รายงานวิจัยฉบับสมบูรณ์

โครงการ การเก็บเกี่ยวและการแยกส่วนเอนไซม์โปรตีเนสจากเครื่องในปลาดุกบิ๊กอุยโดยวิธี Aqueous two-phase system

โดย ผู้ช่วยศาสตราจารย์ ดร.สรรพสิทธิ์ กล่อมเกล้า

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สาขาวิชาวิทยาศาสตร์และเทคโนโลยีอาหาร
คณะเทคโนโลยีและการพัฒนาชุมชน มหาวิทยาลัยทักษิณ

สนับสนุนโดยสำนักงานคณะกรรมการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และ สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

ขอขอบพระคุณสำนักงานคณะกรรมการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัย สำหรับเงินอุดหนุนโครงการวิจัยเรื่อง “การเก็บเกี่ยวและการแยกส่วนเอนไซม์โปรตีเนสจากเครื่องในปลาดุกบิ๊กอุยโดยวิธี Aqueous two-phase system” จำนวน 480,000 บาท ขอขอบพระคุณคณะเทคโนโลยีและการพัฒนาชุมชน มหาวิทยาลัยทักษิณ และคณะอุตสาหกรรมเกษตร มหาวิทยาลัยสงขลานครินทร์ที่ให้การสนับสนุนด้านอุปกรณ์และเครื่องมือวิทยาศาสตร์สำหรับการวิจัยครั้งนี้ และขอขอบพระคุณ ศาสตราจารย์ ดร. สุทธวัฒน์ เบญจกุล นักวิจัยที่ปรึกษา ที่ให้คำแนะนำที่เป็นประโยชน์สำหรับการวิจัยครั้งนี้

ผู้ช่วยศาสตราจารย์ ดร.สรรพสิทธิ์ กล่อมเกล้า
หัวหน้าโครงการฯ

บทคัดย่อ

จากการศึกษากิจกรรมของเอนไซม์โปรตีเนสจากเครื่องในปลาดุกบิ๊กอุย พบว่าเอนไซม์มีพีเอชและอุณหภูมิที่เหมาะสมต่อการย่อยสลายเคซีน เท่ากับ 9.0 และ 50 องศาเซลเซียส ตามลำดับ เอนไซม์โปรตีเนสจากเครื่องในปลาดุกบิ๊กอุยมีความคงตัวในช่วงพีเอช 7-11 เป็นระยะเวลา 30-120 นาที และมีความคงตัวในช่วงอุณหภูมิไม่เกิน 40 องศาเซลเซียส เป็นระยะเวลา 30-120 นาที กิจกรรมของเอนไซม์โปรตีเนสถูกยับยั้งอย่างมีประสิทธิภาพด้วยสารยับยั้งเอนไซม์ทริปซินจากถั่วเหลือง (SBTI) benzamidine phenylmethylsulfonyl fluoride และ *N*-p-tosyl-L-lysine chloromethyl ketone กิจกรรมของเอนไซม์โปรตีเนสลดลงอย่างต่อเนื่องเมื่อความเข้มข้นของเกลือเพิ่มขึ้น (ร้อยละ 0-30) ขณะที่กิจกรรมเพิ่มขึ้นเมื่อความเข้มข้นของแคลเซียมคลอไรด์เพิ่มขึ้น (0-10^{-3} โมลาร์) เอนไซม์โปรตีเนสตัวหลักที่พบในเครื่องในปลาดุกบิ๊กอุยมีน้ำหนักโมเลกุลเท่ากับ 23 และ 20 กิโลดาลตันเมื่อตรวจสอบโดยใช้ SDS-substrate gel จากการศึกษาผลของสารสกัดชนิดต่าง ๆ ต่อการเก็บเกี่ยวเอนไซม์โปรตีเนส พบว่าสารละลาย Tris-HCl ความเข้มข้น 50 มิลลิโมลาร์ พีเอช 7 ที่มี โซเดียมคลอไรด์ ความเข้มข้น 0.5 โมลาร์ และ Brij 35 ความเข้มข้นร้อยละ 0.2 เป็นสารสกัดที่สามารถเก็บเกี่ยวเอนไซม์โปรตีเนสได้สูงสุด ($p<0.05$) จากผลการทดลองแสดงให้เห็นว่าเอนไซม์โปรตีเนสตัวหลักในเครื่องในปลาดุกบิ๊กอุยเป็นเอนไซม์โปรตีเนสชนิดซีรีนที่มีลักษณะคล้ายทริปซิน

จากการศึกษาการเก็บเกี่ยวเอนไซม์ทริปซินจากเครื่องในปลาดุกบิ๊กอุยโดยใช้ Aqueous two-phase system (ATPS) พบว่า น้ำหนักโมเลกุลและความเข้มข้นของพอลิเอทธิลีนไกลคอลรวมทั้งชนิดและความเข้มข้นของเกลือมีผลต่อการแยกโปรตีน ส่วนใหญ่เอนไซม์ทริปซินจะแยกไปยังเฟสพอลิเอทธิลีนไกลคอลซึ่งอยู่ด้านบน ATPS ซึ่งประกอบด้วยพอลิเอทธิลีนไกลคอล (น้ำหนักโมเลกุล 4,000) ที่ระดับความเข้มข้นร้อยละ 20 และ โซเดียมไดไฮโดรเจนฟอสเฟต ความเข้มข้นร้อยละ 20 เป็นสภาวะที่ดีที่สุดในการทำบริสุทธิ์เอนไซม์ทริปซินจากเครื่องในปลาดุกบิ๊กอุย และให้กิจกรรมจำเพาะสูงสุด (30.05 ยูนิต/ไมโครกรัมโปรตีน) และมีความบริสุทธิ์เพิ่มขึ้น 27.3 จากการตรวจสอบความบริสุทธิ์โดยใช้ SDS-PAGE พบว่า เอนไซม์ภายหลังการแยกส่วนมีความบริสุทธิ์มากขึ้นและเมื่อตรวจสอบกิจกรรมเอนไซม์โดยใช้ SDS-substrate gel พบว่า ความเข้มข้นของแถบโปรตีเนสเพิ่มขึ้น แสดงให้เห็นว่าสารสกัดจากเครื่องในมีกิจกรรมจำเพาะเพิ่มสูงขึ้น เอนไซม์ที่ผ่านการแยกส่วนมีกิจกรรมที่เหมาะสมที่พีเอช 9.0 และ อุณหภูมิ 50 องศาเซลเซียส และเอนไซม์มีความคงตัวที่อุณหภูมิไม่เกิน 40 องศาเซลเซียส และมีความคงตัวในช่วงพีเอช 8-12 กิจกรรมของเอนไซม์ลดลงอย่างเด่นชัดเมื่อเพิ่มความ

เข้มข้นของเกลือ (ร้อยละ 0-30) เอนไซม์ทริปซินที่ผ่านการแยกส่วนด้วยวิธี ATPS สามารถย่อยสลายแอกโตไมโอซินธรรมชาติและคอลลาเจนจากเนื้อวัว ดังนั้นการเติมเอนไซม์ทริปซินจากเครื่องในปลาดุกบิ๊กอุยอาจนำมาใช้ในการปรับปรุงความนุ่มของเนื้อ

คำสำคัญ: โปรตีเนส เอนไซม์ทริปซิน เครื่องใน การจำแนกคุณลักษณะ การทำบริสุทธิ์

Abstract

Proteolytic activity from viscera extract of hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) was investigated. Optimal pH and temperature for casein hydrolysis were 9.0 and 50°C, respectively. The enzyme was stable to heat treatment up to 40°C and over a pH range of 7-11 for 30-120 min. The proteolytic activity was effectively inhibited by soybean trypsin inhibitor, benzamidine, phenylmethylsulfonyl fluoride and *N*-p-tosyl-L-lysine chloromethyl ketone. Activities of the viscera extract continuously decreased as NaCl concentration (0-30%) increased while activities increased as $CaCl_2$ concentration (0-10^{-3} M) increased. Based on the proteinase activity of zones separated by electrophoresis, the molecular mass of the major proteinases in hybrid catfish viscera was 23 and 20 kDa. The effect of extraction media on recovery of proteinases was also studied. Extraction of the viscera powder with 50 mM Tris-HCl, pH 7.0 containing 0.5 M NaCl and 0.2% (v/v) Brij 35 rendered a higher recovery of proteinase activity than other extractants tested ($p<0.05$). The results suggested that major proteinases in hybrid catfish viscera were heat-activated alkaline proteinases, most likely trypsin-like serine proteinases.

The partitioning behavior of trypsin from hybrid catfish viscera in aqueous two-phase systems (ATPS) was studies. Factors such as PEG molecular mass and concentration as well as types and concentration of salts affected protein separation. Trypsin partitioned mainly in the top PEG-rich phase. ATPS formed by PEG of molecular weight 4000 (20%, w/w) and NaH_2PO_4 (20%, w/w) showed the best capability for trypsin purification from hybrid catfish viscera. Under such

condition, the highest specific activity (30.05 units/μg protein) and purification fold (27.3) were obtained. SDS-PAGE analysis revealed that the enzyme after ATPS separation was near homogeneity and based on the activity staining, the band intensity of enzyme in ATPS fraction increased, indicating the greater specific activity of the viscera extract. The partitioned enzyme displayed optimal activity at pH 9.0 and 50°C, respectively. The enzyme was stable up to 40°C and within the pH range of 8-12. The enzyme exhibited a progressive decrease in activity with increasing NaCl concentration (0-30%). Partitioned trypsin was able to hydrolyze natural actomyosin (NAM) and collagen extracted from beef meat. Therefore, the addition of trypsin from hybrid catfish viscera might be used to improve meat tenderness.

Keywords: Proteinase, Trypsin, Viscera, Characterization, Aqueous two-phase systems, Purifiation

Executive Summary

1. เอนไซม์โปรตีเนสตัวหลักในเครื่องในปลาดุกบิ๊กอุยเป็นเอนไซม์โปรตีเนสชนิดซีรีนที่มีลักษณะคล้ายทริปซิน เอนไซม์มีกิจกรรมที่เหมาะสมที่พีเอช 9.0 และอุณหภูมิ 50 องศาเซลเซียส เอนไซม์ตัวหลักที่พบมีน้ำหนักโมเลกุลเท่ากับ 23 และ 20 กิโลดาลตัน มีความคงตัวต่อพีเอชในช่วงพีเอชเป็นกลางถึงด่างและคงตัวต่อความร้อนในช่วงอุณหภูมิไม่เกิน 40 องศาเซลเซียส และสูญเสียกิจกรรมเมื่อความเข้มข้นของเกลือเพิ่มขึ้น สารละลาย Tris-HCl ความเข้มข้น 50 มิลลิโมลาร์ พีเอช 7 ที่มีโซเดียมคลอไรด์ความเข้มข้น 0.5 โมลาร์ และ Brij 35 ความเข้มข้นร้อยละ 0.2 เป็นสารสกัดที่สามารถเก็บเกี่ยวเอนไซม์โปรตีเนสได้สูงสุด
2. การใช้ Aqueous two-phase system (ATPS) ซึ่งประกอบด้วยพอลิเอทธิลีนไกลคอล (น้ำหนักโมเลกุล 4,000) ที่ระดับความเข้มข้นร้อยละ 20 และ โซเดียมไดไฮโดรเจนฟอสเฟต ความเข้มข้นร้อยละ 20 เป็นวิธีที่มีประสิทธิภาพในการแยกส่วนและเก็บเกี่ยวเอนไซม์ทริปซินจากเครื่องในปลาดุกบิ๊กอุย ซึ่งทำให้เอนไซม์มีกิจกรรมจำเพาะและมีความบริสุทธิ์เพิ่มสูงขึ้น เอนไซม์ที่ผ่านการแยกส่วนมีพีเอชและอุณหภูมิที่เหมาะสมเท่ากับ 9.0 และ 50 องศาเซลเซียส ตามลำดับ เอนไซม์ทริปซินที่ผ่านการแยกส่วนสามารถย่อยสลายแอกโตไมโอซินธรรมชาติและคอลลาเจนจากเนื้อวัว

CHAPTER 1

PROTEINASES IN HYBRID CATFISH VISCERA:

CHARACTERIZATION AND EFFECT OF EXTRACTION MEDIA

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PROTEINASES IN HYBRID CATFISH VISCERA: CHARACTERIZATION AND EFFECT OF EXTRACTION MEDIA

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ABSTRACT

Proteolytic activity from viscera extract of hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) was investigated. Optimal pH and temperature for casein hydrolysis were 9.0 and 50°C, respectively. The enzyme was stable to heat treatment up to 40°C and over a pH range of 7-11 for 30-120 min. The proteolytic activity was effectively inhibited by soybean trypsin inhibitor, benzamidine, phenylmethylsulfonyl fluoride and *N*-p-tosyl-L-lysine chloromethyl ketone. Activities of the viscera extract continuously decreased as NaCl concentration increased while activities increased as $CaCl_2$ concentration increased. Based on the proteinase activity of zones separated by electrophoresis, the molecular mass of the major proteinases in hybrid catfish viscera was 23 and 20 kDa. The effect of extraction media on recovery of proteinases was also studied. Extraction of the viscera powder with 50 mM Tris-HCl, pH 7.0 containing 0.5 M NaCl and 0.2% (v/v) Brij 35 rendered a higher recovery of proteinase activity than other extractants tested ($p<0.05$). The results suggested that major proteinases in hybrid catfish viscera were heat-activated alkaline proteinases, most likely trypsin-like serine proteinases.

PRACTICAL APPLICATIONS

Hybrid catfish viscera is an abundant and underutilized resource that can be used as a unique proteinase source. Proteinase from various sources catalyzes the hydrolysis of peptide bonds. Thus, it is expected that like other proteinases, hybrid catfish proteinase would be useful in biomedical, food and beverage

application. Moreover, the presented extraction media could be adopted to recover the trypsin-like serine proteinase from hybrid catfish viscera, which is currently a solid waste of Pa-duk-ra industry.

Keywords: Proteinase, Trypsin, Viscera, Catfish, Characterization, Extractant

INTRODUCTION

Proteinases have been known to degrade proteins through hydrolysis of peptide bonds (Klomklao et al., 2006a). Proteinases play a vital role in biotechnology, food processing and other industries as well as in a variety of physiological processes. Proteinases used in industry are mainly derived from plant, animal and microbial sources, whereas their counterparts derived from marine and other aquatic sources have not been extensively used (Simpson, 2000). Marine animals have adapted to different environmental conditions, and these adaptations, together with inter- and intraspecies genetic variations, are associated with certain unique properties of their proteinase, compared with their counterpart enzymes from animals, plants and microorganisms (Simpson, 2000). Some of these distinctive properties include higher catalytic efficiency at low temperature, lower thermal stability and substantial catalytic activity/stability at neutral to alkaline pH (Klomklao et al., 2007a). From this reason, there is a great potential for the recovery and use of proteinases from marine and aquatic sources, particularly from digestive tract. Proteinases from fish digestive organs have been extracted and characterized in several species of fish. Pepsin, trypsin and chymotrypsin are the most important digestive enzymes (Simpson, 2000).

Castillo-Yanez et al. (2004) isolated and characterized pepsin from the Monterey sardine viscera. Pepsins were found in pectoral rattail stomach with the maximal activity at pH 3.0-3.5 and 45°C (Klomklao et al., 2007b). Trypsin and chymotrypsin have been isolated and characterized from the pyloric ceca of tambaqui (Bezerra et al., 2001), viscera of anchovy (Hue et al., 1995), intestine of Nile tilapia (Bezerra et al., 2005). Klomklao et al. (2004) also extracted and characterized proteinases from the spleen of three tuna species. Those proteinases were classified to be trypsin-like serine proteinases with optimal activity at pH 9.0 and 55°C.

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). Pla-duk-ra is highly nutritious and is an excellent source of protein. The Pla-duk-ra processing industry is becoming increasingly important since it is one of the income generators for Phattalung 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 proteinases (Simpson, 2000). Hence, extraction 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, the characteristics of the enzyme obtained might be utilized in food industry or

other industries. However, no information on the properties of proteinases from the hybrid catfish viscera has been reported. The goals of this study were to characterize the proteinases and to study the effect of extraction medias on the recovery of proteinases from hybrid catfish viscera.

MATERIALS AND METHODS

Chemicals

Sodium caseinate, bovine hemoglobin, ethylenediaminetetraacetic acid (EDTA), pepstatin A, soybean trypsin inhibitor, *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, phenylmethylsulfonyl fluoride (PMSF), Brij 35, Triton X-100, Tween 20, L-tyrosine and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid, sodium chloride, tris (hydroxymethyl) aminomethane, acetone and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), Coomassie Brilliant Blue R-250 and *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Fish Sample Preparation

Viscera of hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) were obtained from a local market in Phattalung. 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 immediately frozen and stored at -20°C until used.

Preparation of Viscera Extract

Frozen viscera were thawed using 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 with three volumes of acetone at -20°C for 30 min according to the method of Klomklao et al. (2007a) with a slight modification. 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, defatted viscera powder was suspended in distilled water at a ratio of 1:9 (w/v) and stirred continuously at 4°C for 30 min. The suspension was centrifuged for 30 min at 4°C at 5,000×g using a Sorvall Model RC-B Plus centrifuge (Newtown, CT, USA) to remove the tissue debris. The supernatant was collected and referred to as “catfish viscera extract”.

Enzyme Assay

Proteinase activity of viscera extract from hybrid catfish was determined using hemoglobin and casein as substrates according to the method of An et al. (1994) and Klomklao et al. (2004). Different buffers were used for different pH

conditions: 0.2 M McIlvaine's buffer (0.2 M sodium phosphate-0.1 M sodium citrate) for pHs 2.0-7.5 and 0.1 M glycine-NaOH for pHs 8.0-11.0 (Glass et al., 1989; Munilla-Tella-Moran and Rey, 1996; Hidalgo et al., 1999). To initiate the reaction, 200 μl of 50 times diluted viscera extract was added into assay mixtures containing 200 μl of 2 % (w/v) casein, 200 μl of distilled water and 625 μl of reaction buffer. The enzymatic reaction was terminated by adding 200 μl of 50% (w/v) trichloroacetic acid (TCA). Unhydrolyzed protein substrate was allowed to precipitate for 15 min at 4°C, followed by centrifuging at 7,000×g for 10 min. The oligopeptide content in the supernatant was determined by the Lowry assay (Lowry et al., 1951) using tyrosine as a standard. Activity was expressed as tyrosine equivalents in TCA-supernatant. One unit of activity was defined as that releasing 1 nmole of tyrosine per min (nmol/Tyr/min). A blank was run in the same manner, except the enzyme was added after addition of 50 %TCA (w/v).

pH and Temperature Profiles

Proteolytic activity was measured using substrate-TCA-Lowry assay (An et al., 1994) at pHs 3.0, 5.0, 7.0 (using 0.2 M McIlvaine's buffer) and pH 9.0 (using 0.1 M glycine-NaOH) at various temperatures (25, 30, 40, 50, 55, 60, 65 and 70°C). The optimal pH was determined at 50°C over the pH range of 2.0-11.0 (0.2 M McIlvaine's buffer for pHs 2.0-7.5 and 0.1 M glycine-NaOH for pHs 8.0-11.0).

Effect of Inhibitors

The effect of inhibitors on proteinase activity was determined by incubating viscera extract with an equal volume of proteinase inhibitor solution to obtain the final concentration designated (0.1 mM E-64, 1 mM *N*-ethylmaleimide, 1.0 g/l soybean trypsin inhibitor, 5 mM TLCK, 5 mM TPCK, 1 mM pepstatin A, 5 mM benzamidine, 1 mM PMSF and 2 mM EDTA). After incubation the mixture at room temperature (26-28°C) for 30 min, the remaining activity was measured by substrate-TCA-Lowry method (An et al., 1994). Percent inhibition was then calculated.

Thermal and pH Stability

The viscera extracts were subjected to various temperatures (0-80°C) for 30, 60 or 120 min, followed by cooling in iced water. The remaining activity was measured by substrate-TCA-Lowry method (An et al., 1994) and reported as the relative activity (%) compared with the original activity. The effect of pH on enzyme stability was evaluated by measuring the residual activity after incubation at various pHs for 30, 60 and 120 min at room temperature. Different buffers used included 0.2 M McIlvaine's buffer (0.2 M sodium phosphate-0.1 M sodium citrate) for pH 2.0-7.5 and 0.1 M glycine-NaOH for pH 8.0-11.0.

Effect of NaCl

Effect of NaCl on proteinase activity was studied. NaCl was added to the standard reaction assay to obtain the final concentration of 0, 5, 10, 15, 20, 25 and 30% (w/v). The residual activity was determined at 50°C and pH 9.0 for 15 min using casein as a substrate.

Effect of $CaCl_2$

To study the effect of $CaCl_2$ on proteinase activity, $CaCl_2$ was added to the standard reaction assay to obtain the different final concentration (0, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} and 10^{-2} M). The activity was determined at 50°C and pH 9.0 for 15 min using casein as a substrate.

Activity Staining

Viscera extract was separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by activity staining according to the method of Garcia-Carreno and Haard (1993). Viscera extract was mixed with sample buffer (0.125 M Tris-HCl, pH 6.8 containing 20% (v/v) glycerol) with and without βME at a ratio of 1:1 (v/v). Four μg of proteins were loaded into the gel made of 4% stacking and 12% separating gels. The proteins were subjected to electrophoresis at a constant current of 15 mA per gel by a Mini-Protean II Cell apparatus. After electrophoresis, gels were immersed in 100 ml of 2% (w/v) casein in 50 mM Tris-HCl buffer, pH 7.5 for 1 h with constant agitation at 0°C to

allow the substrate to penetrate into the gels. The gels were then transferred to 2% (w/v) casein in 0.1M glycine-NaOH, pH. 9.0 and incubated at 50°C for 15 min with constant agitation to develop the activity zone. The gels were fixed and stained with 0.125% Coomassie blue R-250 in 45% ethanol and 10% acetic acid and destained in 30% methanol and 10% acetic acid. Development of clear zones on blue background indicated proteolytic activity.

For the inhibitor study, viscera extracts were incubated with an equal volume of proteinase inhibitor solutions to obtain the final concentration designated as describe above for 15 min at room temperature. After incubation, the mixtures were mixed with sample buffer at a ratio of 1:1 (v/v). The mixtures were loaded into the gel and activity staining was performed as previously described.

Effect of Extraction Media on the Recovery of Hybrid Catfish Proteinase

Effect of Extractants on Recovery of Hybrid Catfish Proteinases

Different extraction media including distilled water, 50 mM Na-phosphate, pH. 7.0 and 50 mM Tris-HCl, pH. 7.0 were used to extract proteinases from hybrid catfish viscera. The medium was added into the defatted viscera powder with at a ratio of 1:9 (w/v) and stirred continuously at 4°C for 30 min. The supernatant was recovered by centrifuging the slurry at 5,000×g for 30 min. The proteinase activity and protein content in the extracts were measured and the yield and specific activity of the extracts obtained using different media were compared. The extractant rendering the highest yield was chosen for further steps.

Effect of NaCl Concentration on the Recovery of Hybrid Catfish Proteinases

Defatted viscera powder was suspended in 50 mM Tris-HCl, pH 7.0 containing different NaCl concentration (0, 0.25, 0.5, 0.75 or 1 M) at a ratio of 1:9 (w/v) and stirred continuously at 4°C for 30 min. The supernatant was recovered by centrifuging the slurry at 5,000×g for 30 min. The proteinase activity and protein content in the extracts were measured. The extraction yield and specific activity of the extracts were calculated. The extractant rendering the highest yield was chosen for further steps.

Effect of Surfactant on the Recovery of Hybrid Catfish Proteinases

Defatted viscera powder was suspended in 50 mM Tris-HCl, pH 7.0 containing 0.5 M NaCl and 0.2% (v/v) different surfactants (Brij 35, Tween 20, Triton X-100 and SDS) at a ratio of 1:9 (w/v) and stirred continuously at 4°C for 30 min. The supernatant was recovered by centrifuging the slurry at 5,000×g for 30 min. The proteinase activity and protein content in the extracts were measured. The extraction yield and specific activity of the extracts were calculated.

Protein Determination

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

Statistical Analysis

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

RESULTS AND DISCUSSION

Temperature and pH Profiles of Hybrid Catfish Viscera Extract

The temperature profile of hybrid catfish viscera extract is shown in Fig. 1a. Proteolytic activity assayed at pH 3.0, 5.0, 7.0 and 9.0 increased markedly from 25°C to the highest peak at 50°C. Thereafter, the sharp decrease in activity was observed at higher temperatures. The decrease in activity with temperature above 50°C might be due to the thermal denaturation of enzymes. Generally, high activity was found in the neutral and alkaline pH ranges. The extract showed some activity at acidic pHs, which might result from stomach pepsin. For pH profiles, the extract from viscera of catfish showed two activity peaks. The major peak had the optimum pH at 9.0, while the minor peak showed the highest activity at pH 3.0 (Fig. 1b). The major peak was belonged to alkaline proteinase, while another peak mostly contributed to acid proteinase. Based on the optimum temperature and pH, the major proteinase from catfish viscera was characterized as heat-activated alkaline proteinases. The presence of acid and alkaline proteinases in internal organs of various fish species has been reported. Acidic proteinases from

fish stomach display high activity between pH 2 and 4, while alkaline digestive proteinases are most active at pH between 8 and 10 (Simpson, 2000). Pepsin and trypsin are two main groups of proteinases found in fish viscera. Pepsin is found in fish stomach and is active at acidic condition (Klomklao et al., 2007b), while trypsin is concentrated in pyloric ceca and is active at neutral and alkaline conditions (Klomklao et al., 2004). Acidic proteases from Monterey sardine (*Sardinops sagax carulea*) viscera exhibited optimal activity at 45°C and pH 2.5 when hemoglobin was used as a substrate (Castillo-Yonez et al., 2004). Alkaline proteinases from the intestinal section of discus fish (*Symphysodon aequifasciata*) had an optimum pH ranging from 11.5 to 12.5 when casein was used as a substrate (Chong et al., 2002). Bezerra et al. (2001) reported that alkaline serine proteinase from the pyloric ceca of tambaqui (*colossoma macromum*) exhibited optimal activity at 60°C and pH 9.5 when azocasein was used as a substrate. Heu et al. (1995) also reported that trypsin and chymotrypsin from anchovy viscera had optimal activity at pH 9.0 and 8.0 when casein and synthetic substrate (BAPNA and BTEE) was used as substrate, respectively.

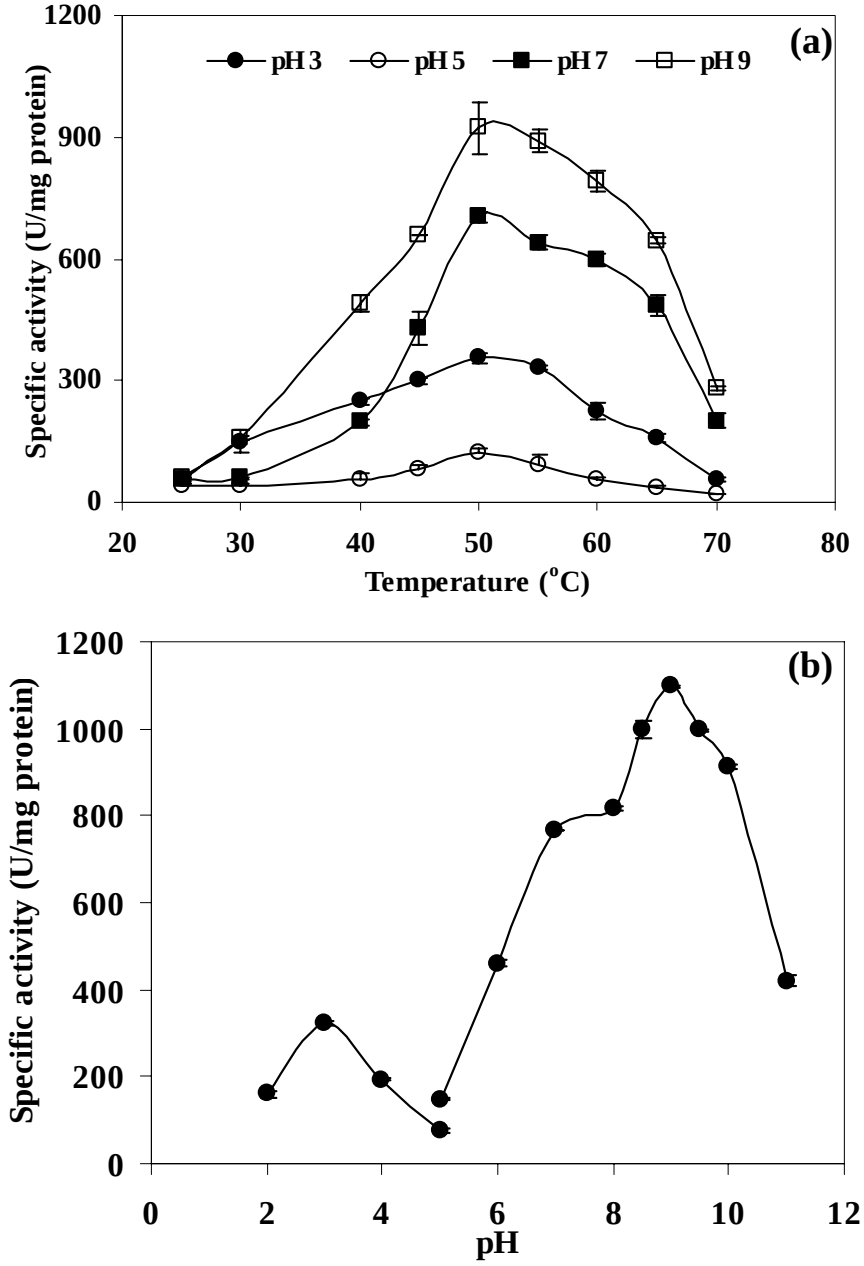

FIG. 1. TEMPERATURE (A) AND pH (B) PROFILES OF PROTEINASES FROM HYBRID CATFISH VISCERA. Bars represent the standard deviation from triplicate determinations.

Thermal and pH Stability

Thermal stability of hybrid catfish viscera proteinase is depicted in Fig. 2a. The enzyme was stable when incubated at temperature up to 40°C for 30-120 min. Nevertheless, the sharp decrease in activity was noticeable at temperature above 50°C. No activity was remained at 80°C, suggesting that complete loss in activity caused by thermal denaturation of proteinases. In general, the stability of proteinases decreased when the heating time was increased. A heating time of 120 min cause the highest loss of activity at every temperature above 40°C used. It was presumed that proteinases from hybrid catfish viscera underwent denaturation during high temperature heating, especially at the longer time. The result was in accordance with Klomklao et al. (2007a) who reported that denaturation of alkaline proteinase from skipjack tuna spleen occurred above 50°C.

For pH stability, the catfish extract was stable in the pH range of 7-11 with the exposure time of 30-120 min, in which residual activity more than 80% was found (Fig. 2b). With an extend incubation time, proteinase activity was lost to a greater extent. The marked decrease in activity was noticeable at pH below 7. The stability of the enzyme at a particular pH might be related to the net charge of the enzyme at that pH (Klomklao et al., 2006b). At extream pHs, strong intramolecular electrostatic repulsion caused by high net charge, results in swelling and unfolding of the protein molecules (Damodaran, 1996). Inactivation of enzyme activity at acidic pH was also reported for the alkaline proteinases and trypsin from skipjack tuna (Klomklao et al., 2007a), and tongol tuna (Klomklao et al., 2006b).

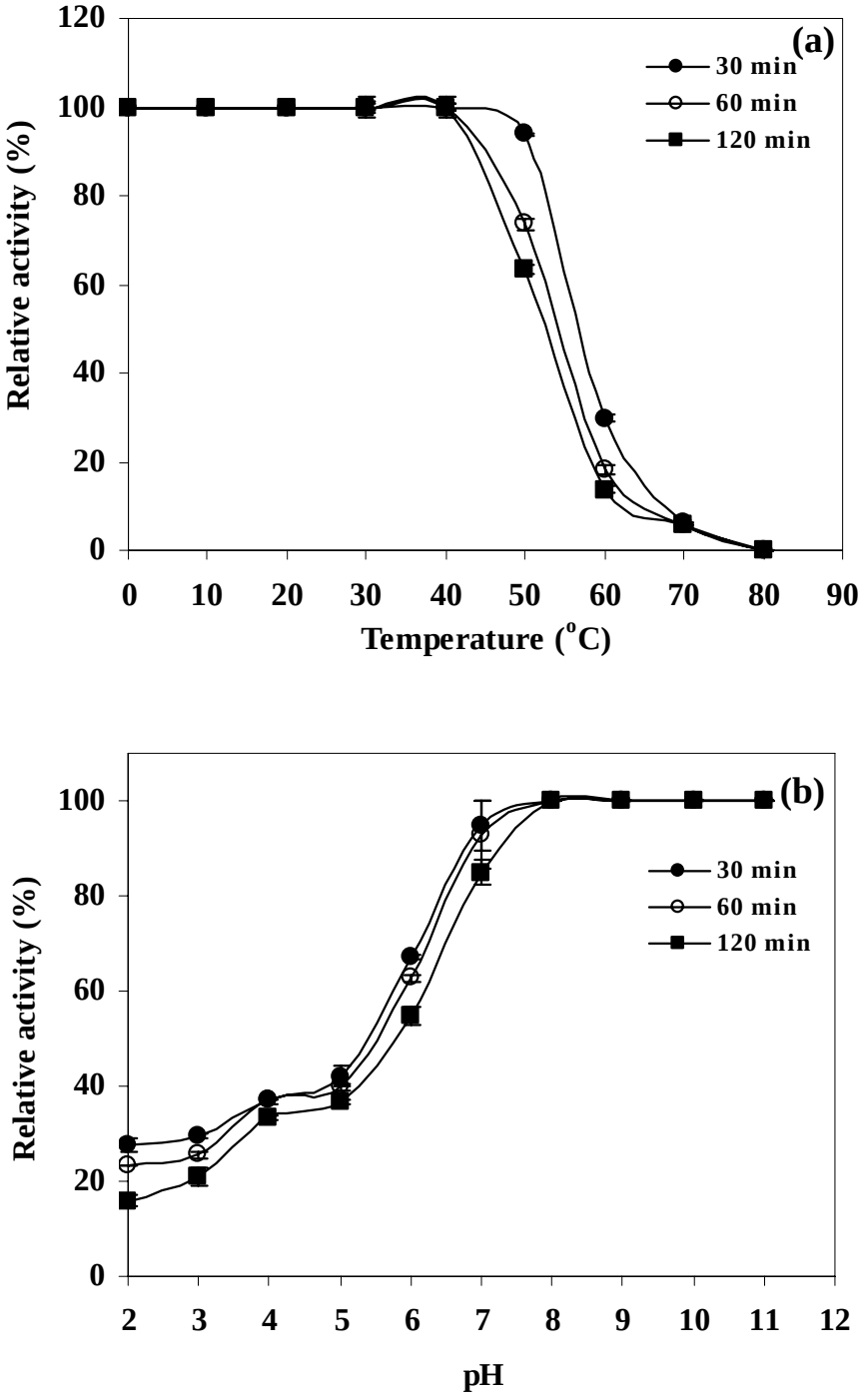

FIG. 2. THERMAL (A) AND pH (B) STABILITY OF PROTEINASES FROM HYBRID CATFISH VISCERA. Bars represent the standard deviation from triplicate determinations.

Effect of Inhibitors

The effect of various inhibitors on proteinase activity from hybrid catfish viscera was determined as shown in Table 1. The proteolytic activity was strongly inhibited by soybean trypsin inhibitor, which are widely used to identify trypsins, PMSF and benzamidine (a serine protease inhibitor) and partially inhibited by TLCK (trypsin inhibitor). Specific inhibitors of cysteine proteinases (E-64, *N*-ethylmaleimide) had no inhibitory effect on proteinase activity from hybrid catfish viscera. Nevertheless, TPCK (a synthetic chymotrypsin inhibitor) EDTA (a metalloprotease inhibitor) and pepstatin A (an aspartic protease inhibitor) showed the negligible inhibitory effect. The result indicated that major proteinases from the viscera of catfish were serine proteinases, particularly trypsin or trypsin-like enzymes. The serine proteinase from the intestine of discus fish was inhibited by soybean trypsin inhibitor, PMSF and TLCK while TPCK and EDTA showed partial inhibition (Chong et al., 2002). Trypsin-like enzyme from tambaqui pyloric ceca was inhibited by some trypsin inhibitors, such as PMSF, benzamidine and TLCK (Bezerra et al., 2001).

TABLE 1. EFFECT OF VARIOUS INHIBITORS ON THE ACTIVITY OF PROTEINASES FROM HYBRID CATFISH VISCERA*

Inhibitors	Concentration	%Inhibition***
Control		0a**
E-64	0.1 mM	0a
N-ethylmaleimide	1 mM	0a
Soybean trypsin inhibitor	1.0 g/l	67.2 ±2.8f
TLCK	5 mM	23.1 ±7.8c
TPCK	5 mM	3.1 ±1.0ab
Benzamidine	5 mM	48.7 ±1.2d
PMSF	1 mM	59.9 ±0.1e
Pepstatin A	1 mM	2.6 ±3.0ab
EDTA	2 mM	6.6 ±0.7b

*Activity was analyzed using casein as a substrate for 30 min at pH 9.0 and 50°C.

**The different letters in the same column denote the significant differences ($p<0.05$).

***Mean ±S.D. from triplicate determination.

Effect of NaCl

The effect of NaCl at different concentrations on proteinase activity in catfish viscera extract is depicted in Fig. 3. The activity decreased with increasing NaCl concentration. No activity was observed in presence of 30% NaCl. The activity loss might be a result of the denaturation of proteinases caused by the "salting out" effect. NaCl at higher concentration possibly competed with the enzyme in water binding, resulting in a stronger protein-protein interaction (Klomklao et al., 2007b). This was possibly associated with the losses in the activity of proteinases. Klomklao et al. (2004) reported that the activity of alkaline proteinases from yellowfin tuna spleen was reduced with the addition of 25-30% NaCl. Yatsunami and Takenaka (2000) found that viscera proteolytic activity of sardine (*Etrumeus micropus*) decreased when the NaCl concentration increased up to 14%. Hernandez-Herrero et al. (1999) also reported that the proteolytic activity of the acid and alkaline proteinases is inhibited strongly in the presence of 15-20% NaCl. From the results, more than 20% of proteinase activity remained in the presence of a high concentration of NaCl (10-20%). Thus, these proteinases may contribute to the hydrolysis of proteins in the salted fish products such as Pla-duk-ra during marinating and storage. This would contribute to flavor development of finished product.

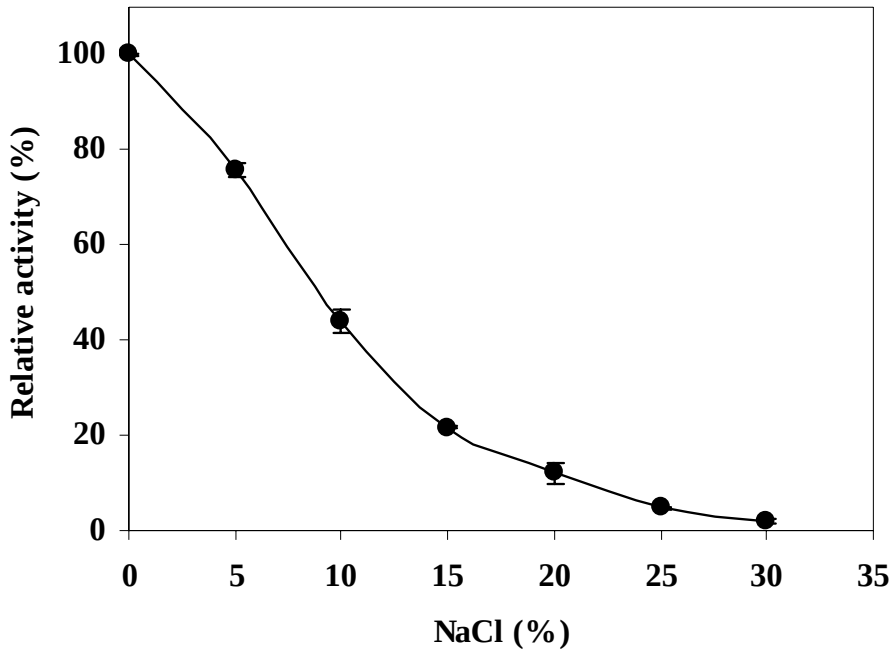

FIG. 3. EFFECT OF NACL CONCENTRATION ON ACTIVITIES OF PROTEINASES FROM HYBRID CATFISH VISCERA. Bars indicate standard deviation from triplicate determinations.

Effect of $CaCl_2$

Proteinase activities of viscera extract from hybrid catfish increased with the addition of calcium chloride (Fig. 4). The highest activity was obtained in the presence of 1 mM $CaCl_2$. It is known that calcium ions promote the formation of active trypsin from trypsinogen and stabilize trypsin against autolysis (Sipos and Markel, 1970). Stabilization against thermal inactivation by calcium was also reported for trypsin from rainbow trout pyloric ceca (Kristjansson, 1991), yellowfin tuna and skipjack tuna spleen (Klomklao et al., 2004). Bode and Schwager (1975) reported that calcium not only protected trypsin against self-digestion, but it also slightly increased its proteolytic activity. Two calcium-binding sites are present in trypsinogen (Kosslakoff et al., 1977). The primary site, with a higher affinity for calcium ions, is common in trypsinogen and trypsin, and the secondary site is only in the zymogen (Kishimura et al., 2006). The binding of calcium to trypsinogen induces a conformational change, which is associated with the formation of an active form. Therefore, calcium ions played an essential role in activation of proteinases from hybrid catfish viscera.

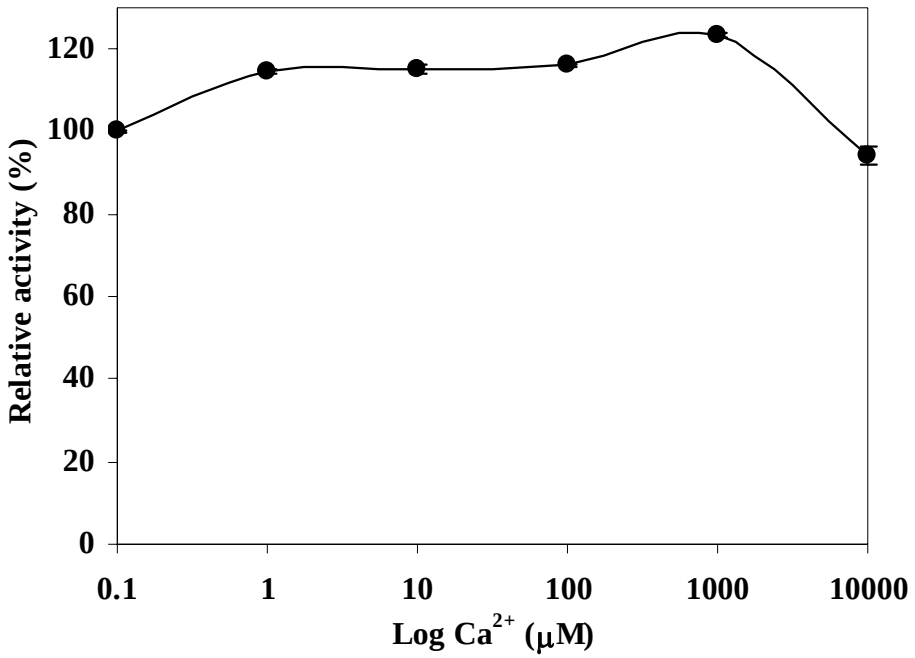

FIG. 4. EFFECT OF $CACL_2$ ON ACTIVITIES OF PROTEINASES FROM HYBRID CATFISH VISCERA. Bars indicate standard deviation from triplicate determinations.

Activity staining

The activity staining of proteinases from hybrid catfish viscera is shown in Fig. 5. Two activity bands were observed as clear zones on the dark background under non reducing condition at the apparent molecular weight of 23 and 20 kDa. Based on the molecular weight, the activity bands were tentatively identified as trypsin and/or trypsin-like serine proteinases. Trypsin consists of a single peptide chain with molecular weight typically of 24 kDa. However, the differences in trypsin maybe owing to the genetic variation among species (Klomklao et al., 2004). The molecular weight of trypsin from Greenland cod pyloric ceca was 23.5 kDa (Simpson and Haard, 1984). Klomklao et al. (2004) reported that the molecular weights of the major activity bands of tongol and yellowfin tuna were 21 kDa.

Under reducing condition (Fig. 5), a loss of proteinase activity on gel was observed. Only the protein band with apparent molecular weight of 23 kDa remained. Nevertheless, the smaller activity band was obtained. It is postulated that the cleavage of disulfide bond by the action of βME could lead to protein denaturation and loss of functionality. Damodaran (1996) reported that proteins that require high structural stability to function as catalysts are usually stabilized by intramolecular disulfide bonds, and their native conformations can be separated into lower-apparent MW proteins by the action of reducing agent. Therefore, the disulfide bond played an essential role in stabilizing the intact structure in the native conformation of proteinases in hybrid catfish viscera.

Fig. 6 showed the effect of inhibitors on the activity bands observed on SDS-substrate gel electrophoresis. The activity bands were effectively inhibited when the extracts was treated with soybean trypsin inhibitor and partially inhibited by TLCK. On the other hand, the activity bands were retained after mixing with E-64, EDTA and pepstatin A. The result confirmed that the major proteinases in hybrid catfish viscera are trypsin or trypsin-like enzyme.

This major viscera proteinase from hybrid catfish will be further purified and characterized.

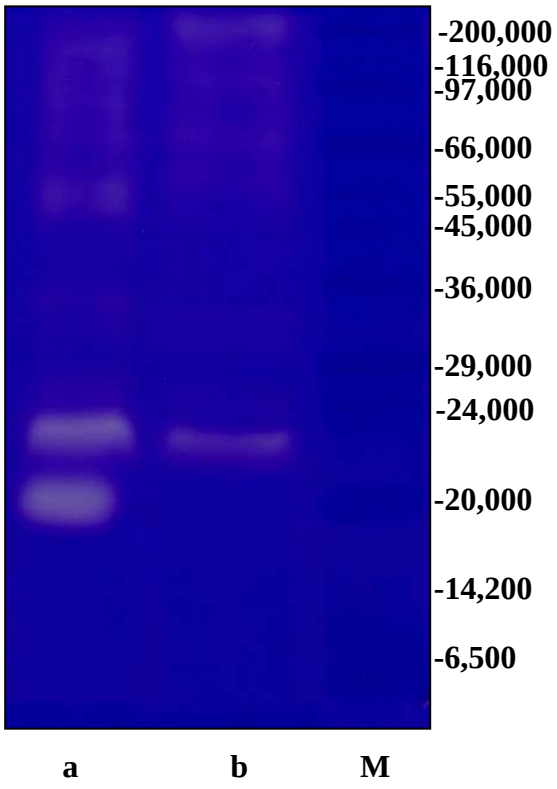

FIG. 5. ACTIVITY STAINING OF VISCERA PROTEINASES FROM HYBRID CATFISH UNDER NON-REDUCING (A) AND REDUCING CONDITION (B). M, MOLECULAR WEIGHT STANDARD.

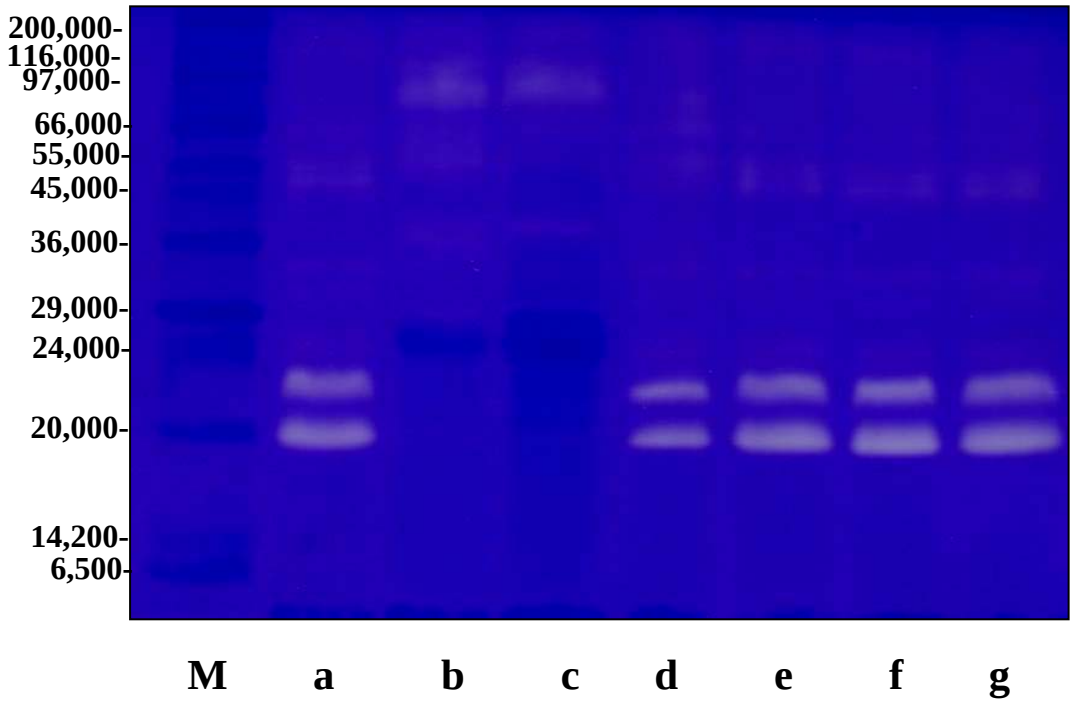

FIG. 6. ACTIVITY STAINING OF VISCERA PROTEINASES FROM HYBRID CATFISH WITH AND WITHOUT PROTEINASE INHIBITORS. M, molecular weight standard; a, control; b:1 g/l soybean trypsin inhibitor; c: 5 g/l soybean trypsin inhibitor; d: 5 mM TLCK; e: 0.1 mM E-64; f: 2 mM EDTA; g: 0.01 mM pepstatin A.

Effect of Extraction Media on the Recovery of Hybrid Catfish Proteinase

Effect of extracting media on proteinase extraction from hybrid catfish viscera is shown in Table 2. Viscera extract using 50 mM Tris-HCl, pH 7.0 showed the higher proteinase activity than those extracted with distilled water and 50 mM Na-phosphate buffer, pH 7.0 when casein was used as substrate ($p<0.05$) The results suggested that Tris-HCl buffer had a greater ability to extract proteinase than Na-phosphate and distilled water. Tris in the buffer might favor the solubilization of proteinase associated with the cell membrane by increasing charge of enzymes and proteins. The repulsion between the enzymes and tissues might lead to the ease of proteinase extraction from the viscera. Trypsins from skipjack tuna spleen and yellowfin tuna spleen were extracted with 10 mM Tris-HCl, pH 7.0 (Klomklao et al., 2006a; 2007a). Kishimura et al. (2006) also used 10 mM Tris-HCl, pH 7.0 for extracting the trypsin from true sardine viscera. From this study, 50 mM Tris-HCl, pH 7.0 was chosen as the extraction medium for hybrid catfish viscera proteinase because the extract had the maximum proteinase activity.

Tris-HCl buffer containing different NaCl concentration were used to extract the proteinases from hybrid catfish viscera (Table 3). When the concentration of NaCl was increased from 0 to 0.5 M, activity, protein content apparently increased ($p<0.05$). However, there was no further increase in the activity with NaCl above 0.5 M. This probably due to the denaturation of hybrid catfish viscera proteinases at high salt concentration. Hence, Tris-HCl buffer containing 0.5 M NaCl was shown to the best extraction medium for catfish viscera proteinase ($p<0.05$).

Table 4 showed the effect of some surfactants on the recovery of proteianse from hybrid catfish viscera. Addition of surfactants in 50 mM Tris-HCl, pH 7 containing 0.5 M NaCl markedly increased the yield of proteinase extracted at 4°C for 30 min. The highest yield or proteinase activity and specific activity was obtained when the hybrid catfish viscera powder was extracted with 50 mM Tris-HCl, pH 7 containing 0.5 M NaCl and 0.2% Brij 35 ($p<0.05$). The yield of proteinase extracted with the aid of Brij 35 was approximately 1.7-fold higher than that of proteinases extracted without Brij 35. The Brij 35 was added to facilitate improved extraction of soluble cell material and to emulsify the small amount of lipid present in hybrid catfish viscera extract to prevent lipid interference with the proteinase activity. Kurtovic et al. (2006) reported that addition of 0.2%(v/v) of Brij 35 to the extract from chinook salmon intestine resulted in a small increase in trypsin activity. From the results, 50 mM Tris-HCl, pH 7.0 containing 0.5 M NaCl and 0.2% (v/v) Brij 35 was chosen as the extraction medium for proteinases in hybrid catfish viscera.

TABLE 2. EFFECT OF EXTRACTING MEDIA ON THE RECOVERY OF PROTEINASE FROM HYBRID CATFISH VISCERA*

Extraction media	Total protein (mg)	Total activity ($\times 10^3$Units)	Specific Activity (U/mg protein)	Proteinase activity (U/g tissue)**
Distilled water	52.38	22.24	424.6±0.63c	14,729.0±21.79a
50 mM Tris-HCl, pH 7.0	67.20	26.01	387.1±4.30b	17,339.4±192.78c
50 mM Na-Phosphate, pH 7.0	64.09	23.86	372.3±1.78a	15,904.5±75.98b

*The defatted catfish viscera powder was extracted in different media at 4°C for 30 min and proteolytic activity was analyzed using casein as substrate for 15 min at pH 9.0 and 50°C.

**Mean±SD from triplicate determinations

The different letters in the same column denote the significant differences ($p<0.05$).

TABLE 3. EFFECT OF NACL CONCENTRATION ON THE RECOVERY OF PROTEINASE FROM HYBRID CATFISH VISCERA*

NaCl Concentration (M)	Total protein (mg)	Total activity ($\times 10^3$Units)	Specific activity (U/mg protein)	Proteinase activity (U/g tissue)**
0	57.30	22.85	398.8±1.79a	15,129.2±67.93a
0.25	58.47	30.31	518.4±2.62c	20,071.9±236.72c
0.5	65.06	32.62	501.4±7.51bc	21,600.1±323.55d
0.75	56.14	27.65	492.5±21.86b	18,308.6±812.61b
1.0	55.30	22.76	411.6±0.62a	15,067.7±121.48a

*The defatted catfish viscera powder was extracted in 50 mM Tris-HCl, pH 7.0 in different NaCl concentration at 4°C for 30 min and proteolytic activity was analyzed using casein as substrate for 15 min at pH 9.0 and 50°C.

**Mean±SD from triplicate determinations

The different letters in the same column denote the significant differences ($p<0.05$).

TABLE 4. EFFECT OF SOME SURFACTANTS ON THE RECOVERY OF PROTEINASE FROM HYBRID CATFISH VISCERA*

Surfactant	Total protein (mg)	Total activity ($\times 10^3$Units)	Specific activity (U/mg protein)	Proteinase activity (U/g tissue)**
Control	63.71	31.28	491.0±16.46a	20,717.5±694.26a
SDS	92.99	49.40	531.2±13.62bc	32,713.3±839.03c
Tween 20	87.18	46.72	535.9±5.25bc	30,943.0±302.82b
Triton X100	88.58	45.70	515.9±10.4b	30,264.0±610.14b
Brij 35	97.01	52.88	545.1±4.8c	35017.2±827.38d

*The defatted catfish viscera powder was extracted in 50 mM Tris-HCl, pH 7.0 containing 0.5 M NaCl (control) in different surfactants at 4°C for 30 min and proteolytic activity was analyzed using casein as substrate for 15 min at pH 9.0 and 50°C.

**Mean±SD from triplicate determinations

The different letters in the same column denote the significant differences ($p<0.05$).

CONCLUSION

Heat-activated alkaline enzyme with the optimal activity at pH 9.0 and 50°C was the predominant enzyme in hybrid catfish viscera extract based on inhibitory study, optimal pH and temperature. Based on inhibitor study, effect of $CaCl_2$ and molecular mass, the major was most likely classified as trypsin or trypsin-like. Major proteinase from hybrid catfish can be successful extracted using 50 mM Tris-HCl, pH 7.0 containing 0.5 M NaCl and 0.2% (v/v) Brij 35 and can be potential novel enzymes for future applications.

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CHAPTER 2

EFFECT OF SALTS AND POLYETHYLENE GLYCOLS ON THE PARTITIONING AND RECOVERY OF TRYPSIN FROM HYBRID CATFISH VISCERA IN AQUEOUS TWO-PHASE SYSTEMS

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EFFECT OF SALTS AND POLYETHYLENE GLYCOLS ON THE PARTITIONING AND RECOVERY OF TRYPSIN FROM HYBRID CATFISH VISCERA IN AQUEOUS TWO-PHASE SYSTEMS

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ABSTRACT

The partitioning behavior of trypsin from hybrid catfish viscera in aqueous two-phase systems (ATPS) was studies. Factors such as PEG molecular mass and concentration as well as types and concentration of salts affected protein separation. Trypsin partitioned mainly in the top PEG-rich phase. ATPS formed by PEG of molecular weight 4000 (20%, w/w) and NaH_2PO_4 (20%, w/w) showed the best capability for trypsin purification from hybrid catfish viscera. Under such condition, the highest specific activity (30.05 units/μg protein) and purification fold (27.3) were obtained. SDS-PAGE analysis revealed that the enzyme after ATPS separation was near homogeneity and based on the activity staining, the band intensity of enzyme in ATPS fraction increased, indicating the greater specific activity of the viscera extract. The partitioned enzyme displayed optimal activity at pH 9.0 and 50°C, respectively. The enzyme was stable up to 40°C and within the pH range of 8-12. The enzyme exhibited a progressive decrease in activity with increasing NaCl concentration.

PRACTICAL APPLICATIONS

This paper describes the separation and recovery of trypsin from hybrid catfish viscera in ATPS and its properties. ATPS provides an efficient and attractive method for partitioning and recovery of trypsin from hybrid catfish viscera. Trypsins from various sources catalyze the hydrolysis of peptide bonds on the carboxyl sides of arginine and lysine. Therefore, it is expected that like other trypsins, trypsin after ATPS separation from hybrid catfish viscera could be useful in biomedical, food and beverage industry application.

Keywords: Aqueous two-phase systems; Trypsin; Proteinase; Purifiation; Separation

INTRODUCTION

Partitioning in an aqueous two-phase system (ATPS) is a selective method used for biomolecule purification (Silva and Franco, 2000). ATPS is formed when combinations of hydrophilic solutes (polymer or polymer and certain salts) display incompatibility in aqueous solution above critical concentrations (Yang et al., 2008). ATPS offers many advantages including low-process time, low-material cost, low-energy consumption, good resolution, high yield and a relative high capacity. In addition, this system is easily scaled-up (Srinivas et al., 1999). Therefore, ATPS have found application in the industrial scale purification of proteins from biomass (Srinivas et al., 1999). The use of ATPS in downstream processing has been focused on the extraction, separation and concentration of various biomolecules includings xylanase (Yang et al., 2008), proteinase (Klomklao et al., 2005), amyloglucosidase (Tanuja et al., 1997), amino acid (Li et al., 1997) etc. However, sometimes, it is used as a potential primary purification technique to reduce the bulk of processing stream, if not the only step to be followed by more selective final purification steps, such as chromatography, electrophoresis, etc. (Klomklao et al., 2005).

Trypsin (EC 3.4.21.4) is a serine endopeptidase that specifically hydrolyzes proteins and peptides at the carboxyl side of arginine and lysine residues. Trypsin plays major roles in biological processes including digestion, activation of zymogens of chymotrypsin and other enzymes (Klomklao et al.,

2007a). Trypsin has been used increasingly since it is both stable and active under harsh conditions, such as temperatures of 50-60°C, high pHs and in the presence of surfactants or oxidizing agents (Klomklao et al., 2007a). Therefore, increasing quantities of highly pure trypsin are required due to its wide range of industrial and scientific uses. Trypsins have been purified and characterized from the viscera of fish such as the spleen of skipjack tuna (*Katsuwonus pelamis*) (Klomklao et al., 2007a) and tongol tuna (*Thunnus tonggol*) (Klomklao et al., 2006) and the pyloric ceca of chinook salmon (*Oncorhynchus tshawytscha*) (Kurtovic et al., 2006). However, traditional methods for purifying trypsin involve several steps including ammonium sulfate precipitation, chromatography, dialysis and filtration, which increase the cost of the processes and reduce the yield (Lima et al., 2002). Thus, it is desirable to develop new methods that improve and may replace totally or partially any stages of the current purification procedures.

Recently, we isolated and characterized the proteinases from hybrid catfish viscera generated during the processing of Pla-duk-ra, Thai dry fermented fish product. Hybrid catfish viscera showed high proteolytic activity and those proteinases were classified to be trypsin which can be recovered and used for industrial application (Klomklao et al., 2008). However, no information regarding the separation and recovery of trypsin from hybrid catfish viscera using ATPS has been reported. The aims of this study were to investigate the feasibility of utilizing ATPS for partitioning and recovery of hybrid catfish viscera trypsin and to find appropriate conditions, concerning PEG molecular mass and concentration as well as types and concentration of salts for efficient partitioning and recovery of trypsin from hybrid catfish viscera.

MATERIALS AND METHODS

Chemicals

Polyethylene glycol (PEG) 1000, 4000, Nα-Benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA), β-mercaptoethbanol (βME), sodium chloride, tris (hydroxymethyl) aminomethane, dimethylsulfoxide, sodium caseinate, magnesium sulfate, dipotassium hydrogen-phosphate, sodium sulfate, sodium dihydrogenphosphate, sodium citrate, ammonium sulfate, sodium dodecyl sulfate (SDS), Coomassie Brilliant Blue R-250 and *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) and bovine serum albumin were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

Fish sample preparation

Viscera of hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) were obtained from a local market in Phattalung, Thailand. 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 immediately frozen and stored at -20°C until used.

Preparation of viscera extract

Frozen viscera were thawed using 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 according to the method of Klomklao et al. (2007b) with a slight

modification. 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 viscera extract, the viscera powder was suspended in distilled water at a ratio of 1:9 (w/v) and stirred continuously at 4°C for 30 min. The suspension was centrifuged for 30 min at 4°C at 5,000×g using a Sorvall Model RC-B Plus centrifuge (Newtown, CT, USA) to remove the tissue debris. The supernatant was collected and referred to as “catfish viscera extract”.

Enzyme assay

Trypsin activity was measured by the method of Benjakul et al. (2000) with a slight modification using BAPNA as substrate. To initiate the reaction, 200 µl of diluted viscera extract were added to the preincubated reaction mixture containing 1000 µl of 0.5 mM of BAPNA in reaction buffer (0.1 M glycine–NaOH, pH 9.0) and 200 µl of distilled water. The mixture was incubated at 50°C for precisely 15 min. The enzymatic reaction was terminated by adding 200 µl of 30% (v/v) acetic acid. The reaction mixture was centrifuged at 8,000×g for 3 min at room temperature (Hettich zentrifugen, Berlin, Germany). Trypsin activity was measured by the absorbance at 410 nm due to p-nitroaniline released. One unit of trypsin activity was defined as the amount causing an increase of 0.01 in absorbance per min.

Preparation of aqueous-two phase systems

ATPS were prepared in 10-ml centrifuge tubes by adding the appropriate amount of PEG, salts and hybrid catfish viscera extract. To study the effect of salts on partitioning the trypsin from hybrid catfish viscera extract using ATPS, different salts including NaH_2PO_4, K_2HPO_4, $MgSO_4$, $Na_3C_6H_5O_7$, $(NH_4)_2SO_4$ and Na_2SO_4 at different concentrations (15, 20 and 25%, w/w) were mixed with 20%PEG1000 in aqueous system. Distilled water was used to adjust the system to obtain the final weight of 5 g. The mixtures were mixed continuously for 3 min using a Vortex mixer (Vortexgenie2, G-560E, Bohemia, New York). Phase separation was achieved by centrifugation for 5 min at 5,000×g. Top phase was carefully separated using a pasteur pipette and the interface of each tube was discarded. Volumes of the separated phases were measured. Aliquots from each phase were taken for enzyme assay and protein determination.

Purification factor (PF), defined as the ratio of specific trypsin activity (SA) of each phase to the initial specific trypsin activity of viscera extract was calculated. Partition coefficient (K_E or K_P), the ratio of enzyme activity or protein concentration in the top phase to that in the bottom phase was also calculated. The volume ratio (V_R) defined as the ratio of volume in the top phase to that in the bottom phase was recorded.

To study the effect of the concentrations (10, 15, 20 and 25%, w/w) of PEG1000 and PEG4000 on partitioning of trypsin in hybrid catfish viscera extract, NaH_2PO_4 at a level of 20% was used in the system. Partitioning was performed as previously described. All experiments were run in duplicate. The ATPS rendering the most effective partitioning was chosen. Phase with high specific activity, was

dialyzed against 10 volumes of 50 mM Tris–HCl, (pH 7.5) for 18 h with three changes of buffer in the first 3 h and five changes in the last 15 h.

Sodium dodecyl sulfate-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 (0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10%β-mercaptoethanol) and boiled for 3 min. The samples (20 µg) were loaded on the gel made of 4% stacking and 15% separating gels and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-Protean II cell apparatus. After electrophoresis, the gels were stained with 0.2% Coomassie Brilliant Blue R-250 in 45% methanol and 10% acetic acid and destained with 30% methanol and 10% acetic acid.

Activity staining

Viscera extract and selected phase with high specific trypsin activity obtained from ATPS were separated on SDS-PAGE, followed by activity staining according to the method of Garcia-Carreno et al. (1993). The samples were mixed with sample buffer (0.125 M Tris-HCl, pH 6.8 containing 20% (v/v) glycerol) with and without βME at a ratio of 1:1 (v/v). Four µg of proteins were loaded into the gel made of 4% stacking and 15% separating gels. The proteins were subjected to electrophoresis at a constant current of 15 mA per gel by a Mini-Protean II Cell apparatus. After electrophoresis, gels were immersed in 100 ml of 2% (w/v)

casein in 50 mM Tris-HCl buffer, pH 7.5 for 1 h with constant agitation at 0°C to allow the substrate to penetrate into the gels. The gels were then transferred to 2% (w/v) casein in 0.1M glycine-NaOH, pH. 9.0 and incubated at 50°C for 15 min with constant agitation to develop the activity zone. The gels were fixed and stained with 0.125% Coomassie blue R-250 in 45% ethanol and 10% acetic acid and destained in 30% methanol and 10% acetic acid. Development of clear zones on blue background indicated proteolytic activity.

Protein determination

Protein concentration was measured by the method of Bradford (1976) using bovine serum albumin as a standard.

pH and temperature profile

Trypsin activity was assayed over the pH range of 4.0-12.0 (0.2 M McIlvaine's buffer for pHs 2.0-7.5 and 0.1 M glycine-NaOH for pHs 8.0-11.0) at 50°C for 15 min. The optimal temperature was determined at pH 9.0 at different temperatures ranging from 20 to 80°C using BAPNA as a substrate.

Thermal and pH stability

The enzyme was incubated at different temperatures (20, 30, 40, 50, 60, 70 and 80°C) for 30 min in a temperature controlled water bath (Precision Scientific Shaking Water bath 25, Chicago, IL, USA). Thereafter, the heat treated samples

were rapidly cooled in an iced bath, and residual activity was assayed using BAPNA as a substrate as previously described.

The effect of pH on enzyme stability was evaluated by measuring the residual enzyme activity after incubation at various pHs for 30 min at 25°C. The compositions of the buffers used for the pH stability study were as described above.

Effect of NaCl

Effect of NaCl on trypsin activity was studied. NaCl was added to the standard reaction assay to obtain the final concentration of 0, 5, 10, 15, 20, 25 and 30% (w/v). The residual activity was determined at 50°C and pH 9.0 for 15 min using BAPNA as a substrate.

RESULTS AND DISCUSSION

Effect of Salts on the Trypsin Partitioning in ATPS

Table 1 shows the effects of type and concentration of salts on the partitioning and recovery of trypsin from hybrid catfish viscera. The trypsin partitioning was assayed in several biphasis systems of 20%PEG1000 with different salts including $MgSO_4$, NaH_2PO_4, K_2HPO_4, $Na_3C_6H_5O_7$, Na_2SO_4, and $(NH_4)_2SO_4$ at different concentrations. No phase separation were found when PEG1000 or salt was used alone (data not shown) suggesting that the combination of either PEG or salt was need for partitioning process. After phase separation, two phases were obtained. The upper phase becomes PEG-rich and the lower phase becomes salt-rich. From all ATPS studied, the trypsin was partitioned mainly in the PEG-rich top phase, principally those with hydrophobic properties (Reh et al., 2002). Therefore, the trypsin yield from the opposite phase (lower phase) was quite low. The result was in agreement with Klomklao et al. (2005) who reported that proteinases from the tuna spleen were partitioned predominantly in the PEG-rich upper phase. Yang et al. (2008) also reported that xylanase was separated in the top polymer-rich phase in ATPS composed of 12.5%PEG and 25%$(NH_4)_2SO_4$.

The maximum SA and PF of trypsin obtained in PEG1000-salts systems depended on the medium composition (Table 1). System of composition 20%PEG1000 and 20%NaH_2PO_4 showed the highest SA (2.24 units/µg protein) and PF (2.8-fold) indicating this system has the best capacity of separating trypsin from hybrid catfish viscera. Thus, 20%NaH_2PO_4 was employed in subsequent study on the effect of PEG molecular mass and concentration on trypsin

partitioning and recovery. The use of 25% (w/v) PEG1500 and 20-25% (w/v) NaH_2PO_4 was effective in β-xylosidase purification (Pan et al., 2001).

Table 1 also shows the distribution of the proteins in ATPS characterized by partition coefficient K. K_E and K_P represented trypsin and protein partitioning, respectively. Phase composition of PEG1000 and NaH_2PO_4 or K_2HPO_4 showed a greater trypsin partition ratio (K_E>25) than those with other salts used. The results indicated that trypsin moved to upper phase upon adding sodium and potassium phosphate. This effect was also observed by Han and Lee (1997) who found that BSA partitioned more to the upper phase as the phosphate concentration increased. Generally, the biomolecule partition coefficients (or ratios) of two substances differ by a factor of 10 or more, their separation can be satisfactorily carried out (Reh et al., 2002). When a single component must be extracted from a mixture, phase system compositions are often manipulated in such a manner that the component partitions into one of the phases, while the other components of the mixture partition into the other phase (Klomklao et al., 2005). Phase composition of 20%PEG1000 and 20%NaH_2PO_4 showed lower K_P (0.26) than others systems, indicated that it caused a shift of contaminant proteins, nucleic acid and other undesirable components to the lower phase. Therefore, the extraction conditions used resulted in the enrichment of specific trypsin activity, which was due to the differential partitioning of the desired trypsin and contaminating enzymes and proteins to the opposite phases.

The effect of salts at different concentrations on the recovery of trypsin from hybrid catfish viscera was also investigated. The trypsin activity decreased with increasing salt concentration led to a poor yield recovery (Table 1). This

result can be rationalized by considering the negative effect of using a high salt concentration. Salts at high concentration possibly competed with enzyme in water binding, resulting in stronger protein-protein interaction, which was probably associated with precipitation (Vojdani, 1996). Klomklao et al. (2005) found that the presence of high concentrations of salts in the reaction medium greatly decreased both the yield and the selectivity towards the proteinase from tuna spleen. Pan et al. (2001) reported that increasing NaH_2PO_4 concentration resulted in a less activity recovery as well as a poorer specific activity. Hence, the type of salt and concentration used were critical factor for recovery and partitioning of trypsin from hybrid catfish viscera in ATPS.

TABLE 1. EFFECT OF PHASE COMPOSITION IN PEG 1000-SALT ATPS ON PARTITIONING OF TRYPSIN FROM HYBRID CATFISH VISCERA

Phase composition (%, w/w)	V_R	K_P	K_E	SA	PF	Yield (%)
20%PEG1000-15%$MgSO_4$	1.15	0.26	1.10	1.49	1.86	46.49
20%PEG1000-20%$MgSO_4$	0.76	0.15	1.00	1.80	2.25	32.82
20%PEG1000-25%$MgSO_4$	0.54	0.19	1.32	1.41	1.76	26.48
20%PEG1000-15%NaH_2PO_4	-	-	-	-	-	-
20%PEG1000-20%NaH_2PO_4	1.37	0.26	25.74	2.24	2.80	59.83
20%PEG1000-25%NaH_2PO_4	1.00	0.61	38.11	1.53	1.91	48.67
20%PEG1000-15%K_2HPO_4	0.80	1.39	38.66	0.95	1.19	74.15
20%PEG1000-20%K_2HPO_4	0.68	1.73	50.96	0.94	1.18	69.56
20%PEG1000-25%K_2HPO_4	0.53	1.08	52.23	1.18	1.48	60.14
20%PEG1000-15%$Na_3C_6H_5O_7$	1.21	0.50	1.97	0.59	0.74	46.12
20%PEG1000-20%$Na_3C_6H_5O_7$	0.89	0.58	4.70	0.42	0.53	43.45
20%PEG1000-25%$Na_3C_6H_5O_7$	0.90	0.78	12.51	0.43	0.54	41.51
20%PEG1000-15%Na_2SO_4	1.40	0.30	0.86	0.61	0.76	45.30
20%PEG1000-20%Na_2SO_4	0.92	0.32	0.92	0.66	0.83	40.62
20%PEG1000-25%Na_2SO_4	1.11	0.76	1.09	0.68	0.85	38.17
20%PEG1000-15%$(NH_4)_2SO_4$	1.02	0.93	4.04	0.68	0.85	52.57
20%PEG1000-20%$(NH_4)_2SO_4$	0.65	1.66	17.74	0.62	0.78	36.19
20%PEG1000-25%$(NH_4)_2SO_4$	0.50	2.40	16.18	0.50	0.63	28.69

(-) No phase separation.

V_R: volume ratio (upper/lower); K_P: partition coefficient of protein; K_E: partition coefficient of trypsin; SA: specific activity (U/μg protein) in the upper phase; PF: purification factor in the upper phase; Yield: recovery yield in the upper phase.

Effect of PEG Molecular Weight on the Trypsin Partitioning in ATPS

The addition of PEGs with different degree of polymerization affected the separation and purification efficiency. Table 2 shows the effects of PEG molecular weight and concentration on the trypsin partitioning in ATPS. Partitioning of trypsin in PEG-Na_2HPO_4 system was strongly dependent on the molecular weight of PEG. All trypsin partitioned into the upper phase in ATPS containing both PEG1000 and 4000 (K_E>1). However, the system containing PEG4000 showed the higher K_E than those with PEG1000. For K_P value, when higher PEG molecular weight was used, the K_P decreased and the lowest K_P (0.03) was observed in ATPS composed of 20%PEG4000 and 20%NaH_2PO_4. The results suggested that the differences in partition parameters can be attributed to the effect of size and concentration of PEG molecule. The highest SA (30.02 units/μg protein) of trypsin was obtained in 20%PEG4000 and 20%Na_2HPO_4 systems, regardless of the particular composition employed. Media containing PEG1000 gave the lower SA of trypsin than systems containing PEG with molecular weight of PEG4000. The results were in agreement with del-Val and Otero (2003) who found that systems containing PEG6000 gave a higher yield of oligosaccharide than those containing PEG4000. Nevertheless, it is well known from studies of protein purification that the partition coefficients of proteins usually increase as the molecular weight of the PEG decreased (Reh et al., 2002). This partitioning behavior is governed by the combination of different factors such as an exclusion effect of the polymer from the protein domain and the forces involved in the polymer-polymer interaction. Reh et al. (2002) reported that most proteins were partitioned to the top phase in phase systems with low molecular weight PEG.

Consequently, factors other than the partition coefficients of the enzymes must play significant roles in the selective partitioning of the enzyme. Protein partitioning behavior showed to be sensitive to the surface hydrophobicity (Tubio et al., 2007). A high hydrophobic character of a biomolecule is a factor that favors the partition equilibrium displacement to the PEG-rich phase (Tubio et al., 2007). Therefore, most trypsin from hybrid catfish viscera partitioned in the top phase when PEG4000 was used might be due to its high hydrophobic properties. Moreover, the surface charge of biological materials is one of the most significant factors affecting the separation by using partitioning. Molecular weight, shape and specific binding sites of biological materials also affect the partition profiles. Electrical interaction and repulsion between charged aqueous phase systems and the proteins affect the partitioning of system (Klomklao et al., 2005; Han and Lee, 1997). Among all ATPS tested, systems comprising 20%PEG4000 and 20% Na_2HPO_4 partitioned the trypsin to the top PEG-rich phase and undesired protein to the bottom salt phase most effectively.

TABLE 2. EFFECT OF PEG MOLECULAR MASS AND CONCENTRATION IN A PEG-NAH_2PO_4 ON PARTITIONING OF TRYPSIN FROM HYBRID CATFISH VISCERA

Phase composition (%, w/w)	V_R	K_P	K_E	SA	PF	Yield (%)
10%PEG1000-20%NaH_2PO_4	-	-	-	-	-	-
15%PEG1000-20%NaH_2PO_4	1.19	0.41	11.87	1.83	1.85	43.64
20%PEG1000-20%NaH_2PO_4	1.42	0.22	24.59	2.39	2.41	58.92
25%PEG1000-20%NaH_2PO_4	1.79	0.21	25.79	5.88	5.94	52.24
10%PEG4000-20%NaH_2PO_4	0.58	0.17	27.34	7.17	7.24	49.49
15%PEG4000-20%NaH_2PO_4	0.62	0.15	35.12	12.08	12.20	53.05
20%PEG4000-20%NaH_2PO_4	1.00	0.03	30.03	30.02	30.32	53.60
25%PEG4000-20%NaH_2PO_4	1.31	0.10	23.68	5.77	5.83	47.58

(-) No phase separation. Abbreviation: see legend of Table 1.

Purification of Trypsin from Hybrid Catfish Viscera

The purification summary of trypsin from hybrid catfish viscera is presented in Table 3. Trypsin was purified by 27.3-fold to homogeneity with a recovery of 52.7% by ATPS process using phase composition of 20%PEG4000-20%NaH_2PO_4. Generally, the method used for trypsin purification from fish digestive organs involved several steps including ammonium sulfate precipitation, size-exclusion and ion-exchange chromatography (Klomklao et al., 2007a), hydrophobic interaction chromatography (Kristjansson, 1991) and affinity chromatography (Kurtovic et al., 2006). Klomklao et al. (2006) reported that trypsin from tongol tuna spleen was purified to 402 fold with a yield of 6.6 by ammonium sulfate precipitation followed by a series of chromatographic separations. In the view of their characteristics, these multi-step methods result in high cost and time consuming purification process. Therefore, ATPS provides the powerful method for the recovery of trypsin from hybrid catfish viscera due to the ease and lower cost.

TABLE 3. PURIFICATION OF TRYPSIN FROM HYBRID CATFISH VISCERA USING 20%PEG1000, 4000-20% NAH_2PO_4 ATPS.

Purification step	Total activity (Units)	Total protein (μg)	Specific activity (Units/μg protein)	Purification fold	Yield (%)
Viscera extract	4251.71	3865.12	1.10	1	100
20%PEG1000-20%NaH_2PO_4	2513.02	1025.70	2.45	2.23	59.11
20%PEG4000-20%NaH_2PO_4	2238.81	74.51	30.05	27.32	52.66

Protein Pattern and Activity Staining of Trypsin from Hybrid Catfish Viscera Partitioned with ATPS

Fig. 1 depicts the SDS-PAGE analysis of protein samples from hybrid catfish viscera extract and various extraction steps using ATPS process. A variety of proteins with different molecular weight was found in the viscera extract. However, a large number of undesirable proteins were removed after separating with ATPS, especially proteins with higher and lower MW. These resulted in a higher purity of interested trypsin. Trypsin obtained by the ATPS of 20%PEG4000-20%NaH_2PO_4 was near homogeneity with estimated molecular mass of 23 kDa. When the proteins or enzymes to be separated differ significantly in their structural properties from others, partitioning can be achieved. The partitioning by ATPS becomes more complicated when those differences are minor (Han and Lee, 1997). The trypsin in viscera extract from hybrid catfish and fraction obtained from ATPS were identified by SDS-substrate polyacrylamide gels (Fig. 2). The apparent MW of the major trypsin activity band was estimated to be 23 kDa. The band intensity was slightly increased after ATPS process. The biggest trypsin activity band was found in system of 20%PEG4000-20%NaH_2PO_4 followed by system of 20%PEG1000-20%NaH_2PO_4 and viscera extract, respectively. The results suggested that the higher specific activity of trypsin loaded into the gel. Klomklao et al. (2005) found that slightly greater proteinase band intensity was observed after partitioning of proteinase from tuna spleen extract using ATPS. From the results, ATPS was demonstrated to be efficient and inexpensive purification step for hybrid catfish viscera trypsin.

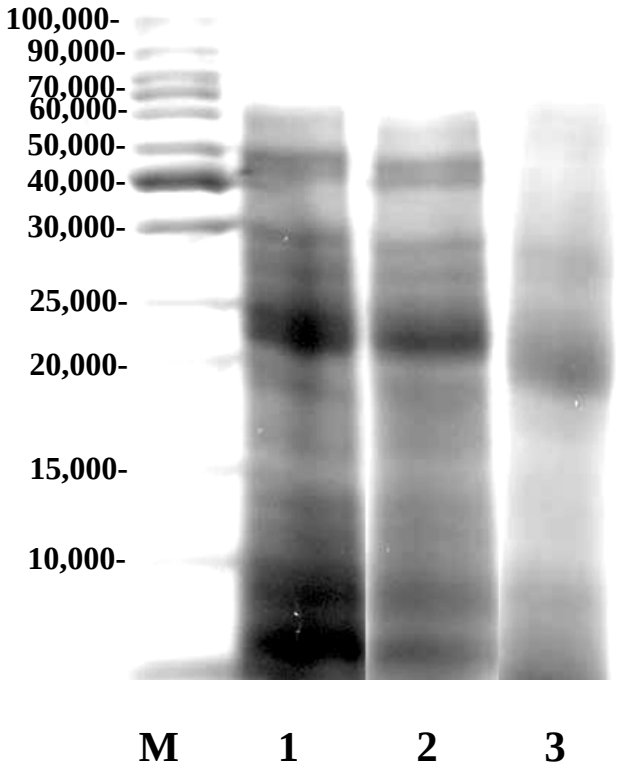

FIG. 1. SDS-PAGE OF VISCERA EXTRACT AND ATPS FRACTIONS FROM HYBRID CATFISH. M, molecular weight standard; lane 1, viscera extract; lane 2, 20%PEG1000-20%NaH_2PO_4 ATPS fraction; lane 3, 20%PEG4000-20%NaH_2PO_4 ATPS fraction.

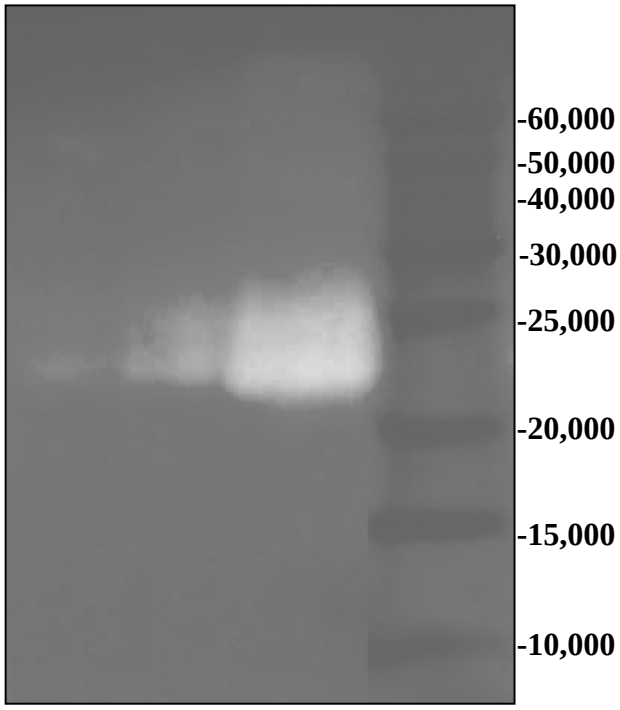

FIG. 2. ACTIVITY STAINING OF VISCERA EXTRACT AND ATPS FRACTIONS FROM HYBRID CATFISH. M, molecular weight standard; lane 1, viscera extract; lane 2, 20%PEG1000-20%NaH_2PO_4 ATPS fraction; lane 3, 20%PEG4000-20%NaH_2PO_4 ATPS fraction.

pH and Temperature Profile

The pH activity curves of partitioned trypsin from hybrid catfish viscera by ATPS are presented in Fig. 3a. The optimum pH for hydrolysis of BAPNA was 9.0 and thus similar to that of trypsin obtained from Atlantic bonito (Klomklao et al., 2007c), and skipjack tuna (Klomklao et al., 2007a). For temperature profile, the maximum activity of the partitioned enzyme was observed at 50°C (Fig. 3b). Above 50°C, the enzyme activity decreased sharply. The loss in activity was presumably caused by thermal denaturation of the enzyme. Trypsin from tongol tuna spleen had an optimum temperature of 65°C (Klomklao et al., 2006). Kurtovic et al. (2006) reported that the maximal activity of trypsin from Chinook salmon was 60°C.

pH and Thermal Stability

Fig. 4a shows the pH stability of hybrid catfish trypsin. The enzyme was stable in the pH range from pH 8.0 to 12.0. At pH value below 8.0, the stability of the enzyme decreased sharply. These properties of the pH stability of hybrid catfish trypsin were similar to those of other fish trypsins (Klomklao et al., 2006; 2007a; 2007c; Kurtovic et al., 2006; Kristjansson, 1991). For thermal stability, catfish trypsin was quite stable from 20 to 40°C and retained approximately 40% of its original activity after incubation at 60°C for 30 min (Fig. 4b). Nevertheless, the trypsin was completely inactivated at 80°C. At high temperatures, the enzyme most likely underwent denaturation and lost its activity (Klomklao et al., 2007a). This finding is consistent with the observation made by De-Vecchi and Coppes

(1996) that the thermal stability of fish trypsin varies with species as well as with experimental conditions.

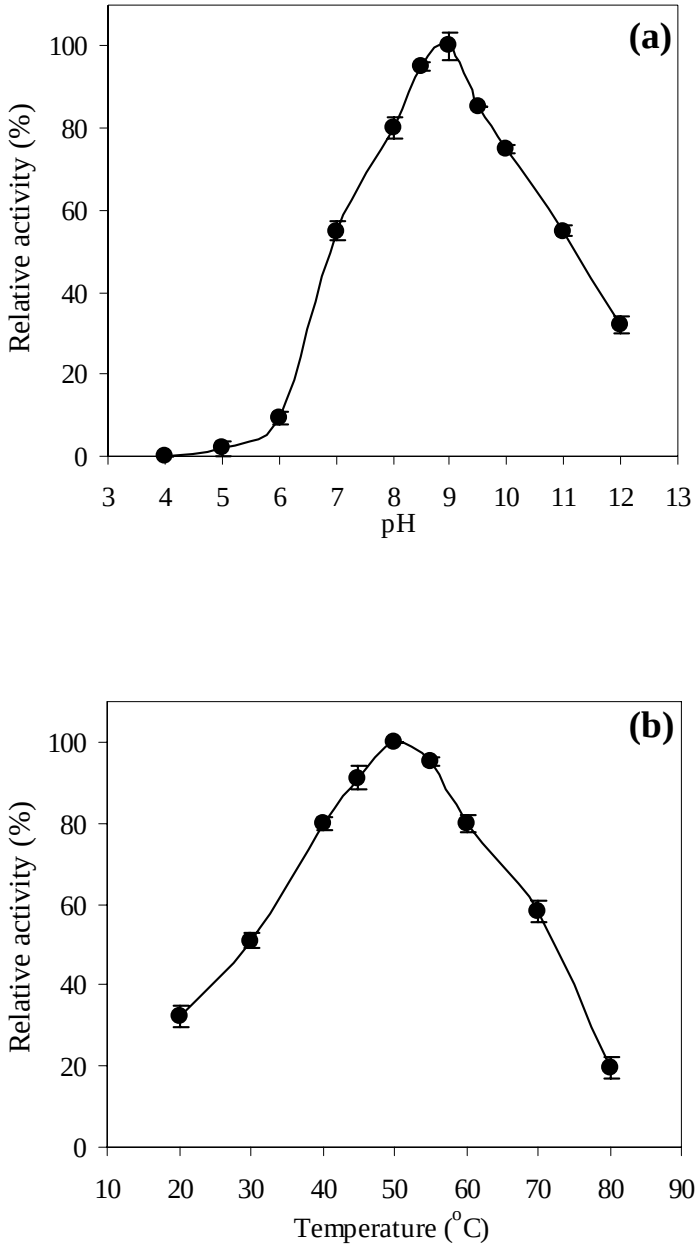

FIG. 3. pH (a) AND TEMPERATURE (b) PROFILES OF PARTITIONED TRYPSIN FROM HYBRID CATFISH VISCERA. Bars represent the standard deviation from triplicate determinations.

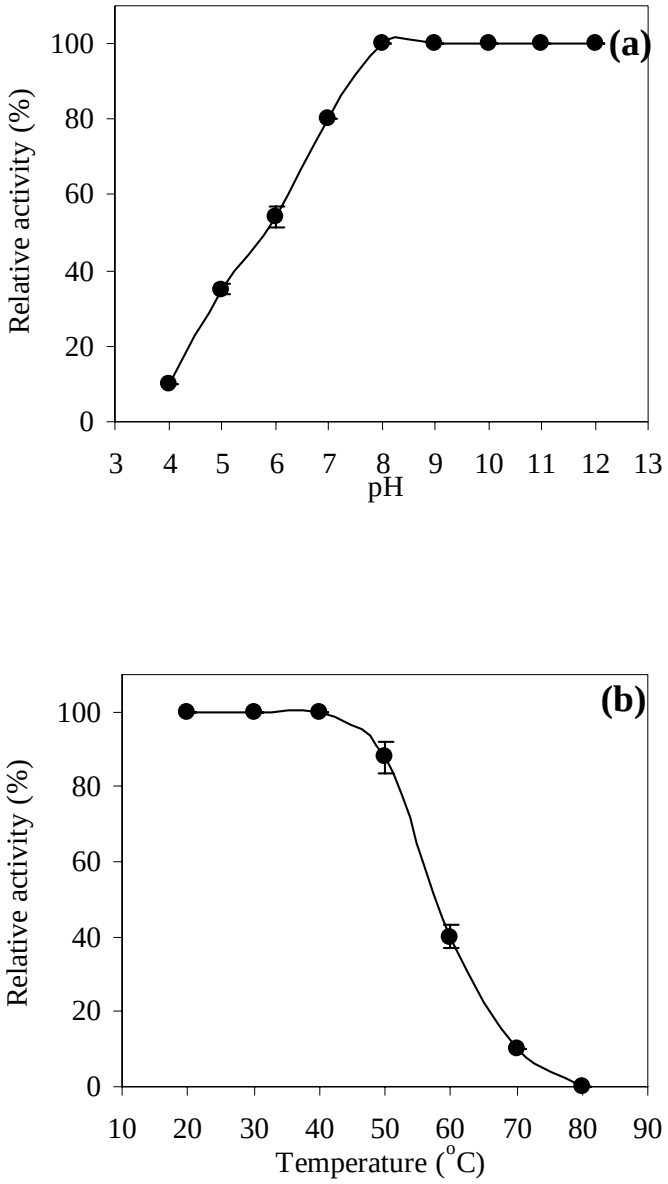

FIG. 4. pH (a) AND THERMAL (b) STABILITY OF PARTITIONED TRYPSIN FROM HYBRID CATFISH VISCERA. Bars represent the standard deviation from triplicate determinations.

Effect of NaCl

The activity of trypsin decreased gradually with increasing NaCl (Fig. 5). The activity at 30% NaCl was about 10% that of control (no NaCl). The decrease in activity might be due to the denaturation of enzymes. The 'salting out' effect was postulated to cause the enzyme denaturation. The water molecule is drawn from the trypsin molecule by salt, leading to the aggregation of those enzymes (Klomklao et al., 2007a). From the results, more than 40% of proteinase activity remained in the presence of a high concentration of NaCl (10-20%). Thus, these proteinases may contribute to the hydrolysis of proteins in the salted fish products such as Pla-duk-ra during marinating and storage. This would contribute to flavor development of finished product.

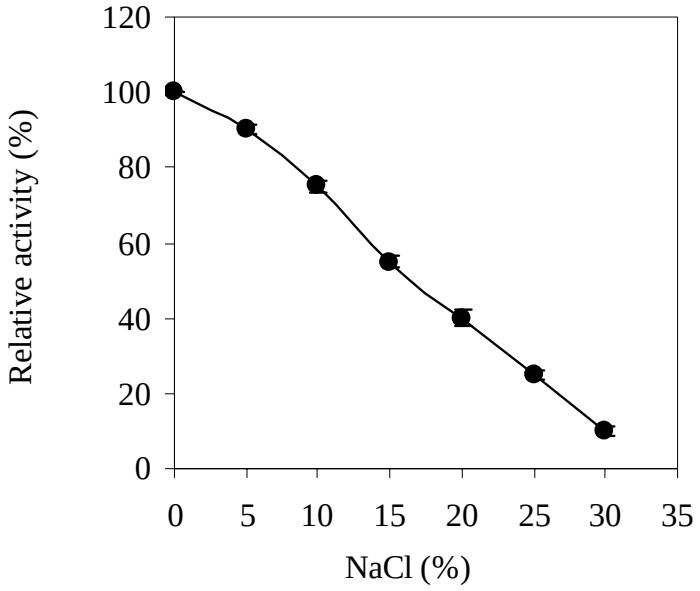

FIG. 5. EFFECT OF NACL CONCENTRATION ON ACTIVITIES OF PARTITIONED TRYPSIN FROM HYBRID CATFISH VISCERA. Bars indicate standard deviation from triplicate determinations.

CONCLUSION

Trypsin with a maximum activity at 50°C and pH 9.0 from hybrid catfish viscera was extracted and purified by partitioning in ATPS. The optimum system was observed at ATPS comprising 20%PEG4000-20%NaH_2PO_4. Compared with traditional methods, this one-step purification was simpler, faster and more efficient. Therefore, ATPS could be used to selectively purify and recovery trypsin from the viscera extract in a single-step operation.

ACKNOWLEDGMENTS

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CHAPTER 3

EFFECT OF TRYPSIN FROM HYBRID CATFISH VISCERA ON PROTEOLYTIC DEGRADATION OF BEEF PROTEINS.

EFFECT OF TRYPSIN FROM HYBRID CATFISH VISCERA ON PROTEOLYTIC DEGRADATION OF BEEF PROTEINS.

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ABSTRACT

The effect of trypsin from the viscera of hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) partitioned by aqueous two-phase systems (ATPS) comprising polyethylene glycol (PEG) with the molecular weight of 4,000 (20% w/w) and NaH_2PO_4 (20%w/w) on hydrolysis of proteins from beef meat was investigated. Partitioned trypsin was able to hydrolyze natural actomyosin (NAM) and collagen extracted from beef meat. Myosin heavy chain and β-component was most susceptible to hydrolysis by trypsin as evidenced by the lowest band intensity. Therefore, the addition of trypsin from hybrid catfish viscera might be used to as the processing aid to improve meat tenderness.

INTRODUCTION

Protein hydrolysis plays an essential role in improving texture from various meats, especially meat tenderization. Tenderness is an important driver of beef consumer satisfaction (Shin et al., 2008). There are several means of tenderizing meat either chemically or physically. Treatment by proteolytic enzymes is one of the popular methods for meat tenderization. Proteolytic enzymes derived from plants, such as papain, bromelain and ficin have been widely used as meat tenderizers in most parts of the world (Naveena et al., 2004). However, these enzymes often degrade the texture of the meat, due to the broad substrate specificity, and develop unfavorable taste due to over-tenderization (Qihe et al., 2006). Therefore, the ideal meat tenderizer would be a proteolytic enzyme with substrate specificity at the relative low pH of meat and would act either at the low temperature at which meat is stored or at the high temperature achieved during cooking (Gerelt et al., 2000).

Trypsin (EC 3.4.21.4) is a serine endopeptidase that specifically hydrolyzes proteins and peptides at the carboxyl side of arginine and lysine residues. Trypsin plays major roles in biological processes including digestion, activation of zymogens of chymotrypsin and other enzymes (Klomklao et al., 2007a). Trypsin has been used increasingly since it is both stable and active under harsh conditions, such as temperatures of 50-60°C, high pHs and in the presence of surfactants or oxidizing agents (Klomklao et al., 2007b). Therefore, the increasing interest has been paid on trypsin due to its wide range applications. Recently, the trypsin from hybrid catfish viscera generated during the processing of Pla-duk-ra, Thai dry fermented fish product, was partitioned and recovered by

aqueous two phase systems comprising PEG 4,000 (20% w/w) and NaH_2PO_4 (20%w/w). Hybrid catfish viscera partitioned by ATPS showed high trypsin activity with high pH and thermal stability. The partially purified trypsin can be applied as meat tenderizer (Klomklao et al., 2009). However, there is no information regarding the hydrolytic activity of hybrid catfish viscera towards muscle proteins, especially from beef. Therefore, this study aimed to investigate the hydrolysis of various beef muscle proteins by trypsin from hybrid catfish viscera.

MATERIALS AND METHODS

Chemicals

Sodium chloride, tris (hydroxymethyl) aminomethane and acetone were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250 and *N*,*N*,*N'*,*N'*-tetramethyl ethylene diamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Polyethylene glycol (PEG) 4000, Nα-Benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA), β-mercaptoethbanol (βME), dimethylsulfoxide and sodium dihydrogenphosphate were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

Sample Preparation

Viscera of hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) were obtained from a local market in Phattalung. 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 immediately frozen and stored at -20°C until used.

Beef meat excised from the shoulder part of a culled cow carcass 2 days after slaughter was obtained from a selected retail butcher shop, Phattalung. The samples were placed in ice with a sample/ice ratio of 1:2 (w/w) and transported to the Department of Food Science and Technology, Thaksin University, Phattalung within 30 min. The beef meat was used for protein extraction.

Preparation of Viscera Extract

Frozen viscera were thawed using 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 according to the method of Klomklao et al. (2007a) with a slight modification. The homogenate was filtered 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, defatted viscera powder was suspended in distilled water at a ratio of 1:9 (w/v) and stirred continuously at 4°C for 30 min.

The suspension was centrifuged for 30 min at 4°C at 5,000×g using a Sorvall Model RC-B Plus centrifuge (Newtown, CT, USA) to remove the tissue debris. The supernatant was collected and referred to as “catfish viscera extract; CVE”.

Partitioning of Trypsin from Viscera by ATPS

Viscera trypsin from hybrid catfish was partitioned and recovered by ATPS according to the method of Klomklao et al. (2005; 2009). CVE (1 ml) was mixed with NaH_2PO_4 at the level of 20% and 20%PEG4000 in aqueous system. Distilled water was used to adjust the system to obtain the final weight of 5 g. The mixtures were mixed continuously for 3 min using a Vortex mixer (Vortexgenie2, G-560E, Bohemia, USA). Phase separation was achieved by centrifugation for 5 min at 5,000×g. Top phase was carefully separated using a pasteur pipette and the interface of each tube was discarded. Aliquots from top phase was dialyzed against 10 volumes of 50 mM Tris–HCl, (pH 7.5) for 18 h with three changes of buffer in the first 3 h and five changes in the last 15 h. Thereafter, the samples were taken for enzyme assay, protein determination and used for further study.

Enzyme Assay

Trypsin activity was measured by the method of Benjakul et al. (2000) with a slight modification using BAPNA as substrate. To initiate the reaction, 200 µl of diluted samples were added to the preincubated reaction mixture containing 1000 µl of 0.5 mM of BAPNA in reaction buffer (0.1 M glycine–NaOH, pH 9.0) and 200 µl of distilled water. The mixture was incubated at 50°C for precisely 15

min. The enzymatic reaction was terminated by adding 200 µl of 30% (v/v) acetic acid. The reaction mixture was centrifuged at 8,000×g for 3 min at room temperature (Hettich zentrifugen, Berlin, Germany). Trypsin activity was measured by the increase in the absorbance at 410 nm due to p-nitroaniline released. One unit of trypsin activity was defined as the amount causing an increase of 0.01 in absorbance per min.

Protein Determination

Protein concentration was measured by the method of Bradford (1976) using bovine serum albumin as a standard.

Preparation of Protein Susbstrates

Natural Actomyosin

Natural actomyosin (NAM) was prepared according to the method of Benjakul et al. (1997) with a slight modification. Beef meat (10g) was homogenized in 1000 ml of chilled (4°C) 0.6 M KCl, pH 7.0 for 4 min using an IKA Labortechnik homogenizer (Selanger, Malaysia). The sample was placed in ice and each 20 s of blending was followed by a 20 s rest interval to avoid overheating during extraction. The extract was centrifuged at 5,000×g for 30 min at 4°C using a Sorvall Model RC-B Plus centrifuge (Newtown, CT, USA). Three volumes of chilled distilled water were added to precipitate NAM. NAM (the pellet) was collected by centrifuging at 5,000×g for 20 min at 4°C.

Collagen

Collagen was prepared according to the method of Kittiphattanabawon et al. (2005) with a slight modification. All preparation procedures were performed at 4°C. To remove non-collagenous proteins, the beef meat was ground and mixed with 0.1 N NaOH at a sample/alkali solution ratio of 1:10 (w/v). The mixture was stirred for 6 h. The alkali solution was changed every 2 h. Then, the pretreated samples were washed with cold distilled water until neutral or faintly basic pHs of wash water were obtained.

Pretreated samples were defatted with 10% butyl alcohol with a solid/solvent ratio of 1:10 (w/v) for 18 h and the solvent was changed every 6 h. Defatted samples were washed with cold water, followed by soaking in 0.5 M acetic acid with a solid/solvent ratio of 1:30 (w/v) for 24 h. The mixture was filtered through two layers of cheese cloth. The residue was re-extracted under the same condition. Both filtrates were combined. The collagen was precipitated by adding NaCl to a final concentration of 2.6 M in the presence of 0.05 M Tris-HCl, pH 7.0. The resultant precipitate was collected by centrifugation at 20,000×g for 60 min. The pellet was dissolved in 0.5 M acetic acid, dialyzed against 0.1 M acetic acid and distilled water, respectively, and then freeze-dried.

Hydrolysis of Different Protein Substrates by Trypsin

Trypsin (0.5 U) was added to the reaction mixture containing 5 mg protein substrates including NAM or collagen, and 825 ml of 0.1 M glycine-NaOH, pH 9.0. The hydrolysis was conducted by incubating the mixture at 50°C for 0, 5, 10,

20 and 30 min. The control was performed by incubating the reaction mixture at 50°C for 30 min without the addition of trypsin. The reaction was terminated by adding preheated solution containing 2% SDS, 8M urea and 2% βME (85°C). The mixture was further incubated at 85°C for 30 min to solubilize total proteins. The solution was centrifuged at 10,000×g for 10 min at room temperature (Hettich zentrifugen, Berlin, Germany) to remove the debris. The supernatant was then subjected to SDS-PAGE analysis.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

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 gel and 7.5% separating gel for collagen sample and 10% separating gel for NAM sample. Electrophoresis was run at a constant current of 15 mA per gel using a Mini-Protean II Cell apparatus. After electrophoresis, the gels were stained with 0.2% Coomassie brilliant blue R-250 in 45% methanol and 10% acetic acid and destained with 30% methanol and 10% acetic acid.

RESULTS AND DISCUSSION

Hydrolysis of Different Protein Substrates by Partitioned Trypsin from the Viscera of Hybrid Catfish

Natural Actomyosin (NAM)

The degradation of NAM by the partitioned trypsin was investigated by SDS-PAGE as shown in Fig. 1. Myosin heavy chain (MHC), the main constituent, was hydrolyzed to the higher extend as the incubation time increased. The marked decrease in band intensity of MHC was observed after incubation at 50°C for 30 min. MHC was degraded rapidly into lower molecular weight products with MW ranging from 170 to 140 kDa within 20 min (Fig. 1). For actin, the degradation increased with increasing the incubation time. However, the degradation rate was lower than that of MHC. Jorgova et al. (1989) reported that bacterial proteolytic enzyme treatment of muscle proteins resulted in the degradation of myosin, thus increasing meat tenderness. Shin et al. (2008) found that the *Sarcodon aspratus* extract and papain caused degradation of MHC extracted from bovine muscle. The degradation of muscle protein plays a major role in determining the tenderness and water holding capacity of meat during postmortem storage (Melody et al., 2004). MHC constitutes approximately 45% of the total myofibrillar proteins, and is the major structural protein in muscle (Schiaffino and Reggiani, 1996). Sawdy et al. (2004) reported a significant correlation between MHC fragments and tenderness in bovine muscle. For the control (without trypsin), a slight degradation of MHC was observed, suggesting the existence of a myofibril-bound proteinase in NAM. From the result, trypsin from hybrid catfish viscera

hydrolyzed myofibrillar proteins effectively, particularly MHC which is the dominant protein in beef muscle. Therefore, trypsin from hybrid catfish viscera could tenderize beef meat, thereby improving the quality and acceptability of treated beef.

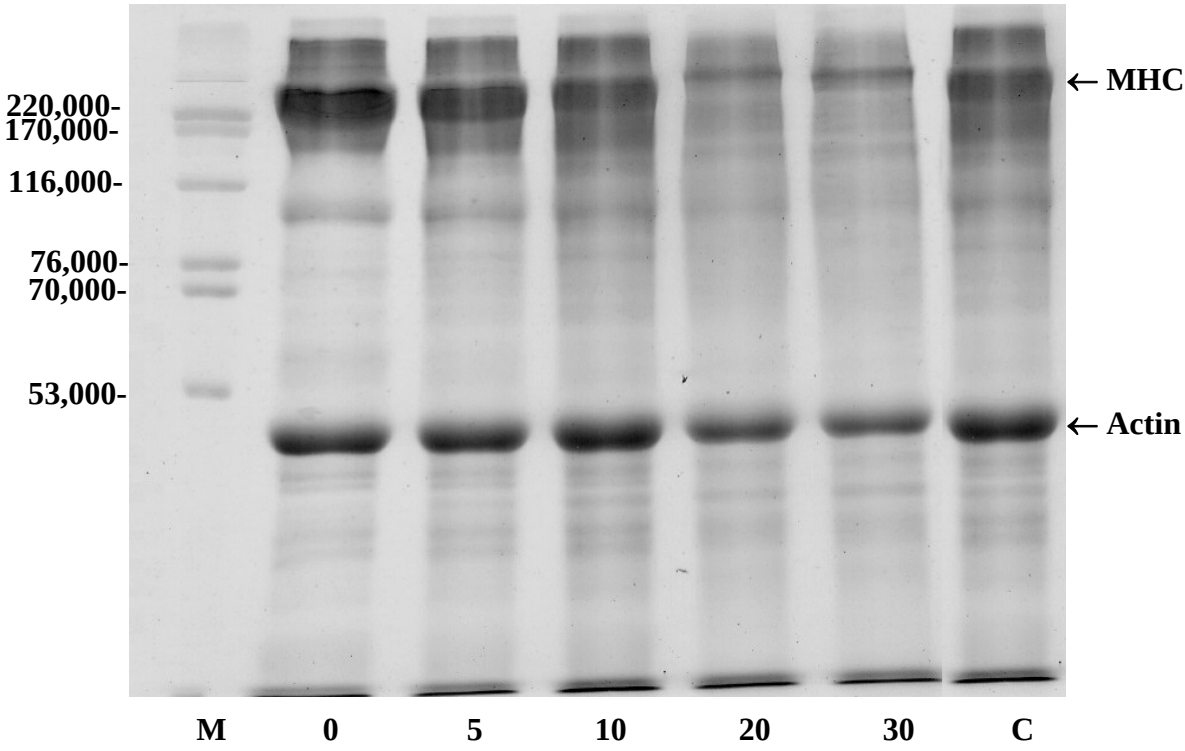

FIG. 1. HYDROLYSIS OF NAM BY TRYPSIN FROM THE VISCERA OF HYBRID CATFISH AT 50°C. M: high-molecular weight standard; C: control (NAM incubated without enzyme addition for 30 min at 50°C). Numbers designate the incubation time (min).

Collagen

Collagen extracted from beef muscle, contained β- and α-compounds as major constituents (Fig. 2). Among all proteins, β-component was most susceptible to hydrolysis, followed by α-compounds. β-component was degraded rapidly within 5 min by the trypsin (Fig. 2). Total disappearance of β-component was observed after 10 min of incubation at 50°C. For α-compounds, both α_1- and α_2-components were hydrolyzed continuously throughout the incubation time of 30 min (Fig. 2). α_2-component was more susceptible to hydrolysis than α_1-component. This suggests that collagen was a good substrate for trypsin from the viscera of hybrid catfish. Yamashita and Konagaya (1991) reported that native collagens were degraded at 20°C by chum salmon cathepsin L but not by cathepsin B. Klomklao et al. (2006) found that trypsin purified from skipjack tuna spleen could not hydrolyze both β- and α- compounds in sardine collagen. Thus, the degradation of collagen depends upon the source of collagen as well as on the types of proteinases. For the control, (without trypsin), the degradation of collagen was not observed. Meat toughness can be subdivided into actomyosin toughness, which is attributable to changes in myofibrillar protein, and background toughness, which is attributable to connective tissues (Qihe et al., 2006). Takagi et al. (1992) reported that collagen and elastin is a significant factor that affects the texture of meat. Use of proteolytic enzymes is one of the popular methods for meat tenderization (Gerelt et al., 200). Papain and bromelain have been widely used as meat tenderizers however both enzymes often degrade the texture of meat and are very expensive (Shin et al., 2008). From the result, trypsin can cleave the

peptide bonds in the β- and α-components. Thus, trypsin from hybrid catfish viscera is a promising substitute for commercial protease enzymes as a potential meat tenderizer.

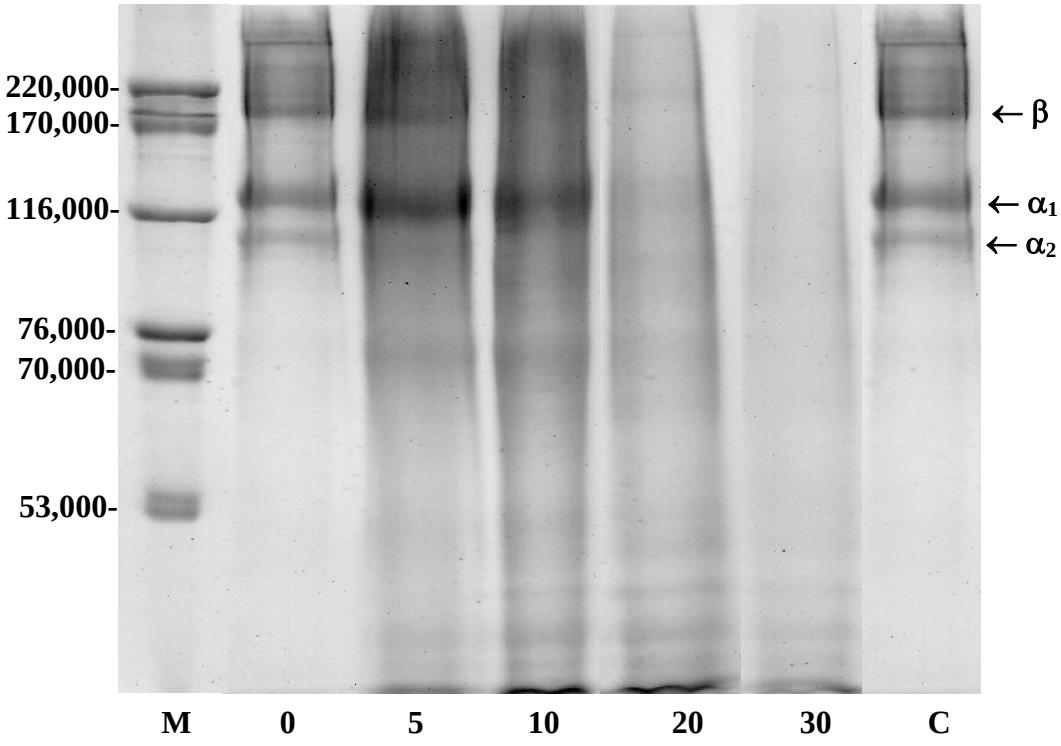

FIG. 2. HYDROLYSIS OF COLLAGEN BY TRYPSIN FROM THE VISCERA OF HYBRID CATFISH AT 50°C. M: high-molecular weight standard; C: control (NAM incubated without enzyme addition for 30 min at 50°C). Numbers designate the incubation time (min).

CONCLUSION

Hybrid catfish viscera trypsin partitioned by ATPS was capable of hydrolyzing myosin heavy chain and collagen effectively. Therefore, trypsin from hybrid catfish viscera can be a potential novel enzyme for further applications, especially for the tenderization of beef meat.

ACKNOWLEDGMENTS

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Output

Publications

1. Klomklao, S., Benjakul, S. and Kishimura, H. 2008. Proteinases in hybrid catfish viscera: Characterization and effect of extraction media. J. Food Biochem. *Accepted.*
2. Klomklao, S., Benjakul, S., Kishimura, H., Osako, K. and Tanaka, M. 2008. Effect of salts and polyethylene glycol on the partitioning and recovery of trypsin from hybrid catfish viscera in aqueous two phase systems. J. Food Biochem. *Accepted.*

Proceedings

1. Klomklao, S., Benjakul, S.and Kishimura, H. 2008. Biochemical characteristics of proteinases from hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) viscera. Food Innovation Asia Conference 2008: FoSTAT-The 10th Agro-Industrial Conference, June 12-13, 2008. BITEC, Bangkok, Thailand.
2. Klomklao, S., Benjakul, S., and Kishimura, H. 2009. Proteinases in hybrid catfish viscera: Characterization and effect of extraction media. 9th the Annual Thailand Research Fund Meeting, October 15-17, 2009. Holiday Inn Resort Reagent Beach, Cha-Am, Petchburi, Thailand.

ภาคผนวก

Biochemical Characteristics of Proteinases from Hybrid Catfish (*Clarias macrocephalus* × *Clarias gariepinus*) Viscera

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Abstract

Proteolytic activity of viscera extract from hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) was studied. The optimal pH and temperature for casein hydrolysis were 9.0 and 50°C, respectively. The enzyme was stable in the pH range of 7-11 with the exposure time of 30-120 min. The activities were also stable when incubated at temperature up to 40°C for 30-120 min. However, the sharp decrease in activity was noticeable at temperature above 50°C. The proteolytic activity was effectively inhibited by soybean trypsin inhibitor, benzamidine, PMSF and TLCK, while E-64, *N*-ethylmaleimide, TPCK, pepstatin A and EDTA showed no inhibition. The effect of NaCl (0-30%) and $CaCl_2$ (10^{-7}-10^{-2} M) on proteolytic activity was also investigated. Activities continuously decreased as NaCl concentration increased. On the other hand, activities increased as $CaCl_2$ concentration increased. The highest activity was obtained with 10^{-3} M $CaCl_2$ addition. The results suggest that major proteinases in viscera of hybrid catfish were trypsin-like serine proteinases.
Keywords: Proteinase, Trypsin, Viscera, Catfish, Characterization

Introduction

Pla-duk-ra is Thai dry fermented fish product, which is composed of eviscerated fish, sugar and salt. The mixture is left to ferment and dry for 5-6 days at room temperature (30-33°C). The Pla-duk-ra processing industry is becoming increasingly important since it is one of the income generators for Thailand, especially in Phattalung. Hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) is one of the raw materials commonly used for Pla-duk-ra production in Thailand. During processing, by-products such as the viscera, head, bones and frames are generated in the large quantity. The discards from fish processing together with fish by-product pose the great disposal problems. However, these abundant underutilized materials are a rich source of enzymes, especially proteinases that may be recovered and applied for commercial use (Simpson, 2000). Therefore, extraction 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, the properties and characteristics of the enzyme obtained might be utilized in food industry or other industries. Therefore, the objective of this study was to extract and to characterize the proteinases from hybrid catfish viscera.

Material and Methods

Proteinases were extracted from hybrid catfish viscera according to the method of Klomklao et al. (2004). Proteinase activity of viscera extract from hybrid catfish was determined using hemoglobin and casein as substrates according to the method of Kunitz (1947) and An et al. (1994) with a slight modification. One unit of activity was defined as that releasing 1 nmole of tyrosine per min (nmol/Tyr/min). Protein concentration was measured by the method of Lowry et al. (1951). For characterization, the enzyme was subjected to the determinations of pH and temperature profile, pH and thermal stability, inhibitor study, effect of NaCl and $CaCl_2$ (Klomklao et al., 2004).

Results and Discussion

1. Temperature and pH profiles of catfish viscera extract

The temperature profile of catfish viscera extract is shown in Fig. 1. Proteolytic activity assayed at pH 3.0, 5.0, 7.0 and 9.0 increased markedly from 25°C to the highest peak at 50°C before rapid inactivation at higher temperatures. For pH profiles, the extract from viscera of catfish showed 2 activity peaks. The major peak had the optimum pH at 9.0, while the minor peak showed the highest activity at pH 3.0 (Fig. 2). The major peak was presumed to be due to alkaline proteinase, while the other peak was postulated to be due to acid proteinase. Based on the optimum temperature and pH, the major proteinase from catfish viscera was characterized as heat-activated alkaline proteinases.

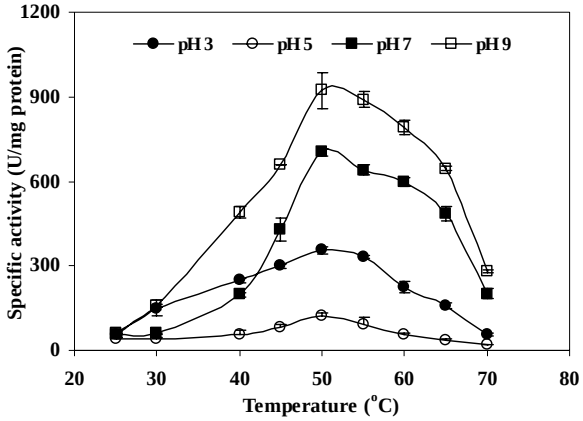

Figure 1. Temperature profiles of proteinases from catfish viscera.

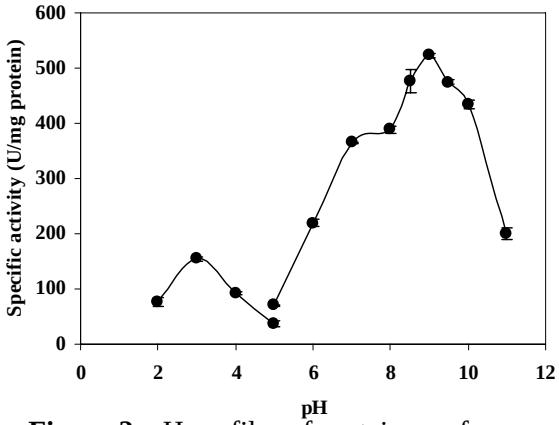

Figure 2. pH profiles of proteinases from catfish viscera.

2. Thermal and pH stability

Thermal stability of catfish viscera proteinase is depicted in Fig. 3. The enzyme was stable when incubated at temperature up to 40°C for 30-120 min. Nevertheless, the sharp decrease in activity was noticeable at temperature above 50°C. No activity was remained at 80°C. For pH stability, the catfish extract was stable in the pH range of 7-11 with the exposure time of 30-120 min, in which residual activity more than 80% was found (Fig. 4). With an extend incubation time, proteinase activity was lost to a greater extent. The marked decrease in activity was noticeable at pH below 7.

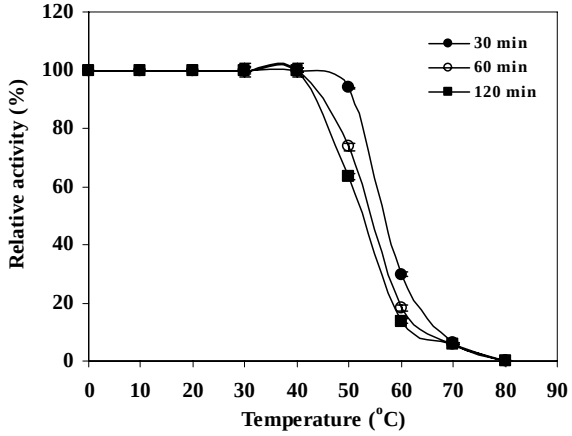

Figure 3. Thermal stability of proteinases from catfish viscera.

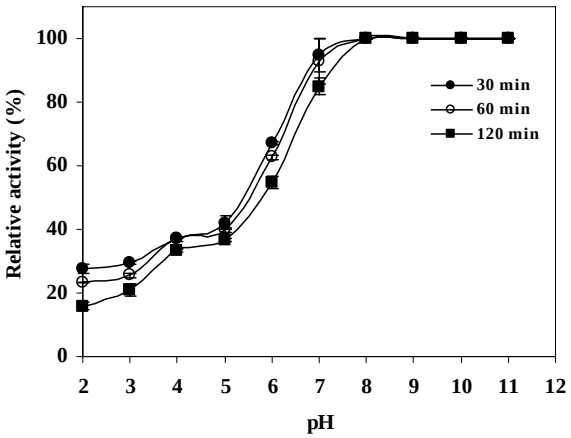

Figure 4. pH stability of proteinases from catfish viscera.

3. Effect of inhibitors on proteinases from catfish viscera

The proteolytic activity was strongly inhibited by soybean trypsin inhibitor, which are widely used to identify trypsins, PMSF and benzamidine (a serine protease inhibitor) and partially inhibited by TLCK (trypsin inhibitor) (Table 1). Specific inhibitors of cysteine proteinases (E-64, *N*-ethylmaleimide) had no inhibitory effect on proteinase activity from catfish viscera. Nevertheless, TPCK (a synthetic chymotrypsin inhibitor) EDTA (a metalloprotease inhibitor) and pepstatin A (an aspartic protease inhibitor) showed the negligible inhibitory effect. The result indicated that major proteinases from the viscera of catfish were serine proteinases, particularly trypsin or trypsin-like enzymes.

4 Effect of NaCl

The effect of NaCl at different concentrations on proteinase activity in catfish viscera extract is shown in Fig. 5. The activity decreased with increasing NaCl concentration. No activity was observed in presence of 30% NaCl. From the results, more than 20% of proteinase activity remained in the presence of a high concentration of NaCl (10-20%). Thus, these proteinases may contribute to the hydrolysis of proteins in the high salt fermented fish products such as fish sauce.

5 Effect of $CaCl_2$

Proteinase activity of catfish viscera extract increased with the addition of calcium chloride (Fig. 6). At a concentration lower than 0.1 μM, calcium had no influence on the activity of catfish viscera extract. When the concentration of calcium was increased from 0.1 μM to 1 mM, activity apparently increased. However, there was no further increase in the activity with calcium chloride above 1 mM. Calcium ions promote the formation of active trypsin from trypsinogen and stabilize trypsin against autolysis (Klomklao et al., 2004). Therefore, calcium ions played an essential role in activation of proteinases from catfish viscera.

Table 1. Effect of various inhibitors on the activity of proteinases from catfish viscera*

Inhibitors	Concentration	%Inhibition***
Control		0a**
E-64	0.1 mM	0a
N-ethylmaleimide	1 mM	0a
Soybean trypsin inhibitor	1.0 g/l	67.2 ±2.8f
TLCK	5 mM	23.1 ±7.8c
TPCK	5 mM	3.1 ±1.0ab
Benzamidine	5 mM	48.7 ±1.2d
PMSF	1 mM	59.9 ±0.1e
Pepstatin A	1 mM	2.6 ±3.0ab
EDTA	2 mM	6.6 ±0.7b

*Activity was analyzed using casein as a substrate for 30 min at pH 9.0 and 50°C. **The different letters in the same column denote the significant differences ($p<0.05$). ***Mean ±S.D. from triplicate determination.

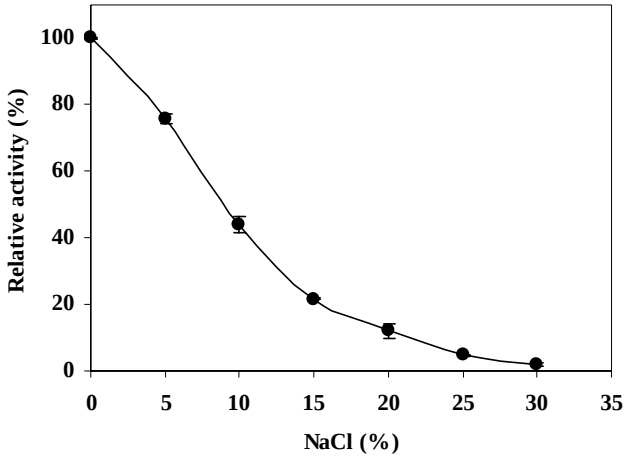

Figure 5. Effect of NaCl concentration on activities of proteinases from catfish viscera.

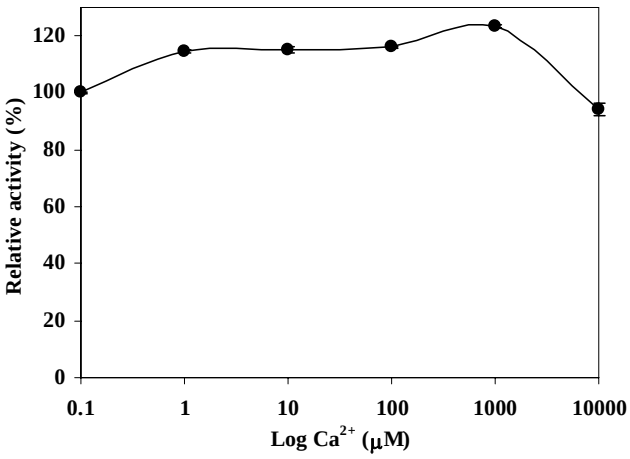

Figure 6. Effect of $CaCl_2$ concentration on activities of proteinases from catfish viscera

Conclusion

Trypsin-like enzyme with the optimal activity at pH 9.0 and 50°C was the predominant enzyme in hybrid catfish viscera extract based on inhibitory study, optimal pH and effect of $CaCl_2$. The proteinases from catfish viscera can be potential novel enzymes for future applications.

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Proteinases in Hybrid Catfish Viscera: Characterization and Effect of Extraction Media

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Abstract

Proteolytic activity from viscera extract of hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) was investigated. Optimal pH and temperature for casein hydrolysis were 9.0 and 50°C, respectively. The enzyme was stable to heat treatment up to 40°C and over a pH range of 7-11 for 30-120 min. The proteolytic activity was effectively inhibited by soybean trypsin inhibitor, benzamidine, PMSF and TLCK. Activities of the viscera extract continuously decreased as NaCl concentration increased while activities increased as $CaCl_2$ concentration increased. Based on the proteinase activity of zones separated by electrophoresis, the molecular mass of the major proteinases in hybrid catfish viscera was 23 and 20 kDa. The effect of extraction media on recovery of proteinases was also studies. Extraction of the viscera powder with 50 mM Tris-HCl, pH 7.0 containing 0.5 M NaCl and 0.2% (v/v) Brij 35 rendered a higher recovery of proteinase activity than other extractants tested ($p<0.05$). The results suggested that major proteinases in hybrid catfish viscera were alkaline heat activated proteinase, most likely trypsin-like serine proteinases.

Keywords: Proteinase, Trypsin, Viscera, Catfish, Isolation, Characterization, Extractant

Outputs

1. Klomklao S, Benjakul S, Kishimura H. *Proteinases in hybrid catfish viscera: Characterization and effect of extraction media.* Journal of Food Biochemistry **2008**; Accepted.

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