CHAPTER 1

INTRODUCTION

Protease inhibitors are proteins or peptides capable of inhibiting catalytic activities of proteolytic enzymes (Choi et al., 2002). Protease inhibitors are widely distributed in many plant materials used as food, especially in legumes, potato, and cereals (Benjakul et al., 2000). Protease inhibitors in plant play essential roles in biological systems regulating proteolytic processes and participate in defense mechanisms against attack by a large number of insects, fungi, and other pathogenic microorganisms (Lopes et al., 2009). Legume seed protease 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 and 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), Australian wattle seed (*Acacia victoriae* Bentham) (Ee et al., 2008), dry bean (Nergiz and Gokgoz, 2007), velvet bean and jack bean (Betancur-Ancona et al., 2008). Recently, Klomklao et al. (2010a) reported that adzuki bean showed high trypsin inhibitory activity. The inhibitor content and type in legumes vary with many factors, e.g. the cultivar, maturity, fermentation, and heat treatment (Benjakul et al., 2000). The presence of trypsin inhibitor affects the protein efficiency ratio for the consumer. Protease inhibitors inhibit the activity of the protein digesting enzymes in the digestive tract,

reducing the body's ability to utilize protein in food. However, legume seeds can be isolated as a source of protease inhibitor and used in a variety of applications such as medicine, agriculture and food technology (Klomklao et al., 2010a).

Mung bean seeds are important in the diet and economy of Thailand. Mung bean is used to make various kinds of sweet, bean jam, sweetened bean soup, vermicelli, and bean sprout. Based on our preliminary study, mung bean seeds showed high trypsin inhibitory activity. Hence, mung bean seeds were a potential source for recovering trypsin inhibitor and used for a plethora of industrial applications. However, no information is available on trypsin inhibitors in mung bean seeds grown in Thailand. This research aimed to extract, purify and characterize the trypsin inhibitor from Thai mung bean seeds.

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. Klomklao et al. (2010a) purified trypsin inhibitor from adzuki bean seeds 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. 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. 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.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

*N*α-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). Sephadex G-50 was obtained from Pharmacia Biotech (Uppsala, Sweden).

3.2 Trypsin inhibitor extract from mung bean seeds

3.2.1 Preparation of defatted seed flour

One batch of mung bean (*Vigna radiata (*L.)R. Wilczek) used for this study was collected and purchased from the local market in Phatthalung, 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 Klomklao et al. (2010a) 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.

3.2.2 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 1 h 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 extracts were determined and the specific inhibitory activity of the extracts obtained using different media were compared. The extraction media used yielding the extract with the highest specific trypsin inhibitory activity, was selected for further steps.

3.2.3 Effect of extraction time on trypsin inhibitor extraction

Defatted seed flour was mixed with distilled water with a solid/solvent ratio of 1:7 (w/v) and shaken for 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.

3.3 Purification of trypsin inhibitor from mung bean seeds

Crude extract was heated at different temperatures (60, 70, 80, 90 and 100°C) for 10 min and then cooled with iced 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 Sephadex G-50 (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 of 3 ml 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.

3.4 Thermal and pH stability of purified trypsin inhibitor

Sephadex G-50 trypsin inhibitor fraction was 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.

3.5 Salt stability

Sephadex G-50 trypsin inhibitor fraction 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.

3.6 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.

3.7 Inhibitory activity of trypsin inhibitor by electrophoresis

Crude extract, heat-treated extract, $(NH_4)_2SO_4$ fraction and Sephadex G-50 fraction without prior heating were separated on SDS-PAGE, followed by inhibitory activity staining using casein as a substrate with the slightly modified method of Garcia-Carreno, Dimes and Haard (1993) and Klomklao et al. (2010b). 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.

3.8 Trypsin inhibitory activity assay

Trypsin inhibitory activity was measured by the method of Klomklao et al. (2010a) with a slight modification using BAPNA as substrate. A solution containing 200 µl of inhibitor solution and 200 µl (20 µg/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.

3.9 Protein determination

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

3.10 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.).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Extraction of trypsin inhibitor from mung bean seeds

The various extraction media were used to extract the trypsin inhibitor from mung bean seeds (Table 1). The highest trypsin inhibitory activity (822.63 units/g seed) and specific trypsin inhibitory activity (31.95 units/mg protein) were obtained when distilled water was used as the extraction medium ($P<0.05$). The specific inhibitory activity was reduced when NaCl was used ($P<0.05$), particularly with the higher concentration. Salt at higher concentration might increase the solubility of protein, leading to the higher concentration of protein in extract. The denaturation of mung bean trypsin inhibitor at high salt concentration was also presumed. In addition, the low specific inhibitory activity was observed when the NaOH was used. In fact, the high inhibitory activity was obtained when the NaOH was used, but the high protein content in the extract was found, causing the reduced specific inhibitory activity ($P<0.05$). This result was in accordance with Benjakul et al. (2000) who reported that solubilization of protein from cowpea and pigeon pea was markedly increased when alkaline solution was used, compared to NaCl and distilled water. Klomklao et al. (2010a) also found that when NaOH was used to extract trypsin inhibitor from adzuki bean, a significant decrease in specific activity was observed, especially with the higher concentration. Factors involved in protein solubility and recovery include protein meal and solvent ratio, particle size of flour, temperature, length of extraction time, pH, ionic strength, type and concentration of extraction medium as well as the hydration characteristics of proteins (Benjakul et al.,

2000; Klomklao et al., 2010a). From the results, distilled water was chosen as the extraction medium for mung bean seed trypsin inhibitor.

The effect of extraction time on the recovery of trypsin inhibitor from mung bean seed was investigated (Fig. 1). The higher extraction efficiency was found when the extraction time increased up to 2 h (Fig. 1a) ($P<0.05$). Increased extraction time allowed the protein, especially trypsin inhibitors, to be more dissolved. The inhibitory activity was slightly increased when the extraction time of 2 h was used (Fig. 1b) ($P<0.05$). However, the decrease in inhibitory activity was observed when the extraction time of 3-5 h was used ($P<0.05$) (Fig. 1b), indicating the less stability of trypsin inhibitor from mung bean during the extraction. This contributed to the loss of specific activity (Fig. 1a). The extraction time of 3 h was optimum for the recovery of trypsin inhibitor from pigeon, whereas 1 h was optimum for cowpea (Benjakul et al., 2000). Klomklao et al. (2010a) reported that an extraction time of 30 min was found to be optimal for adzuki bean trypsin inhibitor extraction and specific inhibitor activity of adzuki bean seed decreased with increasing extraction time. High mechanical shear generated by shaking can cause denaturation of protein (Klomklao et al., 2010c). Also, the incorporation of air bubbles and adsorption of protein molecules to the air-liquid interface can cause the denaturation of protein (Damodaran, 1996). Therefore, the extraction time of 2 h was selected for the extraction of trypsin inhibitor from mung bean seeds.

Table 1.

Effect of different extraction media on the recovery of trypsin inhbitor from mung bean seeds.

Extraction media	Trypsin inhibitor (units/g seed)	Protein (mg/g seed)	Specific activity (units/mg protein)
Distilled water	822.63±6.48b	25.75±0.34a	31.95±0.25e
0.01 M NaOH	627.01±4.27a	60.36±0.51d	10.39±0.07a
0.02 M NaOH	806.53±4.06b	68.82±0.72e	11.72±0.06b
0.15 M NaCl	805.93±17.52b	31.90±0.33b	25.26±0.55d
0.30 M NaCl	605.47±23.68a	45.00±0.23c	13.45±0.53c

*The defatted seed flour was shaken in different media at ambient temperature for 1 h and trypsin inhibitor activity was analyzed using BAPNA as substrate.
**Mean±SD from triplicate determinations
The different letters in the same column denote the significant differences ($P<0.05$).

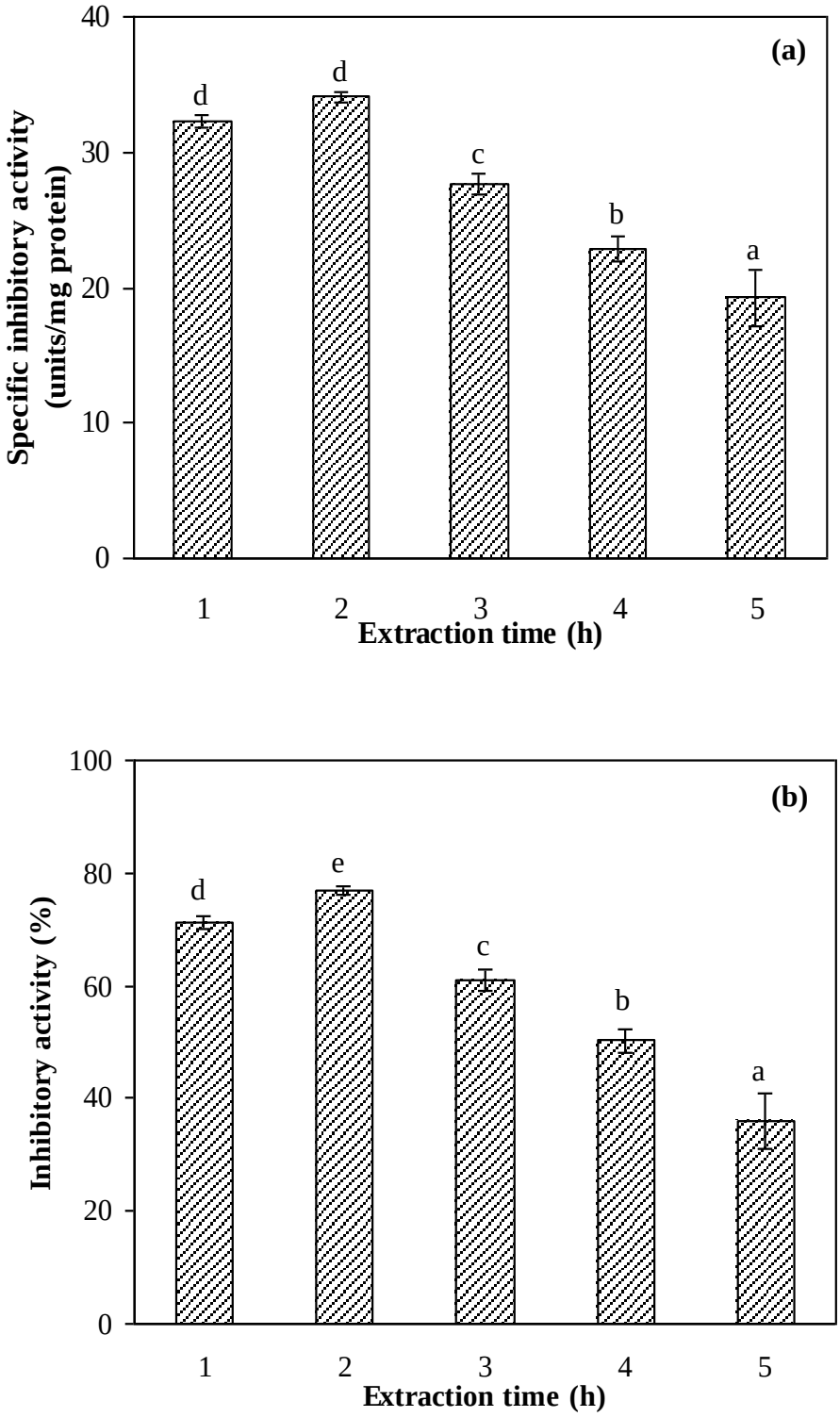

Fig. 1. Effect of extraction time on specific trypsin inhibitory activity (a) and trypsin inhibitory activity (b) of mung bean seed extract. Bar indicate standard deviation from triplicate determinations.

4.2 Purification of trypsin inhibitor from mung bean

The heat treatment of mung bean extract was carried out at different temperatures. The trypsin inhibitory activity was markedly increased after heat treatment at 60°C and the inhibitory activities of mung bean extract were quite constant up to 90°C. A marked decrease in activity was obtained at 100°C (Fig. 2a). For specific trypsin inhibitory activity, it was increased markedly when the heating temperature increased up to 90°C (Fig. 2b). The heating of the plant extract can enrich the trypsin inhibitors, due to the thermal coagulation of the bulk protein (Benjakul et al., 2000). However, when the extracts were heated at 100°C, the specific activity was decreased. Therefore, heat treatment at 90°C for 10 min was introduced to the purification step to rapidly remove some undesired proteins. The heat treatment of extract from mung bean rendered the purification of 2.84-fold (Table 2). Some heat labile protein could be removed, resulting in the higher specific inhibitory activity. Interestingly, the yield was increased to 148.01% from this step. Heat applied might loosen their compact structure that is stabilized by a number of disulfide linkages (Benjakul et al., 2000; Klomklao et al., 2010a). Klomklao et al. (2010a) found that heat-treatment of mung bean extract at 90°C for 10 min resulted in the increased specific trypsin inhibitory activity by 6.46-fold.

Heat-treated extract was further purified by ammonium sulfate precipitation. An ammonium sulfate saturation of 30-65% was shown to be the optimum range for trypsin inhibitor recovery (data not shown). An increase in purity of 3.98-fold was obtained when 30-65% ammonium sulfate was used (Table 2). Prasad et al. (2010) reported that ammonium sulfate precipitation of proteinase

inhibitor from the seeds of black gram at 25-80% resulted in the increase in specific activity by 2.27-fold.

When ammonium sulfate fraction with trypsin inhibitory activity was subjected to gel filtration on Sephadex G-50, a single peak was obtained. Purification fold of 13.51 with a yield of 30.25% was observed (Table 2). Klomklao et al. (2010a) found that the use of gel filtration on Sephacryl S-200 in the final step of the purification process of trypsin inhibitor from the adzuki bean seed led to an increase in trypsin inhibitory activity by 10.91-fold.

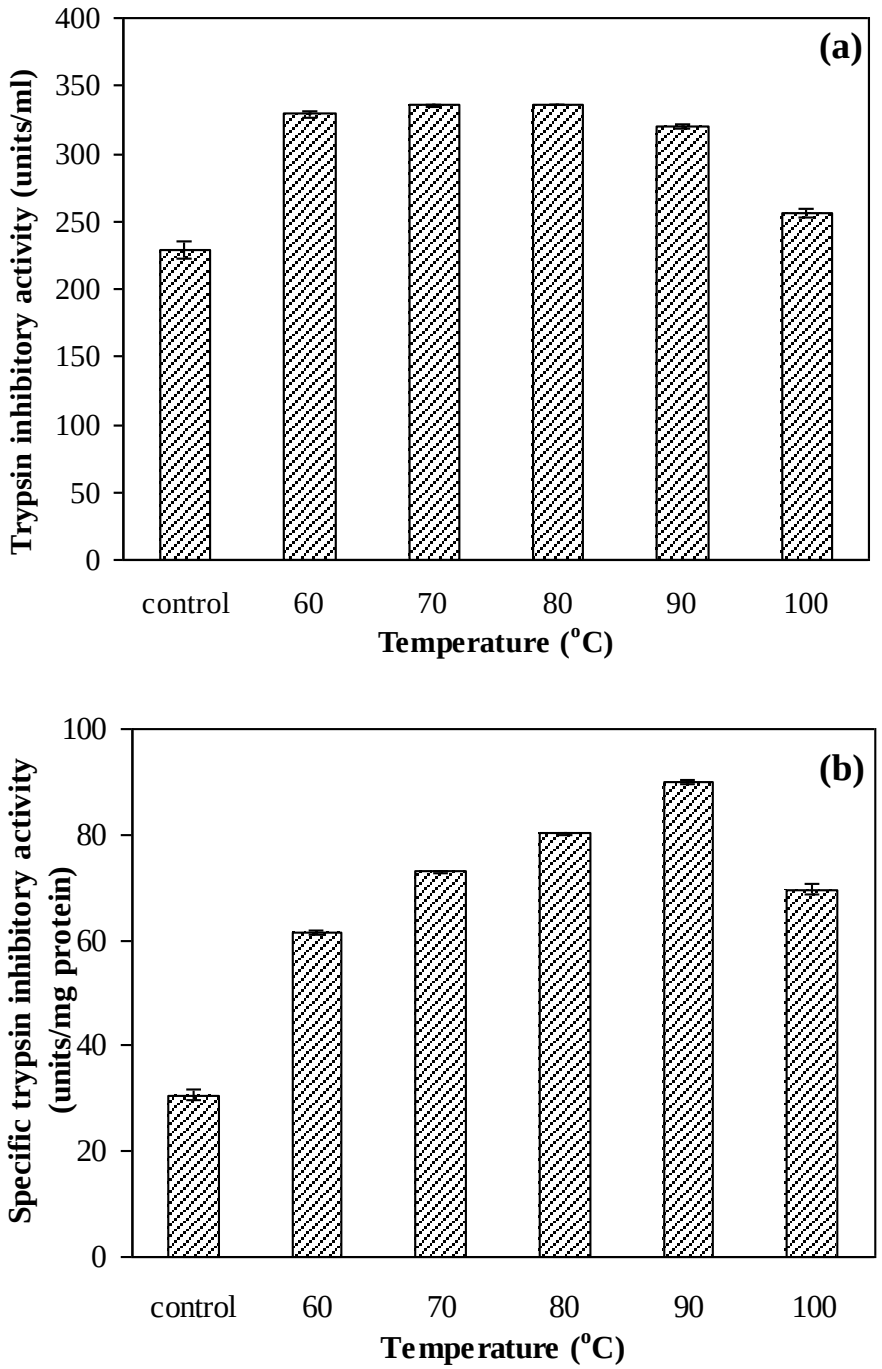

Fig. 2. Effect of heat treatment at different temperatures on trypsin inhibitory activity (a) and specific trypsin inhibitory activity (b) of mung bean seed extract. Bar indicate standard deviation from triplicate determinations.

Table 2.

Purification of trypsin inhibitors from mung bean seeds

Purification steps	Total activity (Units)	Total protein (mg)	Specific inhibitory activity (units/mg protein)	Purity (fold)	Yield (%)
Extract	18931.97	630.52	30.03	1	100
Heat treatment	28021.83	328.75	85.24	2.84	148.01
$(NH_4)_2SO_4$ precipitation (30-65%)	7624.67	63.74	119.62	3.98	40.27
Sephadex G-50	5727.51	14.12	405.63	13.51	30.25

4.3 Protein pattern and activity staining of trypsin inhibitors from mung bean

The proteins from each steps of purification were separated under reducing and nonreducing condition and stained with Coomassie blue after electrophoresis (Fig. 3a). Based on the protein patterns under nonreducing condition, crude extract contained a variety of protein with different molecular weights. The heat treatment directly affected the protein pattern in the mung bean seed extract. Proteins with molecular weight of 30, 32 kDa and proteins with molecular weight higher than 70 kDa were removed by heat treatment at 90°C. Those proteins disappeared with heating process were presumed to be heat labile proteins. After ammonium sulfate precipitation, a large number of proteins were removed, especially proteins with higher molecular weight, with a concomitant increase in protein with molecular weight of 14 kDa. After subjecting to Sephadex G-50, the purified trypsin inhibitor was observed to migrate as a single band at 14 kDa on SDS-PAGE. However, in the presence of reducing agent, the protein band of Sephadex G-50 fraction with molecular weight of 14 kDa disappeared. Two protein bands with molecular weights of 10 and 4 kDa were obtained. These results suggested that the main component in pooled Sephadex G-50 fractions consisted of subunits stabilized by disulfide bond. SDS-PAGE indicated that a large amount of proteins was removed during purification. Subsequently, higher purity of interested trypsin inhibitor was observed as shown in Table 2.

Trypsin inhibitory activities of the mung bean extract and various fractions obtained from purification process were analyzed using SDS-substrate gel (Fig. 3b). It was shown that only one distinct band with apparent molecular size of 14 kDa was found in all fractions under nonreducing condition. The band intensity

slightly increased after heat-treatment, ammonium sulfate precipitation and Sephadex G-50 chromatography. This was due to the higher specific trypsin inhibitory activity loaded onto the gel. Klomklao et al. (2010a) reported that the apparent molecular weight of trypsin inhibitor was estimated to be 14 kDa based on SDS-PAGE and inhibitor activity zones separated by electrophoresis. The molecular mass of trypsin inhibitors from bambara groundnuts was 13 kDa (Benjakul et al., 2000). Two trypsin inhibitory activity bands were observed for cowpea (10 and 18 kDa) and pigeon pea (15 and 25 kDa) (Benjakul et al., 2000). Yoshizaki et al. (2007) reported that the molecular weight of trypsin inhibitor purified from *Calliandra selloi* Macbride seeds was 20 kDa. Two of the families of serine proteinases, the Kunitz and Bowman-Birk type inhibitors, have been the subject of much research, especially in the Graminaceae, Leguminosae and Solanaceae. These families differ from each other in mass, cysteine content, and number of reactive sites (Richardson, 1977). Generally, the Bowman-Birk type of inhibitor has a lower molecular weight compared with the Kunitz type. Kunitz type inhibitors are proteins of molecular weight of >20 kDa, with low cysteine content and a single reactive site, whereas the Bowman-Birk type inhibitors have molecular weight of 8-10 kDa, as well as high cysteine content and two reactive sites (Richardson, 1997). Godbole et al. (1994) found that 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. From the result, the inhibitor from mung bean probably belongs to Bowman-Birk type. However, the amino acid sequence is needed to verify the classification of this inhibitor. After reduction with βME, no activity band was found in all fractions (Fig. 3b). It is postulated that the cleavage of disulfide bond by the

action of βME could lead to protein denaturation and loss of activity. Klomklao et al. (2010a) reported that the band of trypsin inhibitor purified from adzuki bean seeds was not observed under reducing condition. Damodaran (1996) reported that proteins that require high structural stability to function as catalysts 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 mung bean and the fractions was prerequisite for their inhibitory action.

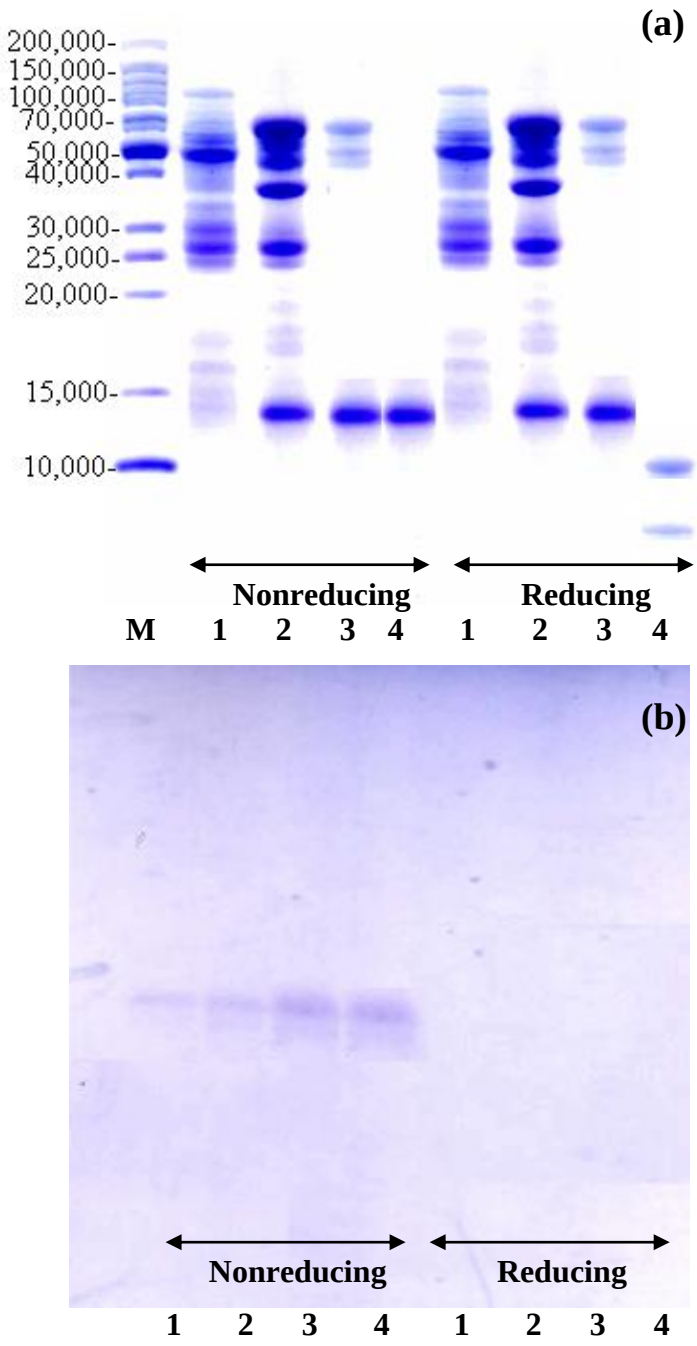

Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (a) and inhibitory activity staining for trypsin (b) of crude trypsin inhibitor from mung bean seed and different fractions under reducing and nonreducing condition. M, molecular weight standard; 1: crude extract; 2:heat-treated extract; 3: ammonium sulfate fraction, 4: Sephadex G-50 fraction.

4.4 Thermal stability of purified trypsin inhibitor

Fig. 4 shows the changes in inhibitory activity of purified trypsin inhibitor from mung bean seed incubated at 90°C for different times. No significant changes in the inhibitory activity of the purified inhibitor was observed up to 50 min ($P>0.05$). A slight decrease in activity was observed when heated at 90°C for 60 min; however, the remaining activity was higher than 90%. Partially purified trypsin inhibitor from three seeds including cowpea, pigeon pea and bambara groundnuts were heat stable up to 30 min at 90°C (Benjakul et al., 2000). Klomklao et al. (2010a) reported that trypsin inhibitor from adzuki bean retained high inhibitory activity toward trypsin after incubation at 90°C for 60 min. Nevertheless, trypsin inhibitor from field beans, peanuts and cereals are heat sensitive (Liener and Kakade, 1969). Also, Vasconcelos et al. (1997) reported that trypsin inhibitors from Brazilian soybeans were destroyed completely by heating at 92°C for 5 min. The differences in thermal stability of the trypsin inhibitor were probably due to differences in the nature of the protein, e.g. conformation and bonding involved (Cheftel et al., 1985). The presence of substances, e.g. crude fiber, phytate and tannin might partially contribute to the higher residual trypsin inhibitory activity after heat treatment (Benjakul et al., 2000). From the results, purified trypsin inhibitors from mung bean seed were very heat stable up to 50 min at 90°C and could be applied in various thermal processes such as the gelation of surimi.

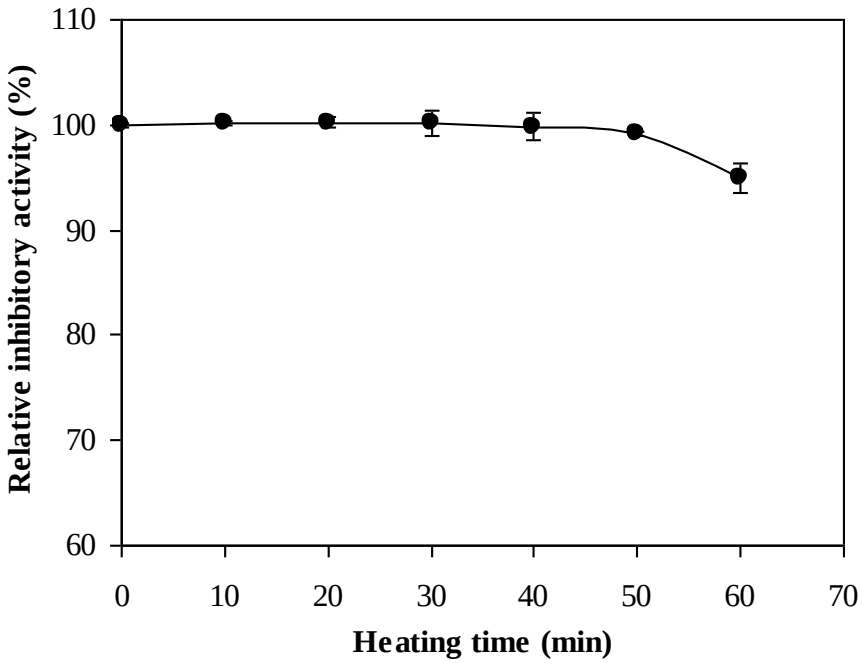

Fig. 4. Thermal stability of Sephadex G-50 trypsin inhibitor fraction from mung bean seed. Bar indicate standard deviation from triplicate determinations.

4.5 pH stability of purified trypsin inhibitor

pH stability of trypsin inhibitor purified from mung bean seeds was investigated (Fig. 5). The purified inhibitor was stable over a broad pH range. However, there was some decrease in activity at low and high pHs. Therefore, the inhibitor was generally stable in the neutral pH ranges. At extreme pH values, strong intramolecular electrostatic repulsion caused by high net charge results in swelling and unfolding of the protein molecules (Damodaran, 1996). Godbole et al. (1994) found that the inhibitor from pigeon pea retained their activity between pH 7 and 10. Benjakul et al. (2000) reported that the inhibitor from pigeon pea and cowpea retained their activity between pH 4 and 10. However, for bambara groundnut, the decrease activity was observed at alkaline pH. Trypsin inhibitor purified from adzuki bean seeds was stable over a wide pH range (4-10) (Klomklao et al., 2010a). Trypsin inhibitors from black cowpea and soybean were stable over a broad pH range, but were most stable at neutral pH and the extract from soybean seed was less stable than that from black cowpea seed in the acidic pH range (Benajkul et al., 1999). The differences in pH stability of trypsin inhibitors from different sources indicated the different molecular properties including bonding stabilizing the structure, the trypsin inhibitor conformation and anatomical locations.

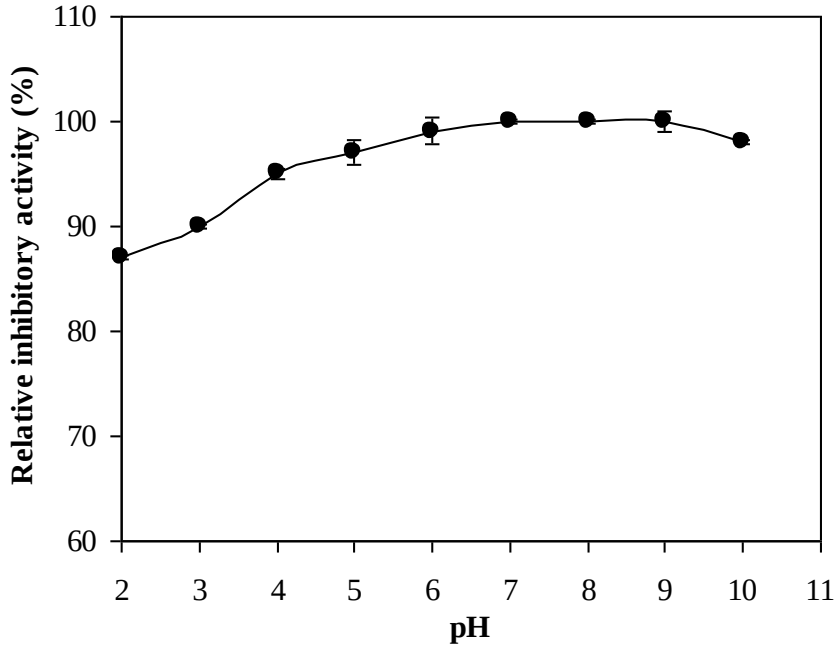

Fig. 5. pH stability of Sephadex G-50 trypsin inhibitor fraction from mung bean seed. Bar indicate standard deviation from triplicate determinations.

4.6 Effect of salt on the stability of purified trypsin inhibitor

The effect of NaCl on the inhibitory activity of purified trypsin inhibitor is depicted in Fig. 6. No marked changes in relative inhibitory activity were found when NaCl was added up to 3.0% ($P>0.05$). Klomklao et al. (2010a) reported that NaCl, at 0-3% concentration, did not affect the inhibitory activity of trypsin inhibitor purified from adzuki bean seeds. From the result, purified trypsin inhibitor showed high salt stability up to 3%. Therefore, purified inhibitor from mung bean can be useful in surimi-based products in which 2-2.5% salt are commonly used without the severe loss in the inhibitory activity.

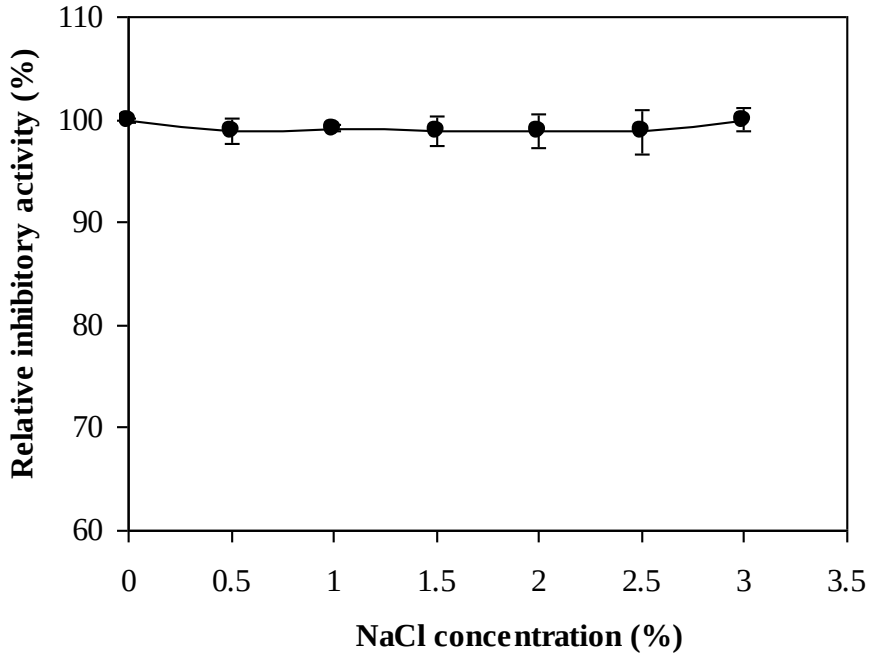

Fig. 6. Effect of salt concentration on the stability of Sephadex G-50 trypsin inhibitor fraction from mung bean seed. Bar indicate standard deviation from triplicate determinations.

CHAPTER 5

CONCLUSION

Mung bean seeds showed high trypsin inhibitory activity against porcine trypsin. The trypsin inhibitor from mung bean was successfully isolated and characterized. The molecular weight of trypsin inhibitor was estimated to be 14 kDa. It was stable to various pHs, heat treatment and was also stable at high salt concentration up to 3%. The purified trypsin inhibitor from mung bean can be used as an alternative additive for improving the quality of surimi gels, especially those suffering with softening caused by trypsin or trypsin-like serine proteinases.

Acknowledgments

This research work was financially supported by Thaksin University through Contract No. 6/2553.

Output

Klomklao, S., Benjakul, S. and Kishimura, H. (2010). Extraction, purification and properties of trypsin inhibitor from Thai mung bean (*Vigna radiate* (L.)R. Wilczek). Submitted to Food Chemistry.

REFERENCES

Abrahamson, M., Mason, R., & Hansson, H. (1991). Human cystatin C: Role of the N-terminal segment in the inhibition of human cysteine proteinases and in its inactivation by leukocyte elastase. *Journal of Biochemistry, 273*, 621-626.

Barrett, A.J., & Starkey, P.M. (1973). The interaction of α2-macroglobulin with proteinases: Characteristics and specificity of the reaction, and a hypothesis concerning its molecular mechanism. *Biochemical Journal, 133*, 709-724.

Benjakul, S., Karoon, S., & Suwanno, A. (1999). Inhibitory effects of legume seed extracts on fish proteinases. *Journal of the Science of Food and Agriculture*, 79, 1875-1881.

Benjakul, S., Visessanguan, W., & Thummaratwasik, P. (2000). Isolation and characterization of trypsin inhibitors from some Thai legume seeds. *Journal of Food Biochemistry*, *24*, 107-127.

Betancur-Ancona, D., Gallegos-Tintore, S., Delgado-Herrera, A., Perez-Flores, V., Ruelas, A.C., & Chel-Guerrero, L. (2008). Some physicochemical and antinutritional properties of raw flours and protein isolates from *Mucuna pruriens* (velvet bean) and *Canavalia ensiformis* (jack bean). *International Journal of Food Science and Technology*, 43, 816-823.

Campos, J.E., Martinez-Gallardo, N., Mendiola-Olaya, E., & Blanco-Labra, A. (1997). Purification and partial characterization of a proteinase inhibitor from terzry bean (*Phaseolus acutifolius*) seeds. *Journal of Food Biochemistry, 21*, 203-218.

Carlson, T.H. (1996). Composition of plasma. In E.C. Rossi, T.L. Simon, G.S. Moss, & S.A. Gould (Eds.), *Principles of Transfusion Medicine* (pp.383-391). Baltimore: Williams & Wilkins.

Chaudhary, N.S., Shee, C., Islam, A., Ahmad, F., Yernool, D., Kumar, P., & Sharma, A.K. (2008). Purification and characterization of a trypsin inhibitor from *Putranjiva roxburghii* seeds. *Phytochemistry, 69*, 2120-2126.

Cheftel, J.C., Cuq, J.L., & Lorient, D. (1985). Amino acids, peptides, and proteins. In O.R. Fennema (Ed), *Food Chemistry* (pp. 245-369). New York: Marcel Dekker.

Choi, J.H., Park, P.J., & Kim, S.W. (2002). Purification and characterization of a trypsin inhibitor from the egg of skipjack tuna *Katsuwonus pelamis*. Fisheries *Science, 68*, 1367-1373.

Christeller, J.T., Farley, P.C., Ramsay, R.J., Sullivan, P.A., & Laing, W.A. (1998). Purification, characterization and cloning of an aspartic proteinase inhibitor from squash phloem exudates. *European Journal of Biochemistry, 254*, 160-167.

Damodaran, S. (1996). Amino acids, peptides, and proteins. In O.R. Fennema (Ed), *Food Chemistry* (pp. 322-429). New York: Marcel Dekker.

Ee, K.Y., Zhao, J., Rehman, A., & Agboola, S. (2008). Characterization of trypsin and α-chymotrypsin inhibitors in Australian wattle seed (*Acacia victoriae* Bentham). *Food Chemistry, 107*, 337-343.

Garcia-Carreno, F.L. (1996). Proteinase inhibitors. *Trends in Food Science and Technology, 7*, 197-204.

Garcia-Carreno, F.L., & Hernandez-Cortes, P. (2000). Use of protease inhibitors in seafood products. In N.F. Haard, & B.K. Simpson (Eds.), Seafood Enzymes (pp. 531-547). New York: Marcel Dekker.

Garcia-Carreno, F.L., Dimes, L.E., & Haard, N.F. (1993). Substrate-gel electrophoresis for composition and molecular weight of proteinase or proteinaceous protinase inhibitors. *Analytical Biochemistry, 214*, 65-69.

Garcia-Carreno, F.L., Toro, M.A.N., Diaz-Lopez, M., Hernandez-Cortes, M.P. & Ezquerra, J.M. (1996). Proteinase inhibition of fish muscle enzymes using legume seed extracts. *Journal of Food Protection*, 59, 312-318.

Godbole, S.A., Krishna, T.G., & Bhatia, C.R. (1994). Purification and characterization of protease inhibitors from pigeon pea (*Cajanus cajan* (L) Millsp) seeds. *Journal of the Science of Food and Agriculture, 64*, 87-93.

Guillamon, E., Pedrosa, M.M., Burbano, C., Cuadrado, C., Sanchez, M.C., & Muzquiz, M. (2008). The trypsin inhibitors present in seed of different grain legume species and cultivar. *Food Chemistry, 107*, 68-74.

Kennedy, A.R. (1998). Chemopreventive agents: Protease inhibitors. *Pharmacology and Therapeutics, 78,*167-209.

Klomklao, S., Benjakul, S., Kishimura, H., Osako, K., & Tanaka, M. (2010a). A heat-stable trypsin inhibitor in adzuki bean (*Vigna angularis*): effect of extraction media, purification and biochemical characteristics. *International Journal of Food Science and Technology, 45*, 163-169.

Klomklao, S., Benjakul, S., Kishimura, H., Osako, K., & Tanaka, M. (2010b). Effect of salts and polyethylene glycols on the partitioning and recovery of trypsin from hybrid catfish viscera in aqueous two-phase systems. *Journal of Food Biochemistry, 34*, 730-747.

Klomklao, S., Benjakul, S., & Kishimura, H. (2010c). Proteinases in hybrid catfish viscera: Characterization and effect of extraction media. *Journal of Food Biochemistry, 34*, 711-729.

Laemmli, U.K. (1970). Cleavage of structure proteins during the assembly of the head of bacteriophage T_4. *Nature, 277,* 680-685.

Liener, I.E., & Kakade, M.L. (1969). Protease inhibitors. In I.E. Liener (Ed.), *Toxic constituents of plant foodstuffs* (pp. 14-25). London: Academic Press.

Lopes, J.L.S., Valadares, N.F., Moraes, D.I., Rosa, J.C., Araujo, H.S.S., & Beltramini, L.M. (2009). Physico-chemical and antifungal properties of protease inhibitors from *Acacia plumosa*. *Phytochemistry, 70*, 871-879.

Lowry, O.H., Rosebrough, N.J., Fan, A.L., & Randall, R.J. (1951). Protein measurement with Folin phenol reagent. *Journal of Biological Chemistry, 193,* 256-275.

Macedo, M.L.R., Matos, D.G.G., Machodo, O.L.T., Marangoni, S., & Novello, J.C. (2000). Trypsin inhibitor from *Dimorphandra mollis* seeds: Purification and properties. *Phytochemistry, 54,* 553-558.

Maity, J., & Patra, B.C. (2003). Isolation and characterization of trypsin inhibitor from the water fern, *Azolla pinnat*a R. Br. *Journal of Food Biochemistry, 27,* 281-294.

Nergiz, C. & Gokgoz, E. (2007). Effects of traditional cooking methods on some antinutrients and in vitro protein digestibility of dry bean varieties (*Phaseolus vulgaris* L.) grown in Turkey. *International Journal of Food Science and Technology*, 42, 868-873.

Otlewski, J., Krowarsch, D., & Apostoluk, W. (1999). Protein inhibitors of serine proteinases. *Acta Biochimica Polonica*, *46*, 531-565.

Prasad, E.R., Dutta-Gupta, A., & Padmasree, K. (2010). Purification and characterization of a Bowman-Birk proteinase inhibitor from the seeds of black gram (*Vigna mungo*). *Phytochemistry*, *71*, 363-372.

Richardson, M. (1977). The proteinase inhibitors of plants and microorganisms. *Phytochemistry, 16*, 159-169.

Ritonja, A., Krizaj, I., Mesko, P., Kopitar, M., Lucovnik, P., Strukelj, B., Pungercar, J., Buttle, D.J., Barrett, A.J., & Turk, V. (1990). The amino acid sequence of a novel inhibitor of cathepsin D from potato. *FEBS Letters, 267*, 13-15.

Salvesen, G., Parkes, C., Abrahamson, M., Grubb, A., & Barrett, A.J. (1989). Human low-Mr kininogen contains three copies of a cystatin sequence that are divergent in structure and in inhibitory activity for cysteine proteinase. *Biochemical Journal, 234*, 429-434.

Steel, R.G.D., & Torrie, J.H. (1980). *Principles and Procedures of Statistics; A Biometrical Approach*. New York: McGraw-Hill.

Thomas, D., Richards, A., & Kay, J. (1989). Inhibition of aspartic proteinases by alpha-2-macroglobulin. *Journal of Biochemistry, 259*, 905-907.

Vacconcelos, I.M., Siebra, E.A., Maia, A.A.B., Moreira, R.A., Neto, A.F., Campelo, G.J.A., & Oliveira, J.T.A. (1997). Composition, toxic and antinutritional factors of newly developed cultivars of Brazilian soybean (*Glycine max*). *Journal of the Science of Food and Agriculture, 75*, 419-426.

Whitaker, J.R. (1994). Principles of Enzymology for the Food Science. (2nd Ed.), New York: Marcel Dekker.

Xavier-Filho, & Campos, F.A.P. (1989). Proteinase inhibitors. In P.R. Cheeke (Ed.), Toxicants of plant origin, Vol III-Proteins and amino acids (pp. 1-27). FL, USA: CRC Press.

Ylonen, A., Rinne, A., Herttuainen, J., Bogwald, J., Jarvinen, M., & Kalkkinen, N. (1999). Atlantic salmon (*Salmo salar* L.) skin contains a novel kininogen and another cysteine proteinase inhibitor. *European Journal of Biochemistry, 266,* 1066-1072.

Yoshizaki, L., Troncoso, M.F., Lopes, J.L.S., Hellman, U., Beltramini, L.M., & Wolfenstein-Todel, C. (2007). *Calliandra selloi* Macbride trypsin inhibitor: Isolation, characterization, stability, spectroscopic analyses. *Phytochemistry, 68,* 2625-2634.