CHAPTER 1

INTRODUCTION

Surimi, a refined form of mechanically deboned fish meat, has unique functionality including gel-forming ability and water-and oil-binding properties and can be used as a valuable ingredient or base component for a broad range of food products. However, proteolytic disintegration of surimi gels mostly occurring at a temperature close to 50-60°C (modori) causes an irreversible destruction of the gel structure of surimi (Benjakul et al., 1999). This shows the detrimental effects on surimi quality by substantially lowering the gel strength giving brittle and nonelastic gels. To alleviate the problems associated with protein degradation caused by the endogenous proteinases, proteinase inhibitors and other food-grade additives have been used to improve the physical properties of surimi gel. Such inhibitors most commonly used include bovine plasma protein (BPP), potato powder (PP), egg white (EW), whey protein concentrate (WPC) (Morrissey et al., 1993; Benjakul et al., 2000). Among commercially available food grade protein additives, BPP was shown to be the most effective gel enhancer. Despite their gel enhancing activity, these food-grade inhibitors have the limitation because they have a negative impact on surimi quality, especially discoloration and off flavor. Similarly, egg white is costly and has an undesirable egg-like odor at levels required for inhibition (Benjakul et al., 1999). Thus, the alternative food-grade proteinase inhibitors are needed in surimi suffering with gel softening such as threadfin bream, bigeye snapper gel, etc.

Plant storage organs such as seeds, fruits, and tubers normally contain relatively large quantities of proteinase inhibitors (El-Shamei et al., 1996). The

quantity of inhibitor depends upon variety and physiological status of the plant and on the level of insect infestations or damage (Richardson, 1977). Proteinase inhibitors in legume seed can have a major impact on nutritional value as they inhibit pancreatic serine proteinases, thus impairing protein digestion. However, legume seeds can be extracted as a potential source of proteinase inhibitor and applied in a variety of applications such as medicine, agriculture and food technology. Proteinase inhibitor can be used to improve texture in various foods, for example, sausages, meat balls, and low-salt fish products (Benjakul et al., 2000, Rawdkuen and Benjakul, 2008). Legume seed proteinase inhibitors normally contain trypsin/chymotrypsin inhibitors which belong to two families, Kunitz and Bowman-Birk (Guillamon et al., 2008). Kunitz-type inhibitors are found mainly in seeds of the Mimosoideae while Bowman-Birk inhibitors are observed mostly in seeds of the Papilionoideae (Xavier-Filho & Campos, 1989). Trypsin and chymotrypsin inhibitory proteins have been isolated and characterized from a variety of legume seeds, e.g. pigeon pea (Godbole et al., 1994), tepary bean (Campos et al., 1997), cowpea, bambara groundnuts (Benjakul et al., 2000), dry bean (Nergiz and Gokgoz, 2007), velvet bean and jack bean (Betancur-Ancona et al., 2008). Recently, Ee et al. (2008) isolated the trypsin and α-chymotrypsin inhibitors from Australian wattle seed (*Acacia victoriae* Bentham). Almeida et al. (2008) reported that *Vicia faba* showed high trypsin inhibitory activity.

Adzuki bean (*Vigna angularis*) is an important traditional pulse crop in eastern Asia. Major consumers include Japan, Korea and China. The adzuki bean is a small bean that has an inherently sweet, nutty taste. Most popular adzuki bean cultivars produce red- or maroon-coloured seeds that resemble mung been seeds, but other seed colours include black, green, grey, yellow, white, and mottled

combinations of various colours. In Japan, adzuki beans are consumed as ‘an’ or ‘ann’ (bean paste) used alone or as a filler for various sweet pastries, and as whole beans boiled and sweetened for snacks and confectionery items. Generally, other legumes can be used to make ‘ann’ however, due to the desirable colour, delicate flavour and characteristic texture of its paste, the adzuki is preferred (Yousif et al., 2002). Based on our preliminary study, adzuki bean seeds contained high trypsin inhibitory activity. Hence, adzuki bean seeds were a potential source for recovering trypsin inhibitor and used for a plethora of industrial applications, especially surimi. Therefore, the objectives of this study were to purify and to characterize the biochemical properties of trypsin inhibitor from adzuki bean seeds. The effect of trypsin inhibitor from adzuki bean seed on proteolysis and gelling properties of threadfin bream was also investigated.

CHAPTER 2

LITERATURE REVIEW

1. Protease inhibitor

An enzyme inhibitor is any substance that reduces the rate of an enzyme catalyzed reaction (Whitaker, 1994). Protease inhibitors mimic the protein substrate by binding to the active site of the protease. Specific inhibitors are active-site-direct substances and combined with the catalytic or substrate-binding site of the enzyme to form a stable complex (Salvesen and Nagase, 1989). Nonspecific inhibitors are rare in nature, and the only one known is a plasma protein, α_2-macroglobulin (α_2M) (Barrett and Starkey, 1973). Chelators that remove cations from metal-dependent proteases, and denaturants that alter catalytic sites are known as inactivators rather than inhibitors (Garcia-Carreno, 1996).

Inhibitors are divided into two types, the irreversible and the reversible inhibitors, based on kinetic considerations (Salvesen and Nagase, 1989). Irreversible inhibitors are generally low-molecular-weight site-directed compounds. The group of irreversible inhibitors includes all compounds that react with an enzyme to form kinetically stable covalent bonds. Most of the known irreversible inhibitors are synthetic substances that are used to determine the class of a protease. Reversible inhibitors are, in general, naturally occurring proteins and the enzyme activity is regenerated by displacement of the inhibitory molecule. Reversible inhibitors can be divided into three distinct types including competitive, non-competitive and uncompetitive inhibition, based on their effect on the slope and intercept of a reciprocal plot of observed initial rates versus initial substrate concentrations

compared to the same reaction in the absence of inhibitors (Salvesen and Nagase, 1989).

2. Natural protease inhibitor

Inhibitors have been isolated from a variety of organisms including bacteria, animal and plants. Their sizes are also extremely variable from 50 residues (e.g. bovine pancreatic trypsin inhibitor) to up to 400 residues (e.g. alpha-1 protease inhibitor). They are strictly class-specific except proteins of α_2M which bind and inhibit most proteases through a molecular trap mechanism.

Protease inhibitors commonly accumulate in high quantities in plant seeds, bird eggs and various body fluids. Protease inhibitors are also found in mammalian and marine animal blood plasma, where they account for more than 10% of total protein (Ylonen et al., 1999). Furthermore, plant seeds such as legumes contain protease inhibitor which can be used to inhibit biological systems (Garcia-Carreno et al., 1996).

3. Classification of protease inhibitors

Protease inhibitors can be broadly separated into two general categories based upon their spectrum of activity: the nonspecific protease inhibitors and the class-specific protease inhibitors. Nonspecific protease inhibitors are capable of inhibiting members of all 4 classes of proteases.

3.1 Cysteine protease inhibitors

These inhibitors act as a protective mechanism against cysteine proteases released into circulation after cell death. The cystatin superfamily contains three families of proteins that are related functionally as cysteine protease inhibitors and evolutionarily by their amino acid sequence identity. These inhibitors occur in all cells and body fluids of mammals and many lower organisms. The interaction of cystatins with cysteine peptidases is a reversible and tight-binding one at the active site, but without formation and cleavage of covalent bonds. The affinity of the cystatins to the lysosomal cysteine proteases is very high. They do not react with serine or other types of proteases (Abrahamson et al., 1991). The cystatins are classified into three families as:

Family I: cystatin A, cystatin B. Synthesized without signal peptides; MW 11-12 kDa; contain no disulfide bonds; occur intracellularly in the cytosol.

Family II: cystatin C, D, S, SN, SA. Synthesized with signal peptides; MW 13-14 kDa; contain disulfide bonds; are secreted and present in the body fluids.

Family III: kininogens. Exist in several forms (L-kininogen, H-kininogen); MW 60-120 kDa; are glycoproteins; contain three cystatin domain; two of which are functional; occur mainly in blood plasma.

3.2 Serine protease inhibitors

Serine protease inhibitors comprise the largest super-family of the class specific protease inhibitors. A feature of all the protease inhibitors in the serpin superfamily is a particular peptide bond, located in a C-terminal domain, that is susceptible to attack by serine proteases (Carlson, 1996). These inhibitors are very

abundant in mammalian plasma and plant cells and play a main role in many physiologic processes (Otlewski et al., 1999).

Antithrombin is a serine protease inhibitor involved in the coagulation cascade. Antithrombin III is one of the three plasma serpins that reacts 2-4 orders of magnitude more rapidly with target proteases in the presence of heparin. It is produced in the liver and endothelial cells and is responsible for 70% of the anticoagulant activity of normal plasma. Anti-thrombin forms a complex between the active site of thrombin and the reactive site of antithrombin. Thrombin or another protease binds to heparin, and brings the active site of the protease into close contact with the reactive site of antithrombin (Carlson, 1996).

Soybean trypsin inhibitors: Protease inhibitors that have been isolated from soybeans are of two types: the Kunitz trypsin inhibitor (TI) and the Bowman-Birk (BB) inhibitor. The first group has an MW between 20 and 25 kDa, with the specificity directed primarily toward trypsin. The inhibitor was shown to combine tightly with trypsin. The BB inhibitor is capable of inhibiting both trypsin and chymotrypsin at independent reactive sites. BB inhibitor has a stable conformation even after disulfide bonds are broken by heating (Kennedy, 1998).

3.3 Aspartic protease inhibitors

The best characterized aspartic proteases from mammals (pepsin, chymosin, cathepsin D and rennin) are all inhibited by pepstatin A. Aspartic protease inhibitors can be found in many sources such as potato, yeast, the nematode *Ascaris*, and squash (Garcia-Carreno and Hernandez-Cortes, 2000). The aspartic protease inhibitors from potato form a multigene family of at least 10 members (Ritonja et al.,

1990). These inhibitors are similar to the soybean trypsin inhibitor family, which also possesses trypsin inhibitory activity. An inhibitor from squash phloem exudates (Christeller et al., 1998) has no similarity with any other known protein, which suggests that it belongs to a new inhibitor family. Squash, as yeast inhibitor, is an aspartic protease inhibitor that does not contain any disulfide bonds and there is no N-glycosylation site. Until recently, α_2-macroglobulin was thought to be the only major inhibitor of aspartic proteases (Thomas et al., 1989).

3.4 Metalloprotease inhibitors

Any substrate that complexes with and/or removes an essential cation from an apoenzyme will be an inhibitor of that enzyme (Whitaker, 1994). Most of the design of class specific inhibitors of metalloproteases has focused on attempts to chelate or bind the catalytic zinc atom. Synthetic inhibitors, therefore, commonly contain a negatively-charged moiety to which is attached a series of other groups designed to fit the specificity pockets of a particular protease (Whitaker, 1994).

An inhibitor can react directly with essential groups of the active site of the enzymes or with specific groups on the enzymes not involved in the active site per se (Whitaker, 1994). The most useful type of inhibitor in elucidation of reaction mechanisms is one that reacts with the active site of enzyme where substrate, cofactor, and/or activator are bound (Garcia-Carreno and Hernandez-Cortes, 2000).

4. Isolation and characterization of protease inhibitor from plants

Protease inhibitors occur in most legumes and cereals, in certain vegetables such as cabbage, cucumbers, potatoes, tomatoes, and spinach. Some fruits, e.g. apples, bananas, pineapple and raisins also contain protease inhibitors. The quantity of inhibitor depends upon variety and physiological status of the plant and on level of insect infestations or damage (Richardson, 1977). Inhibitor of plant origin can inhibit several classes of proteases such as aspartic protease, serine protease, cysteine protease and metalloprotease.

Many inhibitors were isolated in pure form and characterized more or less completely by elucidation of amino acid composition, amino acid sequence, conformation, and chemistry of the reactive sites. A Bowman-Birk inhibitor from the seeds of black gram was isolated by ammonium sulfate fractionation, followed by ion-exchange, affinity and gel filtration chromatography (Prasad et al., 2010). The inhibitor showed a single band in SDS-PAGE under non-reducing condition with an apparent molecular mass of ~8 kDa. The protease inhibitor was stable up to a temperature of 80°C and active over a wide pH range between 2 and 12. Further, upon reduction with dithiothreitol, the purified inhibitor lost its inhibitory activity as well as secondary structural conformation.

Crude extracts from Australian wattle seed and their salt (ammonium sulfate)-precipitated fractions were analyzed for trypsin and chymotrypsin inhibitor activity, using gel electrophoresis and spectrophotometric methods (Ee et al., 2008). Three different bands with molecular weight 30.20, 38.03 and 39.81 kDa were active, with 50% salt-precipitated fraction exhibiting highest activity and number of active bands. The same proteins also appeared to be responsible for both trypsin and

chymotrypsin inhibitor activity. Trypsin inhibitors from cultivars of cowpea, pigeon pea and bambara groundnuts grown in Thailand were isolated and characterized (Benjakul et al., 2000). Extraction of seeds with NaCl rendered a higher recovery of trypsin inhibitor than other solvents tested. The extraction time of 3 h was optimum for the recovery of trypsin inhibitor from pigeon and bambara groundnuts, whereas 1 h was optimum for cowpea. Based on inhibitor activity zones separated by electrophoresis, the molecular mass of the inhibitor from bambara groundnuts was 13 kDa. Two inhibitory bands were observed for cowpea (10 and 18 kDa) and pigeon pea (15 and 25 kDa). Partial purification of inhibitors was achieved by heat-treatment at 90°C for 10 min, followed by ammonium sulfate precipitation with 30-65% saturation. The partially purified inhibitors from four seeds were heat stable up to 30 min at 90°C at pH 7.0. The activities were also retained over a wide pH range at 25°C.

Macedo et al. (2000) isolated trypsin inhibitor from *Dimorphandra mollis* seeds by a combination of ammonium sulfate precipitation, gel filtration, ion-exchange and affinity chromatographic techniques. SDS-PAGE analysis gave an apparent molecular weight of 20 kDa, and isoelectric focusing analysis demonstrated the presence of three isoforms. The partial N-terminal amino acid sequence of the purified protein showed a high degree of homology with various numbers of the Kunitz family of inhibitors. Trypsin inhibitor from water fern was isolated and characterized (Maity and Patra, 2003). The molecular weight of inhibitor was 21 kDa estimated by SDS-substrate electrophoresis. The partially purified inhibitor was heat stable up to 10 min at 90°C. High activity was also retained over a wide pH range (4-8) at 37°C.

Protease inhibitor extracts were prepared from various legume seeds (Benjakul et al., 1999). Black cowpea and soybean seeds showed the highest inhibitory activity against viscera proteases. Both extracts showed high thermal stability, but that from soybean was slightly less stable. The inhibitory activity of both extracts was retained over a broad pH range. A highly stable and potent trypsin inhibitor was purified to homogeneity from the seeds of *Putranjiva roxburghii* belonging to *Euphorbiaceae* family by acid precipitation, cation-exchange and anion-exchange chromatography (Chaudhary et al., 2008). SDS-PAGE analysis, under reducing condition, showed that protein consists of a single polypeptide chain with molecular mass of approximately 34 kDa. The inhibitor retained the inhibitory activity over a broad range of pH (pH 2-12), temperature (20-80°C) and in DTT (up to 100 mM). The complete loss of inhibitory activity was observed above 90°C. Campos et al. (1997) purified proteinase inhibitor from tepary bean seeds using fractional precipitation, gel filtration, ion exchange chromatography and reverse-phase HPLC. The protein showed an apparent molecular weight of 7,100 Da by PAGE. The protein was characterized as a serine-proteinase inhibitor that inhibited trypsin, chymotrypsin and trypsin-like proteinases, but it also inhibited aspartic acid proteinases from different insects. It contained no carbohydrate residues and showed a high stability at 96°C at low pH.

Klomklao et al. (2011) extracted a trypsin inhibitor from mung bean (*Vigna radiate* (L.) R. Wilczek) seeds. Optimal extraction was attained by shaking the defatted mung been seed powder in distilled water. The extraction time affected the inhibitor recovery significantly. The extraction time of 2 h was optimum for recovery of trypsin inhibitor from mung bean seeds. Trypsin inhibitor from mung bean seeds

was purified by heat-treatment at 90°C for 10 min, followed by ammonium sulfate precipitation with 30-65% saturation and gel filtration on Sephadex G-50. It was purified to 13.51-fold with a yield of 30.25%. Molecular weight distribution and inhibitory activity staining showed that the purified trypsin inhibitor had the molecular weight of 14 kDa. However, the purified inhibitor had no activity under reducing condition (βME). The purified inhibitor was heat stable up to 50 min at 90°C. The inhibitory activity was retained over a wide pH range. NaCl, at 0-3% concentration, did not influence the inhibitory activity of purified trypsin inhibitor from mung bean seeds.

5. Application of proteinase inhibitors from legume seeds in surimi

Several protease inhibitors from legume seeds have been used to control proteolysis activity in muscle and also to improve the physical properties of surimi gels (Benjakul et al., 2000).

Oujifard et al. (2012) found that bambara groundnut protein isolate (BGPI) with trypsin inhibitory activity of 6356.3 units/g markedly increased the breaking force and deformation of modori gel as the levels used (0-3 g/100g) increased. Nevertheless, only 0.25g/100 g BGPI increased breaking force and deformation on kamaboko gel and increasing BGPI levels showed detrimental effect on gelation. Whiteness slightly decreased with increasing BGPI levels.

Partially purified proteinase inhibitors from cowpea (*Vigna unguiculata* (L.) Wasp), pigeon pea (*Cajanus cajan* (L.) Millsp.) and bambara groundnuts (*Voandzeia subterranean* (L.) Thou effectively inhibited sarcoplasmic modori-inducing proteinase extracted from threadfin bream muscle in a concentration

dependent manner (Benjakul et al., 2000). Incorporation of these proteinase inhibitors into threadfin bream surimi partially inhibited autolytic degradation and increased the gel force and deformation. However, lightness and whiteness of surimi gels decreased slightly when the proteinase inhibitor was added at a level of 30 kunits/g (Benjakul et al., 2000) Benjakul et al. (1999) prepared the proteinase inhibitor from black cowpea and soybean seeds. The proteinase inhibitor extracts reduced modori-inducing proteinase activity. The sarcoplasmic modori-inducing proteinase activities were more effectively inhibited than the myofibrillar-associated mododi-inducing proteinase.

CHAPTER 3

A HEAT-STABLE TRYPSIN INHIBITOR IN ADZUKI (*VIGNA ANGULAIS*): EFFECT OF EXTRACTION MEDIA, PURIFICATION AND BIOCHEMICAL CHARACTERISTICS

Abstract

Trypsin inhibitor from adzuki bean (*Vigna angularis*) seed was isolated and characterized. Extraction of seed with NaCl at the concentration of 0.15 M showed a higher recovery of trypsin inhibitor than other solvents tested ($p<0.05$). Optimal extraction time for the recovery trypsin inhibitor from adzuki bean seed was 30 min ($p<0.05$). Purification of inhibitor was achieved by heat-treatment at 90°C for 10 min, followed by ammonium sulfate precipitation with 30-65% saturation and size exclusion chromatography on Sephacryl S-200, presenting a yield and purification of 53.9% and 10.91 fold, respectively. The apparent molecular weight of trypsin inhibitor was estimated to be 14 kDa based on SDS-PAGE and inhibitor activity of zones separated by electrophoresis. The purified inhibitor was stable over a broad pH range and retained high inhibitory activity toward trypsin after incubation at 90°C for 60 min. NaCl, at 0-3% concentration, did not affect the inhibitory activity of purified trypsin inhibitor, however, the activity was lost when sample was treated with β-mercaptoethanol prior to electrophoresis.

Introduction

Adzuki bean (*Vigna angularis*) is an important traditional pulse crop in eastern Asia. Major consumers include Japan, Korea and China. The adzuki bean is a small bean that has an inherently sweet, nutty taste. Most popular adzuki bean cultivars produce red- or maroon-coloured seeds that resemble mung been seeds, but other seed colours include black, green, grey, yellow, white, and mottled combinations of various colours. In Japan, adzuki beans are consumed as 'an' or 'ann' (bean paste) used alone or as a filler for various sweet pastries, and as whole beans boiled and sweetened for snacks and confectionery items. Generally, other legumes can be used to make 'ann' however, due to the desirable colour, delicate flavour and characteristic texture of its paste, the adzuki is preferred (Yousif et al., 2002).

Plant storage organs such as seeds, fruits, and tubers normally contain relatively large quantities of proteinase inhibitors (El-Shamei et al., 1996). The quantity of inhibitor depends upon variety and physiological status of the plant and on the level of insect infestations or damage (Richardson, 1977). Proteinase inhibitors in legume seed can have a major impact on nutritional value as they inhibit pancreatic serine proteinases, thus impairing protein digestion. However, legume seeds can be extracted as a potential source of proteinase inhibitor and applied in a variety of applications such as medicine, agriculture and food technology. Proteinase inhibitor can be used to improve texture in various foods, for example, sausages, meat balls, and low-salt fish products (Benjakul et al., 2000, Rawdkuen & Benjakul, 2008). Legume seed proteinase inhibitors normally contain trypsin/chymotrypsin inhibitors which belong to two families, Kunitz and Bowman-Birk (Guillamon et al., 2008).

Kunitz-type inhibitors are found mainly in seeds of the Mimosoideae while Bowman-Birk inhibitors are observed mostly in seeds of the Papilionoideae (Xavier-Filho & Campos, 1989). Trypsin and chymotrypsin inhibitory proteins have been isolated and characterized from a variety of legume seeds, e.g. pigeon pea (Godbole et al., 1994), tepary bean (Campos et al., 1997), cowpea, bambara groundnuts (Benjakul et al., 2000), dry bean (Nergiz and Gokgoz, 2007), velvet bean and jack bean (Betancur-Ancona e al., 2008). Recently, Ee et al. (2008) isolated the trypsin and α-chymotrypsin inhibitors from Australian wattle seed (*Acacia victoriae* Bentham). Almeida et al. (2008) reported that *Vicia faba* showed high trypsin inhibitory activity. Based on our preliminary study, adzuki bean seeds contained high trypsin inhibitory activity. Hence, adzuki bean seeds were a potential source for recovering trypsin inhibitor and used for a plethora of industrial applications. Therefore, the objectives of this study were to purify and to characterize the biochemical properties of trypsin inhibitor from adzuki bean seeds. The effect of extraction media on the recovery of trypsin inhibitor was also investigated.

Materials and methods

Chemicals

Nα-Benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA), trypsin from porcine pancreas, β-mercaptoethanol (βME), sodium chloride, tris (hydroxymethyl) aminomethane, dimethylsulfoxide, sodium caseinate, ammonium sulfate, sodium dodecyl sulfate (SDS), Coomassie Blue R-250 and *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) and bovine serum albumin were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Sephacryl S-200 were obtained from Pharmacia Biotech (Uppsala, Sweden).

Extraction of trypsin inhibitor from adzuki bean seeds

Preparation of defatted seed flour for trypsin inhibitor extraction

One batch of adzuki bean (*Vigna angularis*) used for this study was collected and purchased from the Royal Project Foundation in Bangkok, Thailand. The dry seeds (approximately 5 mm in diameter were selected and ground using a blender. The seed flour was defatted according to the method of Hines et al. (1992) by stirring with 5 volumes of hexane (w/v) for 10 min. The mixture was filtered through Whatman No. 1 filter paper and the sediment was rinsed with hexane 3 times to remove the residual oil in the seed flour. The defatted flour was air-dried at room temperature until dry and free of hexane odour.

Effect of extraction media on trypsin inhibitor extraction

Different extraction media including distilled water, 0.15 M NaCl, 0.30 M NaCl, 0.01 M NaOH and 0.02 M NaOH were used to extract trypsin inhibitor. The defatted seed flour was added to the medium at a ratio of 1:7 (w/v) and shaken (BW201 Shaking bath, Tokyo, Japan) for 30 min at 150 rpm at room temperature. The extract was recovered by centrifuging at 10,000×g for 30 min. The trypsin inhibitory activity and protein content in the extract were determined and the specific inhibitory activity obtained using different media were compared. The extraction media used for solubilizing the trypsin inhibitor, which was able to extract trypsin inhibitor with the highest specific trypsin inhibitory activity, was selected for further steps.

Effect of extraction time on trypsin inhibitor extraction

Defatted seed flour was mixed with 0.15 M NaCl with a solid/solvent ratio of 1:7 (w/v) and shaken for 0.5, 1, 2, 3, 4, and 5 h. At designated time, the mixture was centrifuged at 10,000×g for 30 min and the supernatants were subjected to determination of trypsin inhibitory activity and protein content. The specific trypsin inhibitory activities were then calculated. The extraction time rendering the highest specific trypsin inhibitory activity was chosen for further study.

Purification of trypsin inhibitor from adzuki bean seeds

Crude extract was heated at different temperatures (60, 70, 80, 90 and 100°C) for 10 min and then cooled with ice water. To remove the heat coagulated debris, the extracts were centrifuged at 10,000×g for 10 min at 4°C. The activity and

specific activity of trypsin inhibitor in the supernatant obtained was measured. The heat treatment which gave a supernatant with highest specific activity was chosen for further study.

The heat-treated extract was subsequently subjected to ammonium sulfate precipitation at different ranges, 0-30%, 30-65%, and 65-90% saturation. The protein content and the inhibitory activity of the ammonium sulfate fractions were analyzed. The ammonium sulfate fraction which showed the highest specific activity was selected for further study.

The ammonium sulfate fraction was applied to a column of Sephacryl S-200 (3.9×64 cm) pre-equilibrated with 10 mM Tris-HCl buffer (pH 7.0), and the proteins were eluted with the same buffer at a flow rate of 0.1 ml/min. Fractions were collected and the main trypsin inhibitory fractions were pooled and concentrated by centrifugation using 10 kDa MWCO Amicon Ultra centrifugal filter devices (Millipore Corporation, Billerica, MA) and used for the study as described in this paper.

Thermal and pH stability of purified trypsin inhibitor

Purified trypsin inhibitor extracts from adzuki bean seed were diluted with distilled water to obtain 60-70% inhibition. The solutions were incubated at 90°C for 0, 10, 20, 30, 40, 50 and 60 min and then cooled in iced-water. The residual trypsin inhibitory activity was determined and reported as the relative activity compared to the original activity.

The effect of pH on trypsin inhibitor stability was evaluated by measuring the residual activity after incubation at various pHs for 30 min at room

temperature. Different buffers used included McIlvaine buffers (0.2 M Na phosphate and 0.1 M Na citrate) for pH 2.0-7.0 and 0.1 M glycine-NaOH for pH 8.0-10.0.

Salt stability

Purified trypsin inhibitor was incubated at room temperature for 30 min in the presence of NaCl ranging from 0 to 3%. The mixture was tested for inhibitory activity against trypsin. The residual inhibitory activity was reported.

Sodium dodecyl sulfate-gel electrophoresis

SDS-PAGE was performed according to the method of Laemmli (1970). Protein solutions were mixed at 1:1 (v/v) ratio with the SDS-PAGE sample buffer in the presence or absence of βME and boiled for 3 min. The sample (20 μg) were loaded on the gel made of 4% stacking and 15% separating gels and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-Protean II cell apparatus (Atto Co., Tokyo, Japan). After electrophoresis, the gels were stained with 0.2% Coomassie Brilliant Blue R-250 in 45% methanol and 10% acetic acid and destained with 30% methanol and 10% acetic acid.

Inhibitory activity of trypsin inhibitor by electrophoresis

Crude extract, heat-treated extract, $(NH_4)_2SO_4$ fraction and Sephacryl S-200 fraction were separated on SDS-PAGE, followed by inhibitory activity staining using casein as a substrate with the slightly modified method of Garcia-Carreno et al. (1993). The gels were washed in 2.5% Triton X-100 for 15 min to remove SDS and renature the proteins. The gel was washed with distilled water before soaking in

trypsin solution (0.2 mg/ml) at 0-4°C for 45 min. The gels were then washed again with distilled water and incubated with 1% casein in 0.1 M glycine-NaOH, pH 9.0 for 90 min at 37°C. The gel was washed again with distilled water, fixed and stained with Coomassie blue R-250. After destaining, the bands with inhibitory activity were compared with the control gel and molecular weight markers.

Trypsin inhibitory activity assay

Trypsin inhibitory activity was measured by the method of Benjakul et al. (2000) with a slight modification using BAPNA as substrate. A solution containing 200 µl of inhibitor solution, 200 µl (20µg/ml) porcine pancreas trypsin was preincubated at 37°C for 15 min. Then, 1,000 µl of the mixtures containing 800 µl of 0.5 mM BAPNA and 200 µl of distilled water (prewarmed to 37°C) was added and vortexed immediately to start the reaction. After incubating for 10 min, 900 µl of 30% acetic acid (v/v) was added to terminate the reaction. The reaction mixture was centrifuged at 8,000×g for 5 min (Eppendorf Micro Centrifuge). Residual activity of trypsin was measured by the absorbance at 410 nm due to p-nitroaniline released. One unit of proteolytic activity was defined as an increase of 0.01 absorbance unit/ml.min under the assay condition. One unit of trypsin inhibitory activity was defined as the amount of inhibitor, which reduces the enzyme activity by one unit.

Protein determination

Protein concentration was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Statistical analysis

A completely randomized design was used throughout this study. All data were subjected to analysis of variance (ANOVA) and the differences between means were carried out using Duncan's Multiple Range Test (Steel and Torrie, 1980). Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for Windows; SPSS Inc.).

Results and discussion

Extraction of trypsin inhibitor from adzuki bean seed

Trypsin inhibitor activity, specific trypsin inhibitory activity and protein content extracted from adzuki bean seed using different extraction media are shown in Table 1. The specific activity of adzuki bean seed extract was 10.31 units/mg protein when distilled water was used for extraction. The highest specific activity (13.41 units/mg protein) was obtained when the adzuki bean seed was extracted with 0.15 M NaCl ($p<0.05$). The specific activity of adzuki bean seed extract decreased significantly when the NaCl concentration was increased ($p<0.05$). The decrease was attributed to the significantly increased protein content in the extract ($p<0.05$) and probably due to the denaturation of adzuki bean trypsin inhibitor at high salt concentration. Alkaline solution showed a lower capacity for extracting trypsin inhibitor from adzuki bean seed, as evidenced by the lower specific activity ($p<0.05$), particularly with the higher concentration. This resulted from more protein recovery under alkaline conditions, leading to the decrease in specific activity. The result was in agreement with Benjakul et al. (2000) who reported that extraction of proteins from cowpea and pigeon pea was markedly increased when alkaline solution

was used, compared to NaCl. The increasing of protein content was observed when grass pea was solubilized with NaOH solution (Deshpande and Campbell, 1992). There are many factors involved in protein solubility and recovery including protein meal and solvent ratio, particle size of flour, temperature, length of extraction time, pH, ionic strength, type and concentration of extraction as well as the hydration properties of proteins (Sathe and Salunkhe, 1981). From the results, 0.15 M NaCl was selected as the extractant for adzuki bean seed trypsin inhibitor.

The effect of extraction time on the recovery of trypsin inhibitor from adzuki bean seed is shown in Table 2. An extraction time of 30 min was found to be optimal for adzuki bean trypsin inhibitor extraction. Specific inhibitor activity of adzuki bean seed decreased with increasing the extraction time ($p<0.05$). Longer times resulted in a significant decrease in the inhibitory activity of adzuki bean extract ($p<0.05$). Some trypsin inhibitors in the adzuki bean seed extract probably underwent denaturation during extraction at the longer times, leading to the lower inhibitory activity of the extract. High mechanical shear generated by shaking can cause protein denaturation. Moreover, the incorporation of air bubbles and adsorption of protein molecules to the air-liquid interface can cause the denaturation of protein (Damodaran, 1996). Therefore, not only extraction solvent but also extraction time affected the trypsin inhibitor extraction from adzuki bean seed. From the results, an extraction time of 30 min was chosen as the optimum condition for adzuki bean trypsin inhibitor recovery.

Table 1. Effect of extracting media on the recovery of trypsin inhibitor from adzuki bean seeds

Extraction media	Trypsin inhibitor (units/g seed)	Protein (mg/g seed)	Specific activity (units/mg protein)
Distilled water	380.77±26.35c	36.93±0.45b	10.31±0.71c
0.01 M NaOH	314.74±17.78b	72.34±0.43d	4.35±0.25b
0.02 M NaOH	250.32±42.13a	82.98±0.54e	3.02±0.51a
0.15 M NaCl	458.55±5.90d	34.19±0.08a	13.41±0.17d
0.30 M NaCl	419.74±24.27cd	44.29±0.26c	9.48±0.55c

*The defatted seed flour was shaken in different media at ambient temperature for 30 min and trypsin inhibitory activity was analyzed using BAPNA as substrate.
**Mean±SD from triplicate determinations
The different letters in the same column denote the significant differences ($p<0.05$).

Table 2. Effect of extraction time on the recovery of trypsin inhibitor from adzuki bean seeds

Extraction time (h)	Trypsin inhibitor (units/g seed)	Protein (mg/g seed)	Specific activity (units/mg protein)
0.5	372.40±4.73b	28.90±0.07a	12.89±0.16d
1.0	255.10±14.06a	31.68±0.35b	7.09±0.46c
2.0	222.86±13.46a	32.05±0.24b	6.95±0.42c
3.0	216.32±2.23a	34.23±0.10c	6.32±0.07b
4.0	215.34±15.72a	35.68±0.45d	6.04±0.44ab
5.0	200.00±10.19a	36.11±0.45d	5.54±0.28a

*The defatted seed flour was shaken in 0.15 M NaCl at ambient temperature for various times and trypsin inhibitory activity was analyzed using BAPNA as substrate.
**Mean±SD from triplicate determinations
The different letters in the same column denote the significant differences ($p<0.05$).

Purification of trypsin inhibitor from adzuki bean

The adzuki bean seed extracts were subjected to heat treatment at different temperatures. The trypsin inhibitory activity markedly increased after heat treatment at 60°C and the activity were quite constant up to 90°C (Fig. 1a). However, there was no further increased in the activity after heat treatment at 100°C. The results suggest that the inhibitor from adzuki bean seed was very heat stable up to 90°C for 10 min. For specific trypsin inhibitor activity, the activity was increased markedly when the heating temperature increased up to 90°C (Fig. 1b). A marked decrease in activity was found at 100°C. Hence, heat treatment at 90°C for 10 min was introduced to the purification process to rapidly remove some undesired proteins. An increase in purity of 6.46-fold was obtained (Table 3). Interestingly, the yield was increased to 148.9% from this step. Heat treatment might induce the conformational change of trypsin inhibitor, most likely by destroying the noncovalent bond. The modified configuration of trypsin inhibitor might favour the interaction between trypsin inhibitor and trypsin. Benjakul et al. (2000) obtained an increase in purity of trypsin inhibitor from cowpea by 3.37-fold with heat treatment process. Heat-treated extract was further purified using ammonium sulfate precipitation. An ammonium sulfate saturation of 30-65% was found to be the optimum range for trypsin inhibitor recovery (data not shown). The ammonium sulfate precipitation process was able to fractionate the protein of interest. With 30-65% ammonium sulfate precipitation, a slightly increase in purity of 7.09-fold was obtained (Table 3). Ee et al. (2008) reported that a slightly increase in purity was achieved by using ammonium sulfate precipitation at 50%. To refine the ammonium sulfate fraction, the active fractions

were subjected to gel filtration on Sephacryl S-200 column (Fig. 2). Purification of 10.91-fold with a yield of 53.9% was obtained.

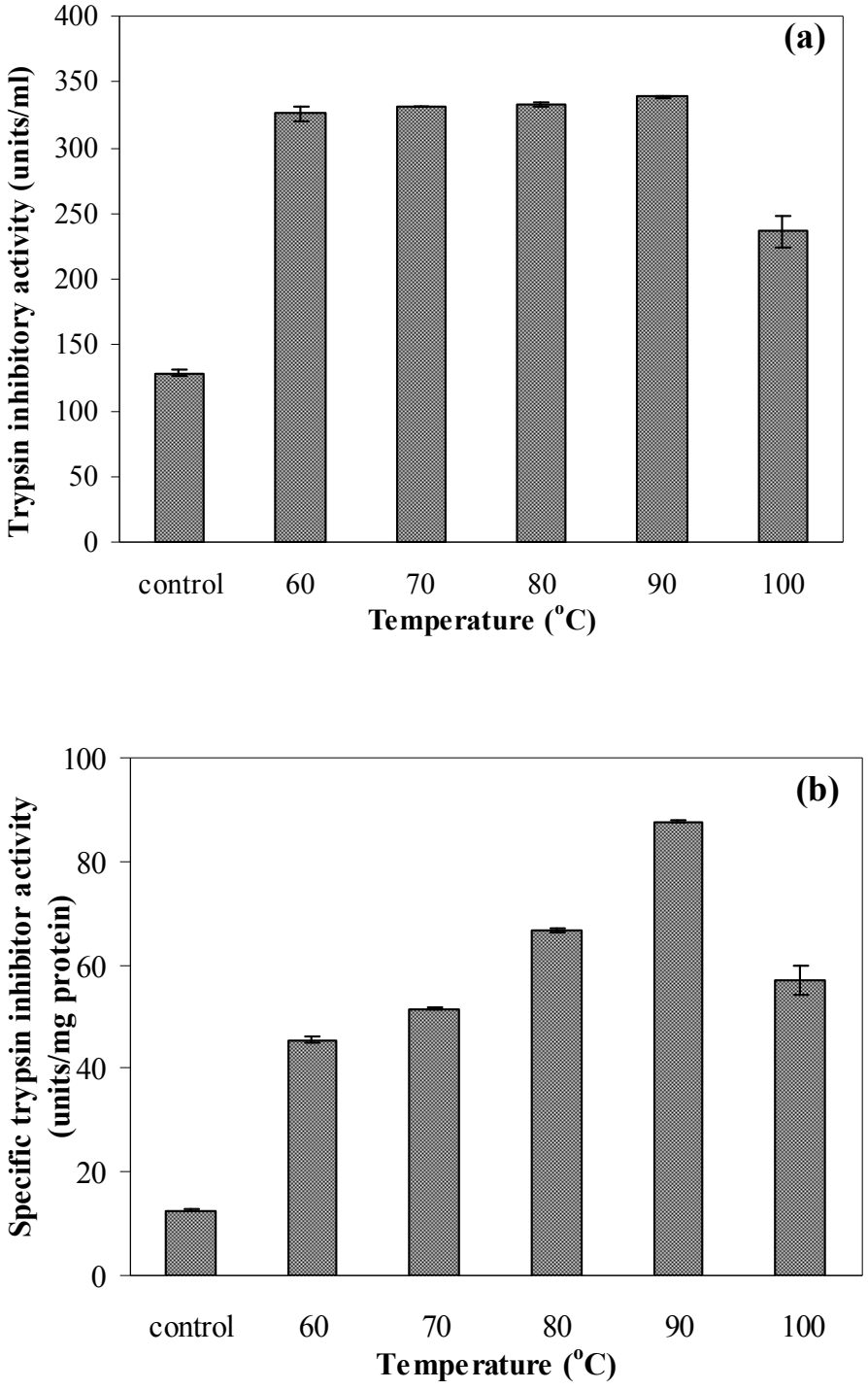

Figure 1. Effect of heat treatment at different temperatures on trypsin inhibitory activity (a) and specific trypsin inhibitory activity (b) of adzuki bean seed extract. Bar indicate standard deviation from triplicate determination

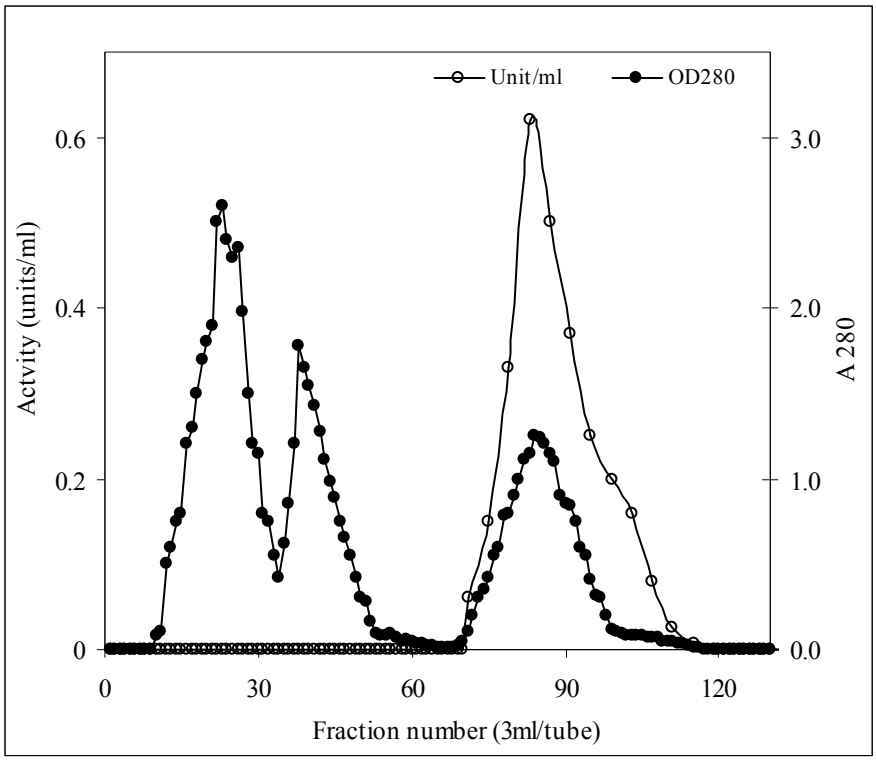

Figure 2. Elution profile of trypsin inhibitor on Sephacryl S-200 column.

Table 3. Purification of trypsin inhibitor from adzuki bean

Purification steps	Total activity (Units)	Total protein (mg)	Specific inhibitory activity (units/mg protein)	Purification fold	Yield (%)
Extract	13,032.00	1031.39	12.64	1	100
Heat treatment	19,416.00	237.79	81.65	6.46	148.9
$(NH_4)_2SO_4$ precipitation (30-65%)	9,596.36	107.01	89.68	7.09	73.6
Sephacryl S-200	7,018.10	50.87	137.96	10.91	53.9

Protein pattern and activity staining of trypsin inhibitors from adzuki bean

The protein components of the adzuki bean seed extract and their fractions from heat treatment, ammonium sulfate precipitation and gel filtration under both reducing and non-reducing conditions are shown in Fig. 3a. As shown in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), based on the protein patterns under non-reducing condition, crude extract contains a number of protein components with different MWs. The major band appeared at apparent molecular weight of 45-50 kDa. A high molecular weight protein (>60 kDa) was found in the crude extract. The heat treatment directly affected the protein pattern in the adzuki bean seed extract. The protein bands with apparent molecular sizes of 50 kDa were decreased when heated at 90°C. Moreover, some protein bands with apparent molecular mass higher than 70 kDa were totally removed by incubation at 90°C. After ammonium sulfate precipitation, a large number of proteins were removed, especially proteins with higher or lower MW. As a result, a higher purity of interested trypsin inhibitor was obtained. When Sephacryl S-200 fraction was analyzed, one protein band with MW of 14 kDa was obtained. Under the reducing condition, the protein band of Sephacryl S-200 fraction with molecular weight of 14 kDa disappeared with the concomitant occurrence of proteins with molecular weight of 8 and 6 kDa. The results suggested that the main component in pooled Sephacryl S-200 fractions consisted of subunits stabilized by disulfide bond. However, two tiny protein bands with molecular weight of approximately 16 kDa were noticeable, suggesting the presence of some contaminating proteins. However, the purification was achieved to a high degree since the trypsin inhibitor constituted greater than 97-98% in the purified fraction.

For trypsin inhibitory activity staining (Fig. 3b), the apparent MW of the trypsin activity band was estimated to be 14 kDa in all fraction under non-reducing condition. Slightly greater band intensity in heat-treated, ammonium sulfate fraction and Sephacryl S-200 fraction was found, suggesting the higher specific trypsin inhibitory activity loaded into the gel. Garcia-Carreno et al. (1996) reported that trypsin, chymotrypsin and papain inhibitors had molecular weights ranging from 14,200-66,000 Da. Benjakul et al. (2000) also reported that the molecular mass of trypsin inhibitors from bambara groundnuts was 13 kDa. Two trypsin inhibitory activity bands were observed for cowpea (10 and 18 kDa) and pigeon pea (15 and 25 kDa) (Benjakul et al., 2000). Generally, the Bowman-Birk type of inhibitor has a lower molecular weight (8-10 kDa) compared with the Kunitz type (>20 kDa). Two protease inhibitors, including trypsin-chymotrypsin inhibitor and trypsin inhibitor (Bowman-Birk type) with molecular weights of 15,000 and 10,500 Da, respectively, were found in pigeon pea (Godbole et al., 1994). From the result, the inhibitor from adzuki bean probably belongs to Bowman-Birk type. However, the amino acid sequence is needed to verify the classification of this inhibitor. Under reducing condition, no activity band was observed in all fractions. It is postulated that the cleavage of disulfide bond by the action of βME could lead to protein denaturation and loss of functionality. Damodaran (1996) reported that proteins that require high structural stability to function as catalyzes are usually stabilized by intramolecular disulfide bonds, and their native conformations can be separated into lower-apparent-MW proteins by the action of reducing agents. Therefore, the intact structure in the native conformation of inhibitor in adzuki bean and the fractions was prerequisite for their inhibitory action.

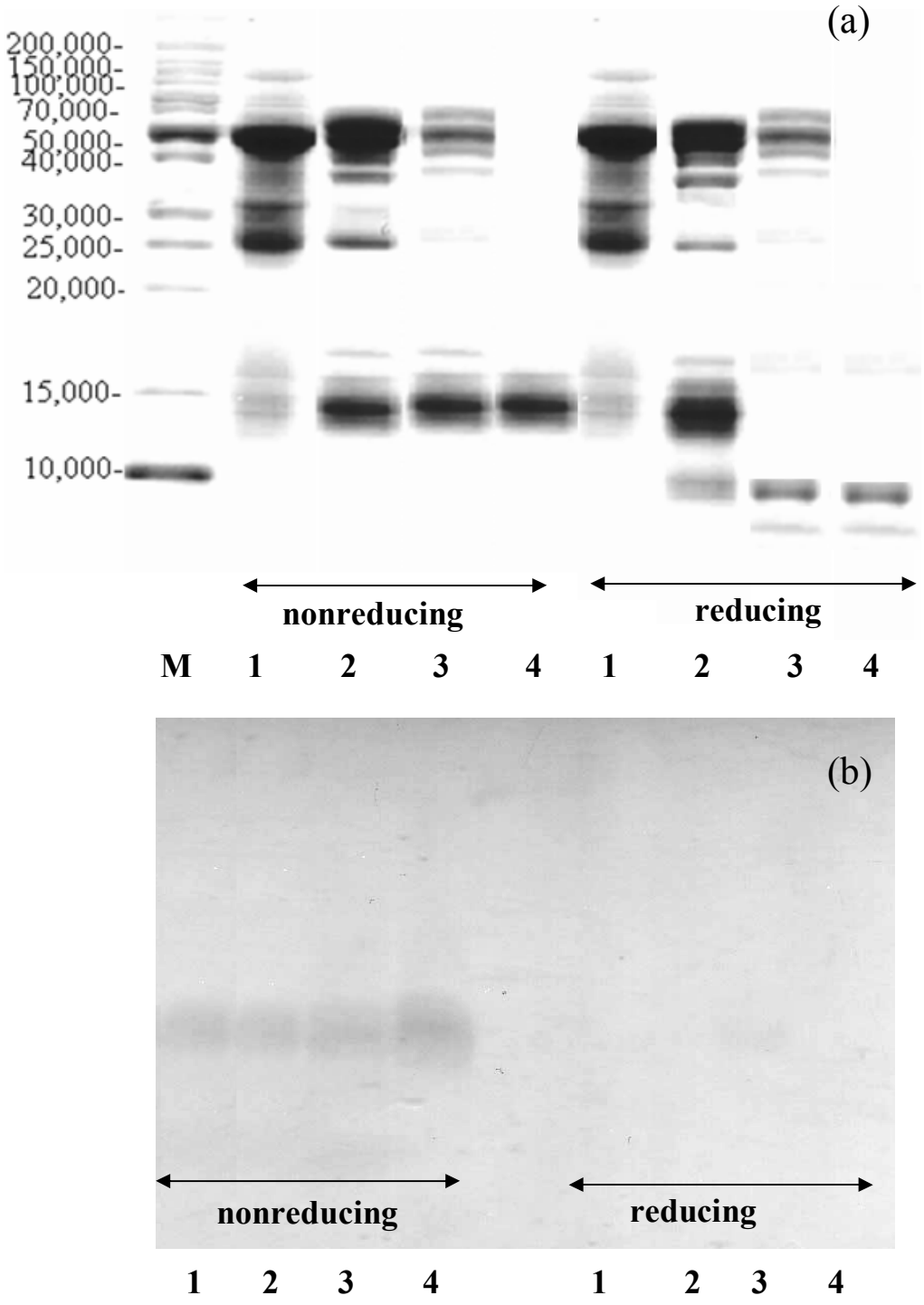

Figure 3. SDS-PAGE (a) and trypsin inhibitory activity staining (b) of isolation of trypsin inhibitor from adzuki bean seed. M, molecular weight standard; 1, crude extract; 2, heat-treated extract; 3, ammonium sulfate fraction, 4, Sephacryl S-200 fraction.

Thermal stability of purified trypsin inhibitor

When purified trypsin inhibitor from adzuki bean seed was incubated at 90°C for different time, no significant changes in the inhibitory activity of the fraction was observed up to 60 min (data not shown). From the results, purified trypsin inhibitor from adzuki bean seed are highly stable to heat and could be applied in various thermal processes such as the gelation of surimi.

pH stability of purified trypsin inhibitor

The pH stability of the purified trypsin inhibitor from adzuki bean seed is depicted in Fig. 4. The inhibitor was stable over a wide pH range. However, slight loss of activity was observed at low pH. Therefore, the inhibitor was generally stable in the neutral and alkaline pH ranges. In general, most proteins are stable within a particular pH range, but denaturation (unfolding) of proteins can occur due to the strong electrostatic repulsion of ionized groups inside the molecules at extreme pH value (Damodaran, 1996). Benjakul et al. (2000) reported that the inhibitor from pigeon pea, cowpea retained their activity between pH 4 and 10. However, for bambara groundnut, the decrease activity was observed at alkaline pH. The differences in pH stability indicated the different molecular properties including bonding stabilizing the structure as well as the trypsin inhibitor conformation among the various species and anatomical locations (Benjakul et al., 2001).

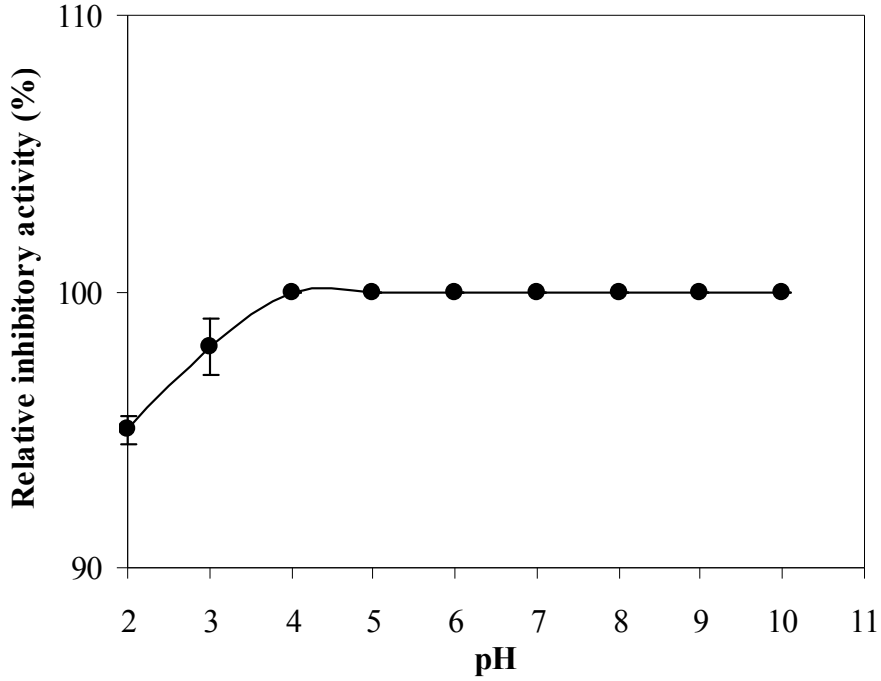

Figure 4. pH stability of purified trypsin inhibitor from adzuki bean seed. Bar indicate standard deviation from triplicate determination.

Effect of salt on the stability of purified trypsin inhibitor

The effect of NaCl on the inhibitory activity of purified trypsin inhibitor was investigated. No marked changes in relative activity were observed when NaCl was added up to 3.0% (data not shown). From the result, purified trypsin inhibitor showed high salt stability up to 3%, which might be useful in surimi-base products in which 2-2.5% salt are commonly used.

Conclusion

Trypsin inhibitor from adzuki bean was successfully extracted by using 0.15 M NaCl. The purified trypsin inhibitor had an MW of about 14 kDa and was stable to various pHs, heating as well as salt up to 3%. The purified inhibitor can be used as a promising proteinase inhibitor in surimi, especially those suffering with softening caused by trypsin or trypsin-like serine proteinase.

Acknowledgments

Authors would like to thank the Thaksin University and the International Foundation for Science, Sweden for financial support.

CHAPTER 4

EFFECT OF TRYPSIN INHIBITOR IN ADZUKI BEAN (*VIGNA ANGULARIS*) ON PROTEOLYSIS AND GEL PROPERTIES OF THREADFIN BREAM (*NEMIPTERUS BLEEKERI*)

Abstract

Trypsin inhibitor (TI) from adzuki bean seed was partially purified by heat-treatment at 90°C for 10 min and it was used to study the impact on proteolysis and gelling properties of threadfin bream (*Nemipterus bleekeri*). TI (0-3%) showed the inhibitory activity against sarcoplasmic proteinases and autolysis of threadfin bream mince and washed mince in a concentration dependent manner. TI was effective in proteolysis prevention as shown by more retained myosin heavy chain (MHC) on SDS-PAGE. Effect of TI (0.5, 1, 2 and 3%) on the properties of threadfin bream surimi was also investigated. Breaking force and deformation of kamaboko gel increased as added levels of TI increased ($P<0.05$). This was associated with lower levels of protein degradation, as evidenced by the decreased in trichloroacetic acid-soluble peptide content ($P<0.05$). However, whiteness slightly decreased ($P<0.05$) with increasing TI levels. Therefore, TI at an appropriate level could be an alternative food-grade inhibitor to improve gel properties of surimi.

Introduction

Proteolytic disintegration of myofibrillar proteins has an adverse effect on gel-forming properties of surimi. The breakdown of myofibrillar proteins inhibits the development of three-dimensional gel network (Benjakul, Visessanguan, Tueksuban, & Tanaka, 2004). Generally, gel-weakening phenomenon or "modori" observed at temperatures above 50°C is a major concern in surimi gel manufacture. This phenomenon is induced by endogenous thermal stable proteinases, which can degrade myosin (An, Peters, & Seymour, 1996). Gel softening varies with species, but is generally caused by two major groups of proteinase, cathepsin and heat-stable alkaline proteinases (An, Seymour, Wu & Morrissey, 1994).

Threadfin bream is widely used in Thailand and other countries in the Southeast Asia as an important raw material for surimi production due to its white color and large availability. However, the surimi from this species is susceptible to modori due to the presence of heat-activated proteinases, which were active at 50-60°C (Kinoshita, Toyohara, & Shimizu, 1990). The proteinases are responsible for gel weakening. To prevent the detrimental effect caused by the proteolytic activity, food-grade inhibitors have been used to protect myofibrillar proteins from proteolysis caused by indigenous proteinases. The most commonly used inhibitors are beef plasma (BPP), egg white, potato powder and whey protein concentrate (Morrissey, Wu, Lin, & An, 1993; Yongsawatdigul, & Piyadhammaviboon, 2004). However, the use of BPP has been prohibited, due to the occurrence of mad cow disease. Also, some BPP preparations result in off-flavors and off-color. Egg white is high cost and has an undesirable egg-like odour, white off-color problems may be encountered when potato powder is used (Rawdkuen, & Benjakul, 2008). Therefore, alternative

food-grade proteinase inhibitors for surimi production are still needed. Legume seeds are rich in proteinase inhibitors (Klomklao, Benjakul, Kishimura, & Chaijan, 2011), which can be used to prevent modori in surimi. Adzuki bean (*Vigna angularis*) is an important traditional pulse crop in eastern Asia. Based on our previous study, adzuki bean seeds contained high trypsin inhibitory activity (Klomklao, Benjakul, Kishimura, Osako, & Tanaka, 2010a). Extraction and production of trypsin inhibitor from adzuki bean seed are able to increase its value and can be used as protein additive in surimi. However, no information regarding the effect of trypsin inhibitor from the adzuki bean (*Vigna angularis*) seeds on the surimi gel has been reported. Therefore, the objectives of this study were to investigate the preventive effects of trypsin inhibitor from adzuki bean seeds on autolysis of threadfin bream and to study its impact on gelling properties of surimi.

Materials and methods

1 Chemicals

Nα-Benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA), trypsin from porcine pancreas, β-mercaptoethanol (βME), sodium chloride, trichloroacetic acid, tris (hydroxymethyl) aminomethane, dimethylsulfoxide, sodium caseinate, ammonium sulfate, sodium dodecyl sulfate (SDS), Coomassie Blue R-250 and *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) and bovine serum albumin were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

2 Trypsin inhibitor extract from adzuki bean seeds

Adzuki bean (*Vigna angularis*) was purchased from the Royal Project Foundation in Bangkok, Thailand. The seeds were ground using a blender. The seed flour was defatted according to the method of Klomklao et al. (2010a). To prepare the extract, defatted seed flour was mixed with 0.15 M NaCl at a ratio of 1:7 (w/v) and shaken (BW 201 Shaking bath, Tokyo, Japan) for 30 min at 150 rpm at room temperature. The extract was recovered by centrifuging at 10,000×g for 30 min at 4°C.

To purify the trypsin inhibitor, crude extract was heated at 90°C for 10 min and then cooled with ice water (Klomklao et al., 2010a). The coagulated debris was removed by centrifuging at 10,000×g for 10 min at 4°C. The supernatant was freeze-dried and kept at 4°C until used. The dry powder was referred to as partially purified trypsin inhibitor (TI).

3 Trypsin inhibitory activity assay

Trypsin inhibitory activity was measured by the method of Klomklao, Benjakul, and Kishimura (2014) using BAPNA as substrate. One unit of trypsin inhibitory activity was defined as the amount of inhibitor, which reduces the trypsin activity by one unit. One unit of trypsin activity was defined as an increase of 0.01 A_{410} due to p-nitroaniline released.

4 Threadfin bream preparation

Threadfin bream (*N. bleekeri*) were caught from the Songkhla Coast along the Gulf of Thailand, stored in ice and off-loaded approximately 24-36 h after capture. Fish were transported in ice to the Department of Food Science and Technology, Thaksin University, Phatthalung within 2 h. Fish were then filleted, vacuum-pakced and frozen at -20°C until used for sarcoplasmic fluid preparation. To prepare fish mince, the fish were filleted and then ground through a 4 mm plate.

5 Preparation of Sarcroplasmic proteinases (Sp)

Sarcoplasmic proteinases (Sp) were prepared by centrifuging 50 g of finely chopped fillets of threadfin bream at 5,000xg for 30 min at 4°C. The supernatant was used as Sp.

6 Washed mince preparation

Washed mince was prepared according to the method of Benjakul, Visessanguan, and Srivilai (2001) with a slight modification. The comminuted flesh was homogenized with 5 volumes of 50 mM NaCl for 2 min using an IKA Labortechnik homogenizer (Selangor, Malaysia). The homogenate was centrifuged at 10,000×g for 10 min at 4°C using a Sorvall Model RC-B Plus centrifuge (Newtown, CT, USA). The washing process was repeated twice. The final precipitate was designated as “washed mince”.

7 Effect of trypsin inhibitor on inhibition of threadfin bream sarcoplasmic proteinases

Sp was mixed with partially purified trypsin inhibitor (TI) to obtain the final concentration of 0.5, 1, 2 and 3% (w/v). Sp (100 μl) was incubated with the solution of TI (100 μl) for 20 min at room temperature. The residual activity was determine using casein-TCA-Lowry method (Klomklao, Benjakul, & Kishimura, 2010b) at 60°C for 30 min. Activity determined in the absence of trypsin inhibitor was used as the control.

8 Effect of TI on autolytic activity

The inhibitory activity of TI against autolysis of mince and washed mince was measured according to the method of Morrissey et al. (1993). TI at levels of 0, 0.5, 1, 2 and 3% (w/w) was added to 3 g of mince and washed mince. The mixture was then incubated in a water bath at 60°C for 2 h and then stopped the reaction by addtion of 27 ml of 5% cold TCA solution. TCA-soluble peptides in the supernatant were analyzed using the Lowry assay (Lowry, Rosebrough, Fan, & Randall, 1951). Inhibition of autolysis is expressed as percent of autolytic activity inhibited, compared to that in the control (without trypsin inhibitor addition).

To monitor the protein pattern, another lot of sample was added with hot 5% SDS solution (85°C) to terminate the reaction and solubilize total protein. All samples were subjected to SDS-PAGE (Laemmli, 1970).

9 Sodium dodecyl sulfate-gel electrophoresis

SDS-PAGE was performed according to the method of Laemmli (1970). Protein solutions were mixed at 1:1 (v/v) ratio with the SDS-PAGE sample buffer and boiled for 3 min. The sample (20 µg) were loaded on the gel made of 4% stacking and 10% separating gels and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-Protean II cell apparatus (Atto Co., Tokyo, Japan). After electrophoresis, the gels were stained with 0.2% Coomassie Brilliant Blue R-250 in 45% methanol and 10% acetic acid and destained with 30% methanol and 10% acetic acid.

10 Protein determination

Protein concentration was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

11. Effect of TI on gel properties of threadfin bream surimi

11.1 Surimi gel preparation

Surimi was prepared according to the method of Benjakul and Visessanguan (2000). Fresh threadfin bream were washed with tap water. The flesh was removed manually and minced into the uniformity. The mince was then washed with cold water (5°C) at a mince/water ratio of 1:2 (w/w). The mixture was stirred gently for 3 min and washed process was repeated twice. Finally the washed mince was subjected to centrifugation using a Model CE 21 K basket centrifuge (Gradniumpiant, Belluno, Italy) with a speed of 700 g for 15 min. The washed mince referred to as ‘surimi’ was kept in ice until used.

11.2 Effect of TI on gel-forming ability of threadfin bream surimi

Surimi prepared was added with 2.5% salt and TI was added at level of 0, 0.5, 1, 2 and 3% (w/w). The moisture content was then adjusted to 80%. The mixture was chopped for 5 min at 4°C to obtain the homogenous sol. The sol was then stuffed into polyvinylidine casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. The paste was incubated at 40°C for 30 min, followed by heating at 90°C for 20 min in a water bath (Memmert, Schwabach, Germany). After heating, all gels were immediately cooled in iced water for 30 min and stored at 4°C overnight prior to analysis. The gel was referred to as "kamaboko gel".

11.3 Texture analysis

Texture analysis of kamaboko gels was carried out using a Model TA-XT2 texture analyser (Stable Micro System, Surrey, UK). Gels were equilibrated at room temperature (25-30°C) before analysis. Five cylindrical samples (2.5 cm in length) were prepared and tested. Breaking force (strength) and deformation (cohesiveness/elasticity) were measured by the texture analyser equipped with a spherical plunger (5 mm diameter), with a depression speed of 60 mm/min and 60% compression.

11.4 Determination of whiteness

Three gel samples from each treatment were subjected to whiteness measurement using a HunterLab (ColorFlex, Hunter Associates Laboratory, VA, USA). Illuminant C was used as the light source of measurement. CIE L^*, a^* and b^*

values were measured. Whiteness was calculated using the following equation (Park, 1994):

$$\text{Whiteness} = 100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{1/2}$$

11.5 Determination of autolysis of surimi gel

Finely chopped gel sample (3 g) was mixed with 27 ml of 5% TCA. The mixture was homogenized for 2 min using an IKA Labortechnik homogenizer (Selangor, Malaysia) at a speed of 11,000 rpm. The homogenate was incubated at 4°C for 1 h and centrifuged at 8,000g for 5 min, using a Mikro 20 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). TCA-soluble peptides in the supernatant were measured according to the Lowry method (Lowery et al., 1951) and expressed as micromole tyrosine/g sample.

12 Statistical analysis

A completely randomized design was used throughout this study. All data were subjected to analysis of variance (ANOVA) and the differences between means were carried out using Duncan's Multiple Range Test (Steel & Torrie, 1980). Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for Windows; SPSS Inc. Chicago, IL, USA).

Results and discussion

1 Effect of TI on inhibition of sarcoplasmic proteinases

Partially purified trypsin inhibitor from adzuki bean seed (TI) showed the inhibitory activity against Sp from threadfin bream muscle in a concentration-dependent manner ($P<0.05$) (Fig.5). A greater degree of inhibition was observed when the levels of TI added increased. These inhibitory activities of TI against threadfin bream Sp was due to the fact that most of the Sp in threadfin bream are trypsin-like serine proteinases (Toyohara, Kinoshira, & Shimizu, 1990). At 3% (w/w), the activity was 76% inhibited. The high inhibition of Sp from threadfin bream muscle indicated the potential use of TI in the prevention of proteolysis during setting/heating for surimi gelation. Benjakul, Karoon and Suwanno (1999) reported that the proteinase inhibitor extracts from black cowpea and soybean reduced Sp from threadfin bream surimi. Toyohara et al. (1990) found that spinash leaf extract showed strong inhibition of the modori phenomenon in threadfin bream surimi.

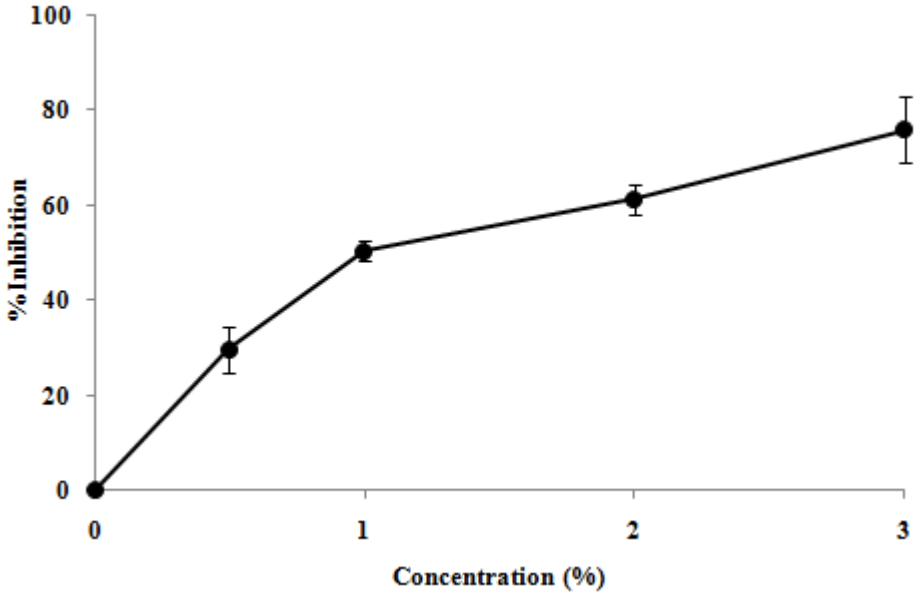

Figure 5. Effect of trypsin inhibitor from adzuki bean at different concentrations on inhibition of threadfin bream sarcoplasmic proteinases. Bars indicate standard deviation from triplicate determination.

2 Effect of TI on autolysis

Figure 6 showed the inhibition of autolysis of mince and washed mince of threadfin bream at 60°C for 2 h in absence and presence of TI at level of 0.5, 1, 2 and 3%. Generally, more inhibition was observed as the concentration of TI increased (P<0.05). At a level of 3%, inhibition of 78 and 82% was observed for mince and washed mince added with TI, respectively. This result indicated that TI was slightly more effective in inhibition of washed mince autolysis compared to unwashed mince. This was possibly due to the fact that washed mince contained a lower amount of proteinases. Sarcoplasmic proteinases were leached out during the washing process (Benjakul, Visessanguan, Thummaratwasik, 2000). However, myofibril-associated proteinases remained in the muscle and were directly associated with gel weakening. From the result, partially purified trypsin inhibitor from adzuki bean functioned as an effective proteinase inhibitor to prevent the degradation of muscle proteins. Oujifard, Benjakul, Ahmad, & Seyfabadi (2012) reported that bambara groundnut protein hydrolysate showed very high efficiency in inhibiting the autolysis of threadfin bream surimi.

Autolysis patterns of mince and washed mince of threadfin bream incubated at 60°C for 2 h, in the absence and presence of TI at different levels, are depicted in Fig. 7. A slight decrease in intensity of myosin heavy chain (MHC) band in mince and washed mince of threadfin bream incubated at 60°C for 2 h without the added TI indicated that myosin degradation was caused by endogenous proteinases (Fig. 7). However, it was retained in mince and washed mince of threadfin bream when TI was added. MHC band intensity in both mince and washed mince increased with increasing the TI concentration. The result indicted that TI could inhibit the

degradation of MHC to some extent, as evidenced by the more retained MHC. Myosin is considered as the most important component contributing to formation of surimi gels. The presence and concentration of intact myosin are related to the gel strength of surimi (Morrissey et al., 1993). Degradation of myosin, a typical characteristic of modori phenomenon, mainly causes a loss of surimi gel strength. Actin was not affected by autolysis. This result is in accordance with Benjakul & Visessanguan (2000) who reported that actin was not cleaved by endogenous proteinase in Pacific whiting muscle, whereas MHC was susceptible to degradation.

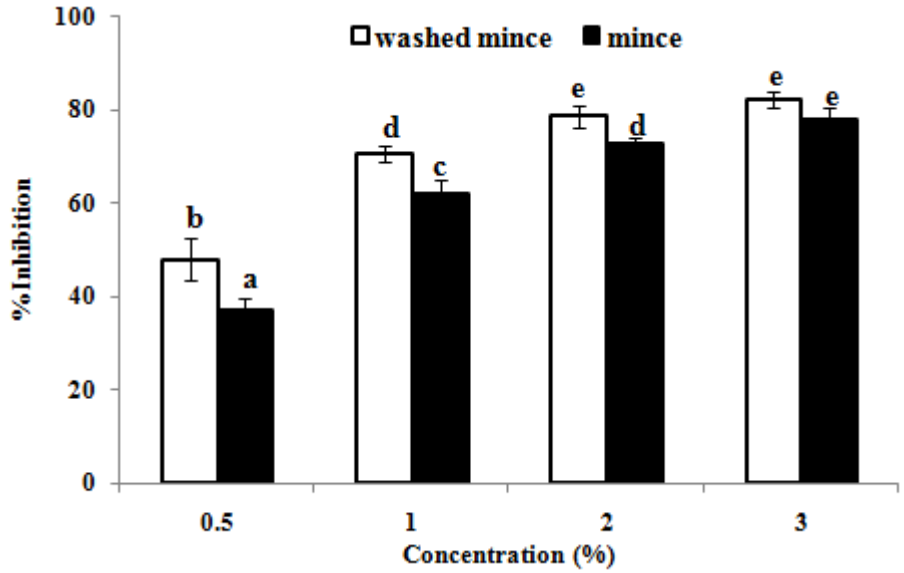

Figure 6. Effect of trypsin inhibitor from adzuki bean at different concentrations on autolytic inhibition of threadfin bream mince and washed mince. Bars indicate standard deviation from triplicate determination. Different letters in the same sample indicate significant differences (P<0.05)

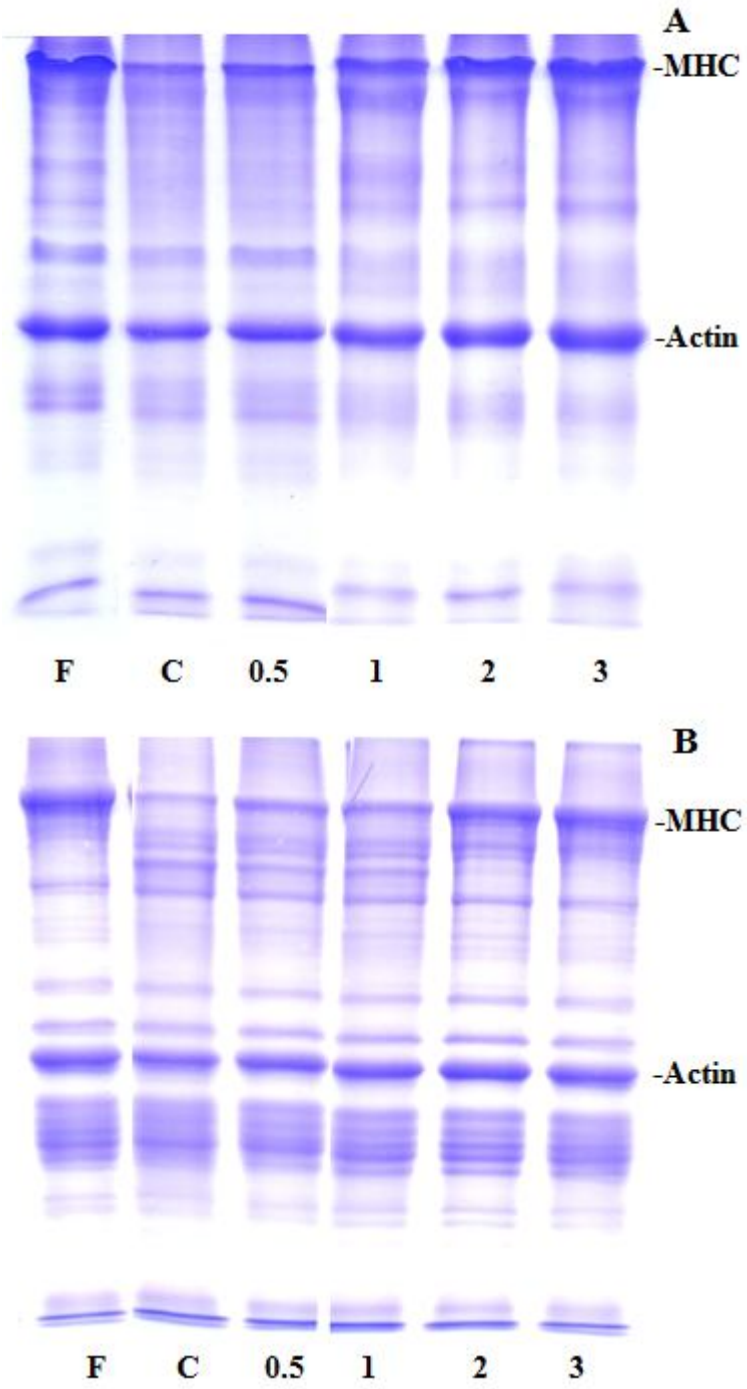

Figure 7. Autolysis pattern of threadfin bream mince (A) and washed mince (B) in the absence and presence of trypsin inhibitor from adzuki bean at different concentrations at 60°C for 2 h. F, mince or washed mince without incubation (control); C, mince or washed mince without TI. Numbers designate the concentration of TI. MHC, myosin heavy chain.

3 Effect of TI on gel properties of threadfin bream surimi

3.1 Texture properties of surimi gel

Breaking force and deformation of kamaboko gel of surimi from threadfin bream containing added TI at the levels of 0-3% are depicted in Fig. 8. The lowest breaking force and deformation were observed in gel containing no TI ($P<0.05$). Breaking force and deformation increased ($P<0.05$) with increasing TI levels. However, no differences in breaking force were found in kamaboko gel added with 2 and 3% TI ($P>0.05$). Breaking force of kamoboko gel, produced from threadfin bream surimi containing 3% added TI, increased by 84.55%, and deformation increased by 29.12%, compared to those of the control gel (without TI). The results revealed that TI was effective in increasing gel strength of threadfin bream surimi. This might be associated with the inhibitory effect of TI on autolysis (Fig. 5-7). Threadfin bream contains serine proteinases as a major indigenous enzyme associated with myofibrillar proteins (Kinoshita et al., 1990). Klomklao et al. (2010a) reported that trypsin inhibitor from adzuki bean seeds inhibited serine proteinases by using trypsin inhibitory assay. Benjakul et al. (2000) reported that incorporation of proteinase inhibitor from cowpea and bambara groundnut into threadfin bream surimi increased the gel force and deformation. Oujifard et al. (2012) found that 0.25% bambara groundnut protein isolate increased breaking force and deformation of kamaboko gel of threadfin bream surimi and increasing bambara groundnut protein isolate levels showed detrimental effect on gelation.

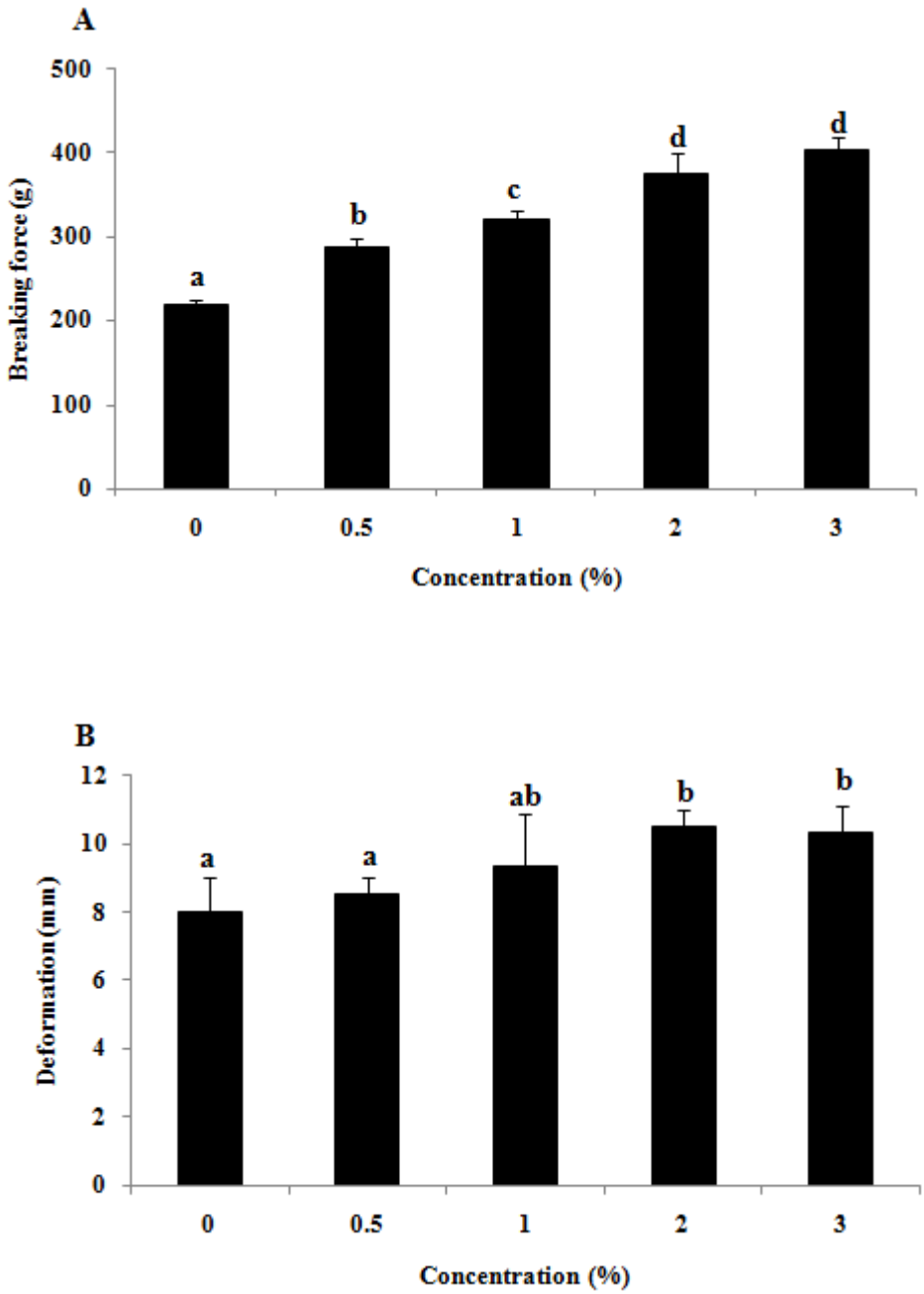

Figure 8. Breaking force (A) and deformation (B) of kamaboko gels containing added trypsin inhibitor from adzuki bean at different levels. Bars indicate standard deviation from triplicate determination. Different letters indicate significant differences (P<0.05)

3.2 Whiteness of surimi gel

Table 4 showed the whiteness of kamoboko gels added with TI at different levels. The addition of TI up to 1% showed no effect on whiteness ($P>0.05$). On the other hand, the slight decrease in whiteness was noticeable in kamaboko gel, as TI levels increased above 1% ($P<0.05$). Undesirable components in the trypsin inhibitor extracts, e.g. pigments, can impart changes in color characteristics of surimi gels (Benjakul et al., 2000). Whiteness of kamaboko and modori gels of threadfin bream surimi slightly decreased with increasing bambara groundnut protein isolate concentration increased (Oujifard et al., 2012). Benjakul et al. (2000) reported that the lightness and whiteness of surimi gels from threadfin bream decreased slightly when the proteinase inhibitor from cowpea was added at a level of 30 kunits/g. From the results, TI at a low level can be used as a protein additive in threadfin bream surimi to improve the gel strength without causing marked changes in whiteness.

Table 4 Whiteness of kamaboko gels containing added trypsin inhibitor from adzuki bean at different concentrations

Concentration of trypsin inhibitor (%)	Whiteness*
0	81.27±0.88b**
0.5	81.13±0.55b
1	80.69±0.52ab
2	79.99±0.12a
3	79.81±0.24a

*Values are given as mean±SD from triplicate determinations.

**Different letters indicate significant differences (P<0.05)

3.3 TCA-soluble peptide content of surimi gel

Fig. 9 showed the degradation of proteins in kamaboko gels without and with TI addition at different levels expressed as TCA-soluble peptide content. TCA-soluble peptide content decreased as TI concentration increased ($P<0.05$). This result suggested that TI had inhibitory activity towards degradation of protein. Adzuki bean contains trypsin inhibitor, which exhibit the most effective inhibitory activity toward trypsin (Klomklao et al., 2010a). The degradation occurred during heat-induced gelation is considered to result from the action of indigenous proteinases (An et al., 1996). Degradation probably occurred during setting at high temperature (40°C), which was close to the temperature of gel softening (50-60°C) (Rawdkuen et al., 2004). From the results, TI effectively reduced the degradation during setting in kamaboko gels as observed by the decrease in TCA-soluble peptides in kamaboko gels added with a high amount of TI. Oujifard et al. (2012) reported that TCA-soluble peptide content was decreased by the addition of bambara groundnut protein isolate to modori gel from threadfin bream. The highest TCA-soluble peptide content was obtained in kamaboko gel without TI addition. This result was in agreement with the lowest breaking force and deformation of the gel without TI addition (Fig. 4). The results reconfirmed that the improved gel strength of surimi from the addition of TI was associated with a reduction in the extent of proteolysis that occurred.

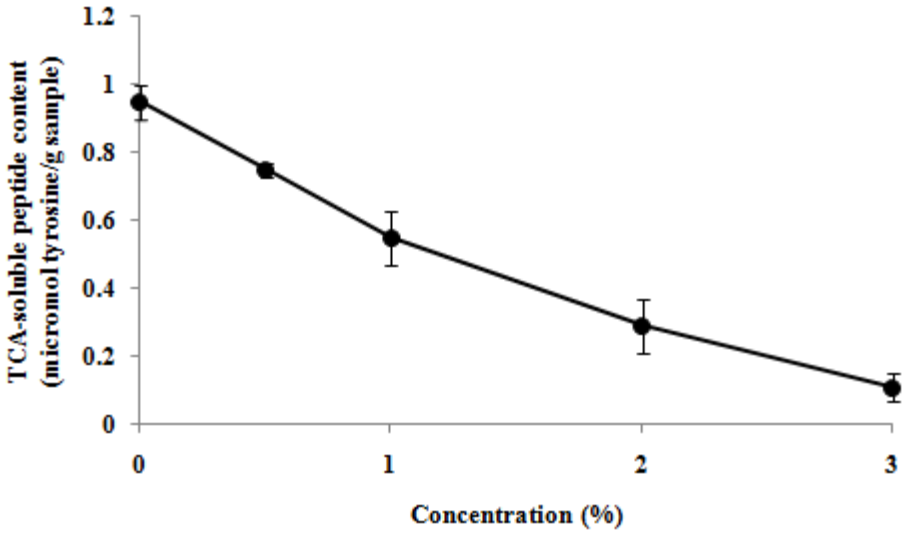

Figure 9. TCA-soluble peptide content in kamaboko gels containing added trypsin inhibitor from adzuki bean at different levels. Bars indicate standard deviation from triplicate determination.

Conclusion

Proteolysis of threadfin bream muscle caused by either heat-activated sarcoplasmic or myofibril-associated proteinases could be partially inhibited by the addition of partially purified trypsin inhibitor from adzuki bean seed. The addition of trypsin inhibitor from adzuki bean at concentration up to 3% (w/w) increased the breaking force and deformation of kamaboko gels. However, trypsin inhibitor addition caused the decreased whiteness, especially with increasing amount added.

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Output

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APPENDIX