coil, were estimated using the Jasco protein secondary structure estimation program, based on the method of Yang et al. (1986).

3.4. Results and Discussion

The purification steps for fermented shrimp paste enzyme are summarized in Figure 7. Crude enzyme extract of fermented shrimp paste was separated on a Superdex 75HR column based on their differential sedimentation in a gravitational field related to their molecular weight, ranging from 3 to 70 kDa. It was well to use gel filtration at this point since that material could be placed directly on a column without the need to remove salts. The enzyme was eluted with 10mM PBS at pH 7.0. As shown in Figure 8a and 8b, six different fractions were eluted from the gel matrix, and the third peak contained the majority of enzyme activity. This active fraction came off the column right before the colouring of fermented shrimp paste, implying it was a low molecular weight protein.

According to Lane 4 in Figure 9, the active fraction (peak 3) collected from gel filtration consisted of at least three different proteins and many impurities. This enzyme fraction was then purified on the basis of the differences in hydrophobicity. A pH 7.0 buffer system was chosen to elute the enzyme from the Sephasil protein C4 column, as preliminary data indicated that separation of enzyme was optimized at neutral pH. As shown in Figure 10a, four fractions, in an increasing order from lowest to highest hydrophobicity, were eluted from the Sephasil Protein C4 column. According to the fibrin plate assay shown in Figure 10b, the second peak was shown to have fibrinolytic

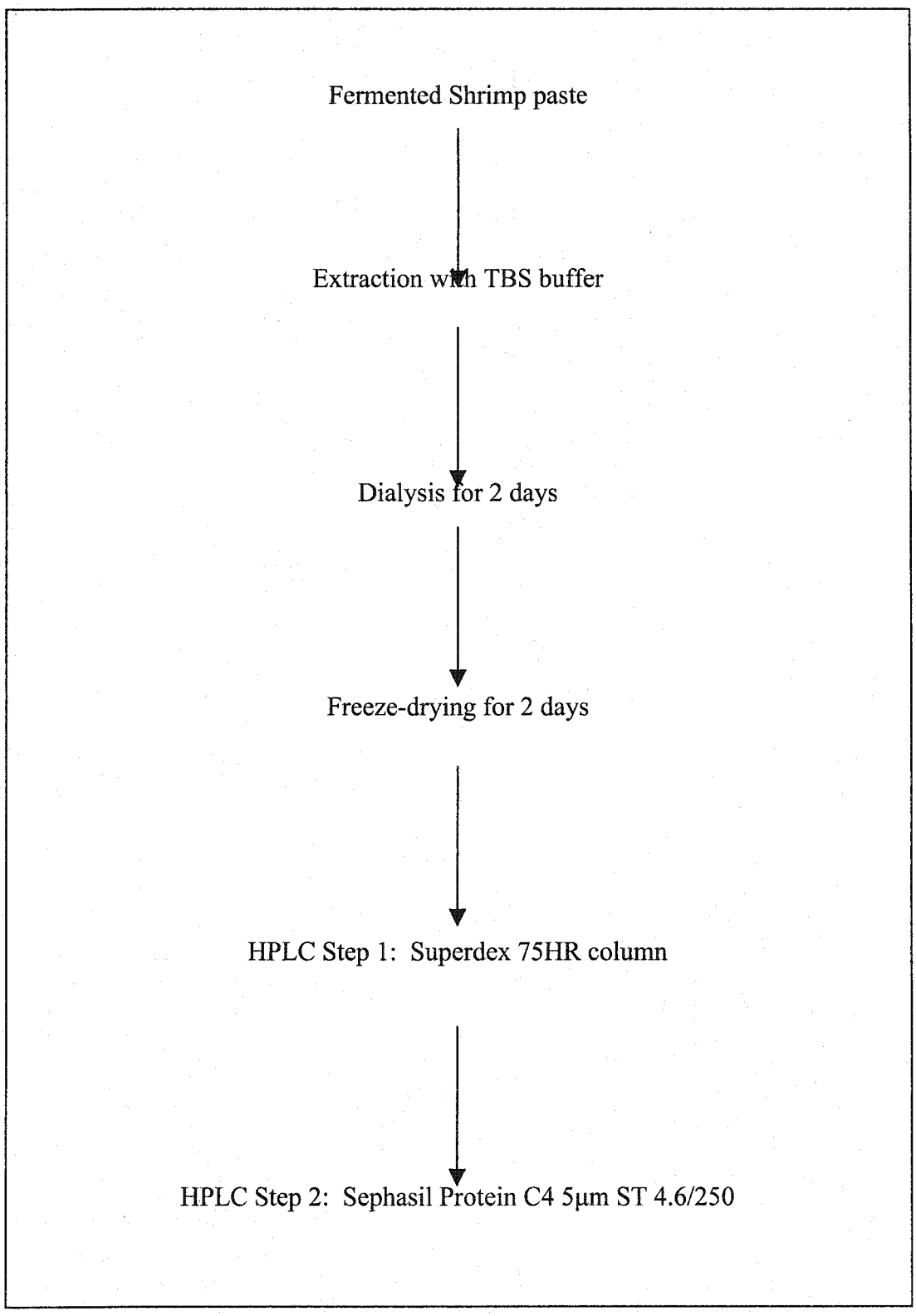

Figure 7 A diagram of purification procedures for fermented shrimp paste enzyme.

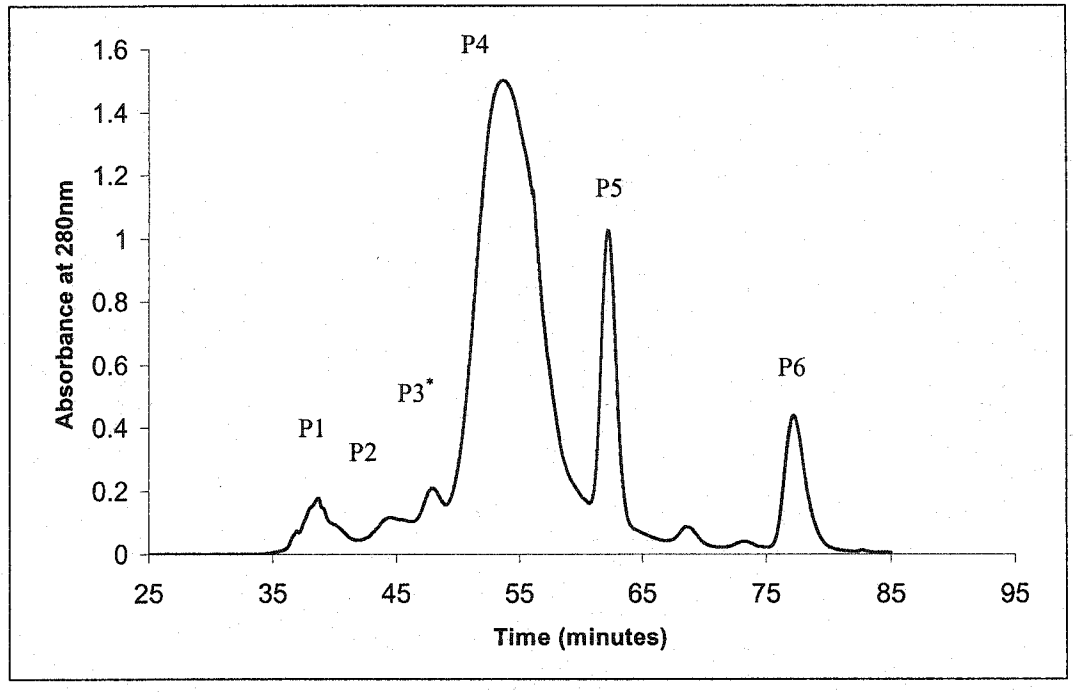

Figure 8a HPLC elution profile of fermented shrimp paste enzyme from Superdex 75HR column. The active enzyme faction is signified by a * in the profile.

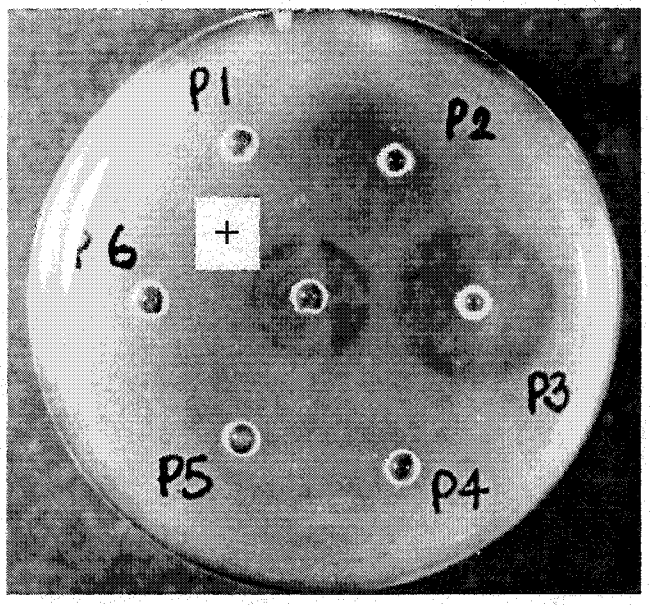

Figure 8b Fibrinolytic activities of the fractions, peak 1 to peak 6, eluted from Superdex 75HR column. Protease plasmin was used as a positive control.

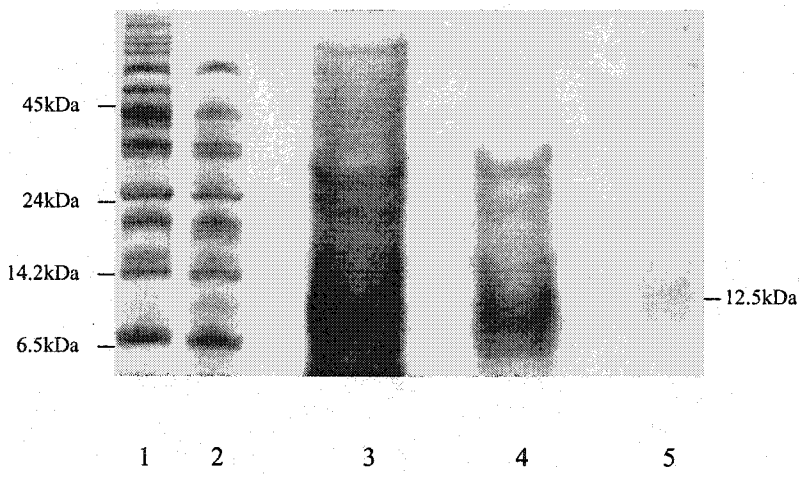

Figure 9 SDS-PAGE of fermented shrimp paste enzyme. Lane 1, wide range molecular weight standard proteins; Lane 2, low molecular weight standard proteins; Lane 3, crude enzyme; Lane 4, partially purified enzyme from gel filtration; Lane 5, pure fibrinolytic enzyme from reversed phase chromatography. Electrophoresis was carried out at 150V on a 15% polyacrylamide gel. Proteins were stained with silver nitrate.

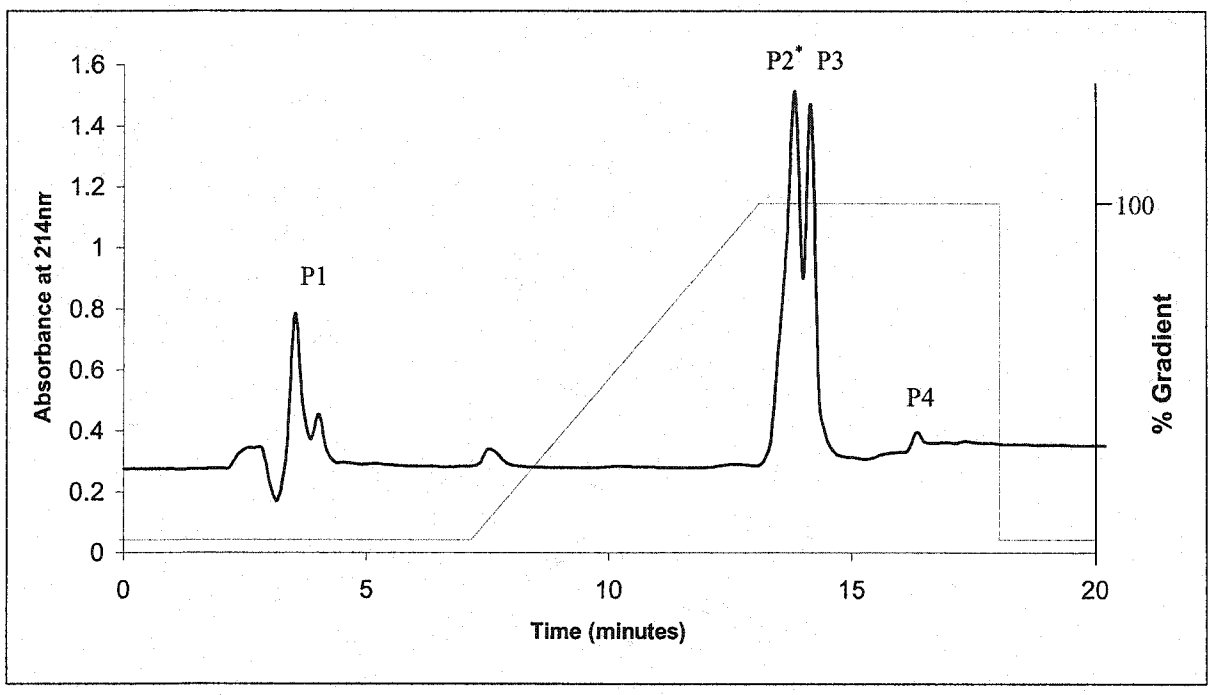

Figure 10a HPLC elution profile of fermented shrimp paste enzyme from Sephasil Protein C4 5µm ST 4.6/250 column. The active enzyme faction is signified by a * in the profile.

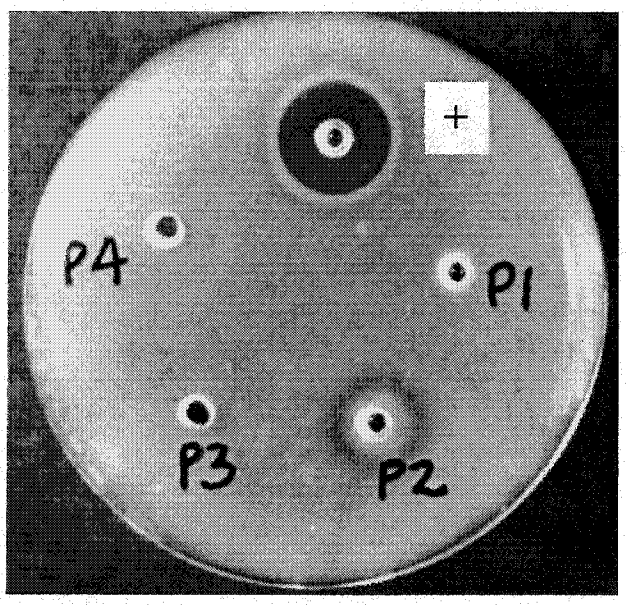

Figure 10b Fibrinolytic activities of the fractions, peak 1 to peak 4, eluted from Sephasil Protein C4 5µm ST 4.6/250 column. Protease plasmin was used as a positive control.

activity. This active enzyme fraction (peak 2) was eluted in extremely hydrophobic condition (100% buffer B), indicating that the enzyme has a highly hydrophobic surface.

According to Lane 5 in Figure 9, fermented shrimp paste enzyme was a monomer with an apparent molecular mass of 12.5kDa on SDS-PAGE. The purification results are summarized in Table 4. The enzyme was purified 12.6-fold with an 18.2% recovery, and it has a specific activity of 2.54Units/mg.

The N-terminal amino acid sequence of the first 15 residues of fermented shrimp paste enzyme is shown in Figure 11. In comparison to other fibrinolytic enzymes that have been discovered, no homology in the N-terminal sequence could be observed. Yet a BLASTp database search showed that fermented shrimp paste enzyme is 63% homology to human titin isoform, which is a muscle protein. Titin is a major constituent of the sacromere in vertebrate striated muscle for controlling the assembly of muscle thick filaments, the muscle elasticity and the generation of passive tension (Silverthorn et al. 1998).

The amino acid composition of fermented shrimp paste enzyme is summarized in Table 5. Neither cysteine nor tryptophan could be observed in the protein structure. This might due to the fact that cysteine and tryptophan are easily destroyed by acid hydrolysis, a process in amino acid analysis. Fermented shrimp paste enzyme was consisted of a high content of proline, which had unique imino bonds. It was suggested that the enzyme was not a compact globular protein since proline residues enhanced random coil and β-turn formations. In other words, the enzyme might have an extended structure with no α–helix or β-pleated sheets in the lateral proximity of proline residues.

Table 4 Purification summary for fermented shrimp paste enzyme.

Procedure	Total Protein (mg)	Total Activity (Units)	Specific Activity (Units/mg)	Purification (fold)	Yield (%)
Crude Enzyme	5.0	0.99	0.20	1	100
Superdex 75 HR gel filtration	0.36	0.39	1.08	5.4	39.4
Sephasil Protein C4 5μm ST 4.6/250	0.07	0.18	2.54	12.7	18.2

Note: Data is calculated as the mean (SEM < 5%). The Units of activity is calculated based on the plasmin standard (3.5Units/mg).

	1			5				10						15	
Fermented shrimp paste (shrimp paste enzyme)	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	A
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

Figure 11 Comparison of N-terminal amino acid sequence of fermented shrimp paste enzyme with other fibrinolytic enzymes. The first 14-15 amino acid residues in the N-terminal of the enzymes are shown (Sumi et al. 1995; Kim et al. 1996; Kim and Kim 1999; Chang et al. 2000; and Jeong et al. 2001;)

Table 5 Amino acid composition of fermented shrimp paste enzyme.

Amino Acids	Mole (%)
Asx	13.47
Glx	10.77
Ser	6.71
Gly	10.62
His	1.58
Arg	3.24
Thr	9.75
Ala	6.90
Pro	12.04
Tyr	2.93
Val	5.21
Met	0.82
Ile	2.52
Leu	4.01
Phe	1.69
Lys	7.74

Secondary structure of the fermented shrimp paste enzyme was obtained using circular dichroism (CD) (Mulkerrin 1996). The observed spectrum of the enzyme and the proportion of secondary structures are shown in Figure 12 and Table 6, respectively. The result indicated that the enzyme was a beta-type of protein with 56% of β-sheet. The CD estimation of 30.7% of random coil and 11.2% of β-bend agreed with that predicted by amino acid composition analysis, as a high percentage of proline residues in the enzyme favored opened and random secondary structures.

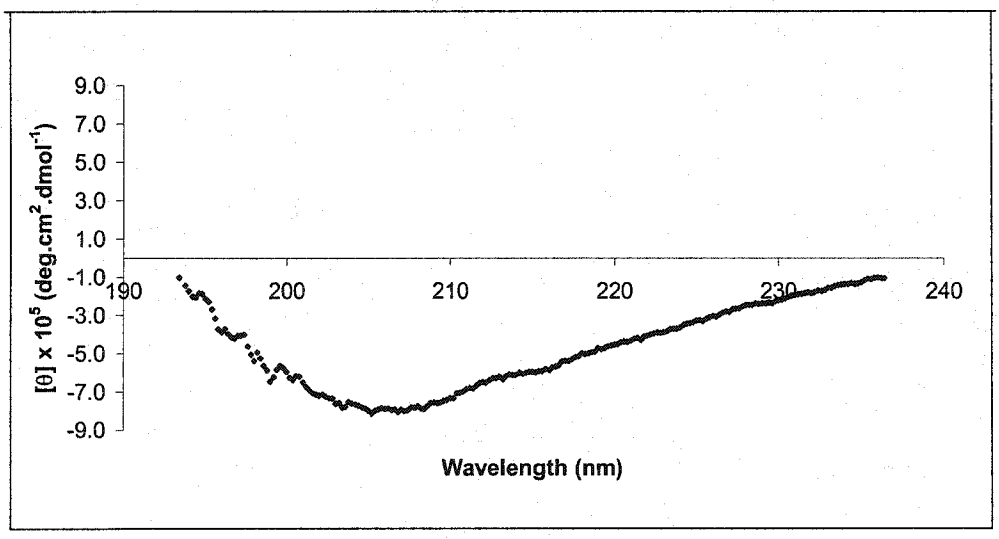

Figure 12 Far-UV spectrum of fermented shrimp paste enzyme. The spectrum was obtained by scanning the sample three times, from 190nm to 250nm. A 10mM of phosphate buffer was used as the blank.

Table 6 Secondary structure of fermented shrimp paste enzyme, calculated as percentages from the CD analysis, using the Jasco protein secondary structure estimation program based on the method of Yang et al., 1986.

Secondary Structure	**Percentage (%)**
α-helix	1.8
β-sheet	56.3
β-turn	11.2
Random coil	30.7

4. EFFECT OF PH ON FIBRINOLYTIC ACTIVITY

4.1. Objectives

To determine the pH stability of fermented shrimp paste enzyme.

4.2. Introduction

Maximum enzymatic activity is attained only within a narrow pH range. The pH optimum of an enzyme is dependent on a number of experimental parameters, such as time of reaction, temperature, concentration of substrate, nature of buffer, ionic strength of medium, and purity of enzyme preparation. Most enzymes undergo irreversible denaturation in very acid and very alkaline solutions, but the pH at which this occurs varies with enzyme (Whitaker 1994). In this section, the pH stability of fermented shrimp paste enzyme was determined by fibrin plate assay.

4.3. Materials and Methods

The pH effect on fibrinolytic activity of the fermented shrimp paste enzyme was assayed from pH 2.0 to 12.0. Crude enzyme in 10mM PBS at pH 7.0 was mixed with different buffers in the range of 2 to 12. The buffers used and their pH ranges were 0.05N HCl (pH 2); 50mM sodium acetate buffer (pH 3 to 5); 50mM phosphate buffer (pH 6 to 8); 50mM carbonate buffer (pH 9 and 10); and 0.05N NaOH (pH 12). The reaction mixtures were incubated for one hour at 37°C, and the enzyme activities were measured by fibrin plate. Maximum activity was expressed as 100% and others were compared to the maximum activity.

4.4. Results and Discussion

The fibrinolytic activity of fermented shrimp paste enzyme was examined in various buffers, ranging from pH 2 to 12. The relative activity of the enzyme is plotted against the pH range as shown in Figure 13. Fermented shrimp paste enzyme was active in a wide range of pH. The fibrinolytic activity of the enzyme was maximized between pH 5.0 to 7.0, indicating that it was a neutral protease. The result was consistent with the amino acids composition analysis in Section 3, which suggested that there were approximately equal amount of acidic and basic amino acids in the enzyme. The enzymes remained active at extremely high and low pH, but the relative activities were decreased to 65% and 56%, respectively.

In accordance with the pH stability of fermented shrimp paste enzyme, it is best to deliver the enzyme in a neutral medium since the enzyme activity is relatively weak at acidic and basic conditions. If fermented shrimp paste enzyme is to be prescribed as pills or capsules, for example, an enteric coating should be used to protect the enzyme from the stomach acid. To obtain its best physiological benefits, fermented shrimp paste should not be consumed with high acidity foods, such as citrus fruits, vinegar, and fermented milk products, or high alkaline foods, like bananas and spinach.

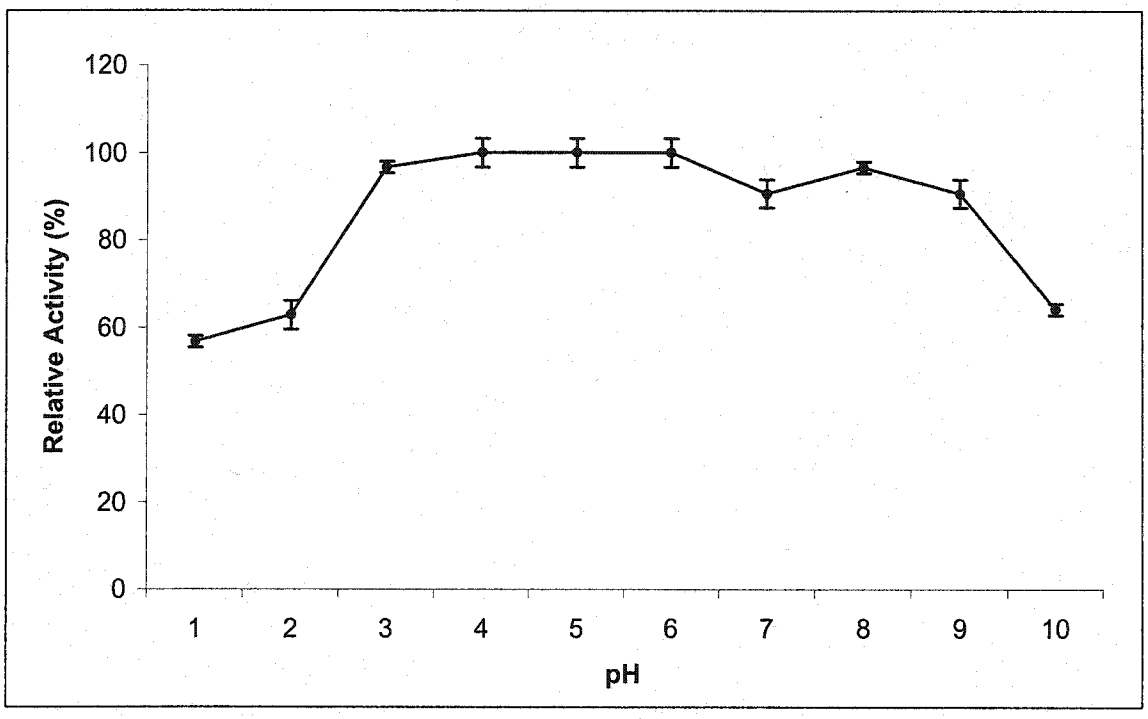

Figure 13 Effect of pH on fermented shrimp paste enzyme at 37°C.

5. EFFECT OF TEMPERATURE ON FIBRINOLYTIC ACTIVITY

5.1. Objectives

To determine the temperature optimum of fermented shrimp paste enzyme.

5.2. Introduction

The reaction temperature is a crucial factor that affects enzymatic reactions in a number of ways. Changes in temperature may exert an effect on stability of enzymes, affinity of enzyme for activators and inhibitors, competing reactions, ionization of prototropic groups of the system, enzyme-substrate affinity, and the velocity of conversion of substrate to product (Whitaker 1994). In this section, the temperature at which fermented shrimp paste enzyme was the most active was measured by fibrin plate assay.

5.3. Materials and Methods

The effect of temperature on fibrinolytic activity was studied at various temperatures at pH 7.0. Crude enzymes in 10mM PBS at pH 7.0 were heated for 10 minutes at different temperatures starting from 30°C up to 90°C (30, 40, 50, 60, 65, 70, 80, and 90°C). The enzymes were applied to fibrin plate, and the relative activities were calculated as a percentage of the maximum activity.

5.4. Results and Discussion

Thermal activity of fermented shrimp paste enzyme was examined after incubation at different temperatures for 10 minutes at pH 7. The relative enzyme activity is plotted against the temperature range as shown in Figure 14. The enzyme activity was optimized between 30°C and 40°C. The enzyme has become less active when temperature rose above 40°C, and it was completely denatured at temperature above 60°C. Some correlation might be drawn between the size and complexity of the enzyme and its susceptibility to heat. The larger and the more complex is an enzyme, the more susceptible it is to high temperature. Since fermented shrimp paste enzyme was determined to be a single polypeptide chains with a molecular mass of 12.5 kDa, it was fairly resistant to heat treatment.

Based on the temperature effect on the fibrinolytic activity of fermented shrimp paste enzyme, the cooking temperature of fermented shrimp paste should be properly controlled. To use fermented shrimp paste as a functional food in preventing heart attack, a low cooking temperature is recommended since fermented shrimp paste enzyme will be denatured at temperature above 60°C. Fermented shrimp paste, for instance, can be used as a dipping sauce or as an ingredient of salad dressing. Like Japanese nattokinase, fermented shrimp paste enzyme can be extracted, and consumed as a nutraceutical in the form of potions or capsules.

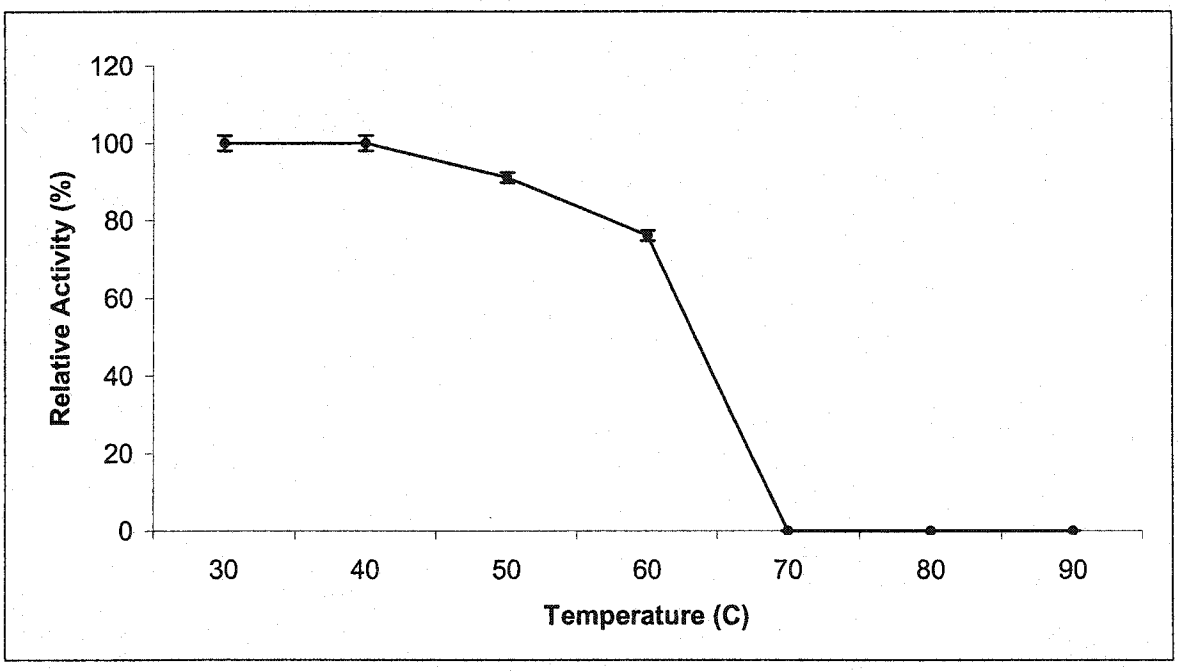

Figure 14 Effect of temperature on fermented shrimp paste enzyme at pH 7.0.

6. EFFECTS OF PROTEASE INHIBITORS AND METAL IONS

6.1. Objectives

To classify fermented shrimp paste enzyme into category of proteolytic enzymes.

6.2. Introduction

Inhibitors are compounds that reduce the rate of an enzyme-catalyzed reaction. In fact, the effect of specific inhibitors on an enzyme system is a powerful tool for investigating the nature of the active site of an enzyme, elucidating the mechanism of enzyme-catalyzed reactions, and establishing the sequences of metabolic pathways. The most useful type of inhibitor in elucidation of reaction mechanisms is one that reacts with the active site on an enzyme where substrate, cofactor, and/or activator are bound (Whitaker 1994). In this section, the effects of several protease inhibitors on the fibrinolytic activity of fermented shrimp paste enzyme were examined so as to categorize the enzyme into protease classes. The effect of divalent ions on the enzyme activity was examined as well.

6.3. Materials and Methods

6.3.1. Inhibition of Enzyme Activity by Protease Inhibitors

Nine protease inhibitors (Sigma Chemical Co.), including phenyl-methyl-sulfonyl fluoride (PMSF), EDTA, Dithiothreitol (DTT), 2,4-dinitrophenol (DNP), trans-epoxysuccinyl-L-leucylamido-(quanidino)-butane (E64), trypsin inhibitor (TLCK), Pepstatin A, 1, 10-phenanthroline, and collagenase inhibitor, were used to characterize fermented shrimp paste enzyme. Each of the inhibitors was dissolved in deionized water

and mixed with pure fermented shrimp paste enzyme (in 10mM PBS at pH 7) at 37°C for 10 minutes. Enzyme activity at two different inhibitor concentrations, 1mM and 2mM, was measured by fibrin plate assay. The relative activity was calculated based on the activity of pure enzyme without any of the protease inhibitors under the same experimental conditions.

6.3.2. Inhibition of Enzyme Activity by Divalent Ions

The effects of various divalent ions, including Mg^{2+}, Cu^{2+}, Ca^{2+}, Zn^{2+}, Hg^{2+}, and Fe^{2+} ions, on fibrinolytic activity were investigated. Metal chlorides (Sigma chemical Co.) were dissolved in deionized water, and mixed with pure enzyme (in 10mM PBS at pH 7.0) at 37°C for 10 minutes. The concentration of the reaction mixture was 1mM and the enzyme activity was determined by fibrin plate assay. The relative activity was calculated based on the activity of the pure enzyme without any metal chlorides under the same experimental conditions.

6.4. Results and Discussion

The effects of protease inhibitors on fibrinolytic activity of fermented shrimp paste enzyme are summarized in Table 7. In general, there are four classes of proteases, including serine proteases, aspartic proteases, cysteine proteases and metalloproteases. Fermented shrimp paste enzyme was not inhibited by PMSF, TLCK, or Pepstatin A, indicating that it was not a serine protease nor an aspartic protease. The enzyme was not a cysteine protease either since it was not affected by E64 and DNP. In addition, the enzyme appeared not to be a metalloprotease since it was not inhibited by metalloprotease inhibitors, like 1, 10-phenanthroline and collagenase inhibitor. Its

Table 7 Effects of protease inhibitors on fibrinolytic activity of fermented shrimp paste enzyme.

Reagents	Relative Activity (%)	
	1mM	2mM
PMSF[a]	96.7	88.9
EDTA	94.7	77.8
DTT[b]	99.3	77.8
DNP[c]	100	100
E64[d]	100	100
TLCK[e]	98.7	100
Pepstatin A	96.7	88.9
1,10-phenanthroline	100	100
Collagenase inhibitor	100	100

Note: Data is calculated as the mean (SEM < 5%) of 2 sets of experiment.
[a]PMSF, phenyl-methyl-sulfonyl fluoride; [b]Dithiothreitol; [c]DNP, 2,4-dinitrophenol; [d]E64, trans-epoxysuccinyl-L-leucylamido-(quanidino)-butane; [e]TLCK, trypsin inhibitor.

activity, however, was slightly inhibited by EDTA, which forms strong complexes with divalent and higher-oxidation-state cations such as ferric ion, zinc ion, copper ion, and calcium ion. Therefore, fermented shrimp paste enzyme was determined to be a novel enzyme, which could not be classified into one of the four classes of protease enzymes, but it shared some similarities with metalloproteases. An inhibition of enzyme activity by DTT predicted that there might be a disulfide linkage in the active site of the enzyme or the enzyme was denatured by DTT.

The effects of cations on enzyme activity were investigated. As shown in Table 8, fermented shrimp paste enzyme was insensitive to calcium ion, mercuric ion, magnesium ion and ferric ion. Yet significant inhibition was observed when the enzyme was incubated with copper ion. In general, the cations perform a specific role in modulation of enzyme activity, while anions play a more general role in modulation of the activity of enzymes. It was hypothesized that copper ion intrudes on fermented shrimp paste enzyme-catalyzed system by binding to carboxyl groups of the enzyme. In other words, carboxyl group could be an essential component of the active site for the enzyme function.

Table 8 Effects of divalent ions on fibrinolytic activity of fermented shrimp paste enzyme.

Divalent ions	Relative Activity (%)
Mg	93.8
Cu	75.0
Zn	93.8
Hg	93.8
Fe	96.9

Note: Data is calculated as the mean (SEM < 5%) of 2 sets of experiment.

7. SUBSTRATE SPECIFICITY OF FERMENTED SHRIMP PASTE ENZYME

7.1. Objectives

To determine the protein substrates for fermented shrimp paste enzyme.

7.2. Introduction

Chemicals that undergo changes in a reaction catalyzed by an enzyme are the substrates of that enzyme. Though enzymes are highly specific for the substrates that they act upon, the degree of substrate specificity varies. Some enzymes act on a group of related substrates, while others on only a single compound (Whitaker 1994). In this section, protein substrates of fermented shrimp paste enzyme were scrutinized by electrophoresis.

7.3. Materials and Methods

7.3.1. Fibrinogen

Pure fermented shrimp paste enzymes in 10mM PBS at pH 7.0 were incubated with 1mg/mL of bovine fibrinogen (Sigma chemical Co.) in a ratio of 1:50 (w/w) at 37 C for various time periods (0, 15, 30, 60, 120 and 180minutes). Fibrinolytic degradation pattern was determined by SDS-PAGE using a 10% polyacrylamide gel. Gel was stained with 0.02% (w/v) Coomassie Brilliant Blue R-250, and destained using a 40% (w/v) methanol and 7% acetic acid solution.

7.3.2. Blood Proteins

Pure fermented shrimp paste enzymes in 10mM PBS at pH 7.0 were incubated with 1mg/mL of each of the four plasma proteins in a ratio of 1:50 (w/w) at 37 C for

three hours. The plasma proteins used were bovine serum albumin (BSA) (Fisher Scientific, NJ), ChromPure bovine immunoglobulin G (IgG) (Sigma Chemical Co.), Thrombin (Th) (Amersham Pharmacia Biotech) and hemoglobin (Hb) (Amersham Pharmacia Biotech). SDS-PAGE of the reduced reaction mixtures was done on a 10% polyacrylamide gel and the proteins were visualized with Comassive Blue.

7.4. Results and Discussion

The degradation pattern of bovine fibrinogen by fermented shrimp paste enzyme is shown in Figure 15. The results indicated that the alpha subunit of the multimeric fibrinogen was resistant to the enzyme digestion since its protein band at 64kDa was intact after three hours of incubation. The beta and gamma subunits, however, were more susceptible to the proteolytic degradation since they were dissociated into three polypeptides with lower molecular weights. Whether these three polypeptide chains belonged to the beta or gamma subunit has yet to be determined.

As fibrinolytic enzyme will be delivered into the circulatory system in order to obtain its beneficial effects, it is important that the enzyme does not breakdown any blood proteins that are not involved in the blood-clotting cascade. Fermented shrimp paste enzyme was incubated with each of the four typical plasma proteins, including bovine serum albumin, immunoglobulin G, thrombin, and hemoglobin, and the degradation patterns of these proteins by the enzyme are shown in Figure 16. The results showed that none of these plasma proteins was broken down by the enzyme after incubation at 37°C for an hour. Thus, fermented shrimp paste enzyme was relatively specific to fibrin or fibrinogen as protein substrates, cleaving beta or gamma subunits without affecting the

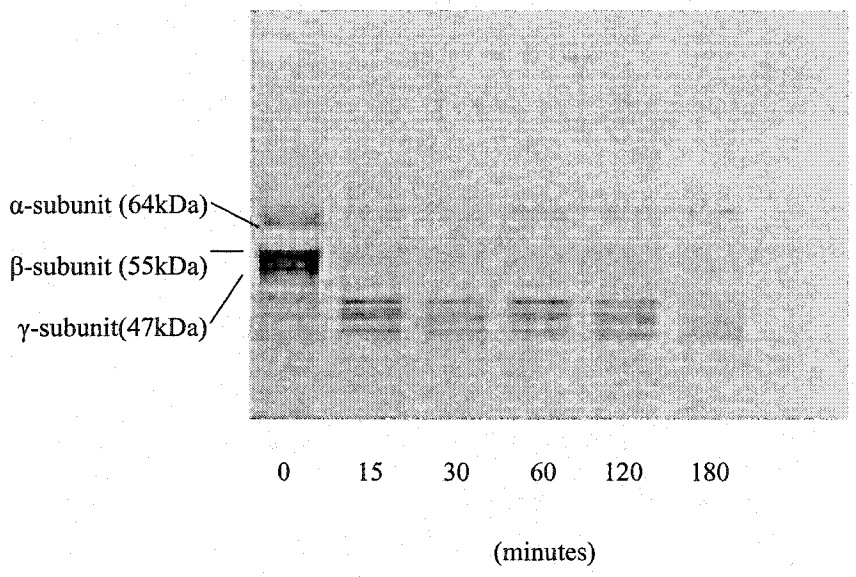

Figure 15 Degradation Pattern of bovine fibrinogen by fermented shrimp paste enzyme. Electrophoresis was carried out on a 10% polyacrylamide gel and the gel was stained with Comassive Blue.

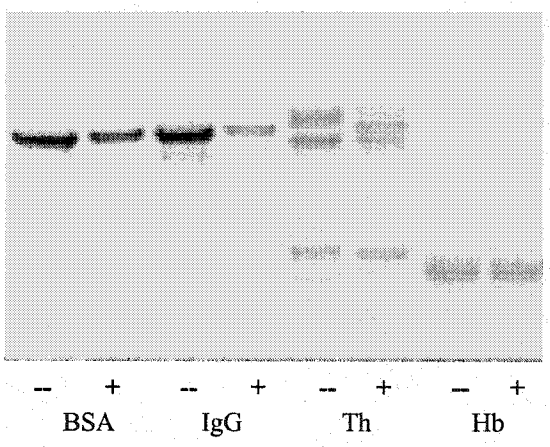

Figure 16 Degradation Pattern of bovine plasma proteins by fermented shrimp paste enzyme. Electrophoresis was carried out on a 10% polyacrylamide gel and proteins were stained with Comassive Blue. The substrate proteins incubated with shrimp paste enzyme at 37°C for 3 hours are specified by "+" signs, and those without enzyme are specified by "--" signs.

alpha subunits of fibrinogen. The enzyme might not interfere with the normal composition of the plasma proteins while it exerted its fibrinolytic activity in the circulatory system.

To further understand the substrate specificity of fermented shrimp paste enzyme, the active site of the enzyme could be examined through the coupling between the enzyme and synthetic protein substrates, such as synthetic thrombin substrate and synthetic plasmin substrate. Based on the nature of the active site of fermented shrimp paste enzyme, the catalytic mechanism, including the binding mechanism and the release mechanism, of the enzyme could be elucidated as well.

8. ANTICOAGULANT ACTIVITY OF FERMENTED SHRIMP PASTE ENZYME

8.1. Objectives

To determine anticoagulant activity of fermented shrimp paste enzyme in addition to its fibrinolytic activity.

8.2. Introduction

Historically, management of heart attack and stoke has relied on the use of anticoagulants such as heparin and warfarin. These drugs reduce the risk of abnormal blood clotting in the body by blocking a third of the coagulation cascade. Recognition that lysis of preformed fibrin could be accomplished *in vitro* by shrimp paste enzyme led to a question of whether shrimp paste enzyme can be an anticoagulant or not. In this section, anticoagulant activity of fermented shrimp paste enzyme was investigated by measuring its effect on human blood clotting time.

8.3. Materials and Methods

8.3.1. Active Partial Thrombin Time (APTT)

Fresh human blood was collected in 3.8% sodium citrate. The serum was centrifuged at 2500xg for 15 minutes. In the assay, a 100μL of human serum was incubated with various amounts of pure enzyme (0, 1.25, 2.5, 5, and 10μg) at 37°C for 1 minute. A 100μL of APTT reagent (Sigma Chemical Co.) was added and the reaction mixture was incubated for another three minutes. The reaction was initiated by adding a 100μL of 20mM $CaCl_2$. Human serum without the addition of fermented shrimp paste enzyme was used as a control.

8.3.2. Prothrombin Time (PT)

Fresh human blood was collected in 3.8% sodium citrate. The serum was centrifuged at 2500xg for 15 minutes. In the assay, a 100μL of human serum was incubated with various amounts of pure enzyme (0, 1.25, 2.5, 5, and 10μg) at 37°C for three minutes. The reaction was initiated by adding a 200μL of ThromboMax with Calcium reagent (Sigma Chemical Co.). Human serum without the addition of fermented shrimp paste enzyme was used as a control.

8.4. Results and Discussion

The anticoagulant activity of fermented shrimp paste enzyme is expressed as active partial thrombin time (APTT) and prothrombin time (PT), which are common clinical tests for measuring defects in the intrinsic and extrinsic pathways of the blood-clotting system, respectively, in Figure 17. APTT of human serum was substantially delayed for more than a minute in the presence of 10μg of fermented shrimp paste enzyme. However, PT was not prolonged evidently even at the highest enzyme concentration. These results indicated that fermented shrimp paste enzymes could act as an anticoagulant as well as a fibrinolytic agent. It might inhibit the blood-clotting cascade by enhancing the anticoagulant activity of naturally occurring blood factors. With its high proteolytic activity, fermented shrimp paste enzyme might also breakdown any one of the blood clotting factors involved in the intrinsic pathway of the cascade, leading to abolition of both thrombin generation and platelet aggregation.

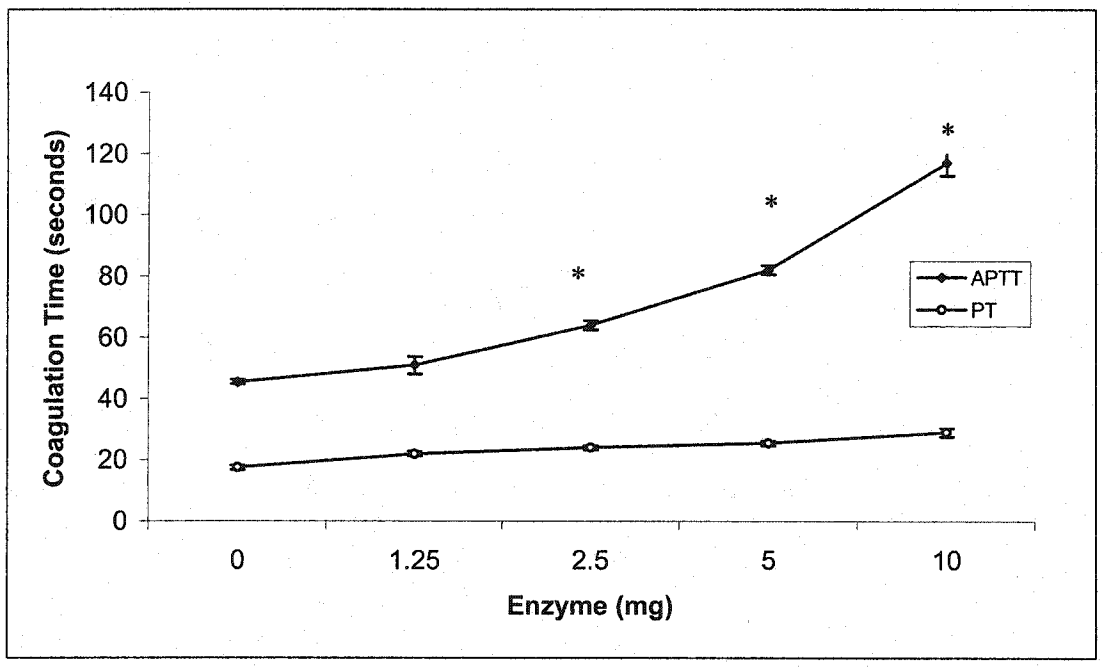

Figure 17 Anticoagulant activity of fermented shrimp paste enzyme. The blood-clotting time was measured by Active Partial Thrombin Time (APTT) and Prothrombin Time (PT). A significant delay in coagulation time ($p<0.05$) is signified by* in the graph.

9. EFFECTS OF DIGESTIVE ENZYMES ON FERMENTED SHRIMP PASTE ENZYME

9.1 Objectives

To determine the effect of digestive enzymes, pepsin and trypsin, on the fibrinolytic activity of fermented shrimp paste enzyme.

9.2 Introduction

To use fermented shrimp paste enzyme as an oral fibrinolytic agent, it is important that it can pass through the gastrointestinal tract into the circulatory system, where it exerts its biological effect. Digestive system is composed of the gastrointestinal (GI) tract, which is a long tube that moves nutrients, water, and electrolytes from the external to the internal environment. Food is masticated in the mouth and digested by enzymes that are secreted either by exocrine glands (salivary glands and the pancreas) or by epithelial cells in the mucosa of the stomach and small intestine. In particular, food proteins, including food enzymes, are broken down by proteolytic enzymes, such as pepsin in the stomach and trypsin in the intestine, into amino acids (Silverthorn 1998). In this section, the effects of two digestive enzymes, pepsin and trypsin, on fermented shrimp paste enzyme was examined by electrophoresis and fibrin plate assay.

9.3. Materials and Methods

Crude fermented shrimp paste enzymes in HCl (pH 2) and PBS (pH 8) were incubated with 4% (w/w) of pepsin and 0.2% (w/w) of trypsin extracted from porcine stomach mucosa (Sigma Chemical Co.), respectively, at 37°C for one hour. Enzyme activities were measured by fibrin plate assay and the hydrolysis patterns were

determined by SDS-PAGE using a 15% polyacrylamide gel. Proteins were stained with silver nitrate.

9.4. Results and Discussion

Fermented shrimp paste was incubated at two different physiological conditions *in vitro*, including an acidic pH with pepsin in the stomach and an alkaline pH with trypsin in the small intestine. According to the reduced SDS-PAGE in Figure 18, fermented shrimp paste enzyme band at 12.5 kDa was intact in both experimental conditions. Also, the enzyme remained active (fibrin plate assay was not shown). Thus, fermented shrimp paste enzyme was resistant to pepsin and trypsin digestion. It was proposed that fermented shrimp paste enzyme might pass through human digestive tract without changing its function and structure. To confirm this preliminary result, however, *in vivo* and animal tests are recommended.

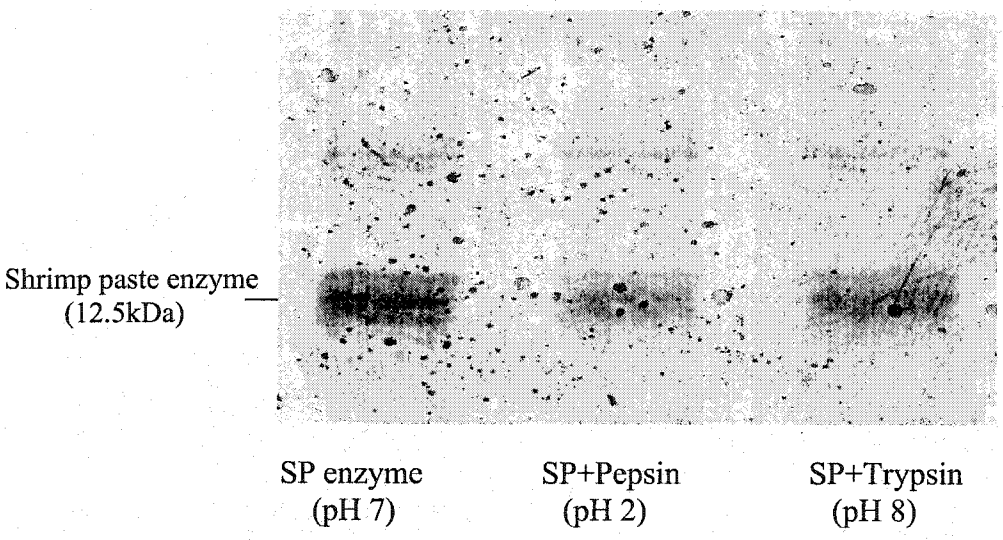

Figure 18 Effects of digestive enzymes, pepsin and trypsin, on fermented shrimp paste enzyme. Electrophoresis was carried out on a 15% polyacrylamide gel and proteins were stained with silver nitrate.

10. CONCLUSION

10.1. Summary of Research

The main objectives of the current research were to explore new sources of fibrinolytic enzymes from Asian fermented food products, to purify these novel enzymes, and to characterize their physiochemical properties.

Thirteen Asian fermented food products were screened for fibrinolytic activity by fibrin plate method. Fermented shrimp paste, a popular Asian seasoning, was shown to have a strong fibrinolytic activity. 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 12.5 kDa, and it was composed primarily of β–sheet and random coils. N-terminal amino acid sequencing indicated that fermented shrimp paste enzyme was novel with no homology to other existing fibrinolytic enzymes. 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. Maximum enzyme activity was observed at 30°C to 40°C, and the enzyme was completely denatured at temperature over 60°C. Fibrinolytic activity of fermented shrimp paste enzyme was not significantly inhibited by the typical protease inhibitors examined in the study, indicating that it was a distinctive enzyme that could not be classified as one of the four proteases. In addition, shrimp paste enzyme was resistant to pepsin and trypsin digestion, and it could substantially delay blood-clotting time *in vitro* using human serum.

10.2. Significance

Cardiovascular diseases, such as heart attack and stroke, are the leading causes of death in North America. The diseases not only affect the elderly but are also the third leading cause of premature death 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 the prevention and the treatment of the diseases. 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.

Fermented shrimp paste enzyme 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, fermented shrimp paste enzyme has a significant potential for food fortification and nutraceuticals applications, such that their use could effectively prevent cardiovascular diseases.

10.3. Future Research Directions

Future research is warranted to verify the origin of fermented shrimp paste enzyme in order to have a better understanding of the classification for the enzyme. It is also important to elucidate the reaction mechanism of the enzyme by studying both the three-dimensional structure and the nature of the active site of the enzyme. *In vitro*, it is suggested that fermented shrimp paste enzyme can be a potent natural agent for oral fibrinolytic therapy or thrombosis prevention. To confirm the physiological functions of the enzyme, the next step will be to examine the intestinal absorption of the enzyme *in vitro* by using human intestinal epithelial cells, and to measure fibrinolytic activity of the enzyme in the blood and the organs using animal model systems.

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