Introduction

Due to the limited biological resources and increased environmental concerns, the interest in full utilization of seafood processing wastes has been increasing. Fish viscera, one of the most important by-products of fishing industry, are known to be a rich source of digestive enzymes, especially proteases with high activity over a wide range of pH and temperature conditions (Gildberg, 1992; Klomklao et al., 2006a). High protease activity of fish viscera enzymes may be interesting for many biotechnological and food processing applications (Haard, 1992). A variety of digestive proteolytic enzymes have been isolated from the internal organs of fish. One of the main digestive proteinases detected in fish viscera, especially in the pyloric ceca and intestine, is trypsins (EC 3.4.21.4). Trypsin, a member of a large family of serine proteinases, specifically hydrolyze proteins and peptides at the carboxyl side of arginine and lysine residues and play major roles in biological process including digestion, activation of zymogens of chymotrypsin and other enzymes (Klomklao et al., 2006a; Cao et al., 2000). Trypsins have been isolated and characterized thoroughly based on their physiochemical and enzymatic properties from several species of fish, e.g. crayfish (*Procambarus clarkii*) (Kim et al., 1994), carp (*Cyprinus carpio*) (Cao et al., 2000), capelin (*Mallotus villosus*) (Hjelmeland and Raa, 1982), tambaqui (*Colossoma macropomum*) (Bezerra et al., 2001), true sardine (*Sardinops melanostictus*) and arabesque greenling (*Pleuroprammus azonus*) (Kishimura et al., 2006a). Recently, Kishimura et al. (2008) isolated and characterized trypsin from the pyloric ceca of walleye pollock (*Theragra chalcogramma*).

Skipjack tuna (*Katusuwonus pelamis*) is one of the most important species commonly used for canning production in Thailand (Klomklao et al., 2004).

Approximately two-thirds of the whole fish are utilized and the remaining involving the viscera becomes the waste. Accordingly, those viscera which are the essential source of potential proteinases can be recovered for further uses. Based on our previous study, skipjack tuna intestine contained high proteolytic activity, which was identified as trypsin-like serine proteinase. However, no information regarding the molecular and biochemical properties of skipjack tuna intestine proteinases has been reported. Our objective was to purify and study the physicochemical and biochemical characteristic of trypsin from skipjack tuna intestine.

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) (Figure 1).

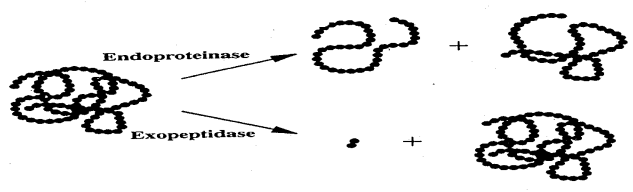

Figure 1. Action of endopeptidases and exopeptidases on protein structure.

Source: An et al. (1996)

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 weight; 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 (Siebert and Botkke, 1963). 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 endoproteinases 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 an optimal 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 BAPNA. In dover sole, the activity at pH 7.0-8.0 was due to trypsin and chymotrypsin-like enzymes, while the optimal 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 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 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 maximum 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 mass 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 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., 2006a). The TR-S and TR-P had maximal activities at around pH 8.0 for hydrolysis of TAME. Optimum temperature 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 as 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. Kishimura et al. (2006b) also isolated and characterized trypsins from the pyloric ceca of yellow tail (*Seriola quinqueradiata*) and brown hekeling (*Physiculus japonicus*) by a series of chromatographic separations including Sephacryl S-200, Sephadex G-50 and DEAE-cellulose. The molecular mass of both enzymes were estimated to be 24 kDa by SDS-PAGE. Both trypsins had maximal activity at pH 8.0 for hydrolysis of TAME. The optimum temperatures for yellow tail and brown hakeling trypsins were 60 and 50°C, respectively. The activities of both trypsins were strongly inhibited by soybean trypsin inhibitor and TLCK. The N-

terminal amino acid sequences of yellow tail trypsin and brown hakeling trypsin were determined as IVGGYECTPHSQAHQVSLNS and IVGGYECPKHSQPHQVSLNS, respectively.

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.

Materials and Methods

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).

2. Fish sample preparation

Internal organs from skipjack tuna (*Katsuwonus pelamis*) were obtained from Chotiwat Industrial Co. (Thailand) Ltd., Songkhla. Those samples (5 kg) were packed in polyethylene bag, kept in ice and transported to the Department of Food Science and Technology, Thaksin University, Phattalung within 30 min. Pooled internal organs were then excised and separated into individual organs. Only intestine was collected, immediately frozen and stored at –20°C until used.

3. Preparation of intestine extract

Frozen intestines 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 thickness of 1-1.5 cm and homogenized into powder in 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. (2006a). 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 until dry and free of acetone odor.

To prepare the intestine extract, intestine 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".

4. Purification of skipjack tuna intestine 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 chromatographed on 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 0.05-0.4 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. Two activity peaks (trypsin A and B) were obtained and pooled fractions from each peak were dialyzed with SB for 10-12 h and then concentrated by lyophilization and used for further study.

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.

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, 55, 60, 65, 70 and 80°C) for 20 min at pH 8.0.

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.

8. Determination of molecular weight

The molecular weight of purified trypsins was determined using size exclusion chromatography on Sephacryl S-200 column. The trypsin separated on size exclusion chromatography was estimated for its molecular weight by plotting available partition coefficient (K_{av}) against the logarithm of molecular weight of the

protein standards. The elution volume (V_e) was measured for each protein standard and the trypsins. 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.).

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.

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.

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 2 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.

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.

13. Determination of N-terminal amino acid sequence

The purified enzymes were 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).

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.

15. Protein determination

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

Results and Discussion

1. Purification of trypsins from skipjack tuna intestine

The results of each purification step are presented in Table 1. After Sephacryl S-200 column chromatography (Fig. 2a), total activity of approximately 58% was remained, while 67.2% of protein was removed. Kishimura et al. (2008) reported that the use of Sephacryl S-200 in the first step of purification process of trypsin from the pyloric ceca of walleye pollock led to an increased in trypsin activity by 18-fold. From the result, purity of 2.0-fold was obtained with Sephacryl S-200 chromatography.

To refine the pooled fraction obtained from previous step, pooled active fractions were subjected to Sephadex G-50 column chromatography. Sephadex G-50 chromatography separated trypsin from other proteins with lower molecular weight (Fig. 2b). Purification fold of 6.5 with a yield of 37% was observed. Sephadex G-50 column chromatography was highly effective in separating trypsin from other proteins, but not for resolving the individual trypsins (Kishimura et al., 2005). Hence, pooled active fractions were subsequently subjected to an ion-exchange chromatography using DEAE-cellulose.

Pooled Sephadex G-50 fractions were dialyzed against 10 mM Tris-HCl, pH 8.0 containing 1 mM $CaCl_2$ (SB), prior to anion exchanger, DEAE-cellulose column. After loading the sample and washing with SB, column was eluted by using a 0.05-0.4 M NaCl linear gradient (Fig. 2c). Two trypsin activity peaks were found (Fig. 2c). These enzymes were assigned as trypsin A and B based on the elution order. Purification fold of 177 and 257 were obtained for trypsin A and B, respectively. Ion exchange chromatography was used to remove the contaminating proteins and to

separate different trypsin isoforms. Kishimura et al. (2005) used DEAE-cellulose in the final step for isolation of two trypsin isozymes from Japanese anchovy viscera, leading to the increases in purity by 37-fold and 73-fold. Cao et al. (2000) also purified two anionic trypsins from carp hepatopancreas by using anion exchange, Q-Sepharose column.

Table 1

Purification of trypsins from skipjack tuna intestine

Purification steps	Total activity (units)*	Total protein (mg)	Specific activity (units/mg protein)	Purity (fold)	Yield (%)
Crude extract	420	5826	0.07	1	100
Sephacryl S-200	242	1910	0.13	2	58
Sephadex G-50	152	33	4.6	65	37
DEAE-Cellulose					
Trypsin A	96.4	7.7	12.5	177	23
Trypsin B	86.4	4.7	18.4	257	21

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

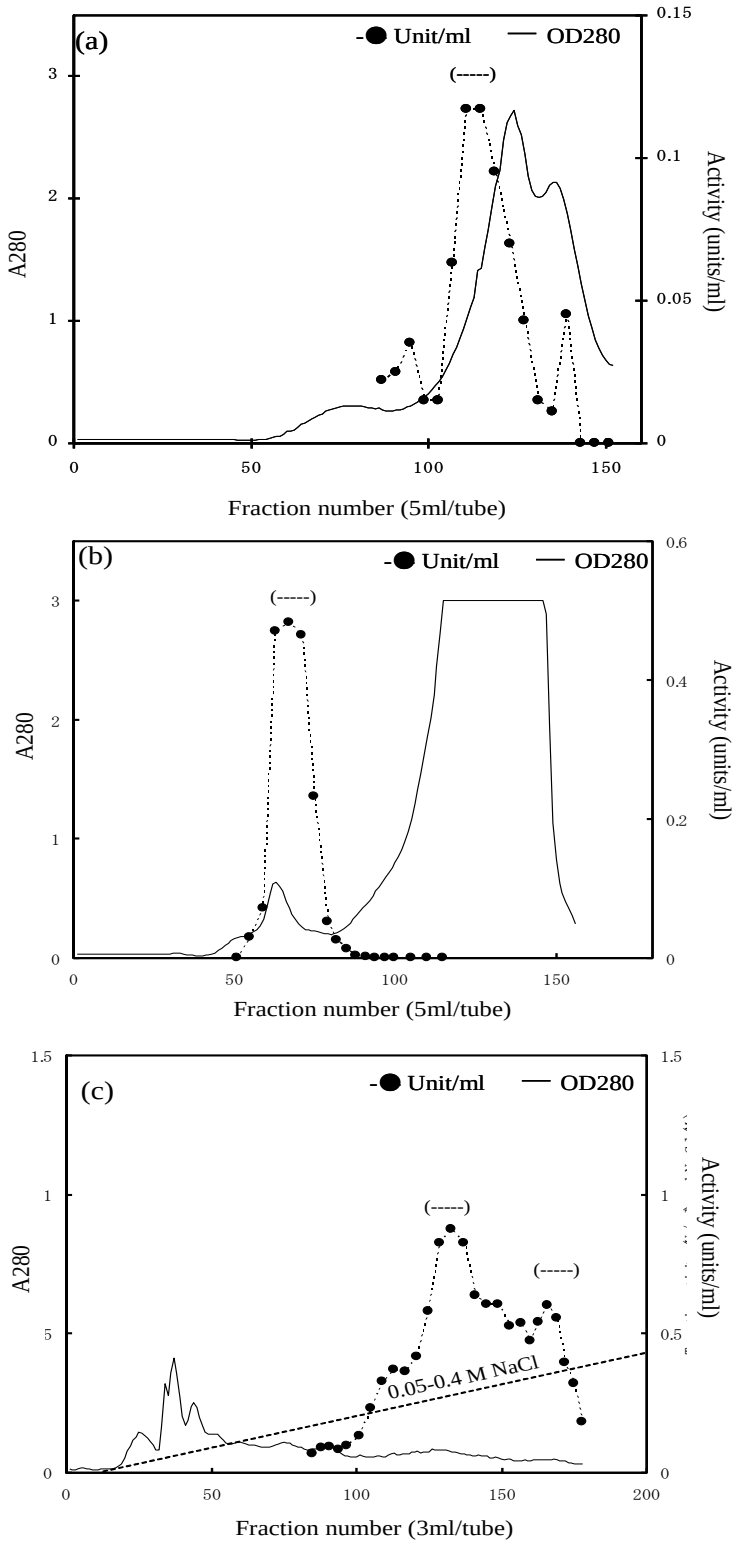

Fig.2. Purification of trypsins from skipjack tuna intestine. (a) Elution profile of trypsins on Sephacryl S-200 column; (b) elution profile of trypsins on Sephadex G-50 column; (c) elution profile of trypsins on the DEAE-cellulose column. Elution was carried out with a linear gradient of 0.05-0.4 M NaCl in SB.

2. Electrophoretic pattern

Trypsin A and B appeared as a single band on the native-PAGE (Fig. 3a) and showed the different mobilities in native-PAGE, indicating the homogeneity of both enzymes. For SDS-PAGE, a single band with the molecular weight of 24 kDa was observed for both enzymes (Fig. 3b). Also, the molecular weight of the enzyme was estimated to be approximately 24 kDa by gel filtration using Sephacryl S-200 (Fig. 4). The results confirm that trypsin A and B are the monomeric protein with a molecular weight of 24 kDa. Generally, fish trypsins have been reported to have molecular weights in the range of 23 to 28 kDa. Two trypsins (I and II) from the pyloric ceca of Japanese anchovy had a molecular weight of 24 kDa as estimated by SDS-PAGE (Kishimura et al., 2005). Kurtovic et al. (2006) reported that purified trypsin from the pyloric ceca of chinook salmon had a molecular weight of 28 kDa by SDS-PAGE. Two trypsins (A and B) from carp hepatopancreas had the molecular weights of 28.5 and 28 kDa by SDS-PAGE and gel filtration, respectively (Cao et al., 2000). Molecular weights of trypsin A and B from spleen of yellowfin tuna were estimated by SDS-PAGE and gel filtration to be approximately 24 kDa (Klomklao et al., 2006b).

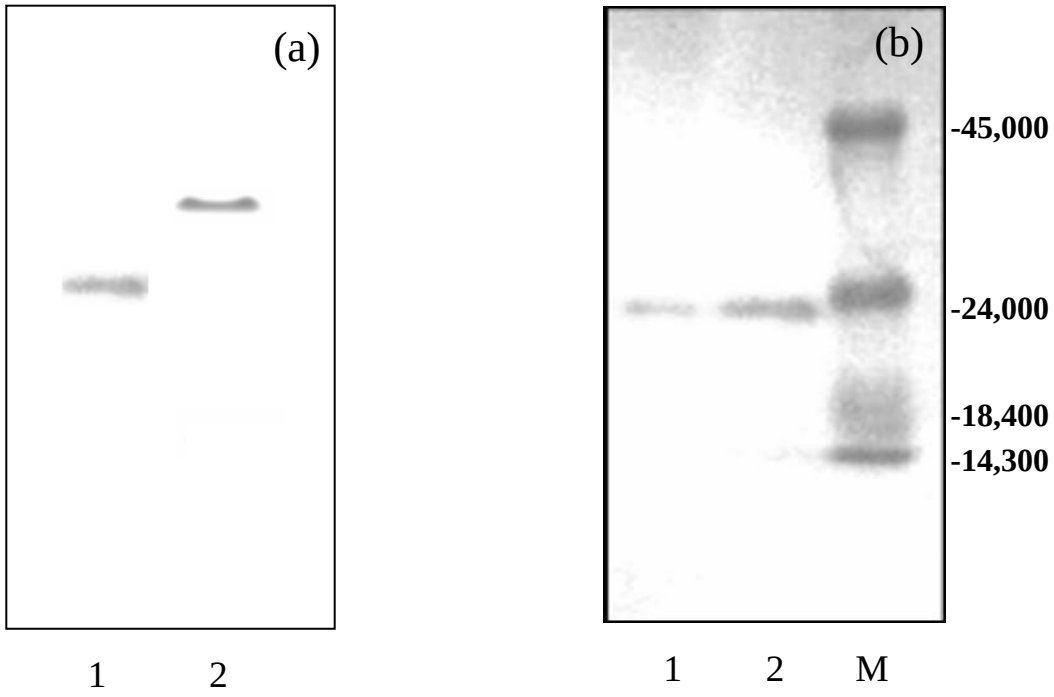

Fig. 3. Protein pattern from native-PAGE (a) and SDS-PAGE (b) of purified trypsin A and B from skipjack tuna intestine. M, molecular weight standard; lane 1, trypsin A; lane 2, trypsin B.

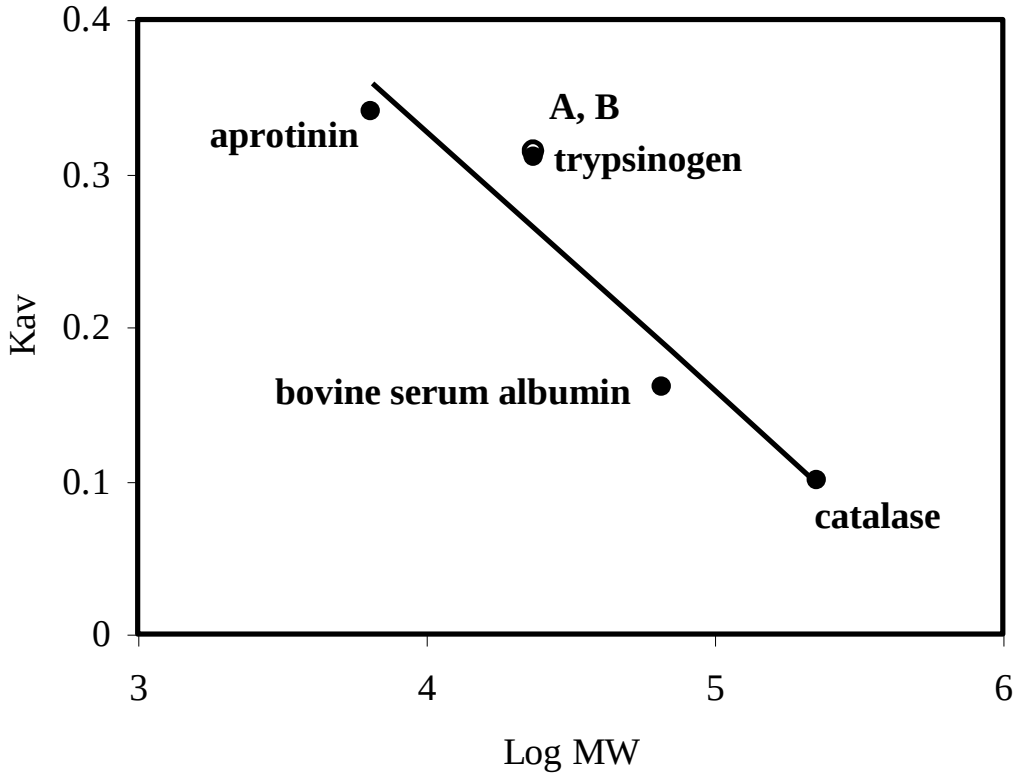

Fig. 4. Calibration curve for the molecular weight determination of the purified trypsin A and B on Sephacryl S-200 chromatography. A, trypsin A; B, trypsin B.

3. Optimal pH and temperature

The effect of pH on the rate of TAME hydrolysis was measured and the results are presented in Fig. 5a. Trypsin A and B exhibited the maximal activity at pH 9.0, which was higher than that of porcine pancreatic trypsin (pH 8.0). Trypsin A, B and porcine trypsin showed the same behavior between pH 4-11. The activities of both trypsins and porcine trypsin were high in pH rang of 7.0-9.0 but considerable loss of activity was observed at very acidic and alkaline pHs. A change in pH affects both the substrate and enzyme by changing the charge distribution and conformation of the molecules (Klomklao et al., 2006b). Most enzymes undergo irreversible denaturation in a very acid and alkaline solution, causing the loss of stability. The optimum pH of both trypsins from skipjack tuna intestine was similar to those of trypsins from yellowfin tuna spleen (Klomklao et al., 2006b) and tongol tuna spleen (Klomklao et al., 2006a).

The activity of trypsin A, B and porcine pancreatic trypsin increased with temperature up to an optimum of 55, 60 and 60°C, respectively, when assayed against TAME (Fig. 5b). A sharp decrease in activity was found at temperature above 60°C, possibly due to the thermal denaturation. Generally, Trypsin A and B showed high activity in the range from 20-50°C while porcine trypsin was more active in the range from 60-80°C. This behavior is in accordance to the fact that mammalian trypsins are more active at higher temperatures than fish trypsins (Simpson, 2000). Studies on trypsins from other fish species like yellowfin tuna, tongol tuna, crayfish and rainbow trout showed similar profiles on activity toward temperature with slight differences on the optimum temperature (Klomklao et al., 2006a; 2006b; Kristjansson, 1991; Kim et al., 1994).

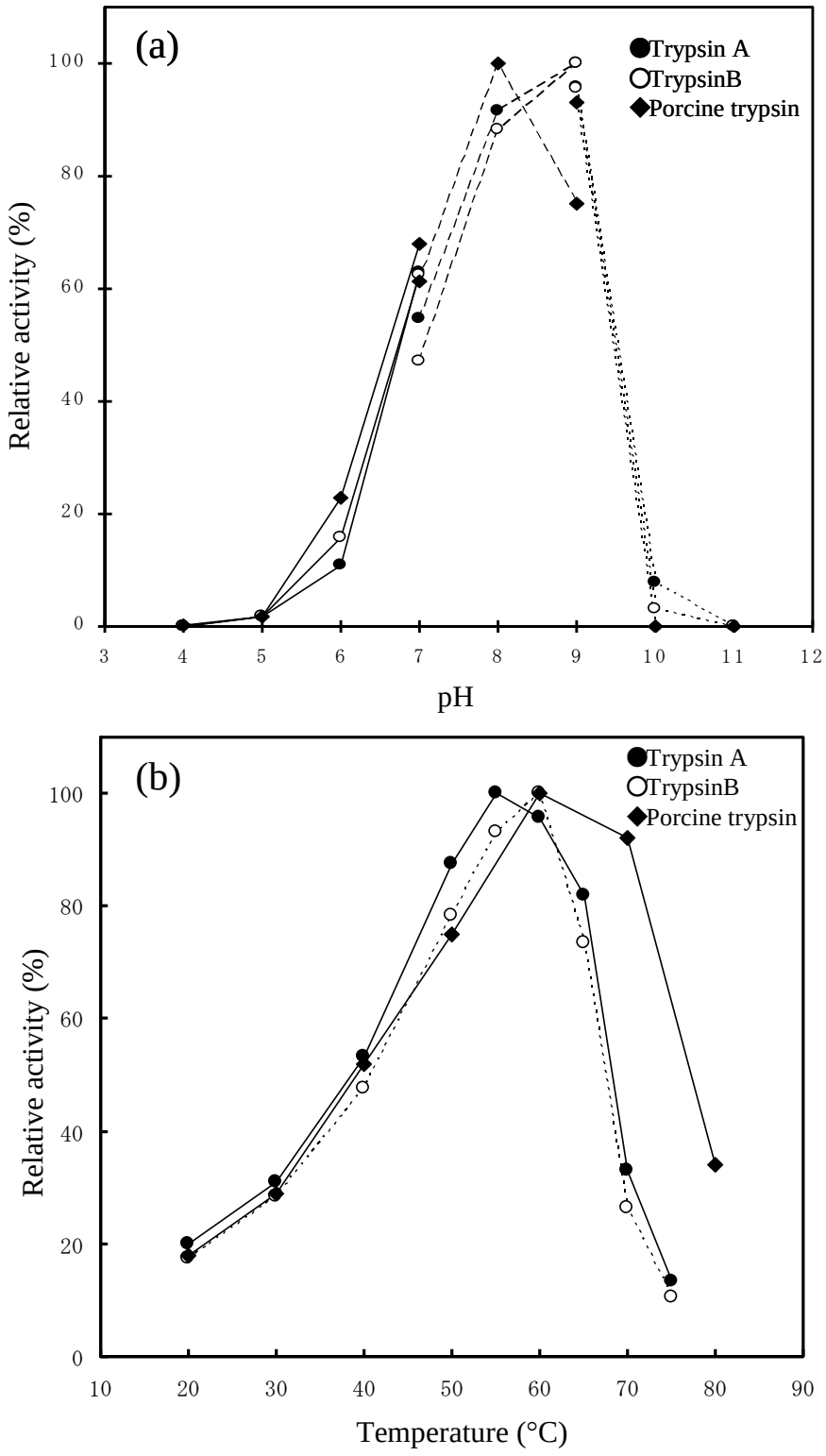

Fig. 5. pH (a) and temperature (b) profiles of purified trypsin A, and B from skipjack tuna intestine and porcine trypsin.

4. pH and thermal stability

The effect of pH on the stability of two trypsins from skipjack tuna intestine and porcine pancreatic trypsin is showed in Fig. 6a. Trypsin A, B and porcine trypsin exhibited different pH stability. Trypsin A and B showed high stability in the pH range of 6.0-11.0, but the inactivation was more pronounce at pH below 6 (Fig. 6a). A completely loss in activity was observed after incubating both trypsins at pH 4, while porcine trypsin has stability at acidic and alkaline pHs. Simpson (2000) reported that trypsin from warm blooded organism are extremely stable at acidic and alkaline pHs while fish trypsins are stable only to alkaline pH. The stability of trypsin at particular pH might be related to the net charge of the enzyme at that pH (Castillo-Yanez et al., 2005). A similar pH effect on activity has been reported for trypsin from several fish species (Kishimura et al., 2006b; Castillo-Yanez et al., 2005; Klomklao et al., 2006a, b).

For thermal stability, both trypsins from skipjack tuna intestine was stable below 55°C, but the activity sharply decreased above 60°C (Fig. 6b). The enzyme was almost completely inactivated at 75°C. At high temperatures, enzymes most likely underwent denaturation and lost their activity (Klomklao et al., 2006a). Trypsins from skipjack tuna intestine exhibited the similar thermal stability to those of other fish species (Kishimura et al., 2006a; Castillo-Yanez et al., 2005; Klomklao et al., 2006a, b) However, the temperature stability of trypsin A and B from skipjack tuna intestine was more unstable than trypsin from porcine trypsin (Fig. 6b). Differences in thermal stability of enzymes might be determined by bonding stabilized enzyme structure. More disulfide linkages as well as stronger hydrophobic interactions in the interior of protein contribute to the greater stability of proteins.

Higher thermostability was associated with a higher number of intramolecular disulfide bonds in protease (Simpson, 2000; Klomklao et al., 2006a).

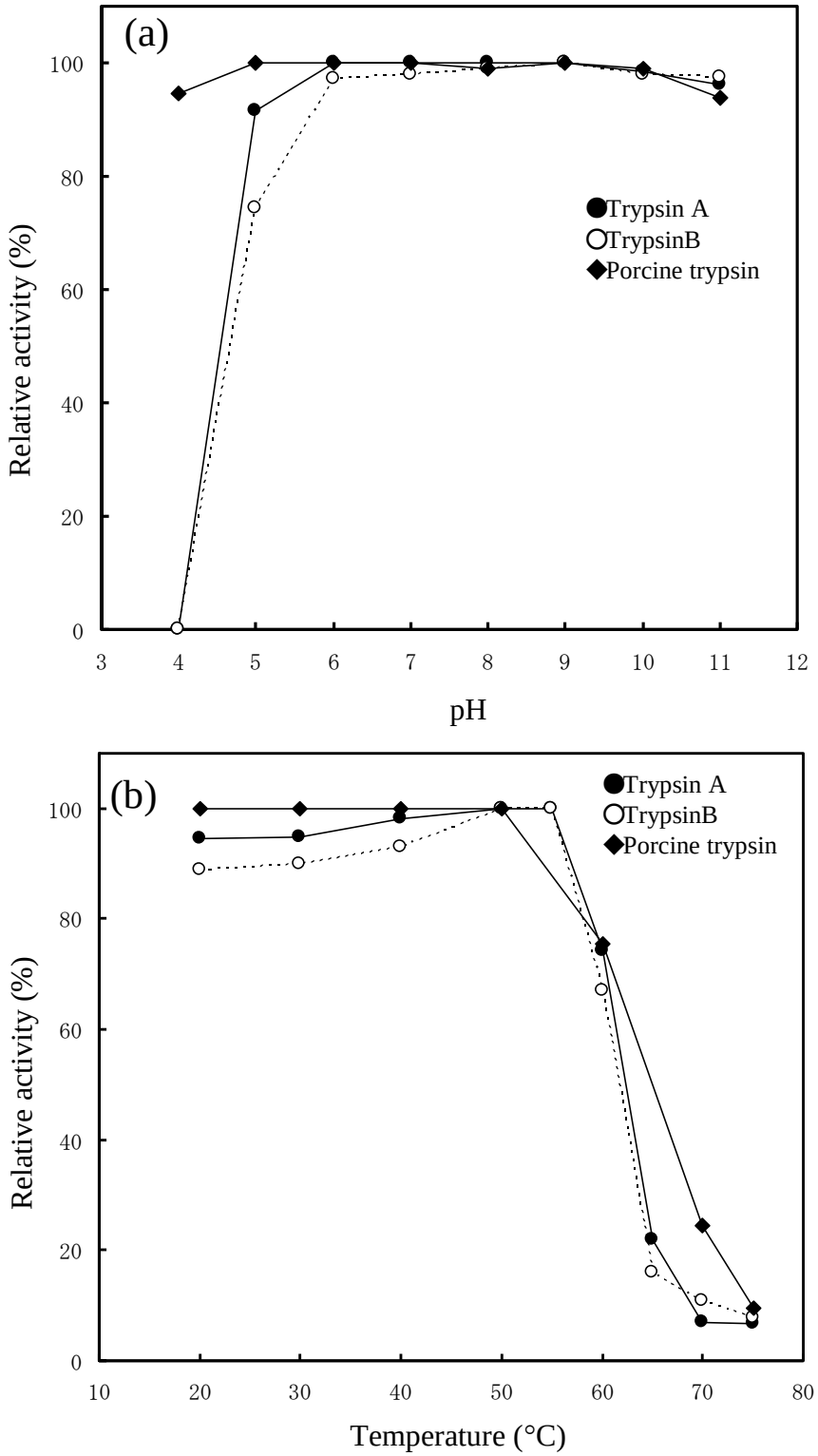

Fig. 6. pH (a) and thermal (b) stability of purified trypsin A and B from skipjack tuna intestine and porcine trypsin.

5. Effect of calcium ions on the thermal stability

The effect of $CaCl_2$ on the stability of the skipjack tuna trypsins was determined in the presence of 10 mM EDTA or 10 mM $CaCl_2$. The total activity was retained throughout 8 h at 30°C in the presence of 10 mM calcium ion, while the activities decreased with increasing time in the presence of 10 mM EDTA (Fig. 7). Trypsin A was rather stable in the presence of 10 mM EDTA than trypsin B, particularly when the incubation time increased. The results indicated that trypsin A and B were stabilized by calcium ion similar to porcine pancreatic trypsin (Fig. 7). Binding of calcium to a single binding site in bovine trypsin significantly stabilized the enzyme against denaturation (Klomklao et al., 2004). In the presence of calcium ions, trypsin is believed to undergo a conformational change, resulting in a more compact structure, which is more resistant to autolysis (Kim et al., 1994). Stabilization against thermal inactivation by calcium ion has also been found for the trypsins from yellowfin tuna (Klomklao et al., 2006b), true sardine and arabesque greenling (Kishimura et al., 2006a). However, calcium ion did not show the enhancing effect on stability of trypsins from sardine (Murakami and Noda, 1981) 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.

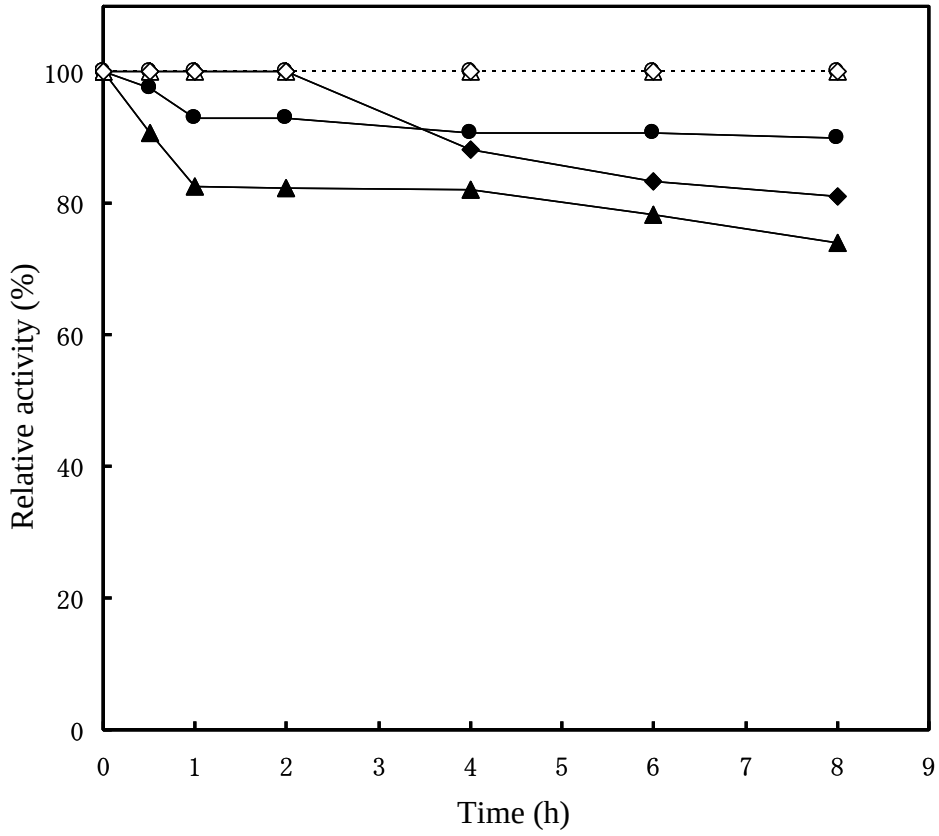

Fig. 7. Effect of calcium ion and EDTA on the stability of purified trypsin A and B from skipjack tuna intestine and porcine trypsin. The enzymes were kept at 30°C for 0-8 h in the presence of 10 mM $CaCl_2$ (open symbols) or 10 mM EDTA (closed symbols), and then the remaining activities at 30°C were detemined. Trypsin A (circle); trypsin B (triangle); porcine trypsin (diamond).

6. Effect of NaCl

The activities of trypsin A and B from skipjack tuna intestine continuously decreased with increasing NaCl (Fig. 8). Trypsin B showed slightly higher activity than trypsin A in the presence of NaCl ranging from 5% to 30%, indicating that trypsin B was more tolerant to NaCl than trypsin A. The activities of trypsin A and B in the presence of 30% NaCl was approximately 35 and 45% of that without NaCl, respectively. The decrease in activity 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., 2004). At 25% NaCl, 40-50% of activities was remained. Therefore, trypsins from skipjack tuna intestine may have a potential in accelerating the hydrolysis of high-salt products, such as fish sauce.

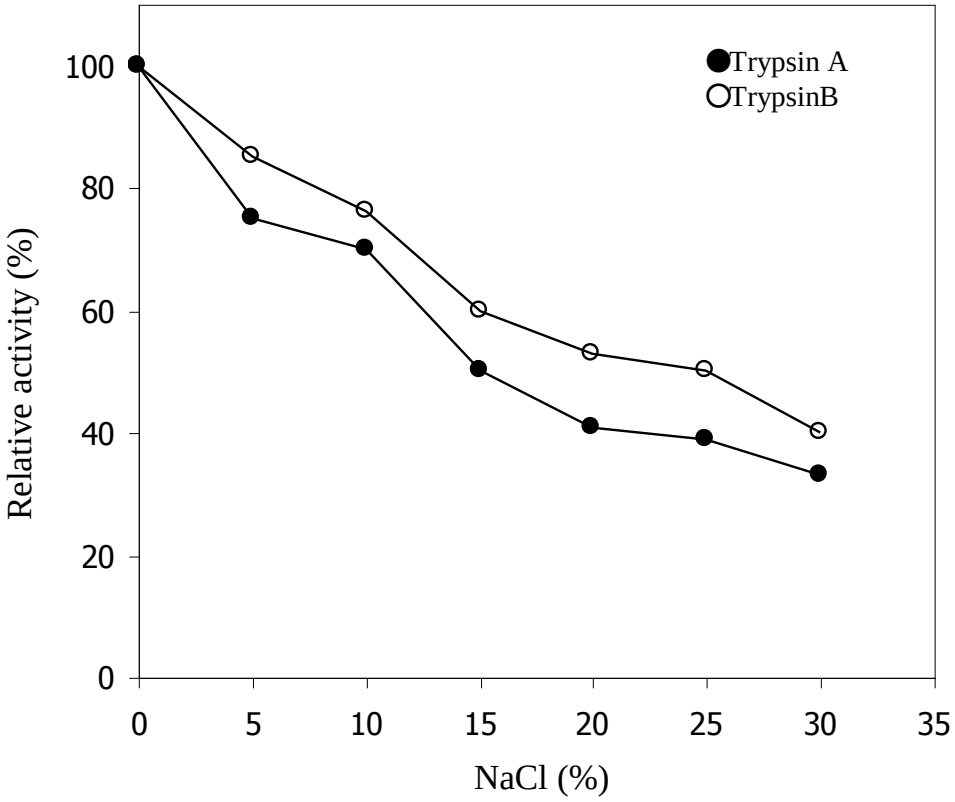

Fig. 8. Effect of NaCl concentrations on activities of purified trypsin A and B from skipjack tuna intestine.

7. Effect of inhibitors

The influence of several well known protease inhibitors on the activity of the skipjack tuna enzymes is summarized in Table 2. Soybean trypsin inhibitor and TLCK strongly inhibited the enzymes. Inhibitors for cysteine and aspartic protease involving E-64, *N*-ethylmaleimide, iodoacetic acid and pepstatin A did not show inhibitory effects towards trypsin activity. However, TPCK, a specific inhibitor for chymotrypsin, and EDTA, which chelates the metal ions required for enzyme, partially lowered trypsin activity. Soybean trypsin inhibitor binds strongly to the active site of trypsin to impede catalysis. Soybean trypsin inhibitor has been shown to inhibit trypsins from carp (Cao et al., 2000), tambaqui (Bezerra et al., 2001), chinook salmon (Kurtovic et al., 2006) and yellowfin tuna (Klomklao et al., 2006b). TLCK is well known as a trypsin specific inhibitor that has been shown to inhibit trypsins from carp (Cao et al., 2000), yellowfin tuna (Klomklao et al., 2006b) and tongol tuna (Klomklao et al., 2006a). TLCK inactivates only trypsin-like enzymes by forming a covalent bond with histidine at the catalytic portion of molecule and then blocking the substrate-binding portion at the active center (Klomklao et al., 2006a). The result confirms that these purified enzymes were serine proteinases, mostly likely trypsin.

Table 2

Effect of various inhibitors on the activity of purified trypins from skipjack tuna intestine*

Inhibitors	Concentration	% Inhibition	
		Trypsin A	Trypsin B
Control		0	0
E-64	0.1 mM	0	0
N-ethylmaleimide	1 mM	0	0
Iodoacetic acid	1 mM	6.6±1.08	4.9±0.05
Soybean trypsin inhibitor	1.0 g/l	88.01±2.1	91.06±1.35
TLCK	5 mM	81.09±0.06	85.98±1.36
TPCK	5 mM	5.78±1.12	9.98±0.87
Pepstatin A	0.01 mM	0	0
EDTA	2 mM	0.88±2.53	1.34±1.04

*Each 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.

8. Kinetic study

Kinetic constants for TAME hydrolysis by skipjack tuna trypsins (Trypsin A and B) were determined using Lineweaver-Burk plots (Table 3). K_m and K_{cat} were 0.31 mM and 82.5 S^{-1} for skipjack tuna intestine trypsin A; 0.22 mM and 69.5 S^{-1} for skipjack tuna intestine trypsin B, respectively. The K_m value of trypsin B was lower than that of trypsin A. This result suggests that trypsin B has higher affinity to TAME, compared with trypsin A. The K_m obtained for skipjack tuna trypsins was similar to those reported for trypsins from yellowfin tuna (Klomklao et al., 2006b), tongol tuna (Klomklao et al., 2006a) and crayfish (Kim et al., 1994). Moreover, the enzyme had slightly lower K_m value than trypsin from bovine, which had K_m in the range of 0.3-0.33 mM (Kim et al., 1994). The result indicates that skipjack tuna trypsins had higher affinity for TAME than bovine trypsin. Additionally, the catalytic efficiency (166.13-315.91 $S^{-1}mM^{-1}$) of trypsin A and B from skipjack tuna intestine was greater than that of mammalian trypsin (Simpson, 2000). From the result, it was suggested that trypsins from skipjack tuna intestine had more flexible structures than trypsin from warm blooded animals.

Table 3

Kinetic properties of skipjack tuna intestine trypsins for the hydrolysis of TAME

Enzyme	K_m (mM)	K_{cat} (S^{-1})	K_{cat} / K_m (S^{-1} mM^{-1})
Trypsin A	0.31±0.01	82.5±0.01	266.13
Trypsin B	0.22±0.02	69.5±0.05	315.91

K_m, K_{cat} values were determined using TAME as a substrate at pH 8.0 and 30°C.

9. N-terminal sequencing

The N-terminal 20 amino acids of trypsin A and B were IVGGYECQAHSQPPQVSLNA and IVGGYECQAHSQPPQVSLNS, respectively (Fig. 9). The result indicates that the N-terminal of the enzymes was unblocked. The N-terminal amino acid sequences of two trypsins were compared with those of other animal trypsins (Fig. 9). It was found that N-terminal of trypsin A and B exhibited the high homology and showed similarity to that of yellowfin tuna spleen and tongol tuna spleen. The highly conserved amino acid sequence between yellowfin tuna tongol tuna and skipjack tuna suggest that they were genetically evolved from a common ancestor. Moreover, the sequences of two trypsins from skipjack tuna intestine and other trypsins started with IVGG after the proteolytic cleavage of in inactive trypsinogen. From the results, the N-terminal sequence clearly showed that trypsin from skipjack tuna intestine was most likely a member of trypsin family. In addition, the present data of the N-terminal amino acid sequence may be useful for designing primers for the cDNA cloning of trypsin.

	5 10 15 20
Trypsin A	I V G G Y E C Q A H S Q P P Q V S L N A
Trypsin B	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. 9. Comparison of N-terminal amino acid sequences of the purified trypsin A and B from skipjack tuna intestine with other enzymes: yellowfin tuna (Klomklao et al., 2006b), tongol tuna (Klomklao et al., 2006a), true sardine , arabesque greenling (Kishimura et al., 2006a), 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).

Conclusion

Two trypsins from skipjack tuna intestine were purified and identified based on molecular weight, substrate specificity, inhibitor study and N-terminal sequencing. Two isoenzymes were salt toterant and could be used in a high-salt environment.

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