CHAPTER 1

INTRODUCTION

Pla-duk-ra is Thai dry fermented fish product, which is produced by mixing eviscerated fish, sugar and salt at the ratio of 25:1:1 (w/w). The mixture is marinated and dried for 5-6 days at room temperature (30-33°C) (Klomklao et al., 2010a). The Pla-duk-ra processing industry is becoming increasingly important since it is one of the income generators for Phatthalung province. Hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) is one of the raw materials commonly used for Pla-duk-ra production. During processing, by-products such as the viscera, head, bones and frames are generated in the large quantity. These solid wastes constitute 60-70% of original raw material which pose the great disposal problems. However, these abundant underutilized materials are a rich source of enzymes, especially proteases (Klomklao, 2008). Hence, isolation and recovery of proteinases from hybrid catfish viscera may contribute significantly to reducing local pollution problem and increase valuable product from catfish processing wastes. Moreover, it might be utilized in food industry or other industries.

Trypsin (EC 3.4.21.4) is known as the serine endoprotease that hydrolyze peptide bonds at the carboxylic end of the amino acid residues arginine (R) and lysine (K) (Klomklao et al., 2009a) and plays major roles in biological process including digestion, activation of zymogens of chymotrypsin and other enzymes (Klomklao et al., 2009a). Trypsins have been isolated and characterized thoroughly based on their physiochemical and enzymatic properties from several fish species, e.g. carp (*Cyprinus carpio*) (Cao et al., 2000), tambaqui (*Colossoma macropomum*)

(Bezerra et al., 2001), true sardine (*Sardinops melanostictus*) and arabesque greenling (*Pleuroprammus azonus*) (Kishimura et al., 2006), chinook salmon (*Oncorhynchus tshawytscha*) (Kurtovic et al., 2006), skipjack tuna (*Katsuwonus pelamis*) (Klomklao et al., 2007), mandarin fish (*Siniperca chuatsi*) (Lu et al., 2008) and walleye pollock (*Theragra chalcogramma*) (Kishimura et al., 2008). Klomkalo et al. (2009b) isolated and characterized trypsin from the pyloric ceca of pectoral rattail (*Coryphaenoides pectoralis*). Recently, hybrid catfish viscera has been reported to possess high proteolytic activity and those proteinases were classified to be trypsin-like serine proteinases with maximal activity at pH 9.0 and 50°C (Klomklao et al., 2010a). However, molecular and the biochemical characteristics of trypsin in hybrid catfish viscera, have not been investigated. The objectives of this study were to purify and to characterize the trypsin from hybrid catfish viscera.

CHAPTER 2

LITERATURE REVIEW

1. Proteolytic enzyme

Protease is the generic name given to those enzymes hydrolyzing the peptide bond in proteins and some synthetic substrate and coded as the EC 3.4.11-99. Proteases, including peptidases and proteinases, are polyfunctional enzymes catalyzing the hydrolytic degradation of proteins (Garcia-Carreno and Hernandez-Cortes, 2000). Proteases can be classified based on their similarities to well characterized proteases, as trypsin-like, chymotrypsin-like, etc., their pH activity profiles as acid, neutral or alkaline proteases, substrate specificity and mechanism of catalysis (Haard and Simpson, 1994).

1.1 Classification of proteases

Proteases are classified according to their source (animal, plant, microbial), their catalytic action (endopeptidase or exopeptidase) and the nature of the catalytic site. In EC system for enzyme nomenclature, all proteases (peptide hydrolyses) belong to subclass 3.4, which is further divided into 3.4.11-19, the exopeptidases and 3.4.21-24, the endopeptidases or proteinases (Nissen, 1993). Endopeptidases cleave the polypeptide chain at particularly susceptible peptide bonds distributed along the chain, whereas exopeptidases hydrolyze one amino acid from N terminus (amino peptidases) or from C terminus (carboxypeptidases).

1.1.1 Endopeptidases

The four major classes of endopeptidases are serine proteinases (EC 3.4.21), cysteine proteinases (EC 3.4.22), aspartic proteinases (EC 3.4.23) and metalloproteinases (EC 3.4.24) (Nissen, 1993). Serine, cysteine and aspartic proteinases have serine, cysteine and aspartic acid side chains, respectively, as a part of the catalytic site. Modification or blocking of this side chain usually leads to complete inactivation of the enzyme and is a standard way of determining the nature of an unknown proteinase (Nissen, 1993). The serine proteinases have maximal activity at alkaline pH, while the closely related cysteine proteinases usually show maximal activity at more neutral pH values. The aspartic proteinases generally have maximal catalytic activity at acidic pH. Among the digestive enzymes, the aspartic proteinase pepsin is secreted in the stomach and the serine proteinases, trypsin and chymotrypsin, are excreted in the duodenum, in accordance with pH values of the digestive tract (acid in the stomach and alkaline in the gut) (Nissen, 1993). The metalloproteinases contain an essential metal atom, usually Zn and have optimal activity near neutral pH. Ca^{2+} generally stabilizes these enzymes and the strong chelating agent, such as EDTA inhibits the activity (Nissen, 1993).

1.1.2 Exopeptidases

The aminopeptidases (EC 3.4.11) are ubiquitous, but less readily available as commercial products, since many of them are intracellular or membrane bound. Carboxypeptidases are subdivided into serine carboxypeptidases (EC 3.4.16), metallocarboxypeptidases (EC 3.4.17) and cysteine carboxypeptidases (EC 3.4.18) according to the nature of the catalytic site (Nissen, 1993).

2. Fish digestive proteinases

Fish viscera or internal organs are a relatively large portion of the animal round mass; approximately 5% (Gildberg, 1992). Fish digestive organs are the important sources of enzymes, especially proteinases. Digestive proteinases from marine animals are produced by the digestive glands of marine animals. Likes the proteinases from plants, animals and microorganisms, digestive proteinases from marine animals are hydrolytic in their action and catalyze the cleavage of peptide bonds with the participation of water molecules as reactants (Simpson, 2000). In addition to its adequate nutritional value, it contains high levels of digestive enzymes, making it a suitable source for recovering proteinases for food application. Digestive proteinases have been studied in several species of fish (Vecchi and Coppes, 1998) and decapods (Garcia-Carreno and Haard, 1993). Proteinases found in the intestine of fish include trypsin, chymotrypsin, collagenase, elastase, carboxypeptidase and carboxyl esterase, and they are normally secreted from the pyloric ceca and pancreas (Haard, 1994). Pepsin and trypsin are two main groups of proteinases found in fish viscera. Pepsin is found in fish stomach and is active at acidic conditions (Gildberg et al., 1990), while trypsin is concentrated in pyloric cecum and active at neutral and alkaline condition (Asgeirsson et al., 1989). Pepsin and trypsin were also detected in the belly cavity adjacent to muscle of fresh fish (Klomklao, 2008). Gildberg (1992) suggested that the leakage of digestive proteinases into the belly cavity of fish can activate collagenases present in the connective tissue as well as directly initiate collagen degradation by digestive collagenase. Certain types of feed in the digestive tract can trigger post-mortem autolysis known as “feedy fish”. Contaminated organ

tissues can be a source of proteinase in minced fish and surimi, which causes the gel softening (modori) (Su et al., 1981).

The distribution of proteinase varies, depending on species and organs. Torrissen (1984) reported that proteinase activity from intestine of rainbow trout (*Salmo gairdneri*) was higher than that of Atlantic salmon (*Salmo salar*). Pyloric ceca of chinook salmon (*Oncorhynchus tschawytscha*) had a higher proteinase activity than that of rainbow trout (Dimes et al., 1994). For discus fish (*Symphysodon aequifasciata*), proteinase activity in intestine was higher than that in stomach (Chong et al., 2002).

2.1 Classification of digestive proteinases from marine animals

2.1.1 Acid/Aspartyl proteinases

The acid or aspartyl proteinases are a group of endopeptidases characterized by high activity and stability at acidic pH. They are referred to as "aspartyl" proteinases (or carboxyl proteinases) because their catalytic sites are composed of the carboxyl group of two aspartic acid residues (Whitaker, 1994). Based on the EC system, all the acid/aspartyl proteinases from marine animals have the first three digits in common as EC 3.4.23. Three common aspartyl proteinases that have been isolated and characterized from the stomach of marine animals are pepsin, chymosin, and gastricsin (Simpson, 2000).

Pepsin is assigned the number EC. 3.4.23.1. It has preferential specificity for the aromatic amino acids, phenylalanine, tyrosine, and tryptophan. In the EC system of classification, chymosin (formerly known as rennin) is assigned the

number EC 3.4.23.4. Chymosin has specificity for the aromatic amino acids, phenylalanine, tyrosine, and tryptophan, similar to pepsin. Gastricsin is assigned a code of EC 3.4.23.3. (Simpson, 2000). Pepsin has an extracellular function as the major gastric proteinase. Pepsin, secreted as a zymogen (pepsinogen), is activated by the acid in stomach to an active form (Clarks et al., 1985). Pepsin-like protease with an optimum pH value of 1.7 was reported to be predominant in the stomach of dover sole (Clarks et al., 1985). Haard (1986) reported that the initial rate of hemoglobin digestion by Atlantic cod pepsin was maximal at 35°C and pH 1.9. Fish pepsins were shown to hydrolyze hemoglobin much faster than casein (Gildberg and Raa, 1983). Gueraed and Le Gal (1987) reported that a hexapeptide is the smallest substrate to be hydrolized by fish pepsins. Most fish species contain two or three major pepsins with an optimum hemoglobin digestion at pH between 2 and 4 (Gildberg and Raa, 1983). Gildberg et al. (1990) found that the affinity of cod pepsin, especially pepsin I towards hemoglobin, was lower at pH 2 than at pH 3.5. Furthermore, pH optimum was highly dependent on substrate concentration. Pepsin I and II showed similar pH optima at pH 3.0 at high concentrations of hemoglobin, whereas pepsin I had a maximal activity at pH 3-4 with low substrate concentration.

2.1.2 Serine proteinases

The serine proteinases have been described as a group of endopeptidase with a serine residue in their catalytic site. This family of proteinases is characterized by the presence of a serine residue, together with an imidazole group and aspartyl carboxyl group in their catalytic sites. The activity is inhibited by diisopropylphosphofluoridate (DFP), through reaction with the hydroxyl group of the

active site serine residue (Simpson, 2000). The proteinases in serine subclass all have the same first three digits: EC 3.1.21. Three major serine proteinases purified and well characterized from the digestive glands of marine animals are trypsin, chymotrypsin, and elastase. Trypsin is assigned the code EC 3.4.21.4. Trypsin has a very narrow specificity for the peptide bonds on the carboxyl side of arginine and lysine. Chymotrypsin is assigned a code of EC 3.4.21.1 and it has a much broader specificity than trypsin. It cleaves peptide bonds involving amino acids with bulky side chains and nonpolar amino acids such as tyrosine, phenylalanine, tryptophan, and leucine. Elastase is designated as EC 3.4.21.11. Elastase exhibits preferential specificity for alanine, valine, and glycine (Simpson, 2000).

Serine proteinases, mainly trypsin and chymotrypsin, play a major role in protein digestion (Martinez and Serra, 1989). Fish trypsins are generally stable at alkaline pH. Purified trypsin from hybrid tilapia (*Tilapia nilotica/aurea*) intestines showed a maximal activity at pH 9.0 and 40°C (Shemy and Levin, 1997). Two trypsin-like enzymes, enzyme I and II, isolated from the gut of capelin had the optimum pH at 8.0-9.0 with the optimum temperature at 42°C (Hjelmeland and Raa, 1982). Simpson et al. (1990) reported that Atlantic cod trypsin was most active at pH 7.5 and 40°C against *N*-α-benzoyl-L-Arg-*p*-nitroanilide (BAPNA). In dover sole, the activity at pH 7.0-8.0 was due to trypsin and chymotrypsin-like enzymes, while the maximal activity at pH 9.5-10.5 was due to elastase (Clarks et al., 1985). The optimum pH for hydrolysis of casein by Greenland cod trypsin was 9.0-9.5. Hjelmeland and Raa (1982) found two trypsins from Arctic fish capelin with molecular mass about 28,000 Da. Greenland cod trypsins had the molecular mass of 23,500 Da (Simpson and Haard, 1984). Trypsin A and B from anchovy had molecular

mass of 27,000 and 28,000 Da, respectively (Martinez et al., 1988). Cohen et al. (1981a) reported that molecular mass of carp trypsin was 25,000 Da. A trypsin-like enzyme was reported to be the major form of protease in the digestive organs of Pacific whiting based on the molecular mass, the inhibition by *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK) and the activity toward specific substrates (Cohen et al., 1981b). Guizani et al. (1991) reported that a trypsin from the pyloric ceca of mullet, *Mugil cephalus*, exhibited optimal activity at a pH of 8.0 and at a temperature of 55 °C. It was stable within a pH range of 7.5-9.0.

The proteolytic activities in the gut of three carnivorous fish species, the deepwater redfish, *Sebaster mentella*, the turbot, *Scophthalmus maximus*, and the gilthead bream, *Sparus aurata*, showed optimal activity at a pH range of 9.5-10.0 with the temperature range of 35-40°C (Munilla-Moran and Saborrido-Rey, 1996). Sabapathy and Teo (1993) studied the distribution of trypsin in rabbitfish, *Siganus canalicutus*, and sea bass, *Lates calcarifer*, digestive tract. Trypsin activity was higher in the rabbitfish, in which the enzyme was detected in all regions of the digestive tract. However, sea bass trypsin was confined to the intestine and pyloric ceca.

2.1.3 Thiol/Cysteine proteinases

The thiol or cysteine proteinases are a group of endoproteinases that have cysteine and histidine residues as the essential groups in their catalytic sites. These enzymes require the thiol (-SH) group furnished by the active site cysteine residue to be intact, hence this group is named “thiol” or “cysteine” proteinases. The thiol proteinases are inhibited by heavy metal ions and their derivatives, as well as by alkylating agents and oxidizing agents (Mihalyi, 1978). The first three digits common

to thiol proteinases are EC 3.4.22. An example of a thiol proteinase from the digestive glands of marine animals is cathepsin B, which is designated as EC 3.4.22.1 (Simpson, 2000).

Sovik and Rustad (2006) reported that cathepsin B from viscera had the maximum activity at 50°C in cod (*Gadus morhua*) and saithe (*Pollachius virens*), at 35°C in tusk (*Brosme brosme*) and ling (*Molva molva*) and at 20°C in haddock (*Melanogrammus aeglefinus*), while cathepsin B in liver had the highest activity at 50°C in saithe (*Pollachius virens*) and tusk (*Brosme brosme*), and at 35°C in cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*). Cathepsin B from hepatopancreas (a digestive gland) of carp (*Cyprinus carpio*) had a temperature optimum of 45°C (pH 6) (Aranishi et al., 1997).

2.1.4 Metalloproteinases

The metalloproteinases are hydrolytic enzymes whose activity depends on the presence of bound divalent cations. Chemical modification studies suggest that there may be at least one tyrosyl residue and one imidazole residue associated with the catalytic sites of metalloproteinases (Whitaker, 1994). The metalloproteinases are inhibited by chelating agents such as 1, 10-phenanthroline, EDTA, and sometimes by the simple process of dialysis. Most of the metalloproteinases known are exopeptidases. They all have a common first three digit as EC 3.4.24. The metalloproteinases have been characterized from marine animals (e.g., rockfish, carp, and squid mantle) but have not been found in the digestive glands except in the muscle tissue (Simpson, 2000).

3. Isolation and characterization of fish digestive proteinases

3.1 Isolation and characterization of aspartic proteinase

Fish pepsins have been isolated, purified and characterized intensively. Gildberg and Raa (1983) purified pepsins from stomach of the Arctic capelin (*Mallotus villosus*) by ammonium sulphate precipitation, DEAE-cellulose and Sephadex G-75, respectively. The purified enzyme had a molecular mass of 25,000 Da and displayed optimal activity at pH 3.7 and 37°C using hemoglobin as a substrate. Pepsin from polar cod stomach was isolated by CBZ-D-phenylalanine-TETA- Sepharose 4B (Arunchalam and Haard, 1985). This proteinase had molecular mass of 40,000 Da and exhibited a maximal activity against hemoglobin at pH 2.0 and 37°C. Furthermore, Gildberg et al. (1990) purified pepsin from stomach of Atlantic cod (*Gadus morha*) by ammonium sulfate fractionation, followed by S-Sepharose. Purification fold of 7.9 with a yield of 8.4% was obtained. The enzyme has isoelectric point of 4.1. The optimal pH for hemoglobin hydrolysis was 3.0.

Cathepsin D is another aspartic proteinase found in fish. Capasso et al. (1999) purified cathepsin D from the liver of Antarctic icefish (*Chionodraco hamatus*) by anion-exchange chromatography followed by affinity chromatography on Concanavalin-A Sepharose. The purified enzyme showed a molecular mass of 40 kDa and displayed optimal activity at pH 3.0 using a synthetic chromogenic substrate, Pro-Pro-Thr-Ile-Phe*Nph-Arg-Leu.

3.2 Isolation and characterization of cysteine proteinase

Cathepsin L from carp hepatopancreas was purified by using ammonium sulfate precipitation and a series of chromatographies, in which the

enzyme had an affinity toward Concanavalin A and Cibacron Blue F3GA (Aranishi et al., 1997). Purification fold of 1,096 with a yield of 1% was observed. Its homogeneity was established by a native-PAGE. Two protein bands corresponding to molecular masses of 30,000 Da and 24,000 Da were found on SDS-PAGE. The enzyme exhibited a maximal activity against Z-Phe-Arg-MCA at pH 5.5-6.0 and 50°C. All tested cysteine proteinase inhibitors, TLCK and chymostatin, markedly inhibited its activity, whereas the other serine proteinase inhibitors and metalloproteinase inhibitor showed no inhibitory activity on the enzyme.

Cathepsin S from hepatopancreas of carp (*Cyprinus carpio*) was purified by ammonium sulfate fractionation, followed by SP-Sepharose, Sephacry S-200 and Q-Sepharose, respectively (Pangkey et al., 2000). Purification fold of 300 with a yield of 0.7% was obtained. The molecular mass of purified proteinase was 37,000 Da estimated by SDS-PAGE. It hydrolyzed Z-Phe-Arg-MCA but not Z-Arg-MCA. The optimal pH and temperature for the hydrolysis of Z-Phe-Arg-MCA were 7.0 and 37°C, respectively. This proteinase activity was inhibited by E-64, leupeptin, 5-5'-dithiobis (2-nitro-benzoic acid) and *p*-tosyl-lys-chloromethylketone.

3.3 Isolation and characterization of serine proteinase

A number of studies on serine proteinases from fish viscera have been carried out. Martinez et al. (1988) purified two trypsin-like enzymes (trypsin A and trypsin B) from the pyloric ceca and intestine of anchovy (*Engraulis encrasicholus*) by ammonium fractionation, affinity chromatography (benzamidine-Sepharose 6B) and ion-exchange chromatography (DEAE-Sepharose), respectively. Molecular masses of type A and type B proteinases were estimated to be 27,000 and 28,000 Da,

respectively. Optimum pHs for type A and type B proteinases were 8-9. Their isoelectric points were about 4.9 (trypsin A) and 4.6 (trypsin B) and they had similar amino acid compositions. Trypsin from the pyloric ceca of Greenland cod was purified by ammonium sulfate fractionation and Sepharose 4B affinity column (Simpson and Haard, 1984). The molecular mass of purified enzyme was 23,500 Da. Trypsin revealed optimum pH and temperature of 7.5 and 40°C, respectively. Enzyme was effectively inhibited by trypsin inhibitor. Trypsin from the pyloric ceca of rainbow trout, *Oncorhynchus mykiss*, was also purified and characterized by Kristjansson (1991). The isolated enzyme had an estimated molecular mass of 25,700 Da. The enzyme was stable in the temperature range of 40 - 50°C and at a pH range of 5.4-8.0. However, the thermal stability was shown to be calcium-dependent.

Purified two anionic trypsins (trypsin A and trypsin B) from the hepatopancreases of carp were prepared (Cao et al., 2000). The purification was carried out using a series of chromatographies including DEAE-Sephacel, Ultrogel AcA54 and Q-Sepharose. Trypsin A was purified to homogeneity with a molecular mass of 28 kDa, while trypsin B showed two close bands of 28.5 kDa and 28 kDa on SDS-PAGE. Trypsin A and B showed optimal activity at 40 and 45°C, respectively, and had the optimum pH of 9.0 using Boc-Phe-Ser-Arg-MCA as a substrate. Both enzymes were effectively inhibited by trypsin inhibitors. Quinones (2000) purified trypsin from pyloric ceca and intestinal tissues of the queen snapper, *Etelis occlatus*. The trypsin displayed optimal activity in a pH range of 8.0 - 9.0. The activity was highest at 50°C for pyloric ceca trypsin and 60°C for the intestinal trypsin. The enzyme was inhibited by soybean trypsin inhibitor. Alkaline proteinase from pyloric ceca of tambaqui (*Colossoma macropomum*) was purified by heat treatment,

ammonium sulfate fractionation and Sephadex G-75 (Bezerra et al., 2001), in which purification fold of 51.2 with a yield of 40% was obtained. The optimum pH and temperature of the enzyme were 9.5 and 60°C, respectively.

Trypsin from the pyloric ceca of Monterey sardine (*Sardinops sagax caerulea*) was purified and characterized by Castillo-Yanez et al. (2005). Electrophoresis analysis determined the molecular mass of the enzyme to be 25,000 Da. The optimum pH for activity was 8.0 and maximal stability was observed between 7.0 and 8.0. Activity was optimal at 50°C and loss in activity was observed at higher temperatures. The purified enzyme was partially inhibited by phenylmethyl sulphonyl fluoride (PMSF) and fully inhibited by the soybean trypsin inhibitor and benzamidine, but was not inhibited by the metallo-protease inhibitor, EDTA. Two trypsins, TR-S and TR-P, were purified from the viscera of true sardine (*Sardinops melanostictus*) and the pyloric ceca of arabesque greenling (*Pleuroprammus azonus*) by gel filtration and anion-exchange chromatography (Kishimura et al., 2006). The TR-S and TR-P had maximal activities at around pH 8.0 for hydrolysis of TAME. Optimum temperatures of the TR-S and TR-P were 60°C and 50°C, respectively. Both TR-S and TR-P were stabilized by calcium ion. The N-terminal amino acid sequences were IVGGYECKAYSQPWQVSLNS and IVGGYECTPHTQAHQVSLNS for TR-S and TR-P, respectively.

Additionally, Kurtovic et al. (2006) purified and characterized a trypsin from the pyloric ceca of chinook salmon (*Oncorhynchus tshawytscha*) by ammonium sulfate fractionation, acetone precipitation and affinity chromatography. The molecular mass of the chinook salmon trypsin was estimated to be 28,000 Da by SDS-PAGE. The chinook salmon enzyme was active over a broad pH range (from 7.5

to at least pH 10.0) at 25°C and was stable from pH 4.0 to pH 10 when incubated at 20°C with a maximal at pH 8.0. The optimum temperature for the hydrolysis of BAPNA by this enzyme was 60°C. It was inhibited by PMSF, soybean trypsin inhibitor and benzamidine.

Purification of collagenase from tuna pyloric ceca was carried out by acetone precipitation, gel filtration chromatography on a Sephadex G-100, ion-exchange chromatography on a DEAE-Sephadex A-50 and gel filtration on a Sephadex G-75 (Byun et al., 2003). The purification and yield were 30.5-fold and 0.023%, respectively. The optimum pH and temperature for the purified collagenolytic enzyme were pH 7.5 and 55°C, respectively. The enzyme was strongly inactivated by metal ions (Hg^{2+} and Zn^{2+}) and serine proteinase inhibitors (PMSF, TLCK and soybean trypsin inhibitor). Purification of collagenolytic proteinase from greenshore crab (*Carcimus maenas*) digestive gland was also carried out by acid precipitation and successive column chromatography (Roy et al., 1996). Purification fold of 304 with a yield of 21.8% was obtained. The molecular mass of the enzyme was estimated to be 23,000 Da by SDS-PAGE and its isoelectric point was 4.0. Optimal temperature and pH were 30°C and 7, respectively. It hydrolyzed native collagen (Type I and III). The proteinase was strongly inactivated by PMSF, soybean trypsin inhibitor and elastatinal.

3.4 Isolation and characterization of metalloproteinase

A collagenolytic metalloproteinase with gelatinase activity from carp hepatopancreas was purified by ammonium sulfate fractionation and gel filtration chromatography (Sivakumar et al., 1999). Purification fold of 20.5 was observed. The

enzyme had a molecular mass of 55 kDa and was active against native type I collagen. Optimum temperature and pH were 25°C and 7-7.5. Activity of active enzyme was strongly inactivated by 10 mM EDTA.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

Ethylenediaminetetraacetic acid (EDTA), pepstatin A, soybean trypsin inhibitor, iodoacetic acid, *N*-p-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenyl-alanine chloromethyl ketone (TPCK), 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), *N*-ethylmaleimide, β-mercaptoethanol (βME), porcine pancreatic trypsin and bovine serum albumin were procured from Sigma Chemical Co. (St. Louis, MO, USA.). Sephacryl S-200 and Sephadex G-50 were purchased from Pharmacia Biotech (Uppsala, Sweden). Diethylaminoethyl (DEAE)-cellulose was obtained from Whatman (Maidstone, England). N^{α}-*p*-tosyl-L-arginine methyl ester hydrochloride (TAME) was purchased from Wako Pure Chemicals (Osaka, Japan). Sodium chloride, tris (hydroxymethyl) aminomethane and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250 and *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA).

3.2 Fish sample preparation

Viscera of hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) were obtained from a local market in Phatthalung. Those samples (5 kg) were packed in polyethylene bag, kept in ice and transported to the Department of

Food Science and Technology, Thaksin University, Phatthalung within 30 min. Pooled internal organs were immediately frozen and stored at -20°C until used.

3.3 Preparation of viscera extract

Frozen viscera were thawed using a running water (26-28°C) until the core temperature reached -2 to 0°C. The samples were cut into pieces with a length of 1-1.5 cm and homogenized with three volumes of acetone at -20°C for 30 min using an IKA homogenizer (Model T25, Selangor, Malaysia) according to the method of Klomklao et al. (2010b). The homogenate was filtrated in vacuo on Whatman No. 4 filter paper. The residue obtained was then homogenized in two volumes of acetone at -20°C for 30 min, and then the residue was air-dried at room temperature. Defatted viscera powder obtained was stored at -20°C until used.

To prepare the viscera extract, viscera powder was suspended in 10 mM Tris-HCl, pH 8.0 containing 1 mM $CaCl_2$ referred to as starting buffer (SB) at a ratio of 1:50 (w/v) and stirred continuously at 4°C for 3 h. The suspension was centrifuged for 10 min at 4°C at 10,000×g (H-200, Kokusan, Tokyo, Japan) to remove the tissue debris, and then the supernatant was lyophilized. Before use, the lyophilized sample (10 g) was dissolved with 50 ml of cold distilled water (4°C) and referred to as "crude extract".

3.4 Purification of hybrid catfish viscera trypsin

All purification processes were carried out in a walk-in cold room (4°C). Fractions obtained from all purification steps were subjected to the measurement of protein content and trypsin activity.

Crude extract was subjected to ammonium sulfate precipitation at 30-70% saturation and allowed to stand for 2 h. The precipitate was collected by centrifugation at 25,000g for 20 min. The pellet obtained was dissolved in a minimal volume of SB and dialyzed with 10 volumes of SB with three changes overnight.

The dialysate was then applied to a Sephacryl S-200 column (3.9×64 cm), which was equilibrated with approximately two bed volumes of SB. Sample was loaded onto column and then eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions of 5 ml were collected and those with TAME activity were pooled, lyophilized and further purified by Sephadex G-50 column.

Lyophilized fractions with TAME activity after Sephacryl S-200 column chromatography were dissolved in distilled water and loaded onto a Sephadex G-50 column (3.9×64 cm) previously equilibrated with approximately two bed volumes of SB. The elution was performed with the same buffer at a flow rate of 0.5 ml/min. Fractions of 5 ml were collected and those with TAME activity were pooled and further purified by anion exchanger DEAE-cellulose chromatography.

Pooled fractions with TAME activity from Sephadex G-50 column chromatography were collected and lyophilized. The lyophilized fractions were dissolved in distilled water and dialyzed against SB for 10-12 h. The sample was then chromatographed on DEAE-cellulose (Whatman, England) column (2.2×18 cm) equilibrated with SB. The sample was loaded onto the column at a flow rate of 0.5

ml/min. The column was washed with SB until A_{280} was less than 0.05 and then eluted with a linear gradient of 0.05-0.5 M NaCl in SB at a flow rate of 0.5 ml/min. Fractions of 3 ml were collected and the fractions with TAME activity were pooled, dialyzed with SB for 10-12 h and then concentrated by lyophilization and used for further study.

3.5 Trypsin activity assay

Trypsin activity was measured by the method of Hummel (1959) using TAME as a substrate. Enzyme solution with an appropriate dilution (20 µl) was mixed with 3.0 ml of 1 mM TAME in 10 mM Tris-HCl buffer, pH 8.0 and incubated at 30°C for 20 min. Production of *p*-tosyl-arginine was measured by monitoring the increment in absorbance at 247 nm. One unit of activity was defined as the amount causing an increase of 1.0 in absorbance per min.

3.6 pH and temperature profile

Trypsin activity was assayed over the pH range of 4.0-11.0 (50 mM acetate buffer for pHs 4.0-7.0; 50 mM Tris-HCl buffer for pHs 7.0-9.0 and 50 mM glycine-NaOH for pHs 9.0-11.0) at 30°C for 20 min. For temperature profile study, the activity was assayed at different temperatures (20, 30, 40, 50, 60, 70 and 80°C) for 20 min at pH 8.0.

3.7 pH and thermal stability

The effect of pH on enzyme stability was evaluated by measuring the residual activity after incubation at various pHs for 30 min at 30°C. Different buffers

used was above mentioned. For thermal stability, enzyme solution was diluted with 100 mM Tris-HCl, pH 8.0 at a ratio of 1:1 (v/v) and incubated at different temperatures (20, 30, 40, 50, 60, 70 and 80°C) for 15 min in a temperature controlled water bath (W350, Memmert, Schwabach, Germany). Thereafter, the treated samples were suddenly cooled in iced water. The residual activity was assayed using TAME as a substrate at pH 8.0 and 30°C for 20 min.

3.8 Determination of molecular mass

The molecular mass of purified trypsin was determined using size exclusion chromatography on Sephacryl S-200 column. The trypsin separated on size exclusion chromatography was estimated for its molecular mass by plotting available partition coefficient (K_{av}) against the logarithm of molecular mass of the protein standards. The elution volume (V_e) was measured for each protein standard and the trypsin. Void volume (V_o) was estimated by the elution volume of blue dextran (M_r 2,000,000). The standards used included aprotinin (M_r 6,500), trypsinogen (M_r 24,000), bovine serum albumin (M_r 66,000) and catalase (M_r 232,000) (Sigma Chemical Co., St. Louis, MO, USA.).

3.9 Effect of $CaCl_2$ on thermal stability

The effect of $CaCl_2$ on thermal stability was determined by heating the enzyme dissolved in 50 mM Tris-HCl, pH 8.0 in the presence of 10 mM EDTA or 10 mM $CaCl_2$, at 30°C for different times (0, 0.5, 1, 2, 4, 6 and 8 h). At the time designated, the samples were cooled in iced water and assayed for remaining activity.

3.10 Effect of NaCl

Effect of NaCl on trypsin activity was studied. NaCl was added into the standard reaction assay to obtain the final concentrations of 0, 5, 10, 15, 20, 25 and 30% (w/v). The residual activity was determined at 30°C and pH 8.0 for 20 min using TAME as a substrate.

3.11 Effect of inhibitors

The effect of inhibitors on trypsin activity was determined according to the method of Klomklao et al. (2004) by incubating enzyme solution with an equal volume of proteinase inhibitor solution to obtain the final concentration designated (0.1 mM E-64, 1 mM *N*-ethylmaleimide, 1 mM iodoacetic acid, 1.0 g/l soybean trypsin inhibitor, 5 mM TLCK, 5 mM TPCK, 1 mM pepstatin A and 10 mM EDTA). The mixture was allowed to stand at room temperature (26-28°C) for 15 min. Thereafter, the remaining activity was measured and percent inhibition was calculated.

3.12 Polyacrylamide 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 (0.125M Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 10% β-mercaptoethanol) and boiled for 3 min. The samples (15 μg) were loaded on the gel made of 4% stacking and 12.5% 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 0.1% Coomassie brilliant blue R-250 in 50% methanol and 7% acetic acid and destained with 7% acetic acid.

Native-PAGE was performed using 12.5% separating gels in a similar manner, except that the sample was not heated and SDS and reducing agent was left out.

3.13 Determination of N-terminal amino acid sequence

The purified enzyme was subjected to SDS-PAGE under reducing conditions and electrophoretically transferred to polyvinylidenedifluoride (PVDF) membrane. After the membrane was briefly stained by Coomassie brilliant blue, the band of protein was applied to a protein sequencer, Procise 492 (Perkin-Elmer, Foster, CA, USA).

3.14 Kinetic studies

The activity was assayed with different final concentrations of TAME ranging from 0.01 to 0.10 mM. The final enzyme concentration for the assay was 0.1 mg/ml. The determinations were repeated twice and the respective kinetic parameters including V_{max} and K_m were evaluated by plotting the data on a Lineweaver-Burk double-reciprocal graph (Lineweaver and Burk, 1934). Values of turnover number (K_{cat}) were calculated from the following equation: $V_{max}/[E] = K_{cat}$, where [E] is the active enzyme concentration.

3.15 Protein determination

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

3.16 Statistical analysis

Data was subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple-range test (Steel and Torrie, 1980).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Purification of trypsin from hybrid catfish viscera

Purification of trypsin from the hybrid catfish viscera is summarized in Table 1. The trypsin purification was started by precipitating the crude extract using 30-70% ammonium sulfate. The ammonium sulfate precipitation process was able to fractionate the enzyme of interest. After this step, the purity was increased by 2.6-fold with 76.7% yield. Klomklao et al. (2009b) reported that ammonium sulfate precipitation of trypsin from the pyloric ceca of pectoral rattail at 30-70% resulted in the increase in specific activity by 6-fold.

Ammonium sulfate-treated fraction was subsequently separated on Sephacryl S-200 column chromatography. Sephacryl S-200 column separated trypsin from other proteins with lower molecular mass (Fig. 1a). Total activity of approximately 56.9% was retained and purification fold of 7.1 was obtained. Size exclusion chromatography was used to remove other proteins in tambaqui pyloric ceca trypsin fraction, leading to the higher purity of trypsin (Marcuschi et al., 2010).

To refine the pooled fraction obtained from previous step, pooled active fractions were subjected to a Sephadex G-50 column (Fig. 1b). Purification of 15.9-fold with a yield of 28.9 was obtained. Klomklao et al. (2010c) found that the use of Sephadex G-50 for the purification of trypsin from Pacific saury pyloric ceca led to an increase in activity by 90.0-fold.

Pooled active Sephadex G-50 fractions were further purified using a DEAE-cellulose column. Pooled Sephadex G-50 fractions were dialyzed against 10

mM Tris-HCl, pH 8.0 containing 1 mM $CaCl_2$ for 24 h at 4°C, prior to loading onto an ion exchanger, DEAE-cellulose. After loading and washing, the column was eluted by a 0.05 – 0.5 M NaCl linear gradient. The single trypsin activity peak was found (Fig. 1c). After DEAE-cellulose chromatography, a large amount of contaminated proteins was removed, resulting in a substantial increase in purification fold. Purity of trypsin was increased by 47.6-fold with the yield of 12.7%. Klomklao et al. (2009a) purified two trypsins from the intestine of skipjack tuna by using anion exchange, DEAE-cellulose column and the purification folds of 177 and 257 were obtained.

Table 1

Purification of trypsin from hybrid catfish viscera

Purification steps	Total activity (units)*	Total protein (mg)	Specific activity (units/mg protein)	Purity (fold)	Yield (%)
Crude extract	526.5	2890	0.18	1.0	100.0
$(NH_4)_2SO_4$ (30-70%)	403.6	854.2	0.47	2.6	76.7
Sephacryl S-200	299.7	233.6	1.28	7.1	56.9
Sephadex G-50	152.0	53.1	2.86	15.9	28.9
DEAE-cellulose	66.8	7.8	8.56	47.6	12.7

*Trypsin activity was assayed at pH 8.0, 30°C for 20 min using TAME as a substrate.

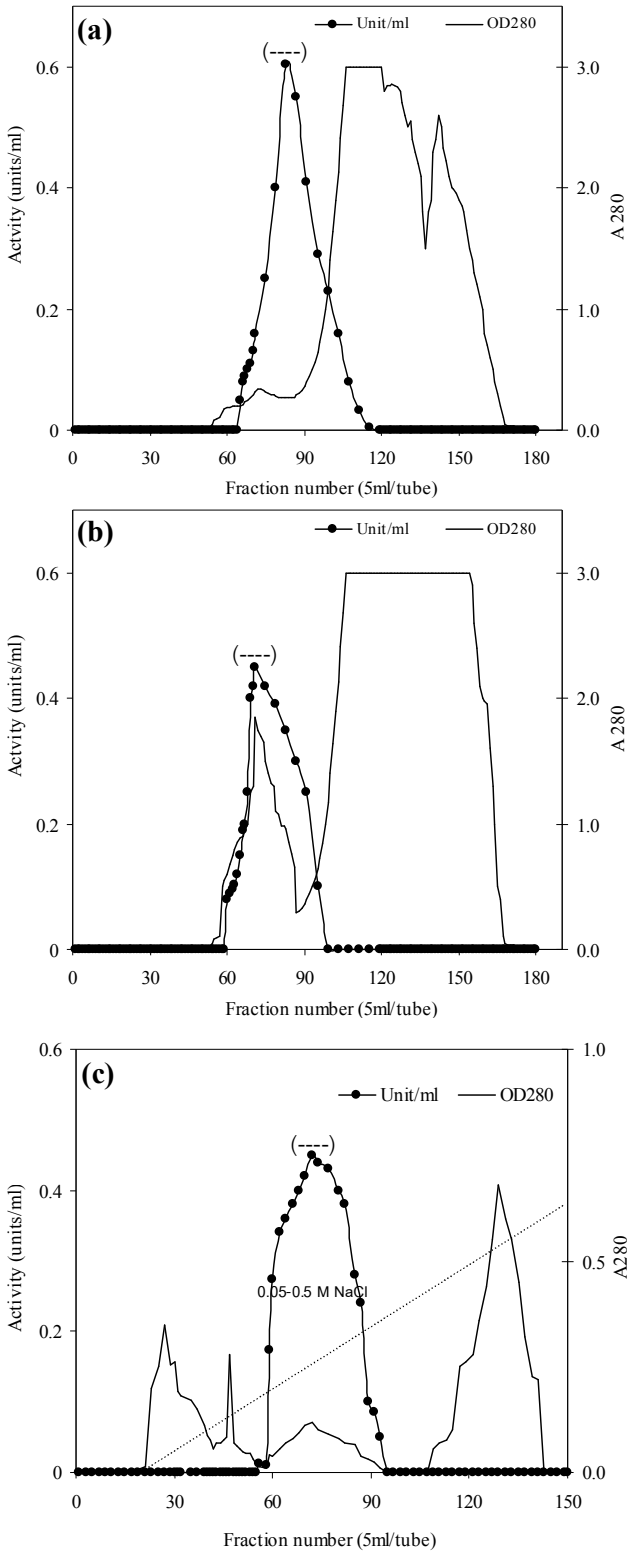

Fig.1. Elution profiles of trypsin from hybrid catfish viscera on Sephacryl S-200 column (a); Sephadex G-50 column (b) and DEAE-cellulose column (c). Elution was carried out with a linear gradient of 0.05-0.5 M NaCl in SB.

4.2 Purity and molecular mass

Purity of the purified trypsin was determined by using native gel-electrophoresis. There was only one protein band (Fig. 2a), indicating the purity of enzyme. Also, trypsin showed a single band on SDS-PAGE. Molecular mass estimated from the mobilities in SDS-PAGE was 24 kDa for trypsin, corresponding with that determined by gel filtration on Sephacryl S-200 (Fig. 3). The results suggested that trypsin is the monomeric protein with a molecular mass of 24 kDa. The molecular mass of trypsin was similar to those of mammalian and fish trypsin. Generally, trypsins have been reported to have molecular masses between 20 and 25 kDa (Klomklao, 2008). However, those from marine organisms have molecular masses in the range of 24-30 kDa. Trypsin from the pyloric ceca of tambaqui had a molecular mass of 23.9 and 27.5 kDa as estimated by mass spectrum and SDS-PAGE, respectively (Marcuschi et al., 2010). The molecular mass of trypsin from the pyloric ceca of Pacific saury was determined to be 24 kDa using Sephacryl S-200 and SDS-PAGE (Klomklao et al., 2010c). Klomklao et al. (2009b) found that the trypsin purified from the pyloric ceca of pectoral rattail had an apparent molecular mass of 24 kDa when analyzed using SDS-PAGE and gel-filtration. Molecular masses of four trypsins from hepatopancreas of crayfish were determined by gel filtration to be approximately 23.8, 27.9, 24.8 and 31.4, respectively (Kim et al., 1994). Cao et al. (2000) reported that the molecular masses of two trypsin enzymes from carp hepatopancreas were approximately 28.5 and 28 kDa by SDS-PAGE and gel filtration, respectively. The differences of molecular mass in trypsins may be due to genetic variation among species (Klomklao et al., 2007). However, the possibility that

these differences are caused by autolytic degradation should not be excluded (Lu et al., 2008).

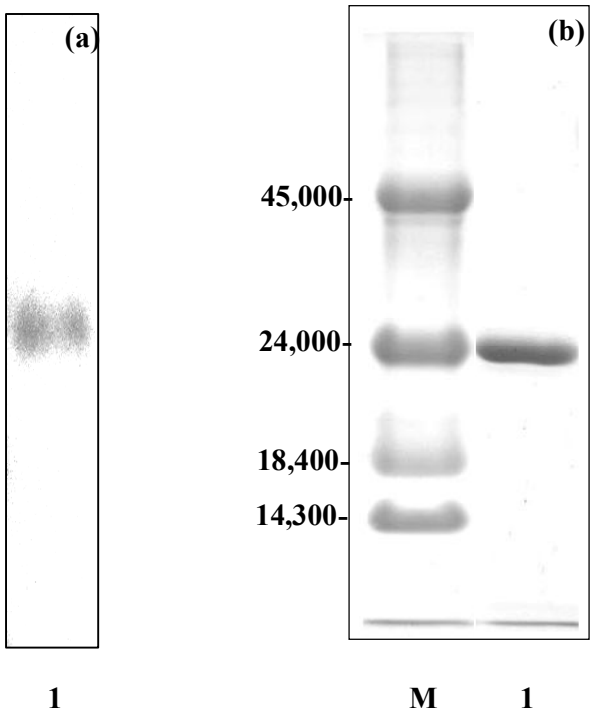

Fig. 2. Protein pattern from native-PAGE (a) and SDS-PAGE (b) of purified trypsin from hybrid catfish viscera. M, molecular mass standard; lane 1, trypsin.

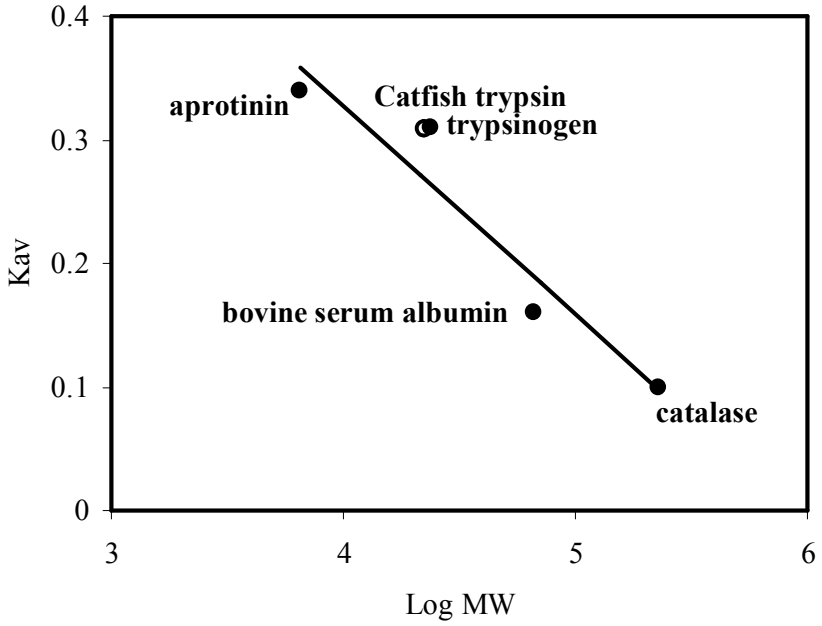

Fig. 3. Calibration curve for the molecular mass determination of the purified trypsin from hybrid catfish viscera on Sephacryl S-200 chromatography.

4.3 pH and temperature profile

The effect of pH on the activity of trypsin from the viscera of hybrid catfish was determined over pH range of 4.0-11.0 as shown in Fig. 4a. The trypsin was highly active between pH 7.0-9.0 with a relative activity more than 50%. The optimal pH for hydrolysis of TAME by trypsin was found at pH 8.0, which was in accordance with those of trypsins from the pyloric ceca of Monterey sardine (Castillo-Yanez et al., 2005), the intestine of Nile tilapia (Bezerra et al., 2005) and the pyloric ceca of chinook salmon (Kurtovic et al., 2006). Activity of trypsin generally decreased at pHs higher and lower than the optimal pH. This was possibly due to the conformational changes of enzyme under harsh condition, resulting in the lower activity (Klomklao et al., 2010c).

Temperature profile of trypsin from the viscera of hybrid catfish is depicted in Fig. 4b. The optimal temperature of trypsin was 60°C when TAME was used as a substrate. The optimal temperature of purified trypsin was similar to that of trypsin from pyloric ceca of chinook salmon with a temperature optimum of 60°C (Kurtovic et al., 2006). Klomklao et al. (2009a) also reported that two isoforms of trypsin from the intestine of skipjack tuna exhibited maximal activity at 55-60°C. However, this optimal temperature of trypsin purified from hybrid catfish viscera was higher than those of trypsins obtained from other species, such as Mandarin fish (35-40°C) (Lu et al., 2008), tambaqui (50°C) (Marcuschi et al., 2010) and pectoral rattail (45°C) (Klomklao et al., 2009b). The differences in enzyme conformation were governed by habitat, environment and genetic (Klomklao, et al., 2006a). A sharp decrease in activity of hybrid catfish trypsin at temperature above 70°C was most likely owing to thermal denaturation.

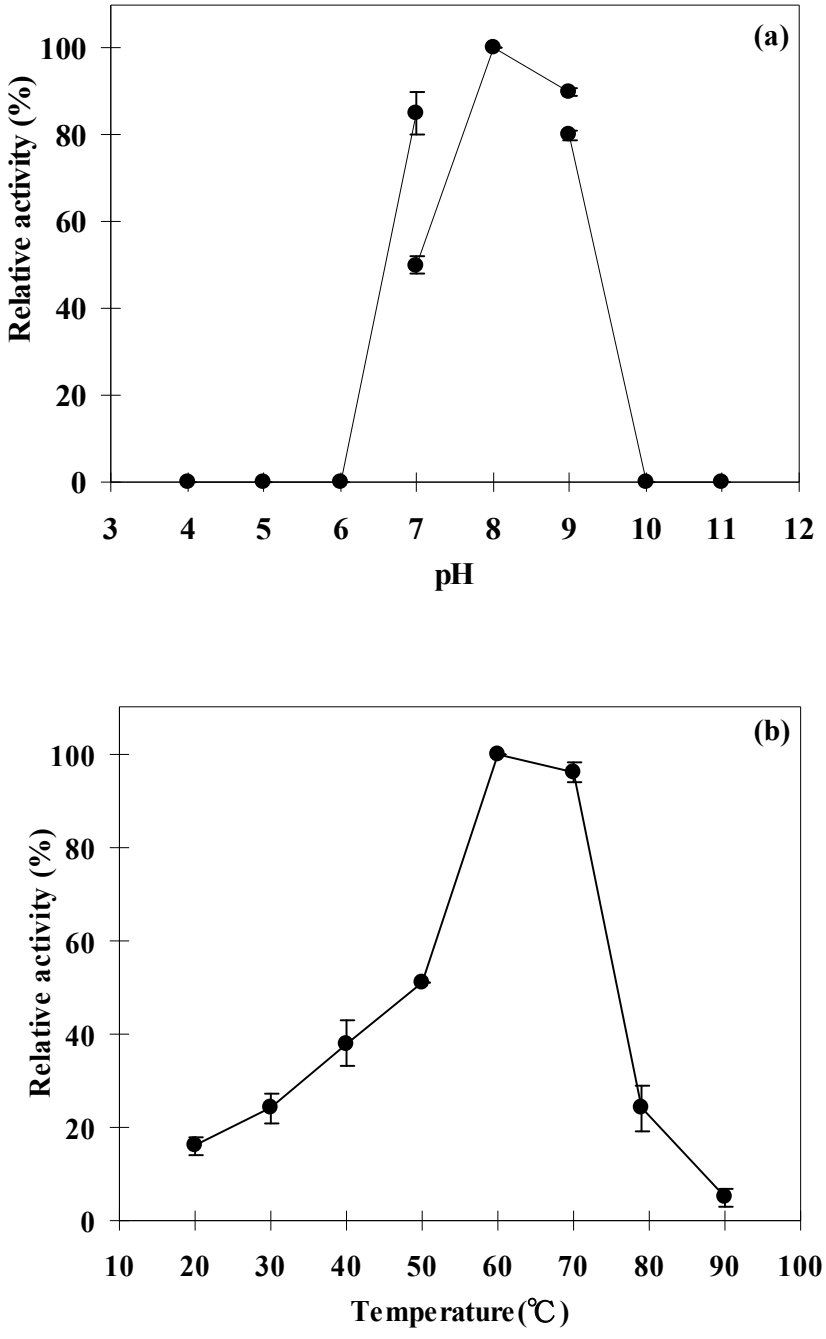

Fig. 4. pH (a) and temperature (b) profiles of purified trypsin from hybrid catfish viscera. Bars indicate standard deviation from triplicate determination.

4.4 pH and thermal stability

The pH stability of trypsin from the viscera of hybrid catfish is shown in Fig. 5a. Hybrid catfish trypsin was stable in the pH ranging from 6.0 to 11.0. However, the marked decrease in activity was noticeable at pH below 5.0. No activity of trypsin was found after incubation at pH 4.0. The stability of trypsin at a particular pH might be related to the net charge of the enzyme at that pH (Castillo-Yanez et al., 2005; Klomklao et al., 2010b). Diminished stability at acidic pHs was observed for trypsin from several fish species (Kishimura et al., 2005; Klomklao et al., 2009a; 2009b; 2010a). The trypsin might undergo the denaturation under the acidic condition, where the conformational change took place and enzyme could not bind to the substrate properly.

Thermal stability of trypsin from the viscera of hybrid catfish is depicted in Fig. 5b. Trypsin purified from hybrid catfish viscera was stable for 15 min when heated up to 50°C (Fig. 5b). No trypsin activities were remained after the heat treatment at 80°C. After heat treatment at 60°C, activities of approximately 60% were retained. At high temperature, enzyme possibly underwent denaturation and lost their activity. Klomklao et al. (2009b) found that the trypsin activity of pectoral rattail pyloric caeca was stable up to 40°C and activity almost disappeared when heated at temperature higher than 70°C. Trypsins from the pyloric ceca of Mandarin fish was stable at temperature ranging from 20 to 45°C for 30 min but activity was rapidly lost at temperature above 45°C (Lu et al., 2008). Kurtovic et al. (2006) reported that trypsin from the pyloric ceca of chinook salmon was unstable at temperatures above 40°C. Enzymes are inactivated at high temperature due to the partial unfolding of the enzyme molecule. The mechanism for increasing thermal stability of proteins appears

to be due to strengthening of hydrophobic interactions and disulfide bond in the interior of the protein molecule (Kim et al, 1994). Numerous disulfide linkages, as well as stronger hydrophobic interactions in the interior of the protein contribute to protein thermal stability. Disulfide bonds may stabilize a folded conformation (Klomklao et al., 2009a). Fish enzyme was less stable than that from the bovine at temperatures above 40°C, possibly caused by the lower number of maximal intramolecular disulfide bonds in fish trypsin, compared with bovine trypsin (Klomklao et al., 2009a).

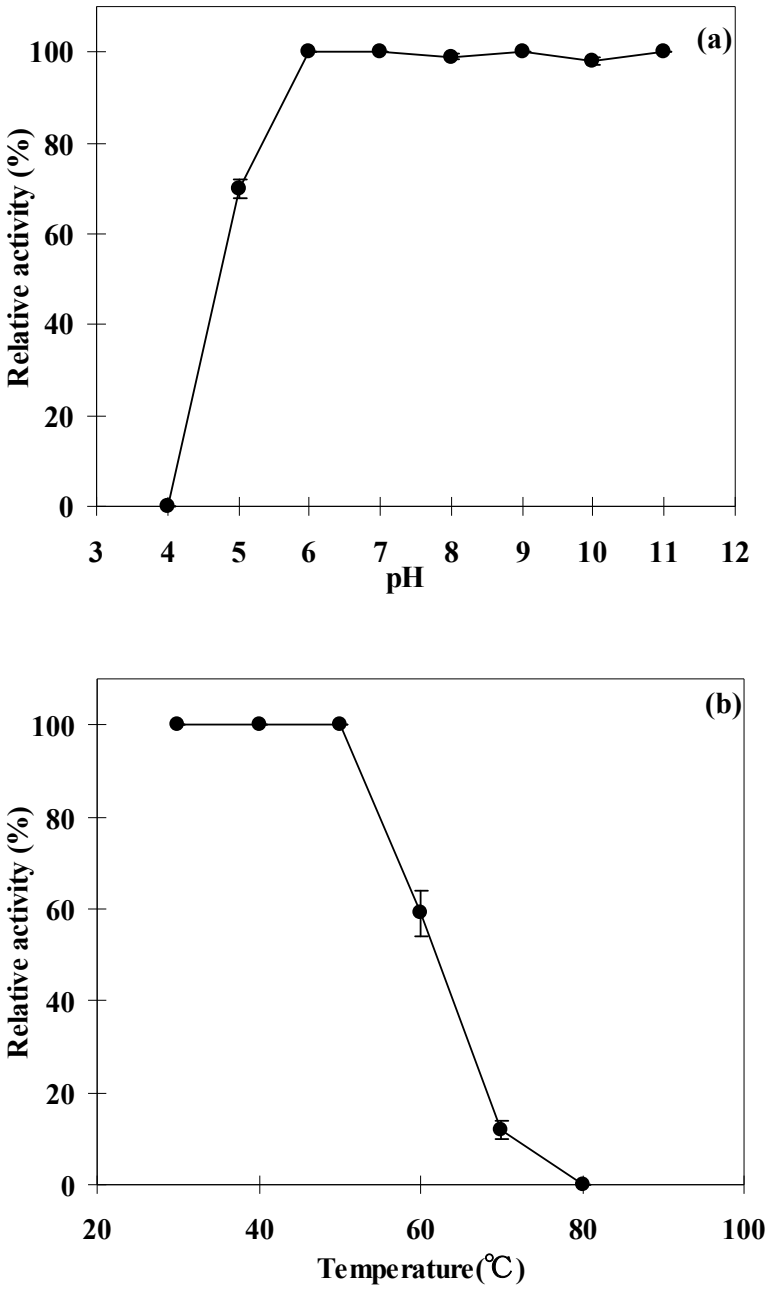

Fig. 5. pH (a) and thermal (b) stability of purified trypsin from hybrid catfish viscera. Bars indicate standard deviation from triplicate determination.

4.5 Effect of calcium ions on the thermal stability

The effect of $CaCl_2$ on the thermal stability of trypsin from hybrid catfish viscera was examined at pH 8.0 and 40°C. Trypsin was highly stable in the presence of calcium (Fig. 6). In the presence of 10 mM EDTA, the activity decreased with increasing incubation time. However, in the presence of 10 mM calcium ion, total activity was remained after 8 h of incubation at 40°C. These results indicated that trypsin from hybrid catfish viscera was most likely stabilized by calcium ion. The presence of calcium ions activates trypsinogen to trypsin and increases the thermal stability of the enzyme. This stabilizing effect is accomplished by a conformational change in the trypsin molecule, resulting in a more compact structure (Simpson, 2000; Klomklao et al., 2004). Stabilization against thermal inactivation by calcium was also reported for the trypsins from true sardine (Kishimura et al., 2006), skipjack tuna (Klomklao et al., 2009a) and Pacific saury (Klomklao et al., 2010c). However, calcium ion did not show the enhancing effect on stability of trypsins from sardine (Murakami and Noda, 1981), capelin (Hjelmeland, and Raa, 1982) and Nile tilapia (Bezerra et al., 2005). These findings suggest a difference in the structure of the primary calcium binding site among different marine fish trypsins.

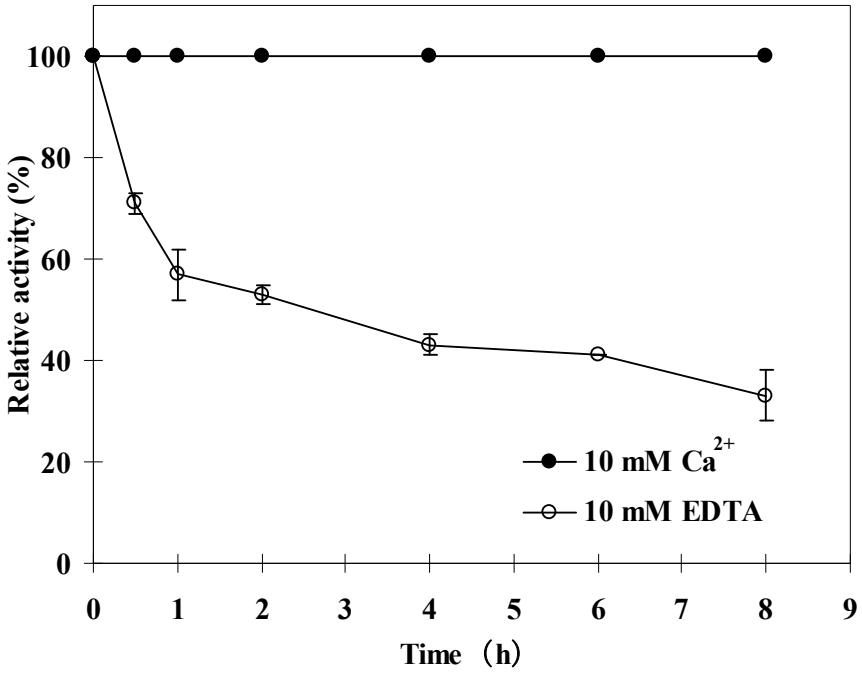

Fig. 6. Effect of calcium ion and EDTA on the stability of purified trypsin from hybrid catfish viscera. The enzymes were kept at 30°C for 0-8 h in the presence of 10 mM $CaCl_2$ or 10 mM EDTA and then the remaining activities at 30°C were determined. Bars indicate standard deviation from triplicate determination.

4.6 Effect of NaCl

Trypsin activity continuously decreased with increasing NaCl (Fig. 7). The activity in the presence of 30% NaCl was approximately 60% of that without NaCl. The decrease in activity might be due to the denaturation of enzymes. It could be described by the salting out phenomenon. An increase in ionic strength causes a reduction in enzyme activity by an enhanced hydrophobic-hydrophobic interaction between protein chains, and the competition for water of ionic salts, leading to the induced enzyme precipitation (Klomklao et al., 2009a). At 25% NaCl, remaining activity of trypsin was approximately 65%. Therefore, trypsin from hybrid catfish viscera may have a poteintial in accelerating the hydrolysis of high-salt products, such as fish sauce.

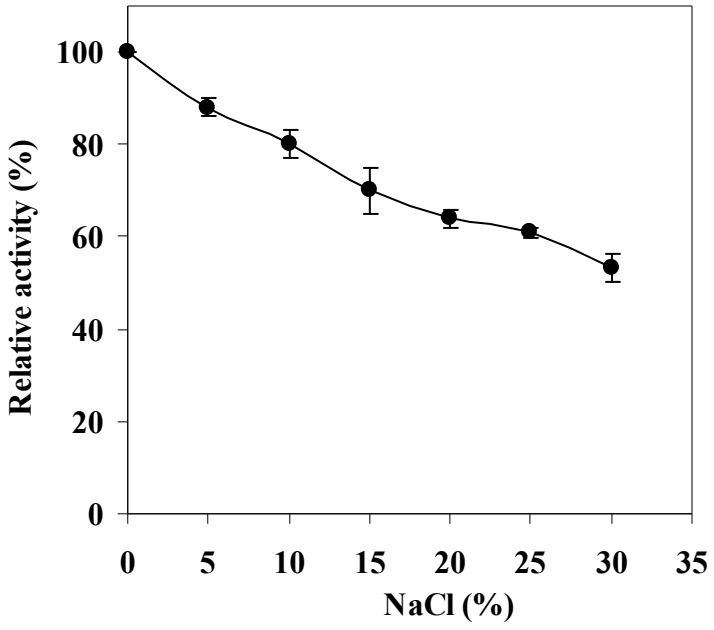

Fig. 7. Effect of NaCl concentrations on activities of purified trypsin from hybrid catfish viscera. Bars indicate standard deviation from triplicate determination.

4.7 Effect of inhibitors

Effect of various inhibitors on activity of trypsin from hybrid catfish viscara was shown in Table 2. Trypsin was markedly inhibited by trypsin inhibitors such as soybean trypsin inhibitor (92.61 %inhibition) and TLCK (89.98 %inhibition). Inhibitors for cysteine- and aspartic proteinases involving E-64, *N*-ethylmaleimide, iodoacetic acid and pepstatin A did not show inhibitory effects towards trypsin activity. No inhibition was also observed when TPCK, a specific inhibitor for chymotrypsin, was used. The result confirms that this purified enzyme was serine proteinase, most likely trypsin. Kurtovic et al. (2006) reported that the activity of trypsin from the pyloric ceca of chinook salmon was inhibited by the general serine proteinase inhibitor, phenyl methyl sulfonyl fluoride, and also by the specific trypsin inhibitors, soybean trypsin inhibitor and benzamidine. The enzymatic activity of trypsin from the pyloric ceca of tambaqu significantly decreased in the presence of TLCK, benzamidine and PMSF (Marcuschi et al., 2010). EDTA, which chelates the metal ions required for enzyme, partially lowered trypsin activity (13.52 %inhibition). Removal of calcium ion might affect enzyme structure, resulting in some losses in activity. The result suggests that trypsin most likely required metal ions as cofactors for activity.

Table 2

Effect of various inhibitors on the activity of purified trypin from hybrid catfish viscera *

Inhibitors	Concentration	% Inhibition
Control		0
E-64	0.1 mM	0
N-ethylmaleimide	1 mM	0
Iodoacetic acid	1 mM	0
Soybean trypsin inhibitor	1.0 g/l	92.61±3.1
TLCK	5 mM	89.98±0.16
TPCK	5 mM	0
Pepstatin A	1 mM	0
EDTA	10 mM	13.52±4.01

*Enzyme solution was incubated with the same volume of inhibitor at 25°C for 15 min and the residual activity was determined using TAME as a substrate for 20 min at pH 8.0 and 30°C.

4.8 Kinetic study

Kinetic constants, K_m and K_{cat} for TAME hydrolysis by purified hybrid catfish viscera trypsin were determined using Lineweaver-Burk plots (Table 3), along with the corresponding values for other fish trypsin. K_m and K_{cat} of hybrid catfish viscera trypsin were calculated to be 0.3 mM and 92.1 S^{-1}, respectively. The K_m obtained for hybrid catfish trypsin was slightly lower than those reported for trypsin from crayfish (Kim et al., 1994). However, the enzyme had higher K_m value than trypsin from Pacific saury (Klomklao et al., 2010c), skipjack tuna (Klomklao et al., 2009a) and pectoral rattail (Klomklao et al., 2009b). Therefore, purified trypsin exhibited the higher affinity to TAME than those from crayfish but less than those from Pacific saury, skipjack tuna and pectoral rattail. The catalytic efficiency (K_{cat}/K_m) of trypsin from viscera of hybrid catfish was 307 $S^{-1}mM^{-1}$ which was close to trypsin from skipjack tuna but lower than trypsin from Pacific saury (Klomklao et al., 2010c) and pectoral rattail (Klomklao et al., 2009b). This results suggest that trypsin from hybrid catfish viscera would be less efficient in transforming the substrate to product. However, the catalytic efficiency of hybrid catfish viscera trypsin was higher than those of mammalian trypsins reported (Simpson, 2000).

Table 3

Kinetic parameters of trypsin from viscera of hybrid catfish and other fish trypsins using TAME as substrate

Enzyme	K_m (mM)	K_{cat} (S^{-1})	K_{cat} / K_m (S^{-1} mM^{-1})
Hybrid catfish	0.30±0.01	92.1±0.01	307
Pacific saury[a]	0.17	200	1176.47
Skipjack tuna[b]	0.22	82.5	266.13
Pectoral rattail[c]	0.15	210	1400
Crayfish[d]	0.33	200	590

K_m, K_{cat} values were determined using TAME as a substrate at pH 8.0 and 30°C. [a]Klomklao et al. (2010c); [b]Klomklao et al. (2009a); [c]Klomklao et al. (2009b); [d]Kim et al. (1994)

4.9 N-terminal sequencing

The N-terminal amino acid sequence of trypsin from the viscera of hybrid catfish was aligned to compare with those of others trypsins as depicted in Fig. 8. The N-terminal amino acid sequence of the first 20 amino acids of purified trypsin was determined to be IVGGYECQAHSQPPTVSLNA (Fig. 8). When N-terminal amino acid sequences of purified trypsin were compared with those of other teleosts, an elasmobranch and three mammals. It was found that the sequence of the purified trypsin displayed high homology to other trypsins, especially the sequence from the first to seventh residues. Generally, the N-terminal region of trypsin-like proteinase, especially from first to seventh residues demonstrate high homology (Cao et al., 2000). However, all fish trypsins had a charged Glu residue at position 6, whereas Thr is most common in mammalian pancreatic trypsin (Fig. 8). Moreover, the sequences of trypsin from hybrid catfish viscera and other trypsins started with IVGG after the proteolytic cleavage of inactive trypsinogen. Trypsin from hybrid catfish viscera was most likely a member of the trypsin family.

	5 10 15 20
Trypsin	I V G G Y E C Q A H S Q P P T V S L N A
Skipjack tuna	I V G G Y E C Q A H S Q P P Q V S L N S
Yellowfin tuna	I V G G Y E C Q A H S Q P H Q V S L N A
Tongol tuna	I V G G Y E C Q A H S Q P H Q V S L N A
True sardine	I V G G Y E C K A Y S Q P W Q V S L N S
Arabesque greenling	I V G G Y E C T P H T Q A H Q V S L D S
Japanese anchovy	I V G G Y E C Q A H S Q P H T V S L N S
Cod	I V G G Y E C T K H S Q A H Q V S L N S
Salmon	I V G G Y E C K A Y S Q T H Q V S L N S
Dogfish	I V G G Y E C P K H A A P W T V S L N V
Dog	I V G G Y T C E E N S V P V Q V S L N A
Porcine	I V G G Y T C A A N S V P Y Q V S L N S
Bovine	I V G G Y T C G A N T V P Y Q V S L N S

Fig. 8. Comparison of N-terminal amino acid sequences of the purified trypsin from hybrid catfish viscera with other trypsins: skipjack tuna (Klomklao et al., 2009a), yellowfin tuna (Klomklao et al., 2006b), tongol tuna (Klomklao et al., 2006a), true sardine , arabesque greenling (Kishimura et al., 2006), Japanese anchovy (Kishimura et al., 2005), cod (Gudmundsdottir et al., 1993), salmon (Male et al., 1995), dogfish (Titani et al., 1975), dog (Pinsky et al., 1985), porcine (Hermodson et al., 1973), and bovine (Walsh, 1970).

CHAPTER 5

CONCLUSION

A 24 kDa trypsin from viscera of hybrid catfish showed high activity in the alkaline pH range and exhibited the maximal activity at 60°C. Ca^{2+} ions stabilized trypsin from thermal denaturation at 40°C. However, loss in activity was observed in the presence of NaCl, especially when NaCl concentration increased. Based on substrate specificity, inhibitor study and N-terminal sequencing, the enzyme purified from hybrid catfish viscera was found to be true member of the trypsin family.

Acknowledgments

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Output

Klomklao, S., Benjakul, S., Kishimura, H. and Chaijan, M. 2011. 24kDa trypsin: A predominant protease purified from the viscera of hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*). Food Chem. 129: 739-746.

Future works

1. Based on biochemical properties, the application of hybrid catfish viscera trypsin in other food or fish processings should be investigated.
2. The stability and preservation of purified hybrid catfish viscera trypsin should be studied.

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