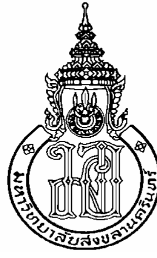

Antioxidative Activity, Functional Properties and the Use as Microbial Media of Protein Hydrolysate from Yellow Stripe Trevally (*Selaroides leptolepis*) Muscle

Vilailak Klompong

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Food Technology

Prince of Songkla University

2008

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Thesis Title	Antioxidative Activity, Functional Properties and the Use as Microbial Media of Protein Hydrolysate from Yellow Stripe Trevally (*Selaroides leptolepis*) Muscle
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ชื่อวิทยานิพนธ์	กิจกรรมการต้านออกซิเดชัน สมบัติเชิงหน้าที่ และการใช้เป็นอาหารเลี้ยงเชื้อจุลินทรีย์ของโปรตีนไฮโดรไลเสตจากกล้ามเนื้อปลาสีกุนข้างเหลือง (*Selaroides leptolepis*)
ผู้เขียน	นางวิไลลักษณ์ กล่อมพงษ์
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บทคัดย่อ

จากการศึกษากิจกรรมการต้านออกซิเดชัน และสมบัติเชิงหน้าที่ของโปรตีนไฮโดรไลเสตจากกล้ามเนื้อปลาสีกุนข้างเหลือง (*Selaroides leptolepis*) ที่ผ่านการย่อยสลายด้วยเอนไซม์อัลคาเลสและเอนไซม์ฟลาโวไซม์ที่ระดับการย่อยสลายต่างๆ (ร้อยละ 5 15 และ 25) พบว่า เมื่อระดับการย่อยสลายสูงขึ้น ปริมาณผลผลิตของโปรตีนไฮโดรไลเสตเพิ่มขึ้น ประสิทธิภาพการจับกับอนุมูลอิสระ (DPPH$^{\bullet}$) รีดิวซิงพาวเวอร์ และประสิทธิภาพการจับโลหะ (Fe^{2+}) ของโปรตีนไฮโดรไลเสตทั้งที่ผ่านการย่อยสลายด้วยเอนไซม์อัลคาเลสและฟลาโวไซม์แตกต่างกัน เมื่อพิจารณาถึงปริมาณผลผลิตและกิจกรรมการต้านออกซิเดชัน พบว่า ระดับการย่อยสลายร้อยละ 15 มีความเหมาะสมสำหรับการผลิตโปรตีนไฮโดรไลเสตที่มีกิจกรรมต้านออกซิเดชัน และประสิทธิภาพการเป็นสารต้านออกซิเดชันแปรผันตรงกับความเข้มข้นของโปรตีนไฮโดรไลเสต โปรตีนไฮโดรไลเสตที่ผ่านการย่อยสลายด้วยเอนไซม์ฟลาโวไซม์มีประสิทธิภาพการต้านออกซิเดชันสูงกว่าโปรตีนไฮโดรไลเสตที่ผ่านการย่อยสลายด้วยเอนไซม์อัลคาเลส ($p<0.05$) ประสิทธิภาพการต้านออกซิเดชันของโปรตีนไฮโดรไลเสตที่ผ่านการย่อยสลายด้วยเอนไซม์อัลคาเลสและฟลาโวไซม์มีความคงตัวที่อุณหภูมิ 90 องศาเซลเซียส เป็นเวลา 10 และ 30 นาที และในช่วงพีเอช 2 ถึง 12 อย่างไรก็ตามประสิทธิภาพการจับกับโลหะ (Fe^{2+}) ของโปรตีนไฮโดรไลเสตลดลงในช่วงพีเอชที่มีความเป็นกรดและด่างสูง โปรตีนไฮโดรไลเสตที่ผ่านการย่อยสลายด้วยเอนไซม์อัลคาเลสและฟลาโวไซม์ที่ระดับการย่อยสลายร้อยละ 15 ปริมาณ 200 ppm สามารถชะลอการสร้างคอนจูเกตไดอีนและสารซึ่งสามารถทำปฏิกิริยากับกรดไทโอบาบิทูริกในระบบเลซิทิน-ไลโพโซม โดยโปรตีนไฮโดรไลเสตที่ผ่านการย่อยสลายด้วยเอนไซม์ฟลาโวไซม์มีประสิทธิภาพการต้านออกซิเดชันสูงกว่าโปรตีนไฮโดรไลเสตที่ผ่านการย่อยสลายด้วยเอนไซม์อัลคาเลส ($p<0.05$) เมื่อนำโปรตีนไฮโดรไลเสตที่ผ่านการย่อยสลายด้วยเอนไซม์อัลคาเลสและฟลาโวไซม์ที่ระดับการย่อยสลายร้อยละ 15 มาแยกส่วนด้วยเจลฟิลเตรชันโครมาโตกราฟีด้วยคอลัมน์

Sephadex G-50 ไอออนโครมาโตกราฟีด้วยคอลัมน์ SP Sephadex C-25 และโครมาโตกราฟีของเหลวแบบสมรรถนะสูง ตามลำดับ พบว่าแฟรกชันที่มีฤทธิ์ต้านออกซิเดชันสูงสุดประกอบด้วยเปปไทด์ที่มีโมเลกุลขนาด 656 และ 617 ดัลตัน เมื่อตรวจสอบด้วยแมสสเปคโตรเมตทรี (MALDI TOF/TOF) โปรตีนไฮโดรไลเสตที่ผ่านการย่อยสลายด้วยเอนไซม์อัลคาเลสและฟลาโวไซม์ที่ระดับการย่อยสลายร้อยละ 15 สามารถป้องกันการทำลายดีเอนเอจากการออกซิเดชันเนื่องจากปฏิกิริยาเฟนตอน เมื่อทดสอบด้วยเทคนิคการคลายตัวของดีเอนเอพลาสมิด (plasmid DNA relaxation assay) โปรตีนไฮโดรไลเสตที่ผ่านการย่อยสลายด้วยเอนไซม์อัลคาเลสและฟลาโวไซม์ที่ระดับการย่อยสลายร้อยละ 15 ประกอบด้วยกรดอะมิโนหลัก ได้แก่ กรดกลูตามิก กลูตามีน กรดแอสพาร์ติก แอสพาราจีน อะลานีน ไลซีน และลูซีน ในขณะที่ไกลซีนเป็นองค์ประกอบหลักในกล้ามเนื้อปลาสีกุนข้างเหลือง ทั้งโปรตีนไฮโดรไลเสตที่ผ่านการย่อยสลายด้วยเอนไซม์อัลคาเลสและฟลาโวไซม์ที่ระดับการย่อยสลายต่างๆ สามารถละลายได้ดี โดยมีค่าสูงกว่าร้อยละ 85 ในพีเอชช่วงกว้าง (2-12) เมื่อระดับการย่อยสลายเพิ่มขึ้นดัชนีกิจกรรมการเกิดอิมัลชัน ดัชนีความคงตัวของอิมัลชัน ความสามารถในการเกิดฟองและความคงตัวของฟองของโปรตีนไฮโดรไลเสตลดลง ($p<0.05$) ที่ระดับการย่อยสลายเดียวกัน สมบัติเชิงหน้าที่ของโปรตีนไฮโดรไลเสตขึ้นอยู่กับชนิดของเอนไซม์ที่ใช้ในการย่อยสลาย

จากการใช้โปรตีนไฮโดรไลเสตที่ผ่านการย่อยสลายด้วยเอนไซม์อัลคาเลสและฟลาโวไซม์ที่ระดับการย่อยสลายต่างๆ เป็นแหล่งไนโตรเจนสำหรับเชื้อจุลินทรีย์เปรียบเทียบกับเปปโตนทางการค้า พบว่า เชื้อแบคทีเรีย ได้แก่ *Staphylococcus aureus* TISTR 118 และ *Escherichia coli* TISTR 780 ที่เลี้ยงในอาหารเหลวประกอบด้วยโปรตีนไฮโดรไลเสตซึ่งผ่านการย่อยสลายด้วยเอนไซม์ฟลาโวไซม์ที่ระดับการย่อยสลายร้อยละ 25 (HF_{25}) มีความหนาแน่นของเซลล์และอัตราการเจริญจำเพาะสูงสุด ในขณะที่เวลาที่ใช้ในการแบ่งตัวสั้นที่สุด ($p<0.05$) สำหรับยีสต์ ได้แก่ *Saccharomyces cerevisiae* TISTR 5017 และ *Candida albicans* PSSCMI 7010 ที่เลี้ยงในอาหารเหลวประกอบด้วยเปปโตนทางการค้ามีอัตราการเจริญสูงกว่าที่เลี้ยงในอาหารที่ประกอบด้วยโปรตีนไฮโดรไลเสตที่ผ่านการย่อยสลายด้วยเอนไซม์อัลคาเลสและฟลาโวไซม์ที่ระดับการย่อยสลายต่างๆ ($p<0.05$) ในขณะที่ไม่มีความแตกต่างของอัตราการเจริญจำเพาะและเวลาที่ใช้ในการแบ่งตัวระหว่างเปปโตนทางการค้าและโปรตีนไฮโดรไลเสตที่ผ่านการย่อยสลายด้วยเอนไซม์อัลคาเลสและฟลาโวไซม์ที่ระดับการย่อยสลายต่างๆ เมื่อเลี้ยงเชื้อรา *Aspergillus oryzae* PSSCMI 3004 ($p>0.05$) ในระหว่างการเลี้ยงเชื้อ *S. aureus* TISTR 118 และ *E. coli* พีเอชของอาหารเหลวที่ประกอบด้วย HF_{25} ลดลงอย่างชัดเจนในช่วง 8 ชั่วโมงแรก ($p<0.05$) ซึ่งส่งผลกระทบโดยตรงต่อขนาดโคโลนีของ *S. aureus* TISTR 118 ($p<0.05$) อย่างไร

ก็ตามการเติมบัฟเฟอร์ลงในอาหารเลี้ยงเชื้อสามารถปรับขนาดโคโลนีของ *S. aureus* TISTR 118 ให้ไม่มีความแตกต่างจากการเลี้ยงด้วยอาหารเหลวที่ประกอบด้วยเปปโตนทางการค้า ($p>0.05$) HF_{25} มีค่าสัดส่วนความสามารถในการให้ผลผลิตสูงกว่าเปปโตนทางการค้า ($p<0.05$) เมื่อเลี้ยงเชื้อ *S. aureus* TISTR 118 และ *E. coli* TISTR 780 จากการใช้ HF_{25} และ เปปโตนทางการค้าในการเลี้ยงเชื้อจากสิ่งแวดล้อม อาหาร และแบคทีเรียก่อโรค พบว่า HF_{25} มีศักยภาพในการเป็นอาหารเลี้ยงเชื้อไม่แตกต่างจากเปปโตนทางการค้า ($p>0.05$) และจากการศึกษาจุลินทรีย์ด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด พบว่าขนาดและรูปร่างของจุลินทรีย์ที่ศึกษาไม่มีความแตกต่างกันเมื่อเลี้ยงใน HF_{25} และ เปปโตนทางการค้า ($p>0.05$)

HF_{25} ประกอบด้วยเถ้า (ร้อยละ 45.73) ไขมัน (ร้อยละ 0.77) และความชื้น (ร้อยละ 4.34) ในปริมาณสูงกว่า แต่ประกอบด้วยโปรตีน (ร้อยละ 42.11) ในปริมาณต่ำกว่าเปปโตนทางการค้า ($p<0.05$) HF_{25} มีสีเข้มกว่าเปปโตนทางการค้าเล็กน้อย ($p<0.05$) HF_{25} ประกอบด้วยกรดอะมิโนจำเป็นในปริมาณสูงกว่าเปปโตนทางการค้า HF_{25} และเปปโตนทางการค้าประกอบด้วยเกลือแร่หลายชนิดในปริมาณต่างกัน HF_{25} และเปปโตนทางการค้าดูดความชื้นได้อย่างรวดเร็วเมื่อความชื้นสัมพัทธ์สูงกว่าร้อยละ 75 จากการทดสอบความคงตัวของ HF_{25} ที่ผ่านการทำแห้งแบบพ่นฝอยระหว่างการเก็บรักษาที่อุณหภูมิห้องเป็นเวลา 12 สัปดาห์เปรียบเทียบกับเปปโตนทางการค้า พบว่า ค่าวอเตอร์แอคติวิตี้ ความชื้น ความเข้มของสี การเกิดสีน้ำตาล ค่าการดูดกลืนแสงที่ 294 นาโนเมตร ค่าความเข้มของฟลูออเรสเซนซ์ ปริมาณสารที่สามารถทำปฏิกิริยากับกรดไทโอบาบิทูริกของ HF_{25} และเปปโตนทางการค้ามีค่าเพิ่มขึ้นเมื่อระยะเวลาการเก็บรักษานานขึ้น ($p<0.05$) HF_{25} และเปปโตนทางการค้ามีค่าการละลายที่พีเอช 5 และ 7 สูงกว่าร้อยละ 93 ตลอดระยะเวลาการเก็บรักษา ทั้ง HF_{25} และเปปโตนทางการค้ามีค่าความขุ่นก่อนและหลังการให้ความร้อนในการนึ่งฆ่าเชื้อไม่แตกต่างกัน ($p>0.05$) ประสิทธิภาพในการเป็นอาหารเลี้ยงเชื้อของ HF_{25} และเปปโตนทางการค้าไม่มีความแตกต่างกัน และมีความคงตัวตลอดระยะเวลาการเก็บรักษาเมื่อเลี้ยงเชื้อ *S. aureus* TISTR 118 และ *E. coli* TISTR 780 ($p>0.05$)

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Author	Mrs. Vilailak Klompong
Major Program	Food Technology
Academic Year	2008

ABSTRACT

Antioxidative activity and functional properties of protein hydrolysates from yellow stripe trevally meat prepared using Alcalase (HA) and Flavourzyme (HF) with different degrees of hydrolysis (DH) (5, 15, 25%) were investigated. As the DH increased, yield was increased. However, 2,2-diphenyl-1-picryhydrazyl (DPPH) radical-scavenging activity, reducing power and metal chelating activity of both HA and HF varied. Based on yield and antioxidative activity, DH of 15% was considered as the most appropriate for production of HA and HF with antioxidative activity. HA with 15% DH (HA_{15}) and HF with 15% DH (HF_{15}) exhibited the antioxidative activity in a concentration dependent manner. HF_{15} generally showed the greater antioxidative activity than did HA_{15} ($p<0.05$). Antioxidative activity of HA_{15} and HF_{15} was stable when heated at 90°C for 10 and 30 min and subjected to a wide pH range (2–12). Nevertheless, metal chelating activity decreased in very alkaline and acidic pH ranges. HA_{15} and HF_{15} at 200 ppm retarded the formation of conjugated diene and thiobarbituric acid reactive substances (TBARS) in lecithin liposome system. HF_{15} possessed the stronger antioxidative activity than did HA_{15} in the system ($p<0.05$). After separation of HA_{15} and HF_{15} by consecutive chromatography including gel filtration using a Sephadex G-50 column, ion-exchange chromatography using a Sephadex C-25 column, followed by high performance liquid chromatography, antioxidative peptides were determined to have the molecular weight of 656 and 617 Da, based on MALDI TOF/TOF. Additionally, HA_{15} and HF_{15} prevented DNA oxidative damage in Fenton reaction system tested by plasmid DNA relaxation assay. HA_{15} and HF_{15} contained glutamic acid and glutamine as the major amino acids, while glycine was the dominant amino acid in yellow stripe trevally

flesh. HA_{15} and HF_{15} were also rich in aspartic acid, asparagine, alanine, lysine and leucine. Both HA and HF had the high solubility, which was higher than 85% over a wide pH range (2–12). When the DH increased, the interfacial activities (emulsion activity index, emulsion stability index, foaming capacity, foam stability) of hydrolysates decreased ($p<0.05$). At the same DH, the functional properties of protein hydrolysate depended on the enzyme used.

When HA with 5, 15 and 25% DH (HA_5, HA_{15}, HA_{25}) and HF with 5, 15 and 25% DH (HF_5, HF_{15}, HF_{25}) were used as nitrogen sources in comparison with commercial peptone (Bacto Peptone), microbial growth was determined. For bacteria, *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*), HF_{25} yielded the highest cell density and specific growth rate (μ_{max}) and the lowest generation time (t_d) ($p<0.05$). For yeasts, *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Candida albicans* (*C. albicans*), Bacto Peptone yielded the higher growth rate than did HA and HF ($p<0.05$), while no differences in μ_{max} and t_d were observed for fungus, *Aspergillus oryzae* (*A. oryzae*) ($p>0.05$). The pH of culture broth containing HF_{25} decreased markedly during the first 8 h of cultivation of *S. aureus* and *E. coli* ($p<0.05$). This directly lowered the colony size of *S. aureus* ($p<0.05$). However, buffered culture broth containing HF_{25} rendered the similar growth and colony size of *S. aureus* ($p>0.05$), compared with the broth containing Bacto Peptone. HF_{25} also showed the higher productivity ratio for *S. aureus* and *E. coli* than did Bacto Peptone ($p<0.05$). When HF_{25} and Bacto Peptone were used as microbial media to determine microbial load of environmental and food samples as well as pathogenic bacteria, HF_{25} generally exhibited similar potential in culturing those microorganisms to Bacto Peptone ($p>0.05$). Scanning electron microscopic study revealed no differences in size and shape of microorganisms cultured in HF_{25} and Bacto Peptone ($p>0.05$).

HF_{25} comprised the higher contents of ash (45.73%), lipid (0.77%) and moisture (4.34%) but lower protein content (42.11%) than did Bacto Peptone ($p<0.05$). HF_{25} powder was slightly darker than was Bacto Peptone ($p<0.05$). HF_{25} contained a higher amount of essential amino acids (44.05%) than did Bacto Peptone (19.34%). HF_{25} and Bacto Peptone contained several minerals at varying levels. At water activity greater than 0.75, the much higher moisture sorption was found in HF_{25} ($p<0.05$). During storage of 12 weeks, slight increases in water activity and moisture

content of HF_{25} and Bacto Peptone were observed ($p<0.05$). Slight decrease in lightness of HF_{25} and Bacto Peptone was found ($p<0.05$). Generally, browning intensity, A_{294} and fluorescent intensity of HF_{25} and Bacto Peptone increased during storage ($p<0.05$). Both samples were soluble at pHs 5 and 7 with the solubility greater than 93% throughout the storage. No change in turbidity of both samples before and after autoclaving was noticeable ($p>0.05$). TBARS of HF_{25} and Bacto Peptone increased ($p<0.05$) during storage. The efficacy of HF_{25} as media for culturing *S. aureus* and *E. coli* was generally equivalent to that of Bacto Peptone and was quite stable during storage ($p>0.05$).

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CHAPTER 1

INTRODUCTION AND LITERATURE REVEIW

1.1 Introduction

Fish have been increasingly demanded among the consumers due to the high nutritive value. Fish can be processed into different finished products with different market values. During processing, solid wastes such as viscera, head, skin, bone, and some muscle tissue are generated and can be as high as 60% of the original raw material. Generally, these wastes have been used as fishmeal or fertilizer (Benjakul and Morrissey, 1997). To meet the need of the fish processing industry, the increasing concern about the pollution has stimulated the interest in converting waste materials to commercially valuable products (Gilmartin and Jervis, 2002; Kurbanoglu and Kurbanoglu, 2004). Apart from those wastes, some under-utilized species have not been fully used due to the dark color of fish flesh as well as the high fat content (Hoyle and Merritt, 1994). Most of harvests are used for fishmeal or fish oil production (Quaglia and Orban, 1987; Benjakul and Morrissey, 1997) and used as animal feed because of the poor functional performance (Mackie, 1982). Value-added products from these species have been intensively studied (Peterson, 1981; Quaglia and Orban, 1987; Rebeca *et al*., 1991).

Hydrolysis processes have been developed to convert under-utilized proteins into more marketable and acceptable forms (Peterson, 1981; Quaglia and Orban, 1987; Gildberg, 1993) which can be widely used in food rather than a lower value animal feed or fertilizer (Haard, 1992; Benjakul and Morrissey, 1997). By developing enzyme technologies for protein recovery and modification, production of a broad spectrum of food ingredients and industrial products may be possible. Protein hydrolysate used as functional ingredients in food systems is existing and promising. Protein hydrolysate from many sources possesses antioxidative activity that can serve as natural antioxidants. Moreover, peptones with high market value can be produced from wastes (Kurbanoglu and Kurbanoglu, 2004). These products may have significant value for the fishery industry as they have somewhat higher market prices

than the common by-products such as fish silage, fishmeal and fertilizer (Kristinsson and Rasco, 2000). Concomitantly, biotechnological expansion creates an increasing demand for microbial growth substrates that constitute a major cost in the production of microbial cells and bioproducts. The nitrogen source tends to be the most expensive medium constituent (Clausen *et al.*, 1985; Guerard *et al.*, 2001). In addition, many microorganisms including most pathogenic bacteria can grow only on peptone-rich media, (Gildberg *et al.*, 1989). At present, commercial nitrogen sources come from casein digests, slaughterhouse waste and vegetable extracts. Despite its ubiquity and low price, fish extract has only been used to minor extent (Vecht-Lifshitz *et al.*, 1990; Strom and Eggum, 1981; Kurbanoglu and Kurbanoglu, 2004). Furthermore, the growing demand for fish vaccine has revealed an interesting market for fish peptones that help fish bacteria grow well (Clausen *et al.*, 1985).

Therefore, production of protein hydrolysate from under-utilized species or fishery wastes could be a means to obtain the prime quality products with the high market value. Even though yellow stripe trevally has been used as food, production of more valuable and marketable products, especially functional ingredients, antioxidants and microbial nutrients, can be the promising means to maximize the utilization of this species. As a consequence, protein hydrolysate with good functionality and antioxidative activity can be produced for further uses in food industry. Also the commercial peptones can be substituted to some extent by fish protein hydrolysate, thus helping dramatically decreased import margin of commercial peptones from overseas. In essence, waste disposal causing the pollution can be minimized.

1.2 Literature Review

1.2.1 Protein hydrolysate

Protein hydrolysate can be defined as proteins that are chemically or enzymatically broken down into peptides of varying sizes. There are a wide variety of uses in food industry, including a substitute for milk proteins because of its high dispersibility, protein supplements and stabilizers in beverages and flavor enhancer in confectionary products. Utilization of protein hydrolysates as functional and

nutritional ingredients and as a source of small peptides and amino acids has increased significantly in the food industry (Jost *et al*., 1987). So far, hydrolysis technology of food proteins to make functional protein ingredients and nutritional supplements has been intensively developed (Kristinsson and Rasco, 2000). Protein hydrolysate can be produced from different protein sources. Its properties, functionality as well as stability were dependent upon many factors involving protein type and composition, degree of hydrolysis, etc. (Adler-Nissen, 1986; Quaglia and Orban, 1990; Sausa *et al*., 2004).

1.2.2 Sources of protein hydrolysate

1.2.2.1 Milk protein hydrolysates

Whey protein hydrolysates are utilized extensively in infant formulas and specialty therapeutic products because of their outstanding nutritional quality and their particular amino acid profile (McDermott, 1987; Turgeon and Gauthier, 1990). Moreover, casein hydrolysate has been used as dairy ingredients in bread making for their nutritional benefits and functional properties including low proof time, high volume and low firmness (Kenny *et al*., 2000). Chobert *et al*. (1988) suggested that the solubility of the casein protein hydrolysates treated with *Staphylococcus aureus* V8 protease was much higher than that of casein. Stephen *et al*. (2001) found that degree of hydrolysis between 10 and 27% improved emulsifying ability of whey protein hydrolysate. However, further hydrolysis led to a marked decrease in emulsifying ability. Emulsion instability was related to apparent molecular weight distribution of casein protein hydrolysates. A relative high amount of peptides larger than 2 kDa positively influenced emulsion stability (Van Der Ven *et al.,* 2001). Moreover, Mahmoud *et al*. (1992) indicated that extensive enzymatic hydrolysis of casein, which is essential to render it virtually nonantigenic for potential use in a hypoallergenic infant formular, resulted in a considerable reduction in emulsifying property of the hydrolysate. Van Der Ven *et al*. (2002) showed that whey protein hydrolysates containing peptides of 3-5 kDa were most strongly related to foam formation. Mutilangi *et al*. (1996) found that heat denaturation diminished the functionality of whey proteins but controlled proteolysis enhanced the functionality of the heat denaturation protein. In general, casein hydrolysate contains the bitter

peptides, leading to the less acceptability. Minagawa *et al.* (1989) found that aminopeptidase T was able to decrease and/or remove the bitterness of such a bitter peptide present in casein hydrolysates and may be useful for improving the tastes of food products.

1.2.2.2 Plant protein hydrolysates

Plant proteins are extracted from abundant and renewable biological resources. In addition, their prices are generally lower than those of animal proteins (Nouri *et al.*, 1997). Plant protein hydrolysate has been used as functional ingredients, dietary food, flavoring agents (Adler-Nissen, 1986) and substrate for the growth of animal cell cultures (Franek *et al.* 2000). Deeslie and Cheryan (1988) demonstrated the feasibilities of producing protein hydrolysates from soy isolate with desired molecular weight and characteristics using the ultrafiltration reactor system. Yeom *et al.* (1994) reduced the bitterness of soy protein hydrolysates by hydrolysis with bromelain.

Sesame is another source for hydrolysate production. Bandyopadhyay and Ghosh (2002) found that sesame protein hydrolysate contained 40-50% protein content. Defatted sesame meal is very important as a protein source for human consumption due to the presence of sulfur-containing amino acids, chiefly methionine. Sesame protein isolate is produced from dehulled, defatted sesame meal and used as the starting material to produce protein hydrolysate by papain. The sesame hydrolysate can be applied as a versatile product, especially in the food, pharmaceutical and related industries.

By extraction of plant materials including soy flour or wheat flour with acetone prior to isolation of protein, a significant decrease in non-protein contaminant was obtained (Franek *et al.*, 2000). Futhermore, acid pretreatment facilitated enzymatic hydrolysis of the protein in defatted soybean flour as evidenced by the increased nitrogen solubility index (Lee *et al.*, 2001). Villanueva *et al.* (1999) produced sunflower protein hydrolysate using an endopeptidase (Alcalase), an exopeptidase (Flavourzyme) or both. Combined use of these proteases generated the highest degree of hydrolysis and highest solubility. Besides, Nouri *et al.* (1997) prepared wheat protein hydrolysates with different degrees of hydrolysis using pepsin.

1.2.2.3 Fish protein hydrolysates

Both fish protein concentrates and fish protein hydrolysates have a potential to be protein supplements in cereal foods and soups (Venugopal and Shahidi, 1995). In some countries, fish protein hydrolysates are used as a milk substitute and as flavoring compounds (Stephens *et al*., 1976). Also, they can be used as a source of peptone for microbial growth (Stephens *et al*., 1976; Jassim *et al*., 1988; Gildberg *et al*., 1989; Vecht-Lifshitz *et al*., 1990; Venugopal and Shahidi, 1995; Kurbanoglu and Kurbanoglu, 2004). Hydrolysates with desirable molecular structure and properties could be produced (Quaglia and Orban, 1990). The production of fish protein hydrolysates normally involves the limited hydrolysis of the ground fish with a protease at the optimum temperature and the pH required by enzymes. Liquefaction of the protein takes place within a few hours, followed by the inactivation of the enzyme. The solubilized proteins are concentrated, usually by spray drying or by isoelectric precipitation (Haard, 1992). Enzymes such as papain, ficin, trypsin, pancreatin, Pronase, Alcalase, or Neutrase can be used to produce a wide range of fish protein hydrolysates (Venugopal and Shahidi, 1995; Benjakul and Morrissey, 1997; Kristinsson and Rasco, 2000). Although pepsin gave the maximal solubilization of threadfin bream, Pronase yielded a product having less bitterness (Kristinsson and Rasco, 2000). Use of proteolytic enzymes from fish itself has been suggested for the production of fish protein hydrolysate (Venugopal and Shahidi, 1995).

Lean fish species are ideal for fish protein hydrolysates preparation. Fish protein hydrolysates prepared from fatty species contain high amount of lipid, and such hydrolysates may require additional treatments such as centrifugation in order to remove the excess fat (Sikorski and Naczk, 1981; Kristinsson and Rasco, 2000). Furthermore, use of washed mince or surimi gave the high quality fish protein hydrolysates (Kristinsson and Rasco, 2000). Rebeca *et al*. (1991) produced fish protein hydrolysate with bacterial proteases, which can be used in the food industry owing to their solubility and nutritional value. Quaglia and Orban (1987) concluded that sardine hydrolysates produced using Alcalase and papain under optimum condition had high nutritional value and high solubility. Gbogouri *et al*, (2004) also reported that salmon head hydrolysate digested by Alcalase had an

excellent solubility at high degree of hydrolysis. Nevertheless, emulsifying capacity, emulsion stability and fat adsorption capacity were better when degree of hydrolysis was low. For sardine hydrolysate prepared using Alcalase, emulsifying properties, surface hydrophobicity and the high molecular weight fraction decreased as degree of hydrolysis increased (Quaglia and Orban, 1990). Benjakul and Morrissey (1997) produced protein hydrolysates from Pacific whiting solid wastes using Alcalase. Freeze-dried hydrolysate was brownish yellow in color and had high protein (79.97% protein). Amino acid composition of freeze-dried hydrolysate was similar to that of Pacific whiting muscle but tryptophan was reduced to 14.74-21.50% of that found in Pacific whiting solid wastes. Guerard *et al.* (2001) used Alcalase to produce protein hydrolysates from yellowfin tuna stomach using the pH-stat method in controlled hydrolysis conditions (Figure 1). Tuna protein hydrolysates performed effectively as nitrogenous source in microbial growth media (Guerard *et al.*, 2001). Clausen *et al.* (1985) prepared an autolysate of fish viscera as the nitrogenous source in a bacterial growth medium. Jassim *et al.* (1988) used fish waste, including heads, bone and fins to prepare media for microorganisms using trypsin. Moreover, Gildberg *et al.* (1989) produced peptone from whole fish, capelin and blue whiting by autolysis with following hydrolysis by Alcalase. The applications of fish protein hydrolysates produced from various enzymes are shown in Table 1. To give a more stable product, a concentrate was prepared by freeze-drying or oven-drying. Trypical yields of solids are 7-9% (w/w) of original mass.

1.2.2.4 Meat protein hydrolysates

Enzymatic modification has been used extensively to improve the functional properties of meat protein to meet the specific needs (Richardson, 1977). Proteolysis alters the properties by changing the molecular size, conformation, solubility and strength of the inter- and intramolecular bonds of the protein molecule (Kinsella, 1976). Smith and Brekke, (1985) indicated that partial proteolysis of mechanically deboned fowl myofibrillar proteins with an acid protease, Milezyme AFP 2000, improved protein solubility and emulsifying capacity. The extent of proteolysis needed for optimum improvement generally varies with the functionality needed.

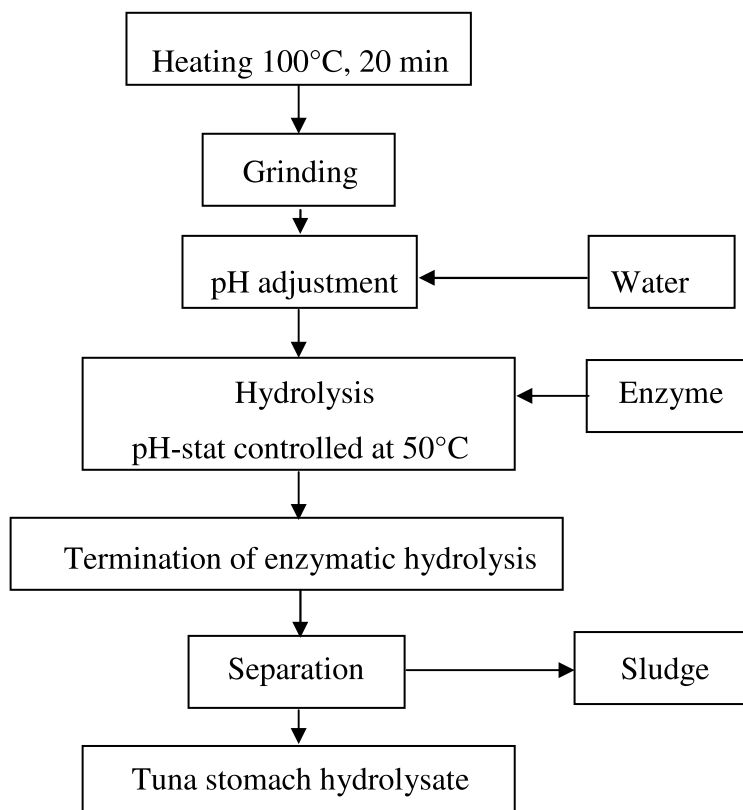


Figure 1. Flow chart for the production of protein hydrolysate from tuna stomach

Source: Guerard *et al.* (2001)

1.2.3 Hydrolysis processes

Chemical and biological methods are used for protein hydrolysis. However, chemical hydrolysis is used commonly in industrial practice. Biological processes using added enzymes are employed more frequently and the enzyme hydrolysis holds the most promise for the future because it renders products of high functionality and nutritive value (Kristinsson and Rasco, 2000). Prior to protein hydrolysis, some treatments, especially defatting or concentrating of the protein, have been employed to ensure the final quality of protein hydrolysate (Sikorski and Naczk, 1981).

The extraction methods are used primarily to concentrate intact protein by the removal of water and oil from the substrate without digestion (Kristinsson and Rasco, 2000). These methods also lessen the problem in preparation of fish protein

Table 1 The applications of fish protein hydrolysates produced from various enzymes

Application	Source	Enzyme	References
Nutritional and functional ingredients	Raw hake	Alcalase	Hale (1972)
	Sardine	Alcalase, Neutrase, papain	Quaglia and Orban (1987)
	Defatted sardine	Alcalase	Sugiyama *et al.* (1991)
	Capelin	Alcalase, Neutrase, papain	Shahidi *et al.* (1995)
	Pacific whiting solid waste	Alcalase, Neutrase	Benjakul and Morrissey (1997)
	Atlantic salmon muscle	Alcalase, Flavourzyme, Corolase	Kristinsson and Rasco (2000)
	Yellowfin tuna stomach	Alcalase	Guerard *et al.* (2001)
	Cod muscle	Alcalase, Flavourzyme	Gilmartin and Jervis (2002)
	Antractic krill meat	Protease from *Bacillus subtilis* and *Aspergillus*	Zhang *et al.* (2002)
	Salmon head	Alcalase	Gbogouri *et al.* (2004)
Nitrogenous source in microbial growth media	Cod viscera	Endogenous enzme	Clausen *et al.* (1985)
	Fish heads, bone and fins	Trypsin	Jassim *et al.* (1988)
	Capelin and blue whiting	Endogenous enzme, Alcalase	Gildberg *et al.* (1989)
	Tuna	Alcalase	Guerard *et al.* (2001)

hydrolysate because of the extreme susceptibility of fat to oxidation (Hoyle and Merritt, 1994). Solven extraction has been frequently used to prepare fish protein concentrate for enzymatic hydrolysis. Isopropanol or azeotropic extraction with ethylene dichloride is commonly used (Sikorski and Naczk, 1981). The final product of isopropanol extraction has a high biological value and is colorless and odorless, with less than 1 % lipids. However, it is not readily soluble or dispersible and has poor emulsification properties. By increasing the temperature of the extraction, the denaturation of protein and a drastic decrease of the functional properties of the product occurred. Additionally, the large proportions of hydrophobic groups are exposed to the solvent and exhibit an extremely low water affinity (Sikorski and Naczk, 1981). Therefore, these techniques jeopardize functional properties and nutritional value of the product (Kristinsson and Rasco, 2000). Although other solvents such as ethanol have been used successfully, they cause some defects (Sikorski and Naczk, 1981). Ethanol in the range of 4-6% resulted in an insignificant decrease in the rate of proteolysis of Caspian and Baltic sprats but was very effective in preventing bacterial spoilage at 60°C (Chernogortsev *et al*., 1966). Moreover, Hoyle and Merritt (1994) prepared fish protein concentrate from raw herring and from herring defatted by ethanol extraction and by cooking and pressing using Alcalase and papain. Fat extraction before hydrolysis lowered degree of hydrolysis, but reduced fishy odor to barely detectable levels. Color and nonenzymic browning of herring hydrolysate with cooking and pressing extraction was darker than those from raw herring and from ethanol extracted herring, respectively, during 3 months of storage at room temperature (Hoyle and Merritt, 1994).

1.2.3.1 Chemical hydrolysis process

Chemical hydrolysis of protein is achieved by cleaving peptides bonds with either acid or base. It is relatively inexpensive and quite simple to conduct. There are, however, many limitations to produce food ingredients. Protein hydrolysis with strong chemicals and solvents is performed at extreme temperatures and pHs and generally yields product with reduced nutritional qualities, poor functionality and restricted to use as flavor enhancer (Kristinsson and Rasco, 2000).

a. Acid hydrolysis

Acid hydrolysis of proteins is used more commonly than hydrolysis under alkaline conditions. Although the process is harsh and hard to control, it is still the preferred method for hydrolyzing vegetable proteins. Production of hydrolyzed vegetable protein, which is widely used for flavor and taste enhancement, requires extensive acid hydrolysis (Kristinsson and Rasco, 2000). Acid hydrolysis of fish protein is carried out using hydrochloric acid or sulfuric acid. After the proteins are completely hydrolyzed at high temperature and high pressure, the neutralization of the digest is conducted. As a consequence, the hydrolysate contains large amount of salt (NaCl), which can make a product unpalatable and interferes with functionality in food systems (Kristinsson and Rasco, 2000). Another drawback of acid hydrolysis is the destruction of tryptophan, which is an essential amino acid. The acid hydrolysis is also widely used to convert the under-utilized and secondary raw materials from fish into fertilizer due to the low production cost and the resulting extensive hydrolysis (Kristinsson and Rasco, 2000).

b. Alkaline hydrolysis

The use of alkaline reactant, primarily sodium hydroxide, to hydrolyze protein often results in poor functionality and more importantly can adversely affect the nutritive value of the hydrolysate. Furthermore, several deleterious reactions occur in alkaline solutions during hydrolysis, leading to the formation of toxic substances, for instance lysinoalanine that is highly undesirable in foods (Linder *et al*., 1995). In addition, alkaline hydrolysis reaction products have an inhibiting effect on proteolytic enzymes, reducing the rate of hydrolysis (Kristinsson and Rasco, 2000).

Fonkwe and Singh (1996) reported that the recovery of mechanically deboned turkey residue using an alkali sodium chloride solution showed the low recovery. Tannenbaum *et al*. (1970) studied the alkaline process for hydrolyzing insoluble fish protein concentrate at alkaline pH (12.5) and 95°C for 20 min. The product had an overall improvement in functionality in comparison with the original fish protein concentrate. Use of the solubilized fish protein concentrate as a milk substitute gave a product far superior to that with fish protein concentrate starting material, which had poor solubility and dispersibility.

1.2.3.2 Biochemical method

Food protein hydrolysates can be prepared by using enzymes to hydrolyze peptide bonds. This can be done by proteolytic enzymes already present in the fish viscera and muscle (endogenous protease), or by adding enzyme from other sources (Kristinsson and Rasco, 2000). Compared with acid and alkaline hydrolysis, the enzymatic procedure can retain higher nutritional value; nevertheless, it is costly. Furthermore, it increases the risk of microbial growth and often leads to the formation of bitter-tasting peptides (Boudrant and Cheftel, 1976).

a. Autolytic hydrolysis

Autolytic hydrolysis, a simple operation, can be used in biochemical production of fish protein hydrolysate. Rate of hydrolysis depends on the action of the digestive enzymes of the fish itself. The end product of autolytic process is practically a fairly viscous liquid teeming with free amino acids and small peptides. Due to several factors including the fish species and season as well as the type and amount of enzyme, it is difficult to control the rate of hydrolysis (Sikorski and Naczk, 1981; Mukundan *et al*, 1986). These variations make it imperative to control the autolytic hydrolysis, and direct the production of hydrolysates such as fish sauces and fish silage with specific molecular properties (Kristinsson and Rasco, 2000). The protein recovery of hydrolysates produced autolytically was considerably lower compared with that produced by commercial enzymes (Shahidi *et al*., 1995).

b. Enzymatic hydrolysis with added enzymes

In order to modify the physicochemical, functional, and sensory properties of the native protein without threatening its nutritive value, enzymatic processes with added enzymes are used to hydrolyze food protein. These processes take place under mild circumstances and do not generate hydrolytic degradation products via racemization reactions occurred with acid and alkaline hydrolysis (Kristinsson and Rasco, 2000). Enzymatic hydrolysis with added enzymes provides many advantages because it allows good control of the process with the desirable properties of resulting products.

(1) Source of enzymes

Commercial enzymes commonly used for protein hydrolysate production are present in Table 2. Alcalase, an alkaline enzyme, has been

Table 2 Commercial Enzymes commonly used for protein hydrolysate production

Enzyme	EC No.	Temperature range (°C)	pH range	Source	Specificity
Alcalase 2.4 LFG	3.4.21.62	55-70	6.5-8.5	*Bacillus licheniformis*	endopeptidase
Neutrase 0.5 L	3.4.24.28	45-55	5.5-7.5	*B. subtilis*	endopeptidase
Protamex	3.4.21.62	35-60	5.5-7.5		protease complex
Alkaline protease	3.4.21.14	60	9.0-10	*B. licheniformis*	endopeptidase
Bromelain		50-60	3-9	pineapple stem	peptidase
Papain		65-80	5-7	*Carica papaya* fruit	peptidase
Corolase 7089	3.4.24.4	<60	5-7.5	*B. subtilis*	endopeptidase
ValidaseTSP	3.4.21.14	45-55	6.5-8	*B. subtilis*	endopeptidase
Corolase N	3.4.24.4	<60	5-7.5	*B. subtilis*	endopeptidase
Corolase PN-L	3.4.23.6	<50	5-8	*Aspergillus sojae*	endo-,exopeptidase
Corolase LAP	3.4.23.18	<70	6-9	*A. sojae*	exopeptidase
Flavourzyme 1000 L	3.4.11.1	50	7.0	*A. oryzae*	endo-,exopeptidase

Source: modified from Gilmartin and Jervis (2002) and Van Der Ven *et al.* (2002)

proven repeatedly to be one of the best enzymes used to prepare functional protein hydrolysate. Sugiyama *et al.* (1991) concluded that defatted sardine fish protein hydrolysates prepared with alkaline proteases, especially Alcalase, had higher solubility, higher tryptophan content and lower molecular weight than those prepared with neutral and acid counterpart. Hale (1972) suggested that the hydrolysis of raw hake by Alcalase at pH 8.5 or above yielded the excellent balance of essential amino acids and high yield of soluble product. Hydrolysates from sardine generated by Alcalase and papain showed the increased nitrogen recovery with augmenting enzyme concentration. At the same hydrolysis time, hydrolysates treated with Neutrase had only 20% nitrogen recovery. Hydrolysates produced using Alcalase and papain also showed better functional properties and high nutritional value than those using Neutrase (Quaglia and Orban, 1987). In addition, Shahidi *et al.* (1995) successfully used Alcalase to produce capelin protein hydrolysates. Alcalase-treated hydrolysate

manifested superior protein recovery, compared with Neutrase and papain and also had the lowest lipid content and extraordinary functional properties. Benjakul and Morrissey (1997) used Alcalase and Neutrase in producing protein hydrolysates from Pacific whiting solid wastes. Optimum activity of Alcalase and Neutrase are at pH 9.5, 60°C and pH 7.0, 55°C, respectively. Alcalase had higher hydrolytic activity than Neutrase. Moreover, Gilmartin and Jervis (2002) produced cod muscle hydrolysate by Alcalase in combination with other commercial enzymes to produce a variety of peptides with low molecular-weight range. Combinations of endopeptidase and exopeptidase effectively converted proteins to amino acids, thus reducing the peptide yield. The marked reduction in molecular weight of detected peptide was found when Flavourzyme and the combination of Alcalase and Flavourzyme were used (Gilmartin and Jervis, 2002). Alcalase was accepted to be the most cost-effective enzyme out of five enzyme preparations tested to cleaved Atlantic salmon muscle proteins (Kristinsson and Rasco, 2000). In addition, Flavourzyme 1000L, Corolase 7089 and Corolase PN-L have shown the extraordinary potential for hydrolyzing fish proteins to render highly functional fish protein hydrolysates (Kristinsson and Rasco, 2000).

(2) Parameters affecting protein hydrolysis

Hydrolysis of fish protein with selected proteolytic enzymes offers the possibility of directing cleavage degree of protein (Kristinsson and Rasco, 2000). Not only the specificity and concentration of enzymes, but also the environmental variables play a vital role in the peptide profile of the final products. Enzymes concentration, processing temperature, pH, reaction time, and substrate/buffer ratio significantly affected the hydrolysis and nitrogen recovery (Benjakul and Morrissey, 1997; Sumaya-Martinez *et al*., 2005). Optimized conditions designate the hydrolysate with different molecular structure and also different functional properties, which can be used in a variety of food formulations. This control of hydrolysis can be accomplished by using highly specific protease (Chobert *et al*., 1988) or by following the degree of hydrolysis (Adler-Nissen and Olsen, 1979; Sumaya-Martinez *et al*., 2005). Enzymatic hydrolysis of stomach proteins from yellowfin tuna (*Thunnus albacares*) wastes by Alcalase showed a linear correlation between the degree of hydrolysis and the enzyme concentration. After addition of extra substrate during the course of hydrolysis, the final degree of hydrolysis obtained

was proportional to the substrate added, suggesting that the concentration of hydrolyzable bonds was one of the main variables controlling the hydrolysis rate (Guerard *et al*., 2001). Nevertheless, commercial batch protein hydrolysis has many disadvantages, for instance, generation of bitterness in protein hydrolysate (Yeom *et al*., 1994), high cost of utilizing tremendous quantities of enzymes, hardship in controlling the extent of reaction that can occur in non homogenous products, low yields and the necessity to inactivate enzymes by pH or heat treatment, which adds to operating costs. Moreover, the enzymes employed in the process cannot be reused. Thereby, the choice of enzymes is commonly determined by the combination of efficacy and economics (Kristinsson and Rasco, 2000)

(3) Bitterness and debitterness process

Bitterness is a common problem in preparation of fish protein hydrolysates due to the formation of peptides containing bulky hydrophobic groups toward their C-terminal. Several methods have been suggested to mask the bitterness of fish protein hydrolysates, including the incorporation of glutamic acid or glutamyl-rich peptides, polyphosphates, gelatin, or glycine into the products (Kristinsson and Rasco, 2000).

Saha and Hayashi (2001) manifested that enzymatic hydrolysis of protein frequently results in bitter taste, which is due to the formation of low molecular weight peptides containing mainly hydrophobic amino acids. To decrease the bitterness, debittering process including the selective separation, adsorption or extraction, such as treatment with activated carbon, extraction with alcohol, isoelectric precipitation, chromatography on silica gel, hydrophobic interaction chromatography, masking of bitter taste with other flavors (Saha and Hayashi, 2001), controlling the degree of hydrolysis (Suh *et al*., 2000), hydrolysis with exopeptidases and plastein reaction, have been developed (Suh *et al*., 2000; Barbosa *et al*., 2004). Moreover, the further enzymatic treatments have proven effective in removing the bitterness of protein hydrolysates. Aminopeptidases show the great promise in removing the bitterness (Saha and Hayashi, 2001). Sugiyama *et al*. (1991) concluded that defatted sardine fish protein hydrolysates prepared with alkaline proteases, especially Alcalase, had the weaker bitterness than those prepared with neutral and acid counterpart. Suh *et al*. (2000) found that active carbon markedly

reduced the surface hydrophobicity and bitterness of corn gluten hydrolysates. Morais *et al*. (2003) and Barbosa *et al*. (2004) suggested that encapsulation in liposomes showed the efficiency in reducing the hydrophobicity and bitter taste of casein hydrolysates and gave a good chemical stability from lipid oxidation during 60 days of refrigerated storage. Plastein reaction has been reported to reduce the bitterness of protein hydrolysate (Williams *et al*., 2001).

1.2.4 Functional properties of protein hydrolysates

Functional property is the behavior of proteins in food systems during processing, storage, preparation and consumption. Modifying and developing functional properties of protein hydrolysates are the major advantages of enzymatic hydrolysis process. Hydrolysis under controlled condition may modify the functional properties of the resulting peptides, increasing their applications (Adler-Nissen, 1986; Quaglia and Orban, 1990; Sausa *et al*., 2004). Enzyme specificity is significant to peptide functionality since it potentially influences the molecular size and hydrophobicity of the hydrolysate (Adler-Nissen, 1986; Turgeon and Gauthier, 1990; Kristinsson and Rasco, 2000). The more the specificity of enzyme, the larger peptides are generated (Kristinsson and Rasco, 2000). The chain length of peptides is also dependent on the extent of hydrolysis, conditions of hydrolysis, concentration of enzyme and the type of protein to be hydrolyzed (Kristinsson and Rasco, 2000). Nouri *et al*. (1997) concluded that limited enzymatic hydrolysis of wheat storage protein yielded hydrolysates with valuable functional properties for food uses.

1.2.4.1 Solubility

Solubility is an indicator of the protein hydrolysate functionality and its applications. Due to the lack of solubility of intact proteins in water over the wide range of pH, enzymatic hydrolysis is very important in increasing the solubility (Kristinsson and Rasco, 2000). Enzymatic breakdown of protein involves a major structural change, in which the protein is slowly hydrolyzed into smaller peptide units. As the degree of cleaving increases, the solubility of protein increases. High solubility of fish protein hydrolysate over a wide range of pH is a substantially useful characteristic for many food applications. Furthermore, it influences the other

functional properties such as emulsifying and foaming properties. However, a very high degree of hydrolysis can have enormously negative effects on the rest of the functional properties (Kristinsson and Rasco, 2000).

Protein hydrolysate prepared by different enzymes had varying solubility profiles (Shahidi *et al.,* 1995). Quaglia and Orban (1987) reported that sardine hydrolysate produced by Alcalase and papain had higher solubility as the molecular weight decreased. Moreover, Sugiyama *et al.* (1991) found that all alkaline proteases gave lower average molecular weight protein hydrolysates, compared with the neutral and acid proteases, thus affecting the solubility of protein hydrolysates. Rebeca *et al*. (1991) reported that the increase in bacterial protease concentration was associated with an increase in soluble nitrogen. Vieir *et al*. (1995) showed that lobster protein hydrolysates cleaved by pepsin had the higher solubility than those produced by papain and fungal protease. Gustavo *et al*. (1995) prepared protein hydrolysates from Brazilian lobster processing waste by enzymatic hydrolysis. They were highly soluble with excellent wettability with no bitterness.

The pretreatments of protein before hydrolysis affected the functionality, especially solubility of protein hydrolysate. Lee *et al*., (2001) showed that mild acid pre-treatment and subsequent enzymatic hydrolysis by using Alcalase and Flavourzyme of defatted soy bean flour yielded higher solubility than defatted soy bean flour. Hoyle and Merritt (1994) suggested that enzymatically hydrolyzed ethanol-extracted herring fish protein hydrolysate had the highest solubility, compared with enzymatically hydrolyzed fish protein hydrolysate without prior ethanol extraction.

1.2.4.2 Water-holding capacity

Water-holding capacity has been defined as the ability of protein to imbibe water and retain it against gravitational force within a protein matrix (Kristinsson and Rasco, 2000). Water-holding capacity of proteins is of great importance to the food industry since water in food system frequently develops the texture. Enzymatic hydrolysis has a considerable influence on this property because it increases polar groups of protein that influence the amount of adsorbed water and moisture sorption isotherm (Kristinsson and Rasco, 2000).

Addition of capelin protein hydrolysate (3%) increased cooking yield and decreased drip loss of comminuted pork, suggesting that capelin protein hydrolysate has potential water binding capacity (Shahidi *et al*., 1995). Onodenalore and Shahidi (1996) reported that 0.5 to 3% shark protein hydrolysate obtained by Alcalase hydrolysis increased the cooking yield of comminuted pork by 2.4 to 9.3%. Moreover, Kristinsson (1998) found that fish protein hydrolysate prepared by Alcalase had the greatest water holding properties in salmon mince patties, compared with those hydrolyzed by other alkaline proteases. Generally, all fish protein hydrolysates showed better water-holding properties than egg albumin and soy protein concentrate.

1.2.4.3 Emulsifying property

Protein hydrolysates are surface active and promote oil-in-water emulsions (Kristinsson and Rasco, 2000). Generally, molecular weight distributions of the hydrolysates play a key role in stabilizing emulsions. At a limit degree of hydrolysis, the hydrolysates have better emulsifying capacity (Kuehler and Stine, 1974; Kristinsson and Rasco, 2000). On the contrary, an excessive hydrolysis causes the loss of this functionality (Shimizu *et al*., 1983). The peptides should have more than 20 amino acids to show good emulsifying properties (Lee *et al.,* 1987; Turgeon and Gauthier, 1990).

Kuehler and Stein (1974) concluded that whey protein hydrolysate cleaved with Prolase possessed large molecular weight peptides with exceptional emulsifying stability and activity. Conversely, Pronase, a broad specific enzyme, yielded smaller peptides with poor emulsifying properties. Mahmoud *et al*, (1992) also indicated that casein hydrolysate cleaved by pancreatin decreased in emulsifying activity as the degree of hydrolysis increased. At 67% degree of hydrolysis, the hydrolysates comprised only amino acids, di- and tri-peptides that decreased their emulsifying activity. In addition, Van Der Ven *et al*. (2001) confirmed that emulsion instability was related to apparent molecular weight distribution of hydrolysates. A relative high amount of peptides larger than 2 kDa increased the emulsion stability. Whey protein hydrolysates with a degree of hydrolysis between 10% and 27% had better emulsifying ability than non-hydrolyzed whey protein concentrate (Euston *et*

al., 2001). Increasing degree of hydrolysis from 27 to 35% led to a marked decrease in emulsion stability. Gauthier and Pouliot (2003) found that whey protein hydrolysate with peptides containing 41 to 60 and 21 to 40 amino acids from β-lactoglobulin were responsible for improved emulsifying properties. Ouaglia and Orban (1990) and Kristinsson (1998) found that enzymatic hydrolysis had a negative impact on the capacity to create and stabilize emulsion as the degree of hydrolysis was intensified for both sardine and salmon protein hydrolysates.

Lipid extraction of raw materials affects the emulsifying property of protein hydrolysate. Dubrow *et al.* (1973) found that emulsifying capacity of fish protein concentrate decreased when the solvent extraction was applied. Removal of lipid from rockfish protein hydrolysate also caused a tremendous loss in emulsifying capacity (Miller and Groninger, 1976). The emulsifying activities and emulsion stability in the <5 kDa fraction of corn gluten hydrolysate prepared by Flavourzyme was higher than those found in 5-10 kDa and >10 kDa fractions (Kim *et al*., 2004).

1.2.4.4 Foaming property

Protein hydrolysates possess the foaming property due to their excellent surface property. Total hydrophobicity or the hydrophobicity of unfolded protein has a significant correlation with foaming characteristics (Townsend and Nakai, 1983). The degree of hydrolysis also influenced the foaming characteristics (Kristinsson and Rasco, 2000). Moreover, the pH of the dispersing medium dramatically influences foaming properties, especially foam stability. Foaming characteristic was highest when pH was close to the isoelectric point of protein (Kinseller, 1981).

Kuehler and Stine (1974) delineated that enzymatic hydrolysis of whey protein increased foaming ability but decreased foam stability. This was ascribed to more air being incorporated into solution of smaller peptides, but the more microscopic peptides do not have the strength needed to maintain the stable foam. Shahidi *et al.* (1995) reported the good foaming properties of capelin protein hydrolysates prepared by Alcalase. However, further hydrolysis could reduce the foaming stability. Casein hydrolysate fraction containing peptides of 3-5 kDa showed

the good foam formation. Conversely, both intact casein and high molecular weight peptides exhibited the high foam stability (Ven *et al*., 2002a).

1.2.4.5. Fat adsorption

Fat-binding capacity of protein correlates with surface hydrophobicity. The substrate specificity of enzymes presumed to play an important role in fat adsorption of resulting hydrolysate (Kristinsson, 1998). The higher bulk density of protein contributes to the more fat adsorption. Hydrolysates from salmon muscle hydrolyzed by various enzymes had different fat adsorption ability. The capacity of a hydrolysate to adsorb fat is an important attribute that not only affects the taste of the product, but also is an important functional property required particularly for the meat and confectionery industry (Kristinsson and Rasco, 2000).

1.2.5 Antioxidants

Antioxidant is defined as any substance which is capable of delaying, retarding or preventing the development of rancidity or other flavor deterioration due to oxidation (Gordon, 2001). In general, antioxidants act by reducing the rate of initiation reaction in the free-radical chain reactions (Schafer *et al*., 2002; Je *et al*., 2005).

1.2.5.1 Classification of antioxidants

a. Primary antioxidants

Primary antioxidants terminate the free-radical chain reaction by donating hydrogen or electron to free radicals and converting them to more stable products. They may also interact with the lipid radicals, forming lipid-antioxidant complexes (Gordon, 2001). Protein hydrolysates from many sources have been reported to possess the primary antioxidative activity (Decker and Crum, 1993; McCarthy *et al.,* 2001; Sathivel *et al.,* 2003).

b. Secondary antioxidants

Secondary or preventive antioxidants function by decomposing the lipid peroxides into stable end products (Rajalakshmi and Narasimhan, 1996). Synergistic antioxidants can be broadly classified as oxygen scavengers and chelators.

Transition metals, such as iron, copper, cobalt, etc in foods affect both the rate of autoxidation and the breakdown of hydroperoxide to volatile compounds. Transition metal ions react very quickly with peroxides by acting as one-electron donors to form alkoxyl radical (Gordon, 2001). Peptides in hydrolysates could chelate the prooxidants, leading to decreased lipid oxidation. Therefore, chelation of transition metal ions by antioxidant or antioxidative peptides retarded the oxidation reaction (Sherwin, 1990).

1.2.5.2 Mode of action of antioxidants

a. Radical scavenger

Antioxidants can retard lipid oxidation by scavenging free radicals, thereby inhibiting the initiation and propagation reactions. Free radical scavengers or chain-breaking antioxidants are capable of accepting a radical from oxidized lipids species such as peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals to form stable end products (Decker, 1998). Antioxidants can scavenge free radical as hydrogen donors or as electron donors (Shimada *et al*., 1992).

b. Peroxide decomposer

Some phenols, amine, dithiopropionic acid and thiopropionic acid function by decomposing the lipid peroxide into stable end products such as alcohol, ketone and aldehyde (Dziezak, 1986; Namiki, 1990).

c. Singlet oxygen quenchers

Singlet oxygen is generated from the triplet state oxygen. The mechanism of converting triplet oxygen to singlet oxygen is initiated by the transfer of the photosensitizer to its electronically excited state due to the absorption of light in the visible or near-UV region. Subsequently, the photosensitizer is able to transfer its excess energy to an oxygen molecule, giving rise to singlet oxygen (Shahidi and Wanasundara, 1992). Singlet oxygen can react with a lipid molecule to yield a hydroperoxide. It reacts about 1,000-10,000 times as fast as normal oxygen with methyl linoleates (Jadhav *et al*., 1996). Lipid oxidation was inhibited by β-carotene because of its capability to quence singlet oxygen (Rajalakshmi and Narasimhan, 1996; Namiki, 1990).

d. Lipoxygenase inhibitor

Lipoxygenase is a non-heme iron-containing enzyme that catalyzes the oxygenation of the 1,4-pentadiene sequence of polyunsaturated fatty acid to produce their corresponding hydroperoxide (Salas *et al*., 1999). Free-radical intermediates occur during lipoxygenase catalysis, leading to cooxidation of easily oxidized compounds (Rajalakshmi and Narasimhan, 1996).

e. Synergists

(1) Chelating agents

Even though chelating agents are not antioxidants, they play a key role in stabilizing the systems. (Jadhav *et al*., 1996). Chelating agents form stable complexes with prooxidant metals such as iron, copper, cobalt, etc via sigma bond formation. It is considered as an effective secondary antioxidant. An unshared pair electron in their molecule structure promotes the chelating action (Rajalakshmi and Narasimhan, 1996; Jadhav *et al*., 1996).

(2) Reducing agents or oxygen scavengers

Reducing agents or oxygen scavengers act by several mechanisms. They may act as hydrogen donors to the radical, thereby regenerating the primary antioxidant or react with free oxygen and remove it in a closed system. (Rajalakshmi and Narasimhan, 1996).

1.2.5.3 Antioxidative activity of protein hydrolysates

Protein hydrolysates from different fish species such as mackerel (Wu *et al*., 2003), herring (Sathivel *et al*., 2003) and tuna cooking juice (Jao and Ko, 2002) have been found to retard lipid peroxidation and are feasible to use as natural antioxidants in foods and biological systems (Jao and Ko, 2002; Mendis *et al*., 2005a). Proteinases used affected the antioxidative activity of protein hydrolysates from tuna cooking juice (Jao and Ko, 2002). Protein hydrolysate from Alaska pollack frame prepared by mackerel intestine crude enzyme exhibited antioxidative activity in a linoleic acid model system (Je *et al*., 2005). Fraction of protein hydrolysate from yellowfin sole frame hydrolyzed by pepsin and mackerel intestine crude enzyme showed a strong antioxidative activity in a linoleic acid model system (Jun *et al*., 2004). Prawn hydrolysate prepared using pepsin showed the most potent antioxidative

activity than those prepared by other enzymes (Suetsuna, 2000). Raw material including whole herring, body, gonad and head had the influences on the antioxidative activity of herring hydrolysate (Sathivel *et al*., 2003). Levels and compositions of amino acids and peptides were reported to determine the antioxidant activities of protein hydrolysates (Wu *et al*., 2003). Antioxidative activity of peptides was also governed by their size and amino acid sequences (Kim *et al*., 2001). The sequence and molecular weight of antioxidative peptides from fish protein hydrolysates are depicted in Table 3. In general, the antioxidative effect of protein hydrolysate was concentration-dependent (Sakanaka *et al*., 2004). Peptide fractions separated from capelin protein hydrolysate possessed a different antioxidant activity and some fractions exhibited a prooxidant effect in a β-carotene-linoleate model system (Amarowicz and Shahidi, 1997).

1.2.6 Peptones

Peptones are defined as protein hydrolysates (Kurbanoglu and Kurbanoglu, 2002) or partially digested proteins, which are soluble in water (Green *et al*, 1977) and are not heat coagulable after heating to 100°C (Talron Biotech. Ltd., 2003). Soluble fish protein hydrolysate from extensive hydrolysis is an excellent nitrogen source for microbial growth. Extensively hydrolyzed fish protein comprises free amino acids and low molecular weight peptides, and thus can be used as microbial peptone (Clausen *et al.,* 1985; Jassim *et al*., 1988; Gildberg *et al.,* 1989; Kristinsson and Rasco, 2000; Guerard *et al*., 2001; Kurbanoglu and Kurbanoglu, 2002). Peptones contain a mixture of amino acids, peptides and proteoses, a heat-stable and acid soluble protein fraction (Innocente *et al*., 1998). The peptides with the molecular weight of 6,500 Da, di-peptides and amino acids from tuna treated with Alcalase were proved to be a suitable nitrogenous source in microbial media (Guerard *et al*., 2001).

Peptones are obtained by various types of digestion such as acid, alkaline or enzymatic hydrolysis. Acid hydrolysis gives a high yield, but ruptures all the proteins and peptides, thus generating only free amino acids. At the same time, it razes some important amino acids such as tryptophan and gives high ash content,

Table 3 The sequence and molecular weight of antioxidative peptides from fish protein hydrolysates

Source	Enzyme	MW (Da)	Sequence	References
Hoki skin gelatin	Trypsin	797	His-Gly-Pro-Leu-Gly-Pro-Leu	Mendis *et al.* (2005b)
Yellowfin sole	Pepsin, Mackerel intestine crude enzyme	1300	Arg-Pro-Asp-Phe-Asp-Leu-Glu-Pro-Pro-Tyr	Jun *et al.* (2004)
Tuna cooking juice	Protease XXIII	751	Pro-His-His-Ala-Asp-Ser	Jao and Ko (2002)
Conger eel	Tryptic enzyme	928	Leu-Gly-Leu-Asn-Gly-Asp-Asp-Val-Asn	Ranathunga *et al.* (2006)
Jumbo squid skin gelatin	Trypsin	880	Phe-Asp-Ser-Gly-Pro-Ala-Gly-Val-Leu,	Mendis *et al.* (2005a)
		1242	Asn-Gly-Pro-Leu-Gln-Ala-Gly-Gln-Pro-Gly-Glu-Arg	
Giant squid muscle	Trypsin	747	Asn-Gly-Leu-Glu-Gly-Leu-Lys,	Rajapakse *et al.* (2005)
		1307	Asn-Ala-Asp-Phe-Gly-Leu-Asn-Gly-Leu-Glu-Gly-Leu-Ala	
Prawn muscle	Pepsin	N/A	Phe-Ile-Lys-Lys, Ile-Lys-Lys, Phe-Lys-Lys	Suetsuna (2000)
Blue mussel	Endogenous enzyme	620	Phe-Gly-His-Pro-Tyr	Jung *et al.* (2005)

N/A: not applicable.

approximately 36% (Gildberg *et al*. 1989; Dufosse *et al*, 1997; Talron Biotech. Ltd, 2003), which limits the applicability of the peptone (Acumedia Manufacturers, Inc., 2003). The problem with high ash content is eliminated if the protein is digested by enzymes under neutral conditions. Although the peptone yield is low, this is a method commonly used (Gildberg *et al*., 1989). The diagram of peptone manufacturing process is shown in Figure 2. The presence of alkaline metals and phosphates can cause the precipitation of the peptones at a neutral pH (Talron Biotech. Ltd, 2003; Acumedia Manufacturers, Inc., 2003).

In general, the proteins used for the production of peptones are of two types, animal proteins such as casein (Tryptone), gelatin, meat (peptone) and vegetable proteins (Phytone) such as soy (Soytone) (Lindquist, 1999). Kurbanoglu and Kurbanoglu (2002) reported that the peptone for microbiological culture media can be produced from ram horn by hydrolyzing with 6 N HCl. The resultant hydrolysate was evaporated and termed as 'ram horn peptone'. Ram horn peptone was comparable with a bacto-tryptone from casein. Kurbanoglu and Kurbanoglu (2004) produced peptone from ram horn waste by treating with acids, followed by neutralization. The ram horn peptone was comparable with a bacto-tryptone from casein and other peptones as a nitrogen source for glycerol production by *Saccharomyces cerevisiae*. With the addition of ram horn peptone to the fermentation medium with an optimum concentration of 4%, the glycerol content was 25% higher than that of bacto-tryptone, 32% higher than that of bacto-peptone and 49% higher than that of fish peptone. The result indicated that ram horn peptone can be used as a promising peptone.

Peptones provide a readily available source of nitrogen for microorganisms that generally do not attack native protein (A. Costantino & C. S.p.A., 2004). Moreover, peptones contain other constituents, which can stimulate growth such as nucleic acid, minerals, vitamins and occasionally carbohydrates as in the case of soy peptone (Talron Biotech. Ltd, 2003). Peptones should be stored in sealed container at 2-30 °C and discarded if not free flowing or if the appearance is changed from the original color (Acumedia Manufacturers, Inc., 2003).

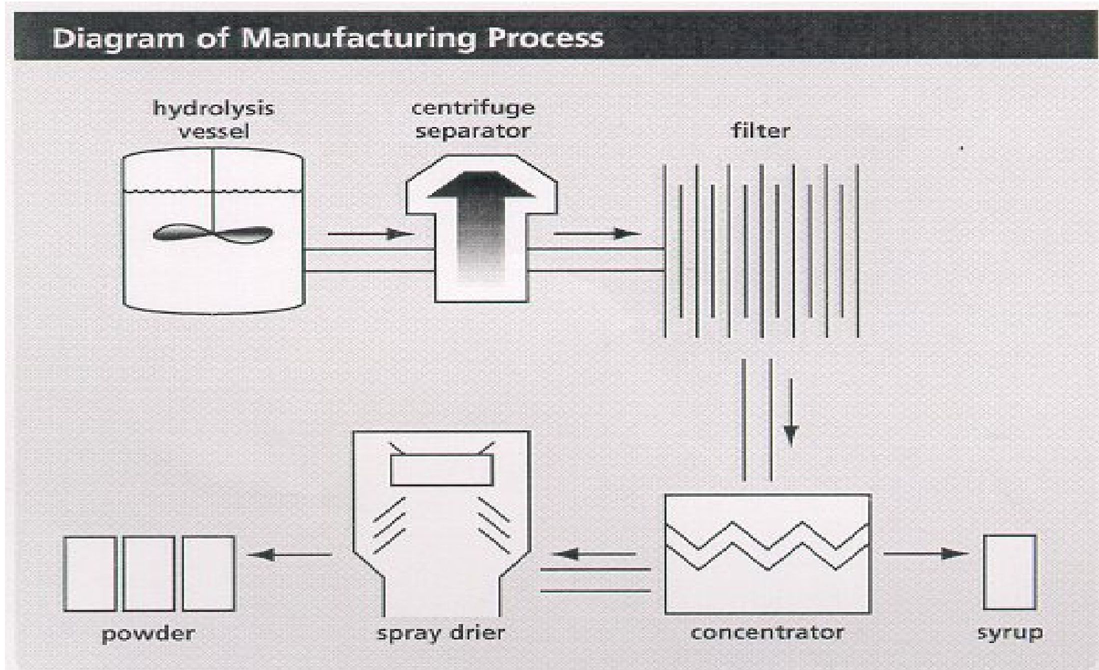


Figure 2 The diagram of peptone manufacturing process

Source: Bridson and Brecker (1970)

1.2.7 Fish peptone and the use for microbial growth

Fish peptone was an extraordinary enrichment ingredient for fish pathogen (Snieszko *et al.*, 1950). Stephen *et al.* (1975) produced microbial media from shrimp waste. After autolytic digestion of shrimp heads and hulls, two peptones were extracted and evaluated for suitability in supporting growth of five genera of bacteria and five genera of fungi. A 0.5% solution of lyophilized shrimp head digest become cloudy when heated at 121°C for 15 min. Nevertheless, the digest advocated the excellent growth of fungi and good growth of bacteria. A heated 0.5% solution of the hull digest was clear and gave the satisfactory growth of both bacteria and fungi. Proximate compositions of various hydrolysates used in microbiological growth media are shown in Table 4. Jassim *et al.* (1988) prepared fish extracts from fish waste by enzymatic digestion and using activated charcoal to reduce odor. An aqueous extract of trypsin-degraded fish wastes is a good substitute for beef extract in culture media. Furthermore, similar colony morphology was observed between organism grown on the nutrient agar and agar containing fish extract. For the fish

Table 4 Proximate compositions of hydrolysates used in microbiological growth media

Component (%) / Sources	Protein	Ash	Fat	Sugar	References
Digested shrimp heads	66.9	14.1	2.7	N/A	Stephens *et al*. (1976)
Digested shrimp hulls	51.4	33.2	1.4	N/A	Stephens *et al*. (1976)
Ram horn	50	47	1.4	2.6	Kurbanoglu and Kurbanoglu (2004)

N/A: not applicable

extract medium enriched with proteose peptone, the growth was luxuriant. Gildberg *et al*. (1989) prepared peptone by a two-step enzymatic hydrolysis of two low-cost fish species, capelin and blue whiting. Approximately 50% of total fish protein was obtained as a completely soluble peptone fraction with neutral pH and low salt content. When comparing with high quality commercial peptones, the fish peptones performed excellently as nitrogen source in bacterial growth media, especially peptone prepared from blue whiting. Freeze-dried tuna protein hydrolysate was also shown to be a nitrogenous source in microbial growth media (Guerard *et al*., 2001). Amino acid compositions of the protein hydrolysates used in microbiological growth media are shown in Table 5. Vecht-Lifshitz *et al*. (1990) showed that industrial fish peptone was an excellent substrate for biomass production in solid and submerged fermentations. De La Broise *et al*. (1998) compared *Escherichia coli* growth kinetics on fish hydrolysates and on casein hydroysates. With casein hydrolysate, the lag phase was shorter and the growth rate and maximum cell density were higher, compared with that grew on fish protein hydrolysates.

Amezaga and Booth (1999) studied the effect of meat peptone type I (Sigma) on the growth of *Escherichia coli* cells under hyperosmosis stress. Peptone enhanced the growth of *E. coli* cells in high-osmolarity medium. The main role of the peptides in peptone was the provision of nutrients rather than the intracellular accumulation of osmolytes. De Freitas and Siqueira (2004) concluded that the amino acids including histidine, cysteine and leucine, and peptone stimulated the fungal growth, while phenylalanine and methionine showed significant growth inhibition.

Table 5 Amino acid compositions of peptone from different sources

Amino acids (%)	Capelin	Blue whiting	Ram horn
Lysine	6.0	6.7	2.1
Histidine	0.8	1.1	0.7
Arginine	3.4	6.1	4.2
Aspartic acid	5.8	7.7	3.6
Threonine	2.2	3.5	1.7
Serine	2.2	3.9	2.6
Glutamic acid	10.7	11.4	7.3
Proline	2.6	3.7	3.5
Glycine	4.5	4.8	4.6
Alanine	4.9	5.5	2.8
Valine	3.9	4.1	2.9
Methionine	2.1	2.6	0.4
Isoluecine	3.1	3.7	1.5
Leucine	5.7	5.9	3.6
Tyrosine	1.2	2.8	1.5
Phenylalanine	2.5	3.4	1.6
References	Gildberg *et al.* (1989)	Gildberg *et al.* (1989)	Kurbanoglu and Kurbanoglu (2004)

1.3 Objectives of study

1. To produce a protein hydrolysate from the meat of yellow stripe trevally with different DHs using Alcalase or Flavourzyme.
2. To study the functional properties and antioxidative activities of protein hydrolysate from yellow stripe trevally.
3. To investigate the potential use of protein hydrolysate from yellow stripe trevally as a nitrogen source for microbial growth and as enumeration media in comparison with commercial Bacto Peptone.

4. To study the moisture sorption isotherm and the storage stability during storage of protein hydrolysate from yellow stripe trevally in comparison with commercial Bacto Peptone.

CHAPTER 2

ANTIOXIDATIVE ACTIVITY AND FUNCTIONAL PROPERTIES OF PROTEIN HYDROLYSATE OF YELLOW STRIPE TREVALLY (*SELAROIDES LEPTOLEPIS*) AS INFLUENCED BY THE DEGREE OF HYDROLYSIS AND ENZYME TYPE

2.1 Abstract

Antioxidative activity and functional properties of protein hydrolysates from yellow stripe trevally (*Selaroides leptolepis*) meat, hydrolyzed by Alcalase 2.4L (HA) and Flavourzyme 500L (HF) with different degrees of hydrolysis (DH) were investigated. As the DH increased, DPPH radical-scavenging activity and reducing power of HA decreased ($p<0.05$) but no differences were observed for HF ($p>0.05$). Metal chelating activity of both HA and HF increased with increasing DH ($p<0.05$). HF generally had a higher ($p<0.05$) chelating activity than had HA at the same DH tested. At low DH (5%), HA exhibited a better DPPH radical-scavenging activity while, at high DH (25%), HF had a higher ($p<0.05$) reducing power. For the functional properties, hydrolysis by both enzymes increased protein solubility to above 85% over a wide pH range (2–12). When the DH increased, the interfacial activities (emulsion activity index, emulsion stability index, foaming capacity, foam stability) of hydrolysates decreased ($p<0.05$), possibly caused by the shorter peptide chain length. At the same DH, the functionalities of protein hydrolysate depended on the enzyme used. The results reveal that antioxidative activity and functionalities of protein hydrolysates from yellow stripe trevally meat were determined by the DH and by the enzyme type employed.

2.2 Introduction

Fish processing by-products and the under-utilized species are commonly recognized as low-value resources with negligible market value. Additionally,

inappropriate disposal is a major cause of environmental pollution. Hydrolysis processes have been developed to convert under-utilized fish and fish by-products into the marketable and acceptable forms (Gildberg, 1993; Quaglia and Orban, 1987), which can be widely used in food rather than as animal feed or fertilizer (Benjakul and Morrissey, 1997).

Functional properties of protein can be improved by enzymatic hydrolysis under controlled conditions (Quaglia and Orban, 1990). Hydrolysis potentially influences the molecular size, hydrophobicity and polar groups of the hydrolysate (Adler-Nissen, 1986; Kristinsson and Rasco, 2000). The characteristics of hydrolysate directly affect the functional properties and the uses as food ingredients (Kristinsson and Rasco, 2000). Hydrolysate has an excellent solubility at high degree of hydrolysis (Gbogouri *et al.*, 2004; Quaglia and Orban, 1987; Shahidi *et al.*, 1995). High solubility of fish protein hydrolysate over a wide range of pH is a substantially useful characteristic for many food applications. Furthermore, it influences the other functional properties, such as emulsifying and foaming properties (Gbogouri *et al.*, 2004; Kristinsson and Rasco, 2000). However, a very high degree of hydrolysis can have enormously negative effects on the functional properties (Kristinsson and Rasco, 2000). Greater emulsifying capacity and emulsion stability were noticeable when DH was low for salmon by-product hydrolysate (Gbogouri *et al.*, 2004) and sardine hydrolysate (Quaglia and Orban, 1990). Shahidi *et al.* (1995) also reported good foaming properties for capelin protein hydrolysates prepared by Alcalase at low DH.

Apart from their functionalities, protein hydrolysates from different sources, such as whey, soy protein (Pena-Ramos and Xiong, 2003), egg-yolk (Sakanaka *et al.*, 2004), prawn (Suetsuna, 2000), tuna cooking juice (Jao and Ko, 2002), yellowfin sole frame (Jun *et al.*, 2004), Alaska Pollack frame (Je *et al.*, 2005), herring (Sathivel *et al.*, 2003), mackerel (Wu *et al.*, 2003) and capelin (Amarowicz and Shahidi, 1997), have been found to possess antioxidant activity. Levels and compositions of free amino acids and peptides were reported to determine the antioxidant activities of protein hydrolysates (Wu *et al.*, 2003). However, there is a little information regarding protein hydrolysates from the meat of yellow stripe trevally (*Selaroides leptolepis*) and their antioxidative activity and functional properties as affected by DH and enzymes used. Therefore, this study aimed to produce a protein hydrolysate from

the meat of yellow stripe trevally, an under-utilized species, with different DHs using two different proteinases and to study their antioxidative activities and functional properties.

2.3 Materials and methods

2.3.1 Chemicals and enzymes

Both 2,2-diphenyl-1-picryhydrazyl (DPPH) and 3-(2-pyridyl)-5,6-bis(4-phenyl-sulphonic acid)-1,2,4-triazine (ferrozine) were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Iron (II) chloride tetrahydrate ($FeCl_2{\bullet}4H_2O$) was procured from Kanto Chemical Co., Inc. (Tokyo, Japan). Alcalase 2.4L and Flavourzyme 500L were obtained from Novozymes (Bagsvaerd, Denmark).

2.3.2 Fish sample preparation

Yellow stripe trevally (*S. leptolepis*), 65 g/fish, off-loaded approximately 24–36 h after capture, were obtained from the fishing port in Satul province along the coast of the Andaman Sea. The fish were placed in ice with a fish/ice ratio of 1:2 (w/w) and transported to the laboratory. Upon arrival, fish were washed and the meat was separated manually. The meat was minced, using a grinder with 0.4 cm diameter holes. The mince was stored in plastic bags in ice until used.

The mince was defatted according to the method of Sikorski and Naczk (1981) with a slight modification. The mince was mixed with isopropanol at a ratio of 1:4 (w/v), homogenized and allowed to stand at room temperature (30–32°C) for 50 min. The supernatant was drained and the residue was defatted at 75°C for 90 min using isopropanol at a ratio of 1:4 (w/v). The supernatant was then removed and the precipitate was air-dried at room temperature (30–32°C). The proximate composition (AOAC, 2000), as well as the solubility (Xiong *et al*., 2000), of both mince and defatted mince, were determined.

2.3.3 Production of protein hydrolysates

Mince (30 g), or defatted mince (7 g), was suspended in 120 ml and 143 ml of distilled water, respectively, in order to obtain the same protein content. The

mixtures were preincubated at 60 or 50°C for 20 min prior to enzymatic hydrolysis using Alcalase or Flavourzyme. The hydrolysis reaction was started by the addition of the enzyme (Alcalase or Flavourzyme) at levels of 0.25, 0.5, 1, 2.5, 5, 7.5 and 10% (w/w). The reaction was conducted at pH 8.5, 60°C for Alcalase and at pH 7.0, 50°C for Flavourzyme for up to 20 min, using the pH-stat method, as described by Adler-Nissen (1986). The pH of the mixture was maintained constant during hydrolysis using 2 M NaOH. DH was then calculated as follows:

$$DH\ (\%) = (BN_b/M_p\ \alpha\ h_{tot}) \times 100$$

where B is the amount of alkaline consumed (ml), N_b is the normality of alkaline, M_p is the mass of the substrate (protein in grammes, % N × 6.25), $1/\alpha$ is the calibration factors for pH-stat, and h_{tot} is the content of peptide bonds (Adler-Nissen, 1986).

At designated DHs, the enzymatic reaction was terminated by placing the samples in a water bath at 90°C for 15 min with occasional agitation. The samples were cooled and the pH values of samples were then adjusted to 7.0 with 6 M HCl or 1 M HCl. Hydrolysates were centrifuged at 2000g for 10 min, using a Biofuge primo centrifuge (Sorvall, Hanau, Germany). Supernatants obtained were freeze-dried using a freeze-dryer (Dura-stop, New York, NY, USA).

Log10 (enzyme concentration) vs. DH was plotted. From the regression equation, the enzyme concentrations required to hydrolyze yellow stripe trevally mince to obtain the desired DHs (5%, 15%, 25%) were calculated.

2.3.4 Proximate analysis

Moisture, protein and fat were determined according to the method of AOAC (2000). The protein and fat contents were expressed on a dry weight basis.

2.3.5 Determination of antioxidative activities

2.3.5.1 2,2-Diphenyl-1-picryhydrazyl (DPPH) radical scavenging activity

DPPH radical-scavenging activity was measured, using the method of Yen and Wu (1999). HA or HF with different DHs were dissolved in distilled water to obtain a concentration of 40 mg protein/ml. To 4 ml of sample solutions, 1.0 ml of 0.2 mM DPPH was added and mixed vigorously. After incubating for 30 min, the absorbance of the resulting solutions was measured at 517 nm using a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). The control was conducted in the same manner, except that distilled water was used instead of sample. DPPH radicalscavenging activity was calculated according to the following equation (Yen and Wu, 1999):

$$\text{DPPH radical-scavenging activity (\%)} = (1 - (A_{517} \text{ of sample}/A_{517} \text{ of control})) \times 100$$

2.3.5.2 Reducing power

Reducing power was determined by the method of Oyaiza (1986). The sample solution (0.5 ml, 40 mg protein/ml) was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. An aliquot (2.5 ml) of 10% trichoroacetic acid was added to the mixture, followed by centrifugation at 3000 rpm (Hettich mikro 20, Tuttlingen, Germany) for 10 min. The upper layer of solution (2.5 ml) was mixed with 2.5 ml of distilled water and 2.5 ml of 0.1% ferric chloride and the absorbance was read at 700 nm. Increased absorbance of the reaction mixture indicates increasing reducing power.

2.3.5.3 Metal-chelating activity

The chelating activity on Fe^{2+} was determined, using the method of Decker and Welch (1990). One millilitre of sample solution (40 mg protein/ml) was mixed with 3.7 ml of distilled water. The mixture was then reacted with 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) for 20 min at room temperature. The absorbance was read at 562 nm. The control was prepared in the same manner except that distilled water was used

instead of the sample. Chelating activity (%) was then calculated as follows (Decker and Welch, 1990):

$$\text{Chelating activity (\%)} = (1 - (A_{562} \text{ of sample}/A_{562} \text{ of control})) \times 100$$

2.3.6 Determination of functional properties

2.3.6.1 Solubility

To determine protein solubility, 200 mg of protein hydrolysate sample were dispersed in 20 ml of deionized water and pH of the mixture was adjusted to 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 with 1 or 6 M HCl and 1 or 6 M NaOH. The mixture was stirred at room temperature for 30 min and centrifuged at 7500g for 15 min. Protein contents in the supernatant were determined using the Biuret method (Robinson and Hodgen, 1940). Total protein content in the sample was determined after solubilization of the sample in 0.5 M NaOH. Protein solubility was calculated as follows:

$$\text{Solubility (\%)} = (\text{protein content in supernatant}/\text{total protein content in sample}) \times 100$$

2.3.6.2 Emulsifying properties

Emulsifying properties were determined according to the method of Pearce and Kinsella (1978). Vegetable oil (10 ml) and 30 ml of 1% protein solution were mixed and the pH was adjusted to 2, 4, 6, 8 and 10. The mixture was homogenized using a homogenizer (Polytron, Luzern, Switzerland) at a speed of 20,000 rpm for 1 min. An aliquot of the emulsion (50 µl) was pipetted from the bottom of the container at 0 and 10 min after homogenization and mixed with 5 ml of 0.1% sodium dodecyl sulfate (SDS) solution. The absorbance of the diluted solution was measured at 500 nm using a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). The absorbances measured immediately (A_0) and 10 min (A_{10}) after emulsion formation were used to calculate the emulsifying activity index (EAI) and the emulsion stability index (ESI) as follows (Pearce and Kinsella, 1978):

$$\text{EAI } (m^2/g) = 2 \times 2.303 \times A_{500}/\ 0.25 \times \text{protein weight (g)}$$

$$\text{ESI (min)} = A_0 \times \Delta t/\Delta A$$

where $\Delta A = A_0 - A_{10}$ and $\Delta t = 10$ min

2.3.6.3 Foaming properties

Foaming capacity and stability of yellow stripe trevally protein hydrolysate were determined according to the method of Sathe and Salunkhe (1981). Twenty milliliters of 0.5% sample solution were adjusted to pH 2, 4, 6, 8 and 10, followed by homogenization at a speed of 16,000 rpm, using a homogenizer (IKA Labortechnik, Selangor, Malaysia) to incorporate the air for 2 min at room temperature. The whipped sample was immediately transferred into a 25 ml cylinder and the total volume was read after 30 s. The foaming capacity was calculated according to the following equation (Sathe and Salunkhe, 1981):

$$\text{Foaming capacity (\%)} = ((A-B)/B) \times 100$$

where A = the volume after whipping (ml); B = the volume before whipping (ml)

The whipped sample was allowed to stand at 20°C for 3 min and the volume of whipped sample was then recorded. Foam stability was calculated as follows:

$$\text{Foam stability (\%)} = ((A-B)/B) \times 100$$

where A = volume after standing (ml); B = volume before whipping (ml).

2.3.7 Statistical analysis

One-way ANOVA was used and mean comparison was performed by Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was carried out using the SPSS statistic programme (Version 10.0) for Windows (SPSS Inc. Chicago, IL).

2.4 Results and Discussion

2.4.1 Effect of defatting on composition and hydrolysis of yellow stripe trevally meat

Chemical compositions of whole mince and defatted mince are shown in Table 6. Defatted mince had a much lower fat content (0.67%) than had whole mince

Table 6. Chemical compositions and properties of yellow stripe trevally mince and defatted mince[a]

Compositions/property (%)	Mince	Defatted mince
Protein[b]	84.2±1.07	96.8±1.02
Fat[b]	3.23±0.27	0.67±0.07
Moisture	79.67±0.52	24.73±0.27
Solubility	45.07±1.96	19.56±0.35

[a] Mean±SD from triplicate determinations.
[b] Dry weight basis.

(3.23%). Coincidentally, a higher protein content was noticeable in the defatted mince. Due to the drying process after defatting, the moisture of the defatted sample was removed to a large extent. The result was in agreement with that of Sikorski and Naczk (1981), who found that isopropanol could remove the fat in fish muscle effectively prior to hydrolysis. Nevertheless, the solubility of defatted mince in KCl was lowered. During isopropanol extraction, proteins are vulnerable to solvent as well as to heat treatment (Mutilangi *et al*., 1996), leading to the exposure of the hydrophobic domain and aggregation of protein (Sikorski and Naczk, 1981). Isopropanol and other alcohols have been known to compete with protein in water binding. As a result, water was more removed from protein molecules in the presence of solvent (Hoyle and Merritt, 1994; Sikorski and Naczk, 1981). Mutilangi *et al*. (1996) reported a decrease in solubility of whey protein concentrate subjected to heat treatment. Protein denaturation was a two stage process initiated by disruption of secondary and tertiary structures, leading to unfolding (Townsend and Nakai, 1983) and aggregation or coagulation (Mutilangi *et al*., 1996). At high temperature, conformational change was irreversible and polymerization, by the formation of intermolecular disulfide bonds, occurred. The aggregation of the denatured molecules is mediated by hydrophobic and sulfhydryl-disulfide interchange reactions (Wong, 1989). From the result, it appears that the defatting process caused denaturation of protein, as evidenced by decreased solubility. Myofibrillar proteins are soluble in high

salt solutions (Wu and Smith, 1987). Therefore, aggregation of proteins might be associated with the lower solubility in KCl.

Hydrolysis of mince and defatted mince, using Alcalase or Flavourzyme, was carried out by the pH-stat method (Figure 3). Rapid hydrolysis was observed within the first 3 min. Thereafter, a slower rate of hydrolysis was found up to 20 min (Figure 3). The typical hydrolysis curves were also reported for Pacific whiting solid wastes (Benjakul and Morrissey, 1997), herring (Liceaga-Gesualdo and Li-Chan, 1999), salmon fillet muscle (Kristinsson and Rasco, 2000), salmon (Gbogouri *et al.*, 2004), capelin (Shahidi *et al.*, 1995) and sardine (Quaglia and Orban, 1987). At the same time of hydrolysis, higher DH was observed for the HA or HF with higher amounts of added enzymes. The result indicates that peptide bonds were more likely cleaved in the presence of a higher amount of enzyme, both Alcalase and Flavorzyme.

When comparing DH of HA or HF produced from whole mince and defatted mince, the hydrolysates obtained from mince possessed a higher DH than did those derived from defatted mince ($p<0.05$). This result was in accordance with Hoyle and Merritt (1994) who found that protein hydrolysates produced from defatted herring had a lower DH than had those from the original herring. Basically, the enzyme interacts rapidly with the insoluble protein particles, and then polypeptide chains that are loosely bound to the surface are hydrolyzed. The more compacted core proteins are cleaved more slowly (Benjakul and Morrissey, 1997). The proteins in defatted mince were most likely denatured, as indicated by a decreased solubility (Table 6). As a consequence, the protein substrates were less susceptible to hydrolysis by the added enzyme. Hoyle and Merritt (1994) found that denatured fish protein possessed poor wettability, thereby reducing the dispersibility and hence accessibility of enzyme to the substrate. During the defatting process, endogenous proteinases in fish muscle might undergo denaturation. The high temperatures used in the defatting process might inactivate endogenous enzymes and hence reduce the rate of hydrolysis, thus leading to a lower DH in hydrolysate from defatted mince. Endogenous enzymes, such as cathepsins in raw fish muscle have been hypothesized to provide an additional proteolytic effect to commercial enzymes during hydrolysis (Mackie, 1982; Ting *et al.*, 1968).

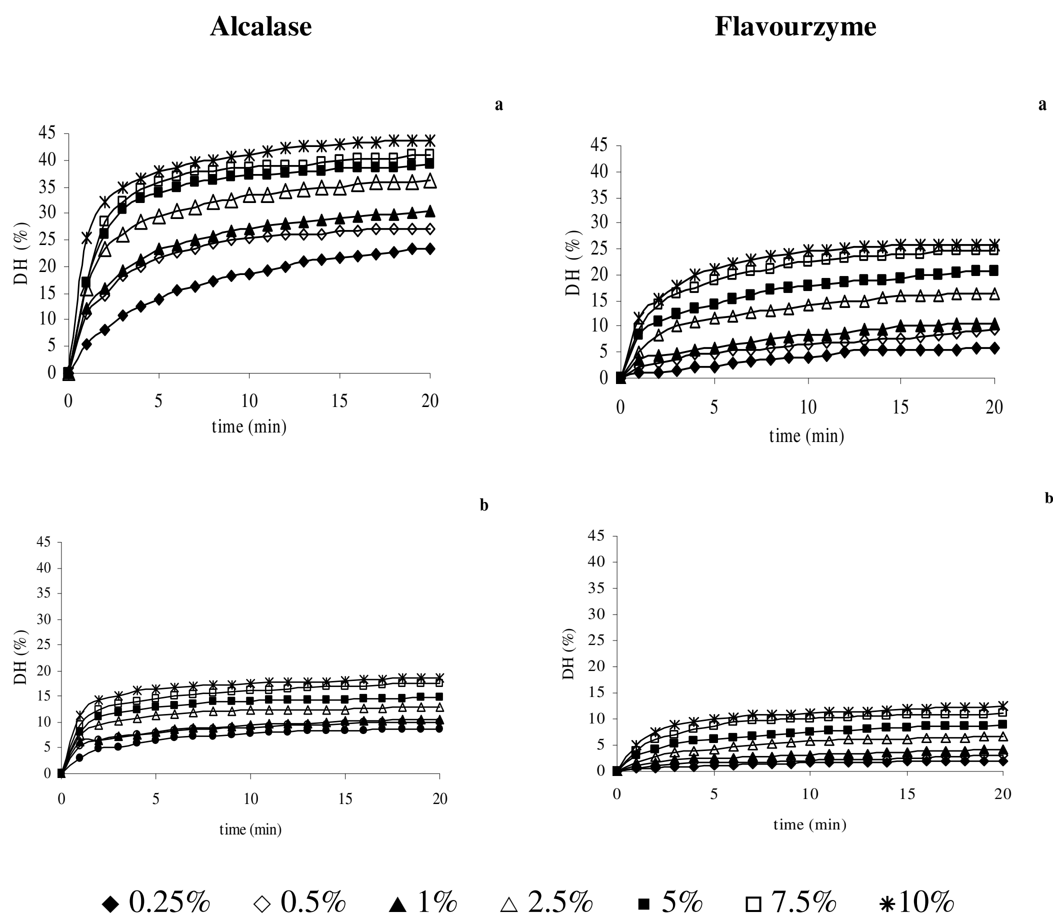
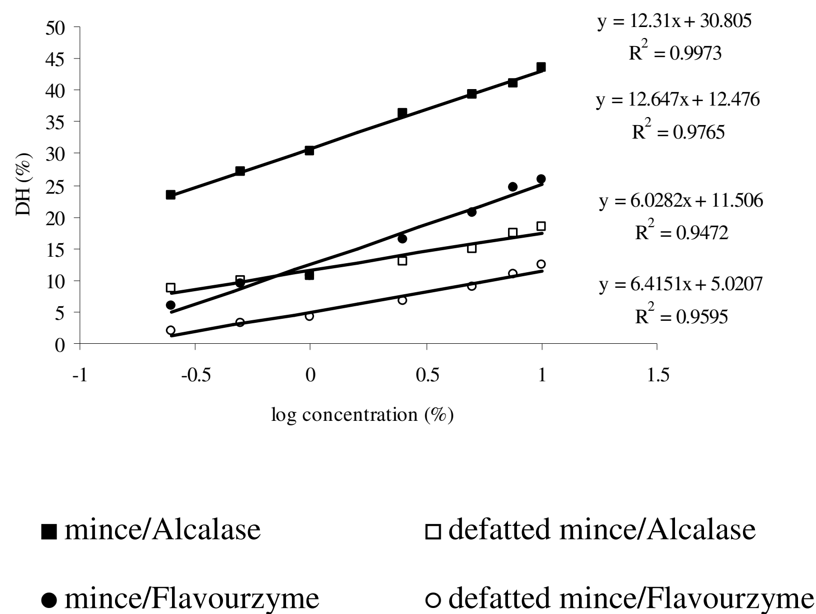


Figure 3. Degree of hydrolysis (DH) of yellow stripe trevally mince (a) and defatted mince (b) during hydrolysis with Alcalase or Flavourzyme at different concentrations

With the same protein substrate and the same amount of enzyme, HA showed a higher DH than did HF over the entire hydrolysis period. The higher DH observed with HA indicates higher proteolytic activity of Alcalase toward yellow stripe trevally muscle proteins, compared to Flavourzyme. Generally, alkaline proteases, including Alcalase, exhibit higher activities than do acid or neutral proteases such as Flavourzyme (Rebeca *et al.*, 1991). Therefore, the susceptibility, to hydrolysis, of yellow stripe trevally muscle proteins depends on the type of enzyme used.

When log10 (enzyme concentration) and DH were plotted, a linear relationship was observed (Figure 4). Similar results were reported by Benjakul and Morrissey (1997) for Pacific whiting solid wastes and by Cheftel *et al.*, (1971) for fish protein concentrate. From this regression, the exact concentration of enzyme required to hydrolyze yellow stripe trevally mince to obtain the specific DH could be calculated. Due to the poor hydrolysis of defatted mince, the original mince without any fat removal was used in further studies.

As the DH increased, the yields of both HA and HF produced from mince increased as indicated in appendix.



■ mince/Alcalase □ defatted mince/Alcalase

● mince/Flavourzyme ○ defatted mince/Flavourzyme

Figure 4. Relationship between DH and log enzyme concentration of Alcalase and Flavourzyme in yellow stripe trevally mince and defatted mince. Different amounts of enzyme were added to the homogenate of mince or defatted mince. The reaction was run for 20 min at pH 8.5, 60°C and pH 7, 50°C for Alcalase and Flavourzyme, respectively.

2.4.2 Effect of DH and enzyme type on antioxidative activity of yellow stripe trevally protein hydrolysate

2.4.2.1 DPPH radical-scavenging activity

DPPH radical-scavenging activities of HA and HF with different DHs are depicted in Figure 5. HA with 5% DH exhibited the highest DPPH radical-scavenging activity ($p<0.05$). As the DH increased, DPPH radical-scavenging activity of HA decreased ($p<0.05$). Nevertheless, no differences ($p>0.05$) were observed for HF with different DH, ranging from 5% to 25%. Additionally, no differences ($p>0.05$) in DPPH radical-scavenging activity were found between HA and HF having DH of 15% and 25%. However, at low DH (5%), HA exhibited a better DPPH radical-scavenging activity than did HF. Antioxidative activity of protein hydrolysates depends on the proteases (Jun *et al*., 2004) and hydrolysis conditions employed (Jao and Ko, 2002; Jun *et al*., 2004; Pena-Ramos and Xiong, 2003). During hydrolysis, a wide variety of smaller peptides and free amino acids is generated, depending on enzyme specificity. Changes in size, level and composition of free amino acids and small peptides affect the antioxidative activity (Wu *et al*., 2003). Jun *et al*. (2004) reported that yellowfin sole hydrolysate, prepared using pepsin at lowest DH (22%), had a higher antioxidative activity, than had those produced using other enzymes, such as Alcalase, α-chymotrypsin, papain, pepsin, Pronase E, Neutrase and trypsin. The molecular mass of the antioxidant was identified as 13 kDa. Wu *et al*. (2003) found that mackerel hydrolysate, with molecular weight of approximately 1400 Da, possessed a stronger antioxidant activity than did those with the molecular weights of 900 and 200 Da. DPPH is a stable free radical with an absorbance maximum at 517 nm in ethanol. When DPPH encounters a proton-donating substance, such as an antioxidant, the radical is scavenged and the absorbance is reduced (Shimada *et al*., 1992). The result reveals that the yellow stripe trevally hydrolysates potentially contained substances which were electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction.

2.4.2.2 Reducing power

As the DH increased, the reducing power of HA decreased ($p<0.05$), but there were no differences ($p>0.05$) for HF with different DH. No

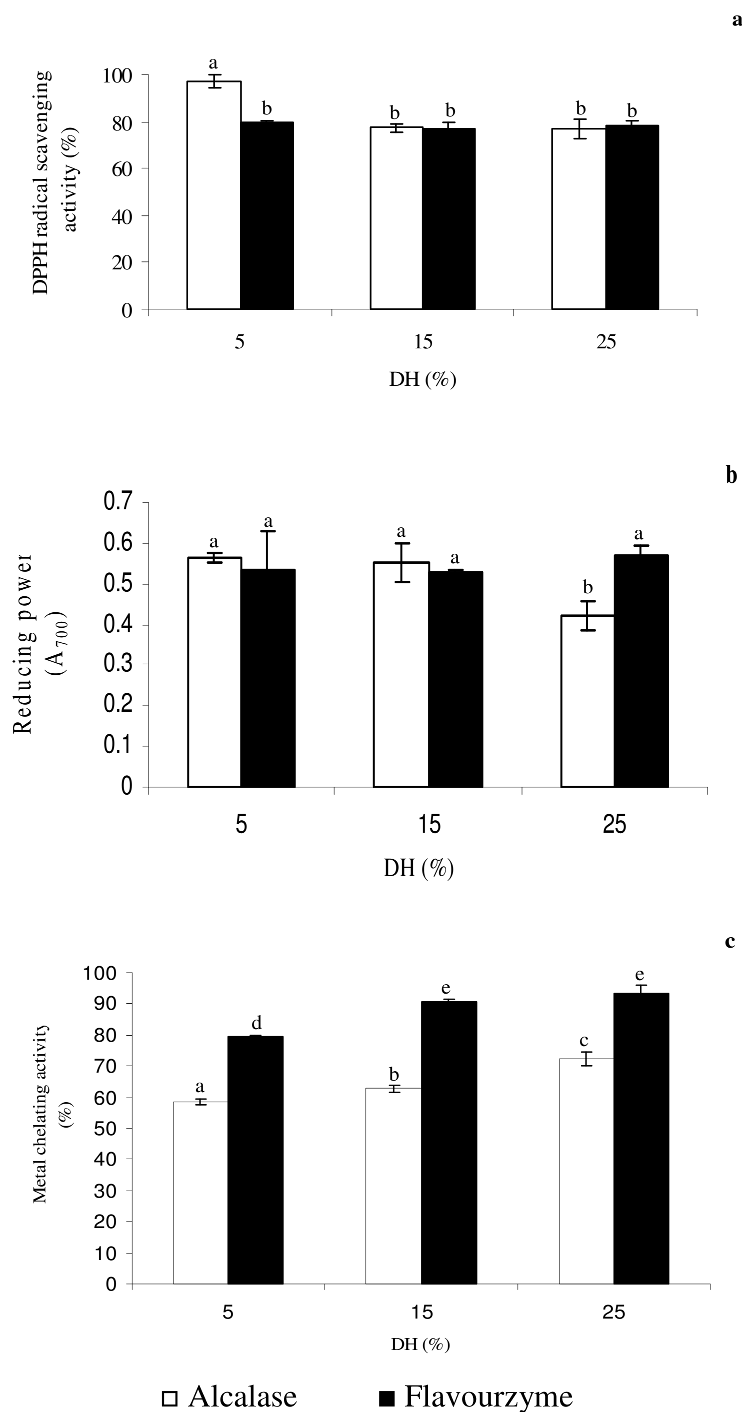


Figure 5. DPPH radical-scavenging activity (a), reducing power (b) and metal-chelating activity (c) of yellow stripe trevally protein hydrolysate produced using Alcalase and Flavourzyme with different DHs. Bars represent standard deviations from triplicate determinations. Different letters within the same parameter indicate the significant differences ($p<0.05$).

changes ($p>0.05$) in reducing power were found between HA and HF with DH of 5% and 15%. However, at high DH (25%), HF showed a higher reducing power than did HA ($p<0.05$). From this result, it appears that protein hydrolysate from yellow stripe trevally meat could function by donating electrons to the free radicals. Moreover, the reducing power of yellow stripe trevally protein hydrolysate was found to depend on the DH and enzyme used.

2.4.2.3 Metal-chelating activity

Metal-chelating activity of both HA and HF increased with increasing DH ($p<0.05$). At the same DH, HF showed a higher chelating activity than did HA ($p<0.05$). A higher degree of cleavage of peptide bonds rendered hydrolysates with higher metal-chelating activities. Hydrolyzed protein from capelin was also found to possess antioxidant activity (Amarowicz and Shahidi, 1997). Therefore, increased metal-chelating activity could be increased through hydrolysis with certain enzymes. Peptides in hydrolysates could chelate the prooxidants, leading to decreased lipid oxidation. Transition metals, such as Fe, Cu, Co, in foods affect both the rate of autoxidation and breakdown of hydroperoxide to volatile compounds. Transition metal ions react very quickly with peroxides by acting as one-electron donors to form alkoxyl radical (Gordon, 2001). Therefore, chelation of transition metal ions by antioxidant or antioxidative peptide would retard the oxidation reaction (Sherwin, 1990). From the results presented here, the peptides in HA and HF could act both as primary and secondary antioxidants. Size and sequence of amino acids in the resulting peptides most likely determine the antioxidant activity of protein hydrolysates (Chen *et al*., 1998).

2.4.3 Effect of DH and enzyme type on functional properties of yellow stripe trevally protein hydrolysate

2.4.3.1 Solubility

The solubilities of HA and HF with different DH in the pH range of 2–12 are shown in Figure 6. All hydrolysates were soluble over a wide pH range with more than 85% solubility. In general, the degradation of proteins to smaller peptides leads to more soluble products (Chobert *et al*., 1988; Gbogouri *et al*., 2004;

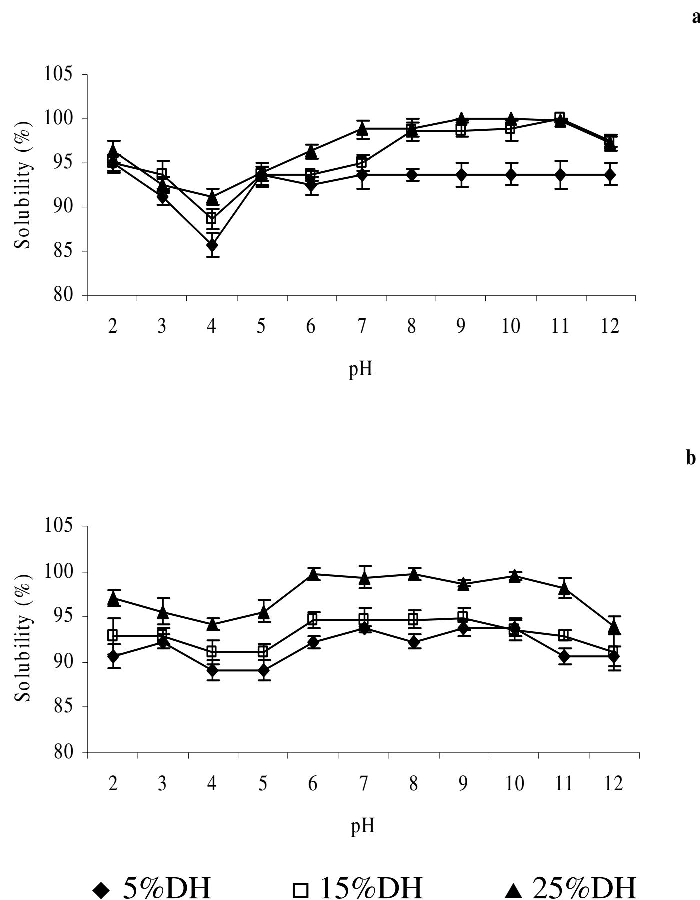


Figure 6. Solubility of yellow stripe trevally protein hydrolysates prepared using Alcalase (a) and Flavourzyme (b) with different DHs as influenced by pHs. Bars represent standard deviations from triplicate determinations.

Linder *et al*., 1996). Both HA and HF, with high DH, had higher solubilities than had those possessing lower DH. This lends further support to the findings of Gbogouri *et al*. (2004), Shahidi *et al*. (1995) and Quaglia and Orban (1987) who reported that hydrolysates had an excellent solubility at high degrees of hydrolysis. The balance of hydrophilic and hydrophobic forces of peptides is another crucial influence on solubility increments (Gbogouri *et al*., 2004). Enzymatic hydrolysis potentially affects the molecular size and hydrophobicity, as well as polar and ionizable groups of protein hydrolysates (Mutilangi *et al*., 1996; Turgeon and Gauthier, 1990). The smaller peptides from myofibrillar proteins are expected to have proportionally more polar residues, with the ability to form hydrogen bonds with water and augment

solubility (Gbogouri *et al.*, 2004). As a consequence, hydrolysates with smaller peptides, i.e. higher DH, were more soluble. The solubilities of HA and HF were quite low at pH 4, whereas solubilities above 90% were noticeable at other pHs tested. Salmon by-product hydrolysates also showed the lowest solubility at pH 4 (Gbogouri *et al.*, 2004). The pH affects the charge on the weakly acidic and basic sidechain groups and hydrolysates generally show low solubility at their isoelectric points (Chobert *et al.*, 1988; Linder *et al.*, 1996). Solubility variations could be attributed to both net charge of peptides, that increase as pH moves away from pI, and surface hydrophobicity, that promotes the aggregation via hydrophobic interaction (Sorgentini and Wagner, 2002). Due to the high solubility of the muscle hydrolysate over a wide pH range, it was presumed that products had a low molecular weight and were hydrophilic in nature (Sorgentini and Wagner, 2002).

2.4.3.2 Emulsifying properties

Emulsifying activity index (EAI) and emulsion stability index (ESI) of both HA and HF with various DH (5%, 15%, 25%) are shown in Figure 7. EAI and ESI of both HA and HF decreased ($p<0.05$) with increasing DH. At low DH (5%), hydrolysates exhibited strong emulsifying properties. With a limited degree of hydrolysis, the hydrolysates have exceptional emulsifying activity and stability (Kristinsson and Rasco, 2000). Higher contents of larger molecular weight peptides or more hydrophobic peptides contribute to the stability of the emulsion (Mutilangi et al., 1996). On the other hand, excessive hydrolysis brings about the loss of emulsifying properties (Gbogouri *et al.*, 2004; Kristinsson and Rasco, 2000; Mahmoud *et al.*, 1992; Quaglia and Orban, 1990). The peptides with low molecular weight may not be amphiphilic enough to exhibit good emulsifying properties (Chobert *et al.*, 1988). The mechanism to generate the emulsion system is attributed to the adsorption of peptides on the surface of freshly formed oil droplets during homogenization and the formation of a protective membrane that inhibits coalescence of the oil droplet (Dickinson and Lorient, 1994). Hydrolysates are surface-active materials and promote oil-in-water emulsion because of their hydrophilic and hydrophobic groups with their associated charges (Gbogouri *et al.*, 2004; Rahali *et al.*, 2000). Thus, hydrolysates with a higher DH had poorer EAI and ESI due to their

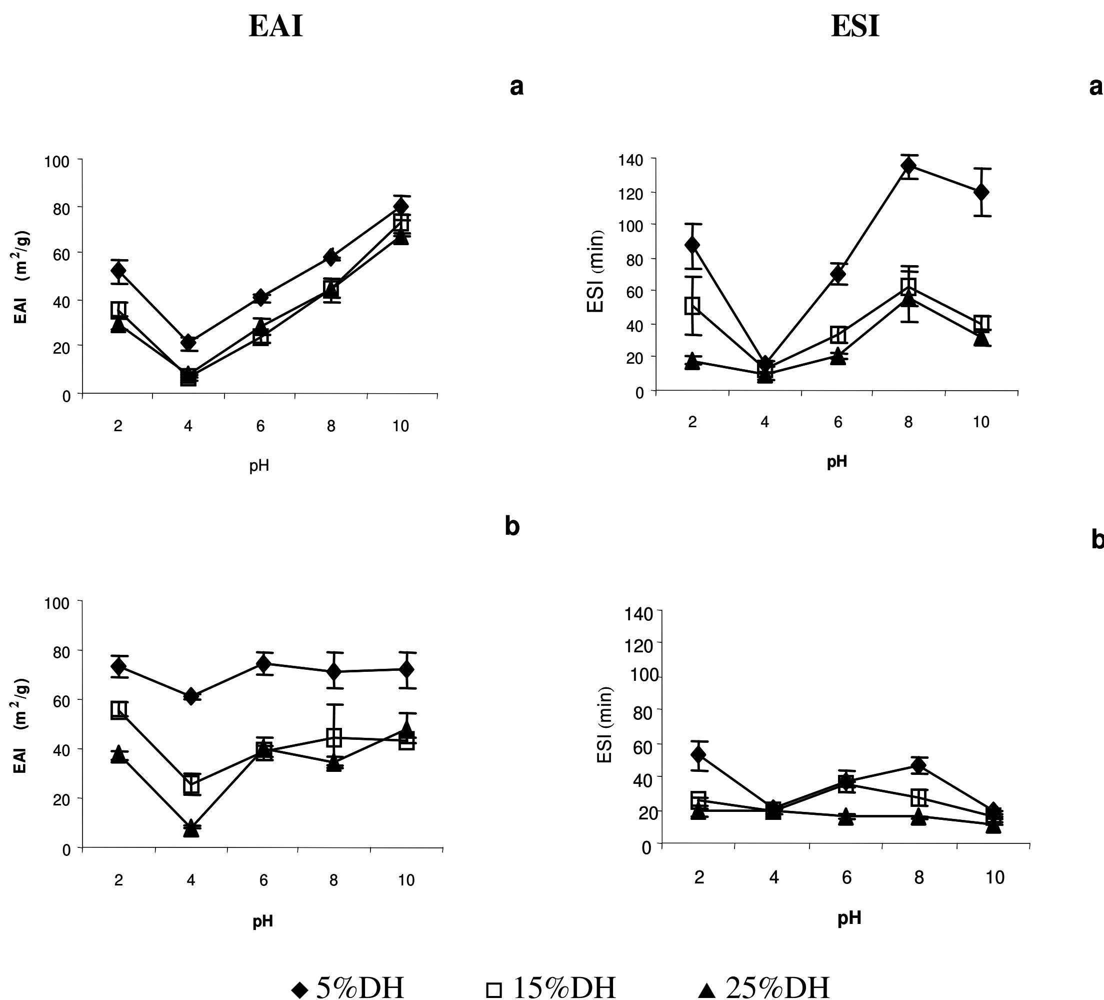


Figure 7. Emulsifying activity index (EAI) and emulsion stability index (ESI) of yellow stripe trevally protein hydrolysates prepared using Alcalase (a) and Flavourzyme (b) with different DHs as influenced by pHs. Bars represent standard deviations from triplicate determinations.

small peptide size. Small peptides migrate rapidly and adsorb at the interface, but show less efficiency in decreasing the interface tension since they cannot unfold and reorient at the interface like large peptides to stabilize emulsions (Gbogouri *et al.*, 2004; Rahali *et al.*, 2000). Apart from peptide size, amphiphilicity of peptides is important for interfacial and emulsifying properties. Rahali *et al.* (2000) analyzed amino acid sequence at an oil/water interface and concluded that amphiphilic character was more important than was peptide length for emulsion properties. The

flexibility of protein or peptide structure may also be a vital factor governing the emulsifying properties (Kato *et al.*, 1985). When considering the effect of pH on EAI and ESI, the lowest EAI and ESI were found at pH 4, with coincidental decrease in solubility (Figure 7). Since the lowest solubility occurred at pH 4, peptides could not move rapidly to the interface. Additionally, the net charge of peptide could be minimized at pH 4. The higher EAI of hydrolysates accompanied their higher solubility (Mutilangi *et al.*, 1996). Hydrolysates with high solubility can rapidly diffuse and adsorb at the interface. At the same DH, HF had a better EAI than had HA. Conversely, HA showed a higher ESI than did HF. EAI and ESI generally increased as pH moved away from pH 4. This effect was more pronounced with HA, thus suggesting that the sequence and composition of amino acids in peptide between HA and HF might be different, leading to varying charge of the resulting peptides at a particular pH. Emulsifying properties were influenced by specificity of enzyme (Gauthier *et al.*, 1993).

2.4.3.3 Foaming properties

As DH increased, both HA and HF displayed a lower foaming capacity and foam stability ($p<0.05$) (Figure 8). Shahidi *et al.* (1995) reported good foaming properties for capelin protein hydrolysates at low DH (12%). Further hydrolysis could reduce the foaming stability since the more microscopic peptides do not have the strength needed to maintain a stable foam (Shahidi *et al.*, 1995). High molecular weight peptides are generally positively related to foam stability of protein hydrolysates (Van Der Ven *et al.*, 2002). Hydrophobicity of unfolded proteins has been shown to correlate with foaming characteristics (Townsend and Nakai, 1983). Foam formation is governed by three factors, including transportation, penetration and reorganization of molecules at the air–water interface. To exhibit good foaming, a protein must be capable of migrating rapidly to the air–water interface, unfolding and rearranging at the interface (Halling, 1981). Dickinson (1989) and Mutilangi *et al.* (1996) suggested that the foaming capacity of protein was improved by making it more flexible, by exposing more hydrophobic residues and by increasing capacity to decrease surface tension. For the adsorption at the air–water interface, molecules should contain hydrophobic regions (Mutilangi *et al.*, 1996). Foam stability depends

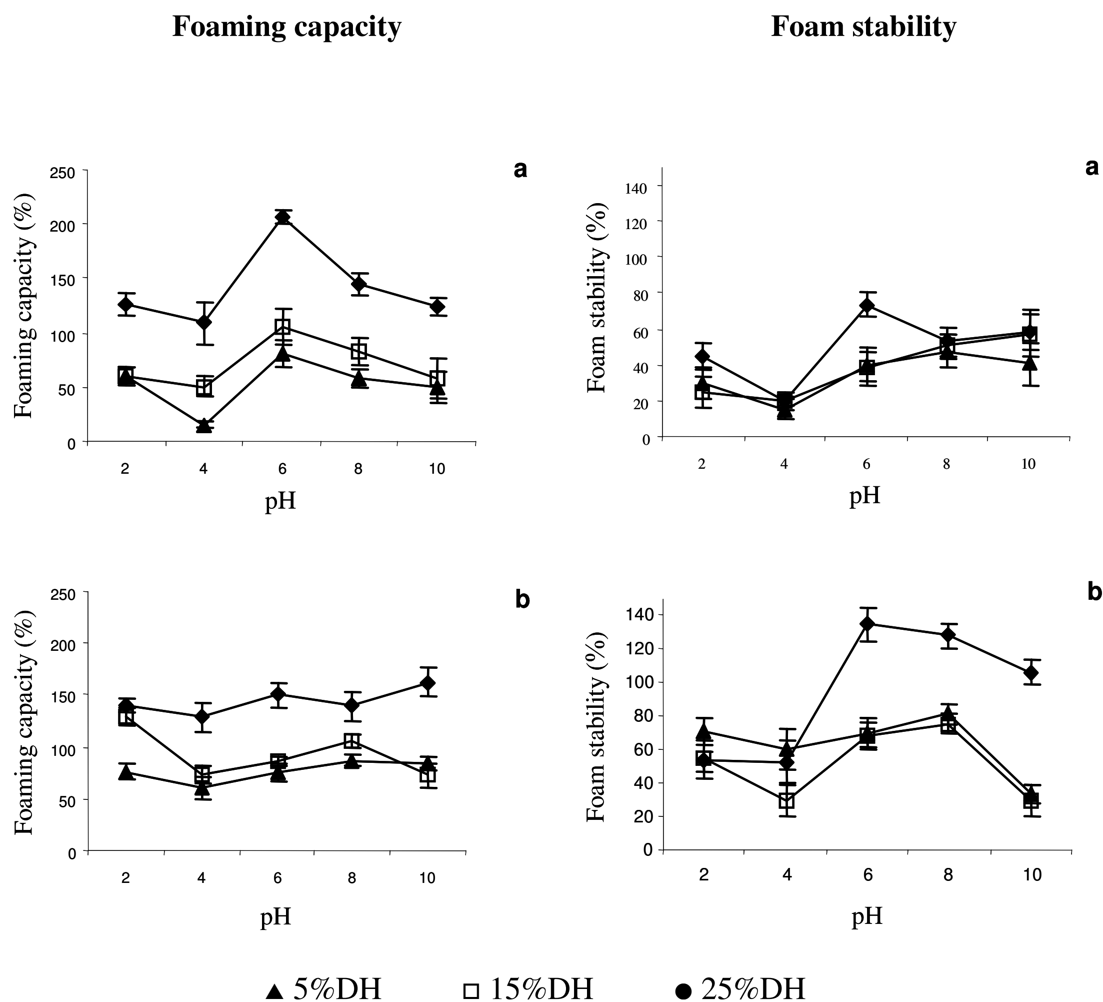


Figure 8. Foaming capacity and foam stability of yellow stripe trevally protein hydrolysates prepared using Alcalase (a) and Flavourzyme (b) with different DHs as influenced by pHs. Bars represent standard deviations from triplicate determinations.

on the nature of the film and reflects the extent of protein–protein interaction within the matrix (Mutilangi *et al.*, 1996). Foam stability is enhanced by flexible protein domains, which enhance viscosity of the aqueous phase, protein concentration and film thickness (Phillips *et al.*, 1994). The foaming properties of both HA and HF hydrolysates were affected by pH. Foaming capacity tended to decrease at pH 4. The foaming capacity of HA reached a maximum at pH 6 with a slight decrease at alkaline pH. Therefore, net charge should influence the adsorption of the proteins at the air–

water interface. When the net charge was increased, the foaming property was enhanced (Sorgentini and Wagner, 2002; Townsend and Nakai, 1983). The lowest foaming properties of proteins also coincided with the lowest solubilities at their isoelectric pH (Pearson, 1983). For foam stability, the lowest value was found at pH 4 for both HA and HF. A decreased foam stability was also observed in HF with DH of 15% and 25% at pH 10. The low foam stability was concomitant with the low solubility at pH 4. Protein solubility makes an important contribution to the foaming behaviour of protein. The pH of the dispersing medium dramatically influences foaming properties, especially foam stability (Townsend and Nakai, 1983). Foam stability depends principally on the nature of the film and reflects the extent of protein–protein interaction within the matrix (Mutilangi *et al*., 1996). The decreased foam stability at very acidic or alkaline pH might be due to the repulsion of peptides via ionic repulsion. At the same DH, HF exhibited a superior foam stability to HA ($p<0.05$). Therefore, the size and charge of peptides may be different for hydrolysates produced by different enzymes. HF most likely contained larger peptides which could form flexible films around the air bubbles, as evidenced by a higher foam stability.

2.5 Conclusions

Antioxidant activity of protein hydrolysates from yellow stripe trevally meat varied with DH and enzyme used. Emulsifying and foaming properties of the protein hydrolysates were also dictated by both factors. Additionally, the functionality of hydrolysates was affected by pH. Therefore, yellow stripe trevally protein hydrolysate can be used in food systems as a natural additive possessing antioxidative properties.

CHAPTER 3

COMPARATIVE STUDY ON ANTIOXIDATIVE ACTIVITY OF YELLOW STRIPE TREVALLY PROTEIN HYDROLYSATE PRODUCED FROM ALCALASE AND FLAVOURZYME

3.1 Abstract

Antioxidative activities of protein hydrolysates from yellow stripe trevally (*Selaroides leptolepis*) prepared using Alcalase 2.4 L (HA) and Flavourzyme 500 L (HF) with the degree of hydrolysis of 15% by pH-stat method were determined. Both protein hydrolysates exhibited the antioxidative activity in a concentration dependent manner. HF generally showed the greater antioxidative activity than HA ($p<0.05$) as indicated by the higher 2,2-diphenyl-1-picryhydrazyl (DPPH) radical scavenging activity, reducing power and metal chelating activity. Antioxidative activity of both hydrolysates was stable when heated at 90°C for 10 and 30 min and subjected to a wide pH range (2–12). Nevertheless, metal chelating activity decreased in very alkaline and acidic pH ranges. HA and HF at 200 ppm retarded the formation of conjugated diene and thiobarbituric acid reactive substances (TBARS) in lecithin liposome system. HF possessed the stronger antioxidative activity than HA ($p<0.05$). However, α-tocopherol at 200 ppm showed the higher antioxidative activity in the system.

3.2 Introduction

Fish processing by-products and the under-utilized species are commonly recognized as low-value resources with negligible market value. Inappropriate disposal of those by-products is a major cause of environmental pollution (Quaglia and Orban, 1987; Gildberg, 1993). Full utilization of the limited fishery resources would be a promising approach to reduce such a problem as well as to gain the maximized benefit. Protein hydrolysates from different fish species such as mackerel

(Wu *et al*., 2003), herring (Sathivel *et al*., 2003), tuna cooking juice (Jao and Ko, 2002) and black soy bean (Shih *et al*., 2002) have been found to show antioxidative activity against peroxidation of lipids or fatty acids and are feasible to use as natural antioxidant in foods and biological systems. Protein hydrolysate can be prepared by enzymatic hydrolysis from fish muscle, especially those with low market value such as yellow stripe trevally (Klompong *et al*., 2007a). Proteinases used can affect the functional properties and antioxidative activity of the protein hydrolysate obtained (Jao and Ko, 2002; Jun *et al*., 2004). Protein hydrolysate from Alaska pollack frame prepared by mackerel intestine crude enzyme exhibited antioxidative activity in a linoleic acid model system (Je *et al*., 2005). Fraction of protein hydrolysate from yellowfin sole frame hydrolysed by pepsin and mackerel intestine crude enzyme showed a strong antioxidative activity in a linoleic acid model system (Jun *et al*., 2004). Prawn hydrolysate prepared using pepsin showed the most potent antioxidative activity than those prepared by other enzymes (Suetsuna, 2000). Raw material including whole herring, body, gonad and head had the influences on the antioxidative activity of herring hydrolysate (Sathivel *et al*., 2003). Levels and compositions of free amino acids and peptides were reported to determine the antioxidant activities of protein hydrolysates (Wu *et al*., 2003).

Antioxidative activity of protein hydrolysate varied with the systems used (Wu and Brewer, 1994; Frankel *et al*., 1997). Egg-yolk protein hydrolysates showed a strong antioxidative activity in a linoleic acid oxidation system and in cookies containing linoleic acid. In addition, the antioxidative effect of protein hydrolysate was concentration-dependent (Sakanaka *et al*., 2004). Peptide fractions separated from capelin protein hydrolysate possessed a different antioxidant activity and some fractions exhibited a prooxidant effect in a β-carotene-linoleate model system (Amarowicz and Shahidi, 1997). Recently, protein hydrolysates from yellow stripe trevally have been produced successfully using Alcalase (HA) and Flavourzyme (HF) (Klompong *et al*., 2007a). Nevertheless, a little information regarding the characteristic and antioxidative activity of hydrolysate prepared using both enzymes has been reported. The objective of this study was to investigate the antioxidative activity of protein hydrolysate from yellow stripe trevally muscle prepared using

Alcalase and Flavourzyme in a model system and the effect of concentration, temperature and pH on their activities.

3.3 Materials and methods

3.3.1 Materials

2,2-diphenyl-1-picryhydrazyl (DPPH), 3-(2-pyridyl)-5,6-bis (4-phenyl-sulphonic acid)-1,2,4-triazine (ferrozine), 3-sn-phosphatidyl choline from soybean (L-α-lecithin, hydroxygenated) and β-mercaptoethanol were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA.). α-Tocopherol was obtained from Sigma-Aldrich (Steinheim, Germany). Iron (II) chloride tetrahydrate ($Fe•Cl_2$) was procured from Kanto Chemical Co., Inc. (Tokyo, Japan). Alcalase 2.4L and Flavourzyme 500L were obtained from Novozymes (Bagsvaerd, Denmark).

3.3.2 Fish sample preparation

Yellow stripe trevally (*Selaroides leptolepis*) with the size of 65g/fish, off-loaded approximately 24–36 h after capture, was obtained from the fishing port in Satul province along the coast of the Andaman Sea. The fish was placed in ice with the fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University. Upon the arrival, the fish was washed and the meat was separated manually. The meat was minced using grinder with the 0.4 cm diameter holes. The mince was stored in ice until use.

3.3.3 Production of protein hydrolysates from yellow stripe trevally

Mince (60 g) was suspended in 240 ml of distilled water. The mixtures were homogenized using a homogeniser (IKA, Labortechnik, Selangor, Malaysia) at a speed of 11 000 rpm for 1 min. The homogenates were preincubated at 50 or 60°C for 20 min prior to enzymatic hydrolysis using Alcalase and Flavourzyme, respectively. The hydrolytic reaction was started by addition of enzymes at levels of 0.05% (w/w) for HA and 1.58% (w/w) for HF, based on the protein content of mince. The reaction was conducted at pH 8.5, 60°C for HA and at pH 7.0, 50°C for HF using the pH-stat method as described by Adler-Nissen (1986) until the DH of 15% was obtained. The

constant pH of mixture during hydrolysis was maintained using 2 M NaOH. DH was calculated as follows:

$$DH\ (\%) = (BN_b/M_p\ \alpha\ h_{tot}) \times 100$$

where B is the amount of alkaline consumed (ml), N_b is the normality of alkaline, M_p is the mass of the substrate (protein in grams, %N×6.25), 1/α is the calibration factors for pH-stat and h_{tot} is the content of peptide bonds (Adler-Nissen, 1986).

At designated DH, the samples were taken and the enzymatic reaction was terminated by placing the samples in a water bath at 90°C for 15 min with occasional agitation. The samples were cooled and the pH of samples was then adjusted to 7.0 with 6 M HCl or 1 M HCl. Hydrolysates were centrifuged at 2000g (relative centrifugal field) for 10 min using a centrifuge (Sorvall, Biofuge primo, Norwalk, CT, USA). Supernatants obtained were freeze-dried using a freeze-dryer (Dura-stop, NY, USA). Protein hydrolysate powder prepared by Alcalase and Flavourzyme was kept in polyethylene bag under vacuum at room temperature in desiccator until use. The hydrolysates obtained were subjected to Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970).

3.3.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of hydrolysates was carried out according to the method Laemmli (1970) using 4.5% stacking gel and 20% resolving gel. HA and HF with different DHs were dissolved in deionized distilled water to obtain the concentration of 50 mg protein/ml. To 100 µl of sample solution, 100 µl of sample buffer with and without β-mercaptoethanol were added and mixed well. Electrophoresis was run at constant voltage of 200 V. After separation, the protein bands were stained with solution containing 50% methanol, 10% glacial acetic acid and 0.25% coomassie brilliant blue. Protein standards with a broad range of molecular weight (Bio-Rad, Hercules, CA) including myosin (MW 204 kDa), β-galactosidase (MW 119 kDa), bovine serum albumin (MW 100 kDa), ovalbumin (MW 52 kDa), carbonic anhydrase (MW 37 kDa), soybean trypsin inhibitor (MW 29 kDa), lysozyme (MW 19 kDa) and aprotinin (MW 7 kDa) as well

as myosin heavy chain/actin standards (Bio-Rad) were used for molecular weight estimation.

3.3.5 Effect of concentration of protein hydrolysate on antioxidative activities

HA or HF with 15% DH, which exhibited the highest antioxidative activity, compared with those with DHs of 5 and 25% (Klompong *et al*., 2007a), were dissolved in distilled water to obtain the concentration of 10, 20, 30 and 40 mg protein/ml. Antioxidative activity of protein hydrolysates at different concentrations was measured by monitoring the DPPH radical scavenging activity, reducing power and metal chelating activity.

3.3.6 Effect of temperatures on antioxidative activity of protein hydrolysate

HA or HF with 15% DH were dissolved in distilled water to obtain the concentration of 40 mg protein/ml. The sample solutions were placed in a temperature controlled water bath at 30, 40, 50, 60, 70, 80 and 90°C for 10 and 30 min. At the time designated, the samples were cooled rapidly in ice water. Antioxidative activity of protein hydrolysates was determined as previously described.

3.3.7 Effect of pH on antioxidative activity of protein hydrolysate

HA or HF with 15% DH at a concentration of 40 mg protein/ml were prepared using distilled water as a medium. The 5 ml of sample solutions were adjusted to pHs 2, 4, 6, 8, 10 and 12 with 1 or 6 M HCl and 1 or 6 M NaOH. The pHs of sample solutions were then adjusted to 7.0 with 1 M phosphate buffer. The final volume of all solutions was brought up to 20 ml using the distilled water. Residual antioxidative activity of protein hydrolysates was determined as mentioned above.

3.3.8 2,2-diphenyl-1-picryhydrazyl radical scavenging activity

The DPPH radical scavenging activity was measured using the method of Yen and Wu (1999). To 4 ml of sample solutions, 1.0 ml of 0.2 mM DPPH was added and mixed vigorously. After incubating for 30 min, the absorbance of the resulting

solutions was measured at 517 nm using a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). The control was conducted in the same manner except that distilled water was used instead of sample. The DPPH radical scavenging activity was calculated according to the following equation (Yen and Wu, 1999):

DPPH radical scavenging activity (%) = (1 – (A_{517} of sample/A_{517} of control)) ×100

3.3.9 Reducing power

Reducing power was determined by the method of Oyaizu (1986). The 0.5 ml of sample solutions (40 mg protein/ml) was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. An aliquot (2.5 ml) of 10% trichoroacetic acid was added to the mixture, followed by centrifugation at 3000 rpm for 10 min using a centrifuge (Hettich mikro 20, Tuttlingen, Germany). The upper layer of solution (2.5 ml) was mixed with 2.5 ml of distilled water and 2.5 ml of 0.1% ferric chloride. The absorbance was then measured at 700 nm. Increased absorbance of the reaction mixture indicates the increasing reducing power.

3.3.10 Metal chelating activity

The chelating activity on Fe^{2+} was determined according to the method of Decker and Welch (1990). One ml of sample solution (40 mg protein/ml) was mixed with 3.7 ml of distilled water. The mixture was then reacted with 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM 3-(2-pyridyl)-5,6-bis(4-phenyl-sulphonic acid)-1,2,4-triazine (ferrozine) for 20 min at room temperature. The absorbance was read at 562 nm. The control was prepared in the same manner except that distilled water was used instead of sample. Chelating activity (%) was then calculated as follows (Decker and Welch, 1990):

Chelating activity (%) = (1 – (A_{562} of sample/A_{562} of control)) ×100

3.3.11 Lecithin liposome system

Antioxidative activities of HA and HF were determined in lecithin liposome system. Lecithin was suspended in deionized water at a concentration of 8

mg/ml by stirring with a glass rod and sonicating for 15 min using a sonicator (Model Transsonic 460/H, Elma, Germany). HA and HF solutions were added to the 30 ml of lecithin liposome systems to obtain a final concentration of 200 ppm. After addition of HA or HF, the liposome suspension was sonicated again for 2 min. To initiate the assay, 10 μL of 30 mM cupric acetate was added. The mixtures were shaken at a speed of 120 rpm at 37°C in the dark using a shaker (UNIMAX 1010, Heidolph, Germany). The control (without HA or HF) and the system containing 200 ppm α-tocopherol were also prepared. Liposome oxidation was monitored every 6 h for totally 36 h by determining conjugated dienes (Frankel *et al.*, 1997) and thiobarbituric acid reactive substances (TBARS) (Lee and Hendricks, 1997; Duh, 1998).

3.3.12 Statistical analysis

One-way analysis of variance (ANOVA) was used and mean comparison was performed by Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was carried out using SPSS statistic program (Version 10.0) for Windows (SPSS Inc. Chicago, IL, USA).

3.4 Results and discussion

3.4.1 Peptide patterns of fish protein hydrolysates

The peptide patterns of HA and HF with 5, 15 and 25% DHs are shown in Figure 9. The lower MW peptides were obtained when DHs were increased. During hydrolysis, enzymatic breakdown of protein involves a major structural change, in which the protein is slowly hydrolyzed into smaller peptide units (Kristinsson and Rasco, 2000). At the same DH, HF generally contained the higher MW peptides than did HA. At DH of 5%, peptides or proteins with MW higher than 60 kDa including myosin heavy chain (MHC) were found in HF. Nevertheless, those peptides/proteins were not remained in HA. Peptide with MW of 65 kDa was dominant in HA, whereas peptide with MW of 70 kDa was predominant in HF. When DH was increased to 15%, the peptide in HA with MW of 65 kDa totally disappeared. However, the peptide with MW of 70 in HF was retained to some extent. At DH of 25%, no peptides or protein bands were found, indicating the intensive hydrolysis of muscle

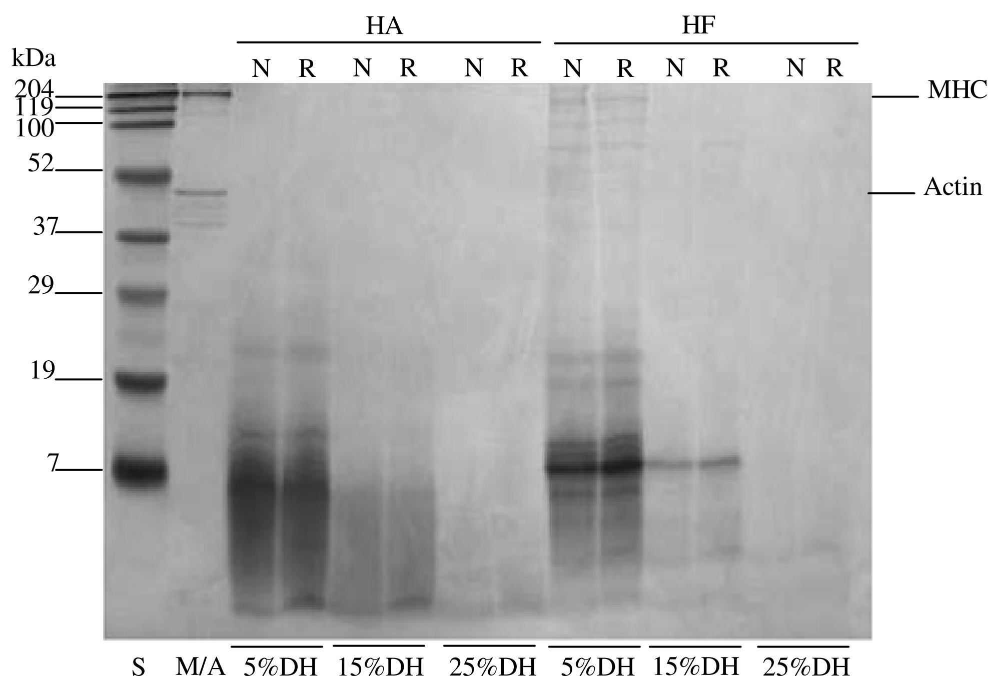


Figure 9. SDS-PAGE patterns of yellow stripe trevally protein hydrolysate prepared using Alcalase and Flavourzyme with different degrees of hydrolysis. S: molecular weight standard; M/A: myosin /actin standard; N: non-reducing condition; R: reducing condition.

proteins. From the result, it was noted that different enzymes showed the different specificity towards the protein substrate. Enzyme specificity contributed to not only molecular size (Adler-Nissen, 1979; Kristinsson and Rasco, 2000) but also antioxidative activities (Suetsuna, 2000; Sathivel *et al.*, 2003; Jun *et al.*, 2004) of resulting hydrolysates.

No differences in peptide patterns were observed between protein hydrolysates prepared using the same enzyme in the presence and the absence of a reducing agent, β-mercaptoethanol. Reducing agents are used to reduce disulfide bonds within a protein subunit or between subunits (Smith, 2003). The result showed that no disulfide bonds were present in peptides or proteins of HA and HF.

3.4.2 Effect of the concentration of fish protein hydrolysate on antioxidative activity

The antioxidative activities as determined by DPPH radical scavenging activity, reducing power and metal chelating activity of HA and HF with different concentrations are depicted in Figure 10. Antioxidant activities generally increased as the concentration of protein hydrolysates increased ($P<0.05$). Sakanaka *et al*., (2004) also found that the antioxidative activity of egg-yolk protein hydrolysates was dose-dependent. From the result, at the same concentration used, HF exhibited the higher DPPH radical scavenging activities than that in HA ($P<0.05$) (Figure 10a). The differences in DPPH radical scavenging activities between HA and HF might be associated with the differences in size of proteins or peptides (Figure 9). The DPPH radical scavenging activity of both HA and HF increased sharply at the concentration ranging from 0 to 10 mg/ml. Thereafter, the slight increase in DPPH radical scavenging activity was observed up to 40 mg/ml. Similar results were obtained with reducing power (Figure 10b), in which the marked increase in reducing power was noticeable in the concentration range of 0–10 mg/ml and the rate of increase was lower at the concentration above 10 mg/ml. The greater reducing power indicated that both hydrolysates could donate the electron to the free radical, leading to the prevention or retardation of propagation. In the propagation step, the free radical reacts with oxygen to form peroxy radical, which reacts with more unsaturated lipids to form hydroperoxide. Furthermore, the lipid free radical formed can react with oxygen to form peroxy radical. Hence, oxidation is a radical chain process (Wong, 1989). However, the continuous increase in metal chelating activity (Figure 10c) of HA and HF was found with increasing concentrations up to 40 mg/ml. Metal ion is an effective prooxidant, which can accelerate the initiation process (Gordon, 2001). As a consequence, the ability of HA and HF in chelating those metal ions could lead to the prevention of lipid oxidation. From the result, both HA and HF could function as both primary and secondary antioxidant via scavenging the free radical and chelating the metal ions.

Despite of the same DH (15%) and concentration used, the differences in antioxidative activities were found between HA and HF. Therefore, the antioxidative

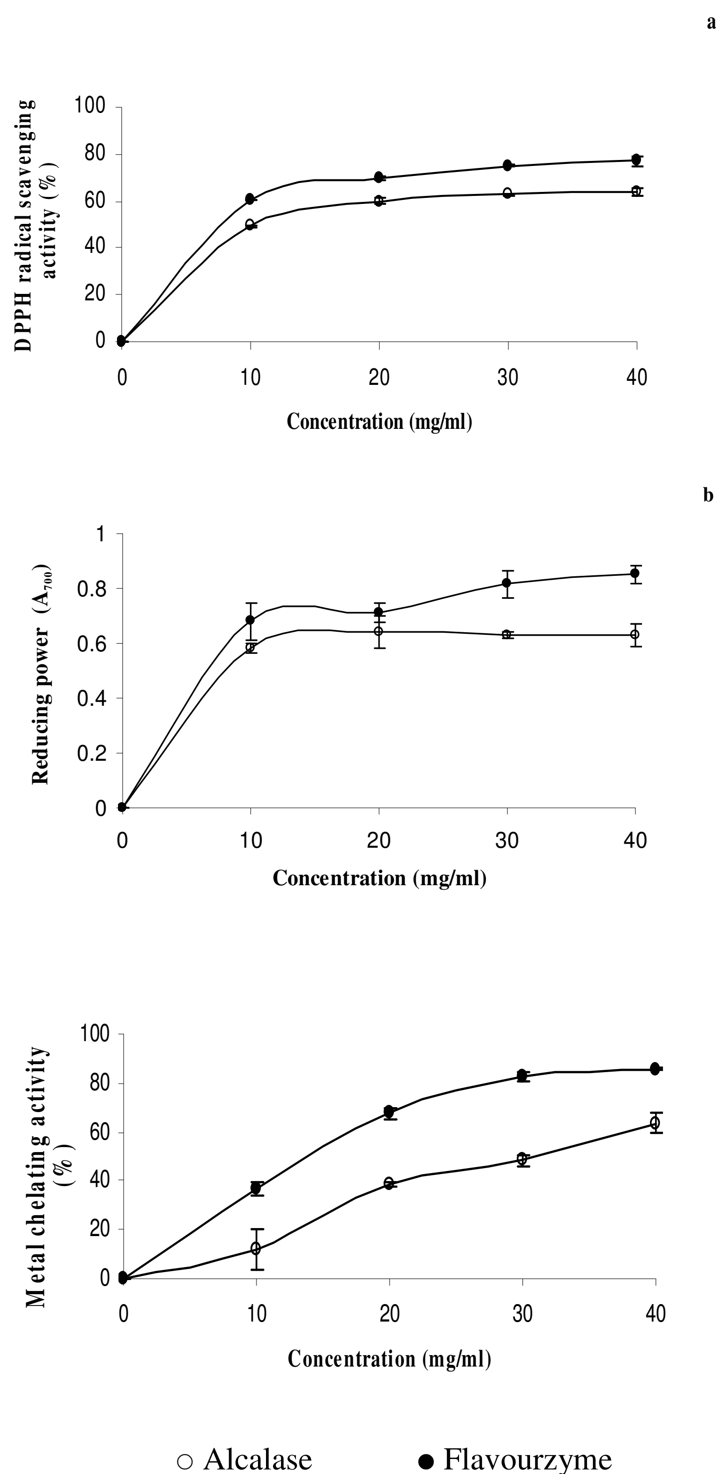


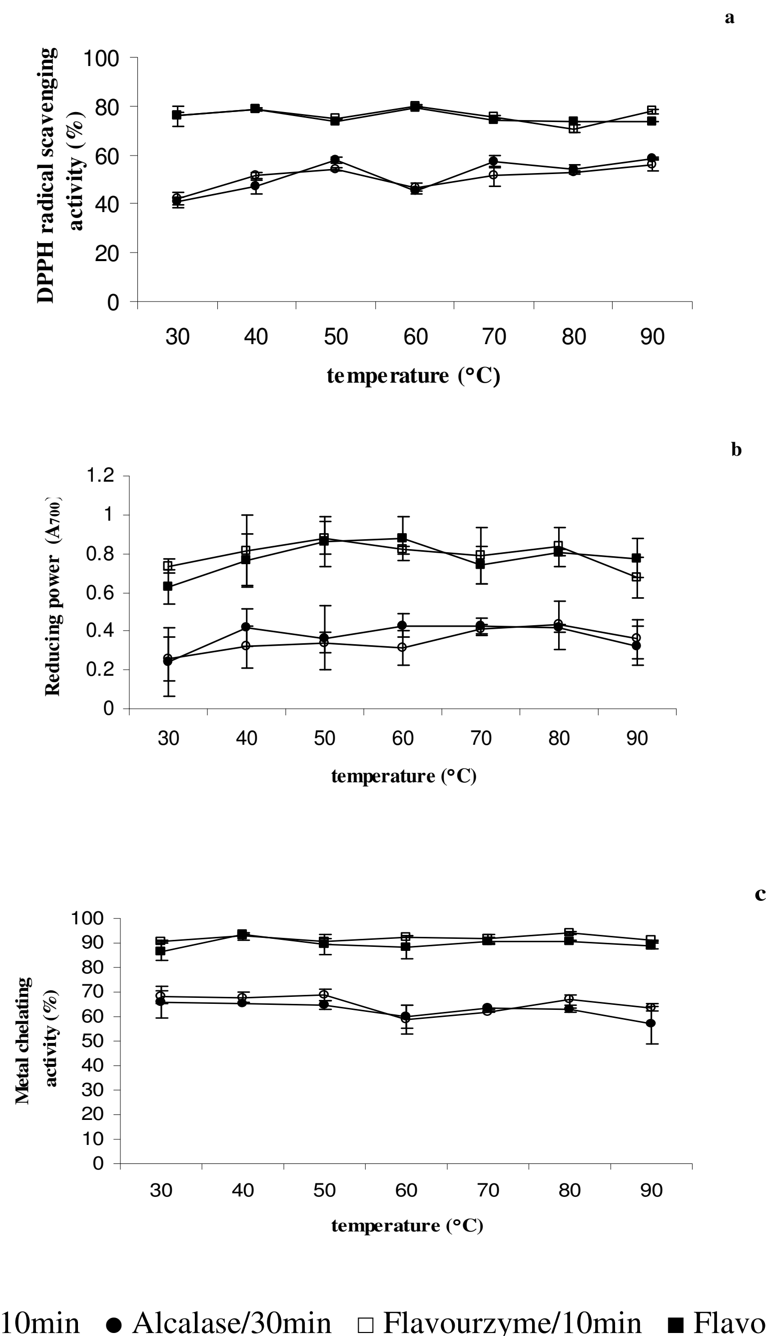
Figure 10 Effect of the concentrations on DPPH radical scavenging activity (a), reducing power (b) and metal chelating activity (c) of yellow stripe trevally protein hydrolysate prepared using Alcalase or Flavourzyme. Bars represent standard deviation from triplicate determinations.

activity of protein hydrolysate from yellow stripe trevally meat was governed by enzymes used. The peptides with different molecular size (Figure 9) might contribute to the different antioxidative activity between both hydrolysates. These results were in agreement with Suetsuna (2000) who reported that prawn protein hydrolysate produced using different enzymes showed the varying antioxidative activity. Different proteinases used also resulted in the differences in antioxidant activities of protein hydrolysate from whey and soy (Pena-Ramos and Xiong, 2003), from yellowfin sole frame (Jun *et al*., 2004) and protein hydrolysate from whole herring, body, gonad and head of herring (Sathivel *et al*., 2003). From the results, both Alcalase and Flavourzyme most likely cleaved the peptide bonds in the yellow stripe trevally muscle at different positions, leading to the different products with varying antioxidative activity.

Polypeptide fragments from myofibrillar proteins are susceptible to attack by peptidase, which will lead to the production of smaller peptides and release of free amino acids. The levels and compositions of free amino acids and small peptides were changed during the hydrolysis depending on enzyme specificity (Wu *et al*., 2003). Alcalase is endopeptidase capable of hydrolyzing proteins with broad specificity for peptide bonds and prefer for a large uncharged residue. Flavourzyme is the endo- and exopeptidase enzyme mixture, which can produce both amino acids and peptides (Ven *et al*., 2002). Wu *et al*. (2003) reported that levels of free amino acids, anserine, carnosine and peptides of the mackerel hydrolysate using protease N were much higher than those by autolysis.

3.4.3 Effect of temperature on antioxidative activity of protein hydrolysate

Hydrolysates prepared by both enzymes, HA and HF, showed the high DPPH radical scavenging activity, reducing power and metal chelating activity after heating for 10 and 30 min in the temperature ranging from 30 to 90°C (Figure 11). The result indicated that both HA and HF were stable to heat treatment. Low MW peptides generated with enzymatic hydrolysis were more likely stable to heating process. Low MW fish protein hydrolysates were influenced by heat minutely, whereas proteins with second, tertiary and quarternary structure were affected greatly by heat (Mutilangi *et al*., 1996). Protein denaturation was a two-stage process initiated



○ Alcalase/10min ● Alcalase/30min □ Flavourzyme/10min ■ Flavourzyme/30min

Figure 11. Effect of temperatures on DPPH radical scavenging activity (a), reducing power (b) and metal chelating activity (c) of yellow stripe trevally protein hydrolysate prepared using Alcalase incubated for 10 min and 30 min or prepared using Flavourzyme incubated for 10 min and 30 min. Bars represent standard deviation from triplicate determinations.

by disruption of secondary and tertiary structures, leading to unfolding (Townsend and Nakai, 1983) and aggregation or coagulation (Mutilangi *et al*., 1996). Good antioxidants should be stable under physiological conditions. From the result, no differences in the DPPH radical scavenging activity, reducing power and metal chelating activity were observed between protein hydrolysates subjected to heating for 10 and 30 min (Figure 11). The result suggested that both hydrolysates could be used as the antioxidants in heat-processed foods, in which the antioxidative activities still remain after heating and can further function during heating, storage and consumption.

3.4.4 Effect of pH on antioxidative activity of protein hydrolysate

The DPPH radical scavenging activity and reducing power of both HA and HF were stable over a wide pH range (Figure 12). However, the DPPH radical scavenging activity of HA tended to decrease in high alkalinity (pH 12). Peptides with the short chains and amino acids in protein hydrolysate are not much affected by charge modification governed by pH changes. Basically, protein hydrolysate is soluble over a wide pH range, showing low influence by pH, whereas native proteins with tertiary and quarternary structure are affected considerably by pH (Gbogouri *et al*., 2004). Nevertheless, metal chelating activity of both HA and HF decreased in high alkaline and acidic pH ranges ($p<0.05$). The results suggested that peptides with metal chelating activity might undergo the conformation changes at high alkaline and acidic pHs, leading to the loss in their ability in chelating the metal ions. Thus, it was likely that peptides possessing metal chelating property might be different from those with DPPH radical scavenging activity and reducing power in term of configuration and amino acid composition. Some peptides with metal chelating activity might maintain secondary and tertiary structure, which underwent the changes caused by repulsion at very acidic or alkaline pHs. Therefore, both HA and HF could be used in foods with the wide pH ranges, in which they could function as the primary antioxidant.

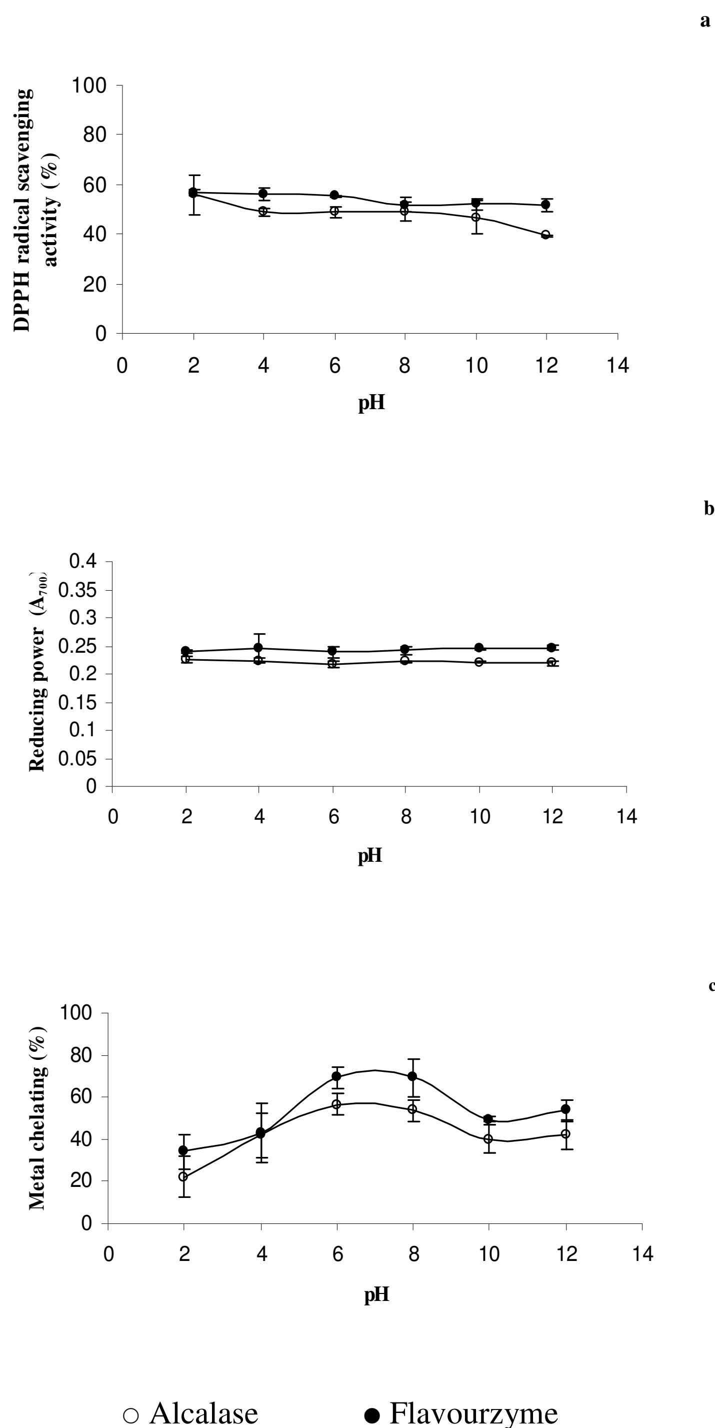


Figure 12. Effect of pHs on DPPH radical scavenging activity (a), reducing power (b) and metal chelating activity (c) of yellow stripe trevally protein hydrolysate prepared using Alcalase or prepared using Flavourzyme. Bars represent standard deviation from triplicate determinations.

3.4.5 Antioxidative activity of protein hydrolysate in lecithin liposome system

Both HA and HF at 200 ppm were able to delay the formation of conjugated diene (Figure 13a) and TBARS (Figure 13b) in lecithin liposome system. However, α-tocopherol at the same level had a greater antioxidative activity, compared with HA and HF. Both hydrolysates could inhibit the early stage lipid oxidation (formation of conjugated diene or hydroperoxide) as well as retard propagation of the oxidation process (degradation of hydroperoxide to TBARS). The concentration of conjugated diene in all samples significantly increased, followed by the decrease. The formation of conjugated diene occurs at the early stages of lipid oxidation (Frankel *et al.*, 1997) and hydroperoxides are expected to decompose to secondary products. The decrease or reaching a stagnant level in conjugated diene was generally accompanied by an increase in TBARS (Pena-Ramos and Xiong, 2003). From the results, the formation of conjugated diene was retarded in the presence of both HA and HF. However, HF was generally more effective in inhibiting the lipid oxidation than HA as shown by the lower conjugated diene formation throughout the incubation period of 30 h.

TBARS in liposome systems without antioxidants (the control) increased markedly after incubation for 6 h at 37°C. For the systems containing HA, HF or α-tocopherol, TBARS increased after 12 h of incubation. As compared with the control system, those comprising HA and HF had the longer induction period. However, α-tocopherol had the longest induction period. A longer induction period indicates a stronger antioxidative activity (Wu *et al.*, 2003). α-Tocopherol does not protect lipids from singlet oxygen that initiates lipid peroxidation via a non free radical mechanism by reacting directly with unsaturated lipids to form lipid hydroperoxides, but inhibits free radical formation in phospholipids (Schafer *et al.*, 2002). It inhibits free radical-mediated lipid peroxidation that has three major components including initiation, propagation and termination until it is depleted. Upon the depletion of α-tocopherol, lipid oxidation accelerates at the same rate as if α -tocopherol has not been present (Schafer *et al.*, 2002). α-Tocopherol is a donor antioxidant (reductant) that will react with peroxyl radical. Thus, it serves as a chain-

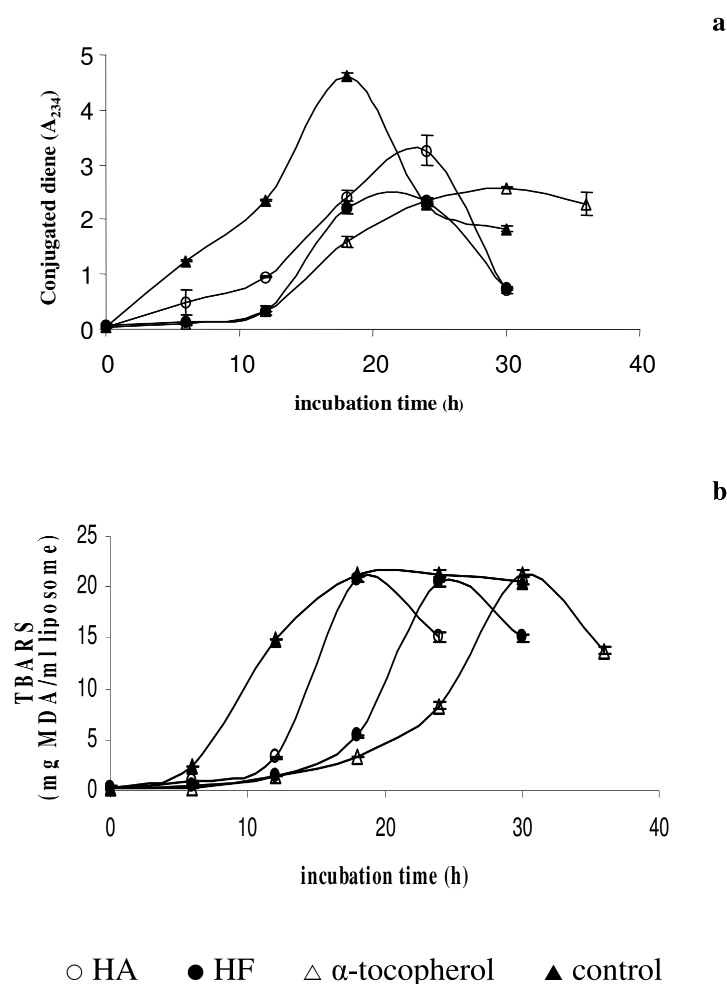


Figure 13. The formation of conjugated diene (a) and TBARS (b) in lecithin liposome system containing yellow stripe trevally protein hydrolysate prepared using Alcalase (HA) or prepared using Flavourzyme (HF), α-tocopherol at 200 ppm in comparison with the control. Bars represent standard deviation from triplicate determinations.

breaking antioxidant, inhibiting the propagation cycle of lipid peroxidation (Schafer *et al.*, 2002; Je *et al.*, 2005).

Both hydrolysates showed antioxidative activity in liposome systems and HA showed the lower antioxidative activity than HF in the liposome systems. Hydrolysis with Flavourzyme augmented the antioxidative activity of yellow stripe trevally meat more effectively than with Alcalase ($p<0.05$). Chen *et al.* (1998) also

found that antioxidative activity of soy proteins can be increased through hydrolysis with certain enzymes, as some peptides possessed a stronger antioxidative activity. These results were in agreement with Pena-Ramos and Xiong (2003) who found that whey and soy protein hydrolysates produced using Protamax could inhibit TBARS formation in an iron-catalyzed liposome system. Sakanaka *et al*. (2004) reported that egg-yolk protein hydrolysate could be a suitable natural antioxidant for preventing the oxidation of polyunsaturated fatty acids.

3.5 Conclusions

The antioxidative activity of protein hydrolysate from yellow stripe trevally meat was governed by enzymes used. Antioxidative activity of protein hydrolysate prepared by Alcalase and Flavourzyme was stable in a wide pH range and to heat treatment up to 90°C. Both protein hydrolysates could retard the lipid oxidation in liposome system effectively, but efficacy was less than α-tocopherol.

CHAPTER 4

AMINO ACID COMPOSITION, PREVENTION OF DNA DAMAGE AND CHARACTERISTIC OF ANTIOXIDATIVE PEPTIDES FROM PROTEIN HYDROLYSATES FROM YELLOW STRIPE TREVALLY (*SELAROIDES LEPTOLEPIS*)

4.1 Abstract

Hydrolysates from yellow stripe trevally meat with 15% degree of hydrolysis (DH) obtained using Alcalase (HA) and Flavourzyme (HF) following pH-stat method were characterized. Both hydrolysates, HA and HF, contained glutamic acid and glutamine as the major amino acids, while glycine was the dominant amino acid in fish flesh. Hydrolysates were also rich in aspartic acid, asparagine, alanine, lysine and leucine. Essential amino acid/non-essential amino acid ratios were 0.55, 0.63 and 0.62 for flesh, HA and HF, respectively. After separation on a Sephadex G-50 column, antioxidative activity of hydrolysate fractions was determined as Trolox equivalent antioxidant capacity. The fraction of HF with molecular weight (MW) of 1.77 kDa exhibited the strongest antioxidative activity, compared with other fractions. Among all fractions of HA, the one having a MW of 2.44 kDa showed the highest antioxidative activity. Thereafter, the selected Sephadex G-50 fractions from both HA and HF were further chromatographed using a Sephadex C-25 column, followed by HPLC. MW of antioxidative peptides from HA and HF determined by MALDI TOF/TOF was 656 Da and 617 Da, respectively. However, peptides isolated from both HA and HF possessed a lower antioxidative activity than Trolox at the same concentration ($p<0.05$). HA and HF prevented DNA oxidative damage in Fenton reaction system tested by plasmid DNA relaxation assay. These results indicate that hydrolysates from yellow stripe trevally can be used as an important source of amino acids and serve as alternative natural antioxidants.

4.2 Introduction

Fish processing by-products and the under-utilized species are commonly recognized as low-value resources with negligible market value. Additionally, their inappropriate disposal is a major cause for environmental pollution. To handle these problems, hydrolysis processes have been developed to convert such resources into marketable and acceptable forms (Quaglia and Orban, 1987; Gildberg, 1993), which can be used in food rather than animal feed or as fertilizer (Benjakul and Morrissey, 1997).

Protein hydrolysates from different fish species such as mackerel (Wu *et al*., 2003), herring (Sathivel *et al*., 2003), tuna cooking juice (Jao and Ko, 2002) and yellow stripe trevally (Klompong *et al*., 2007a) have been found to retard lipid peroxidation and are feasible to use as natural antioxidants in foods and biological systems (Jao and Ko, 2002; Mendis *et al*., 2005a). Proteinases used can affect the functional properties and antioxidative activity of protein hydrolysates obtained (Jao and Ko, 2002; Klompong *et al*., 2007a). Prawn hydrolysate prepared using pepsin showed the most potent antioxidative activity compared to those prepared by other enzymes (Suetsuna, 2000). Levels and compositions of amino acids and peptides were reported to determine the antioxidant activities of protein hydrolysates (Wu *et al*., 2003). Antioxidative activity of peptides was also governed by their composition and amino acid sequences (Kim *et al*., 2001). The amino acid sequence of the peptide separated from casein protein hydrolysate showing the most potent antioxidative activity was Tyr-Phe-Tyr-Pro-Glu-Leu (Suetsuna *et al*., 2000). The peptide His-Gly-Pro-Leu-Gly-Pro-Leu with a MW of 797 Da from tryptic hydrolysate of hoki (*Johnius belengerii*) skin gelatin acted as an antioxidant against linoleic acid peroxidation (Mendis *et al*., 2005b).

Recently, protein hydrolysates from yellow stripe trevally have been produced successfully using Alcalase and Flavourzyme (Klompong *et al*., 2007a; b). However, the characterization of antioxidative peptides of hydrolysate from yellow stripe trevally prepared using both enzymes has not been conducted. The objective of this study was to characterize the antioxidative peptides from protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) produced by Alcalase and Flavourzyme.

4.3 Materials and Methods

4.3.1 Enzymes/chemicals

Alcalase 2.4L and Flavourzyme 500L were obtained from Novozymes (Bagsvaerd, Denmark). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were procured from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Sephadex G-50 was purchased from Pharmacia (Uppsala, Sweden). SP Sephadex C-25 and gel filtration calibration kit (aprotinin, ribonuclease A and carbonic anhydrase) were obtained from GE Healthcare (Uppsala, Sweden).

4.3.2 Fish sample collection and preparation

Yellow stripe trevally (*Selaroides leptolepis*) with the size of 65g/fish, off-loaded approximately 24-36 h after capture along the coast of the Andaman Sea, were obtained from the fishing port in Satul province, Thailand. Fish were placed in ice with the fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 2 h. Upon arrival, fish samples were washed and the meat was separated manually. The meat was minced using a grinder with the 0.4 cm diameter holes. The mince was stored in ice until use.

4.3.3 Production of protein hydrolysates from yellow stripe trevally

Mince (60 g) was suspended in 240 ml of distilled water. The mixture was homogenized using a homogenizer (IKA Labortechnik, Selangor, Malaysia) at a speed of 11,000 rpm for 1 min. The homogenate was pre-incubated at 50 or 60°C for 20 min prior to enzymatic hydrolysis using Flavourzyme and Alcalase, respectively. The hydrolytic reaction was started by addition of 0.05% Alcalase (w/w) or 1.58 % Flavourzyme (w/w) based on the protein content of the mince (Klompong *et al.*, 2007a). The reaction was conducted at pH 8.5, 60°C for Alcalase and at pH 7.0, 50°C for Flavourzyme using the pH-stat method (Adler-Nissen, 1986) for 20 min in order to obtain a degree of hydrolysis (DH) of 15 % as described by Klompong *et al.*

(2007a), indicating that 15% of peptide bonds of mince were cleaved by enzymatic hydrolysis.

To terminate enzymatic reaction, the reaction mixtures were heated in a water bath at 90°C for 15 min with occasional agitation. The samples were cooled and the pH of the samples were subsequently adjusted to 7.0 with 6 or 1 M HCl. Hydrolysates were centrifuged at 2000g for 10 min using a centrifuge (Biofuge primo, Sorvall, Norwalk, CT, USA). Supernatants obtained were freeze-dried using a freeze-dryer (Dura-stop, New York, USA). Protein hydrolysate powder prepared by using Alcalase (HA) and Flavourzyme (HF) were kept in polyethylene bags under vacuum at 4°C in a desiccator until use. The yields of HA and HF powder were 20.10% and 28.86% of mince. Based on whole fish, the yields were 8.04 and 11.55 %, respectively. Both hydrolysates obtained were subjected to analyses.

4.3.4 Tris-tricine gel electrophoresis

Tris-tricine gel electrophoresis of hydrolysates was carried out using precast Tris-tricine gel (10–20% resolving gel, 4% stacking gel). HA and HF were dissolved in deionized water to obtain a concentration of 50 mg protein ⁄ml. To 100 µl of sample solution, 100 µl of Tris-tricine sample buffer were added and mixed well. Electrophoresis was run at a constant voltage of 200 V with 10 mM Tris-tricine running buffer pH 8.3. After separation, the protein bands were stained with a solution containing 50% methanol, 10% glacial acetic acid and 0.25% coomassie brilliant blue. Destaining was carried out using a solution containing 30% methanol and 10% glacial acetic acid. Broad range protein standards (Bio-Rad, Hercules, CA) and myosin heavy chain⁄actin standards (Bio-Rad, Hercules, CA) were used for MW estimation.

4.3.5 Amino acid analysis

HA and HF were placed in a 6 x 50 mm corning tube, previously heated at 525°C for 6 h. The tubes were placed into a hydrolysis vessel for vapor hydrolysis. To the hydrolysis vessel, 250 µl of 6 M HCl with 0.1% phenol were added. The vessel was flushed 3 times with nitrogen and evacuated for 2 min. The vessel was heated for 24 h at 110±2 °C in an oven (Precision Scientific, Thelco

Laboratory, Champaign, IL, USA). The tubes were removed, wiped to remove the excess acid, and dried in a speed-vac for 30 min. After the removal of acid, samples were derivatized according to the vendor's recommendations (Waters Corporation, Milford, MA, USA) using AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) reagent. Thereafter, the samples were transferred to autosampler vials equipped with micro inserts (National Scientific, Rockwood, TN, USA) and heated gently for 10 min. Analysis of samples was performed using a Beckman HPLC (Beckman Coulter, Fullerton, CA, USA) (126 pump, 166 Detector, 507 autosampler, and "System Gold" data system) equipped with a Waters AccQTag amino acid analysis column. Calibration was performed using an amino acid mixture standard (Pierce, Rockford, IL, USA).

4.3.6 Plasmid DNA relaxation assay

Plasmid relaxation assay was determined by a modified version of the method of Ishikawa *et al*. (2004). DNA strand damages were measured by converting circular double-stranded supercoiled plasmid DNA into nicked circular and linear forms. Reactions were performed in 50 µl of solution containing 10 µl of supercoiled pUC18 plasmid DNA (750 ng), 10 µl of 10 mM phosphate buffer (pH 7.8), 5 µl of 3.5% hydrogen peroxide, 5µl of 100 µM ferric chloride and various amounts (10, 20, 30, 40 µg) of HA and HF. The mixtures were incubated at 37ºC for 30 min, and the reactions were stopped by adding 1 µl of 5 mM EDTA (ethylenediaminetetraacetic acid). Reaction mixtures (20 µl) were mixed with 5 µl of loading buffer containing 30% glycerol and 0.25% bromophenol blue. The mixture (15 µl) was loaded onto a 1% agarose gel. Electrophoresis was conducted using a constant voltage of 110 V. The gel was stained using ethidium bromide solution for 10 min. The DNA bands were visualized under UV light and captured by a CCD camera (Genegenious, Singene, UK).

4.3.7 Isolation of antioxidative peptides

HA or HF (10 mg) was dissolved in 1 ml of 50 mM sodium phosphate buffer (pH 7.0) and loaded onto a Sephadex G-50 gel filtration column (1.5×100 cm),

previously equilibrated with the same buffer. The elution was performed with the same buffer at a flow rate of 0.6 ml/min. Fractions of 3.6 ml were collected and subjected to the measurement of A_{220} and A_{280}. Prior to antioxidative activity assay, protein content of each peak of A_{220} was quantified by BCA (bicinchoninic acid) protein assay (Smith *et al*., 1985), diluted to different concentrations (0.02, 0.05, 0.1 mg/ml) using deionized water and tested for antioxidative activity by TEAC (Trolox equivalent antioxidant capacity) assay. MWs of antioxidative peptides isolated on size exclusion chromatography were estimated by plotting available partition coefficient (K_{av}) against the logarithm of MW of the protein standards. The elution volume (V_e) of each protein standard and the antioxidative fractions was measured. Void volume (V_o) was obtained from the elution volume of blue dextran (M_r 2,000,000 Da). The strongest antioxidative activity fractions from HA and HF were then subjected to cation-exchange chromatography.

Antioxidative fractions were loaded onto a SP-Sephadex C-25 column (3×20 cm) previously equilibrated with 20 mM sodium phosphate buffer (pH 4). The column was washed with the same buffer and eluted with a linear gradient of NaCl concentration from 0 to 2 M at a flow rate of 0.6 ml/min. Fractions of 3.6 ml were collected and subjected to the measurement of A_{220} and A_{280}. Peaks of A_{220} were pooled and tested for antioxidative activity by TEAC assay. The highest antioxidative fractions from HA and HF were then injected into a Vydac 218TP54 (4.6×250mm) reversed phase C_{18} column (Grace Vydac, Deerfield, IL, USA) and separated using a linear gradient of acetonitrile (0-40% v/v) containing 0.1% trifluoroacetic acid. The most potent antioxidative activity peaks from both HA and HF were finally isolated on a Zorbax SB C_{18} (2.1×150mm) reversed phase HPLC column (Agilent, Santa Clara, CA, USA) using a linear gradient of acetonitrile (0-20% v/v) containing 0.1% trifluoroacetic acid.

4.3.8 Measurement of antioxidative activity by trolox equivalent antioxidant capacity (TEAC)

Prior to antioxidative activity assay, protein content of HA and HF fractions were quantified by BCA (bicinchoninic acid) protein assay (Smith *et al*., 1985). Both HA and HF fractions were diluted to different concentrations (0.02, 0.05,

0.1 mg/ml) using deionized water. Antioxidative activity was measured using TEAC assay as described by Miller *et al*. (1993) with a slight modification. To 100 µl of sample solutions, 3 ml of $ABTS^{\bullet+}$ were added and mixed well. After incubating for 15 min, the absorbance of the resulting solutions was measured at 734 nm using a spectrophotometer (DU 640 Beckman, Pasadena, CA, USA). Trolox, a more polar form of α–tocopherol, at a concentration of 0, 0.05, 0.1, 0.15 and 0.2 mg/ml was used as a standard. TEAC value was calculated according to the following equation (Miller *et al*., 1993):

$$\text{TEAC value} = (\text{Slope of sample}) / (\text{Slope of Trolox})$$

where slope was obtained from the plot between A_{734} of sample or Trolox and the concentrations of sample or Trolox.

4.3.9 Determination of molecular weight of purified antioxidative peptides

The molecular weights of the purified antioxidative peptides obtained from Zorbax SB C_{18} reversed phase HPLC column were determined using a MALDI TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA, USA). The analyzed solutions were mixed on MALDI plates (Applied Biosystems, Framingham, MA) at a ratio of 1:1 with 5mg/ml alpha-cyano-4-hydroxycinnamic acid matrix in an aqueous solution containing 60% acetonitrile and 0.1% trifluoroacetic acid. The MS spectra were acquired in positive ion mode at a range of peptide mass from 800 to 4000 Da.

4.3.10 Statistical analysis

One-way ANOVA was used and mean comparison was performed by Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was carried out using SPSS statistic program (Version 11.0) for Windows (SPSS Inc. Chicago, IL, USA)

4.4 Results and Discussion

4.4.1 Amino acid composition of protein hydrolysates from yellow stripe trevally muscle

Amino acid compositions of HA and HF with 15% DH are shown in Table 7. Amino acid compositions of HA and HF were generally similar to those of the original fish flesh used. HA, HF as well as flesh contained low levels of methionine and cysteine. Conversely, all samples were rich in glutamic acid, glutamine, aspartic acid, asparagine and alanine. Lysine, an essential amino acid, was also found at high levels in both HA and HF. Both HA and HF had higher glutamic acid, glutamine, arginine, threonine, proline, tyrosine, valine, methionine and leucine contents, but lower glycine, histidine and phenylalanine contents, compared with the original fish flesh. For glycine, a 50% decrease was found after hydrolysis.

Some differences in amino acid profile between HA and HF maybe associated with the existing differences in the hydrolysis conditions such as enzyme type, pH and temperature of hydrolysis process. Shahidi *et al*. (1995) reported that the composition of protein hydrolysates from capelin hydrolysate depended on the types of enzyme used. Wu *et al*. (2003) indicated that the levels and compositions of free amino acids and small peptides were changed during hydrolysis, depending on enzyme specificity. The differences in amino acid composition might be due to the removal of unhydrolyzed debris mainly collagen and stroma with unique amino acid profiles. As a result, hydrolysate obtained in the form of supernatant had a slightly different amino acid composition. Collagen contains glycine at about 1/3 of its total amino acids (Miller, 1988) and is generally resistant to hydrolysis and most likely was removed after hydrolysis. As a consequence, much lower glycine content was obtained in the resulting hydrolysate. Benjakul and Morrissey (1997) manifested that there were some differences in amino composition among Pacific whiting solid waste, muscle and hydrolysate obtained because of the existing differences in protein compositions of raw material.

Essential amino acid/non-essential amino acid ratios of flesh were increased after hydrolysis. The ratios were 0.55, 0.63 and 0.62 for flesh, HA and HF,

Table 7 Amino acid composition of flesh and yellow stripe trevally protein hydrolysates prepared using Alcalase (HA) and Flavourzyme (HF) with 15% DH

Amino acids (%) [x]	Flesh	HA	HF
Asp+Asn	10.01	9.55	9.40
Ser	5.46	5.21	5.15
Glu+Gln	9.88	13.77	13.89
Gly	16.48	8.87	8.65
His [a]	5.49	3.62	2.98
Arg	2.64	3.50	3.88
Thr [a]	2.92	5.35	5.40
Ala	9.64	9.49	9.46
Pro	3.33	3.81	3.84
Cys/2	1.43	1.47	1.53
Tyr	5.62	5.70	6.11
Val [a]	2.77	3.61	3.38
Met [a]	1.76	2.58	1.87
Lys [a]	8.45	8.35	8.72
Ile [a]	4.31	4.14	4.49
Leu [a]	6.72	8.38	8.58
Phe [a]	3.08	2.61	2.66

[a] Essential amino acids.
[x] Duplicate determinations.

respectively. Therefore, hydrolysis process might be used for enhancing the nutritional value of proteins. Additionally, round scad protein hydrolysate has been reported to contain a high essential amino acid/non-essential amino acid ratio (Thiansilakul *et al*, 2007). HA and HF contained greater amounts of proline than did the flesh. This might contribute to the increased bitterness of the hydrolysates. Hydrolysis of the peptide bonds in proteins can increase the number of hydrophobic sites by exposing the interior of protein molecules contributing to bitterness (Ludescher, 1996). Saha and Hayashi (2001) reported that enzymatic hydrolysis of

proteins frequently resulted in bitter taste due to the formation of low-molecular-weight peptides containing basically hydrophobic amino acids.

HF had no bitterness, while HA exhibited slight bitterness when tested by panelisted (data not shown). HF was produced by Flavourzyme, which contains both endopeptidase and exopeptidase. Exopeptidase was been reported to remove hydrophobic amino acids at the termini of bitter peptides, resulting in the lowered bitterness (Saha and Hayashi, 2001). HA and HF contained greater amounts of leucine, valine, tyrosine and methionine than did the intact flesh. Glutamic acid, glycine and aspartic acid found in the flesh, HA and HF are recognized as important constituents providing the taste of fish and shellfish (Jung *et al*., 2005). The difference in antioxidative activity between different hydrolysates from mackerel might be governed by amino acid composition (Wu *et al*., 2003). Aromatic amino acids consisting of tyrosine, histidine, tryptophan and phenylalanine (Chen *et al*. 1998; Rajapakse *et al*., 2005), hydrophobic amino acids comprising valine, leucine and alanine (Kim *et al*., 2001; Rajapakse *et al*., 2005) and also methionine (Mendis *et al*., 2005a) were reported to play a vital role in antioxidative activities.

4.4.2 Protein patterns of protein hydrolysates from yellow stripe trevally muscle

Protein patterns of HA and HF with 15% DH are shown in Figure 14. No MHC (myosin heavy chain) was remained in HA, while a small band of MHC was observed in HF. The result suggested that MHC was more prone to hydrolysis by Alcalase than Flavourzyme. No actin was found in both HA and HF. For protein or peptides with a MW less than 30 kDa, HF generally contained peptides with higher MW than HA. Peptides with a MW of 7-8 kDa were dominant in HF, whereas peptides with a MW lower than 7 kDa were prevalent in HA. Enzymatic breakdown of protein involves a major structural change, in which the protein is slowly hydrolyzed into smaller peptide units (Hevia and Olcott, 1977). Thus, different enzymes exhibited different specificity towards the protein substrate. Enzyme specificity contributed to not only molecular size (Adler-Nissen, 1986; Kristinsson and Rasco, 2000), but also antioxidative activities (Sathivel *et al*., 2003; Suetsuna, 2000; Jun *et al*., 2004; Klompong *et al*., 2007a) of the resulting hydrolysates.

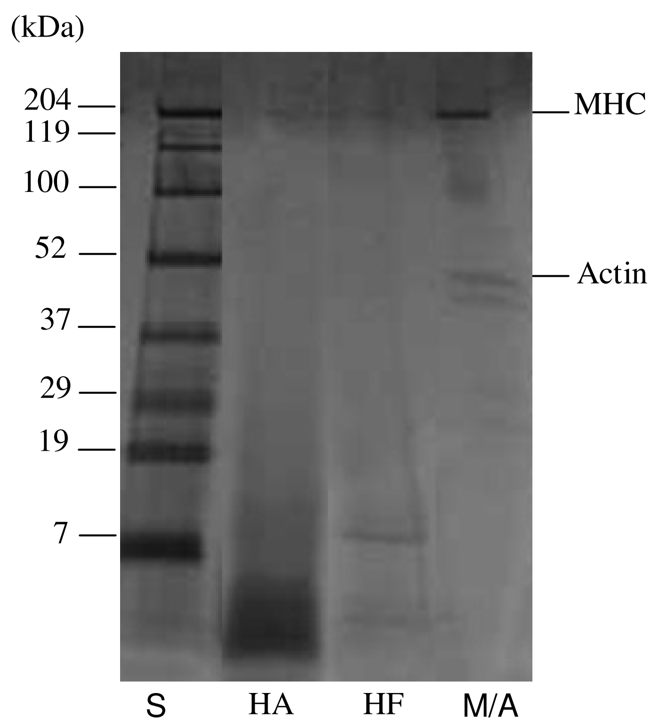


Figure 14. Protein patterns of yellow stripe trevally protein hydrolysate prepared using Alcalase and Flavourzyme with 15% DH. S: molecular weight standard; M/A: myosin heavy chain (MHC)/actin standard.

4.4.3 Effects of protein hydrolysates from yellow stripe trevally muscle on plasmid DNA relaxation

The effect of HA and HF with a DH of 15% on DNA relaxation is shown in Figure 15. The electrophoretogram revealed that pUC18 supercoiled DNA was converted into the nicked form in the Fe(II)+H_2O_2 Fenton reaction system. Fe(II) ions can react with H_2O_2, producing hydroxyl radicals (•OH) by Fenton reaction (Cairo *et al.*, 2002), leading to the relaxation of supercoiled DNA associated with single strand breaks (Toyokuni *et al*, 1992). Relaxed form and supercoiled form of DNA can be separated by agarose gel electrophoresis due to their unique electrophoretic mobilities. Relaxed form appeared when DNA was attacked by hydroxyl radicals (Surguladze *et al.*, 2004). The generation of hydroxyl radicals could be estimated by plasmid DNA relaxation assay (Ishikawa *et al.*, 2004). For the control treatment

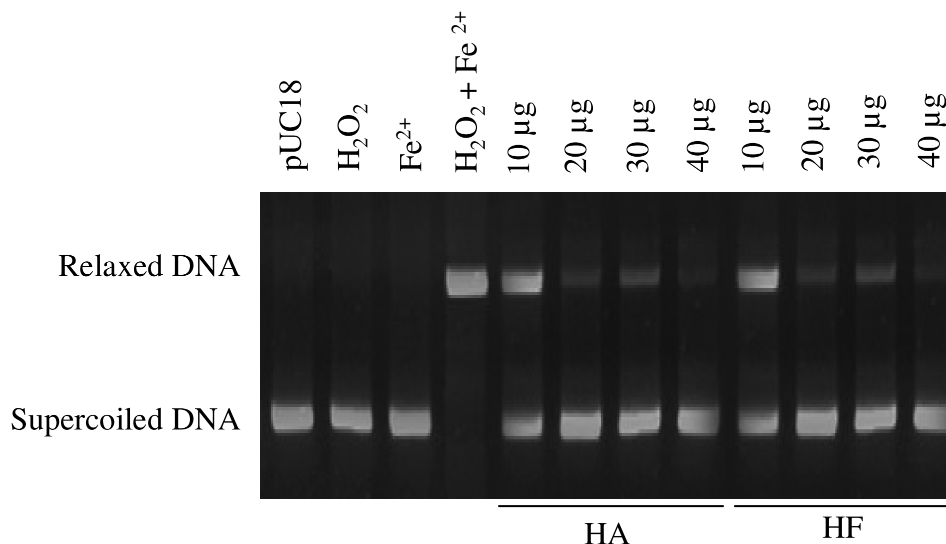


Figure 15. Electrophoretogram of *in vitro* plasmid DNA in Fenton reaction system containing yellow stripe trevally protein hydrolysate prepared using Alcalase (HA) and Flavourzyme (HF) with 15% DH. pUC18: supercoiled pUC18 plasmid DNA. Numbers designate the amount of HA and HF (μg).

(pUC18), H_2O_2 and Fe^{2+} alone did not cause any damage of DNA as indicated by the remaining supercoiled DNA bands.

When Fe^{2+} and H_2O_2 were combined, significant cleavage or breakage of DNA was noticeable. The rates of DNA nicking by free radicals decreased as the concentration of HA and HF increased. In the presence of both HA and HF at a level of 40 μg, the relaxation forms of DNA were rarely detectable. The result showed that HA and HF inhibited DNA relaxation in a dose-dependent manner. Iron-catalyzed hydroxyl radicals might be scavenged by HA and HF. HA and HF were previously found to act as metal chelators (Klompong *et al.*, 2007b). Thus, radicals might be generated to a lower extent as Fe^{2+} was chelated by HA or HF. Additionally, Klompong *et al.* (2007a) found that both HA and HF exhibited radical scavenging activity in a dose-dependent manner. Therefore, HA and HF were effective in preventing DNA damage in the Fenton reaction system and were protective against oxidative damage to DNA. Ishikawa *et al.* (2004) reported that egg yolk phosvitin

inhibited hydroxyl radical formation from Fenton reaction, leading to prevention of DNA damage. Generation of reactive oxygen species (ROS) damaged DNA by generating DNA strand breaks but quenching of ROS with the antioxidant can prevent the damage (Sordet *et al*., 2004). Surguladze *et al*. (2004) also reported that DNA was nicked by a free radical mechanism and the rate of nicking correlated with the iron content and was strongly inhibited by radical scavengers and chelators.

4.4.4 Isolation and characterization of antioxidative peptides from protein hydrolysates from yellow stripe trevally muscle

After separation of HA and HF using Sephadex G-50 column, 4 peaks with MW of 57, 35, 2.44 and 0.47 kDa for HA and 3 peaks with MW of 36, 1.77 and 0.47 kDa for HF were obtained (Figure 16a). Absorbance at 220 nm indicates peptide bonds and 280 nm represents aromatic rings (Ranathunga *et al*., 2006). Fraction C of HA possessed greater A_{220} and A_{280} than did fraction B of HF. Relatively high A_{220} and A_{280} were noticeable for both fractions, which might indicate the presence of a high content of peptide bonds and aromatic compounds (tyrosine and tryptophan) of these fractions (Amarowicz and Shahidi, 1997). For all fractions, A_{220} was greater than A_{280}. Those fractions exhibited varying antioxidative activities as determined by TEAC assay (Figure 16b). The highest TEAC value of HF was found in fraction B with a MW of 1.77 kDa, compared with other fractions ($p<0.05$). For HA fractions, fraction C with a MW of 2.44 kDa showed the strongest antioxidative activity ($p<0.05$), followed by fraction B (35 kDa), D (0.47 kDa) and A (57 kDa), respectively. However, fraction C of HA possessed a stronger antioxidative activity than did fraction B of HF ($p<0.05$). Peptides with different amino acid compositions, sequences (Chen *et al*, 1998) and sizes were thought to exhibit different antioxidative activity (Mendis *et al*. 2005b; Thiansilakul *et al*., 2007).

The Sephadex G-50 fractions with the strongest antioxidative activity from both HA (fraction C) and HF (fraction B) were subsequently subjected to cation-exchange chromatography on a SP-Sephadex C-25 column. After elution, 3 peaks of HA and 5 peaks of HF were collected and pooled (Figure 17a). Peak A of both HA and HF indicated the unbound fractions and peaks B and C of HA and peaks B, C, D and E of HF represented the bound fractions, which were eluted with a 0-2 M NaCl

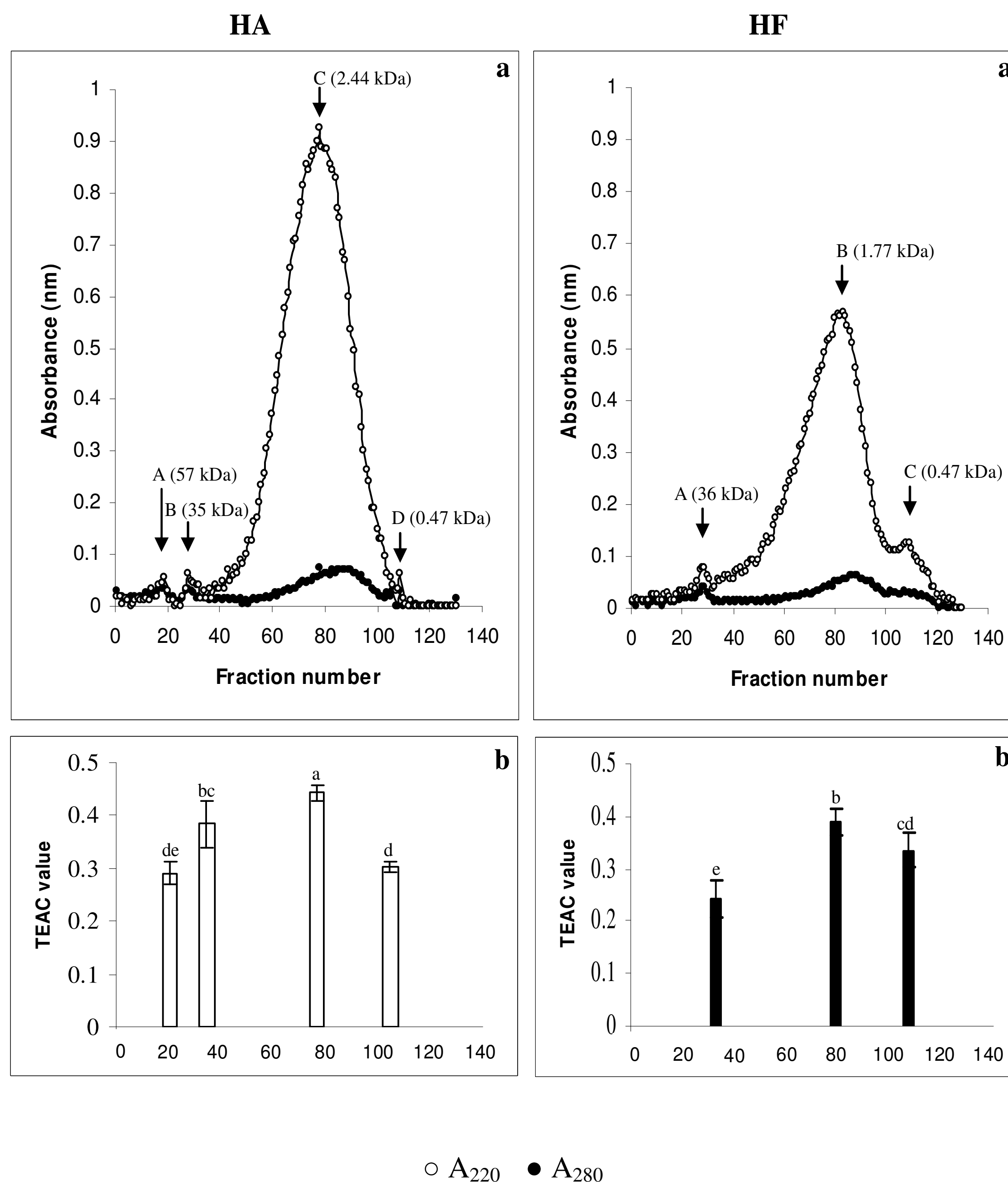


Figure 16. Separation of antioxidative peptides from yellow stripe trevally protein hydrolysate prepared using Alcalase (HA) and Flavourzyme (HF) by Sephadex G-50 column. A_{220} and A_{280} were monitored (a). Fractions showing antioxidative activity (TEAC value) are indicated by arrow signs (b). Bars represent standard deviations from triplicate determinations. Different letters on the bars of both HA and HF indicate the significant differences ($p<0.05$).

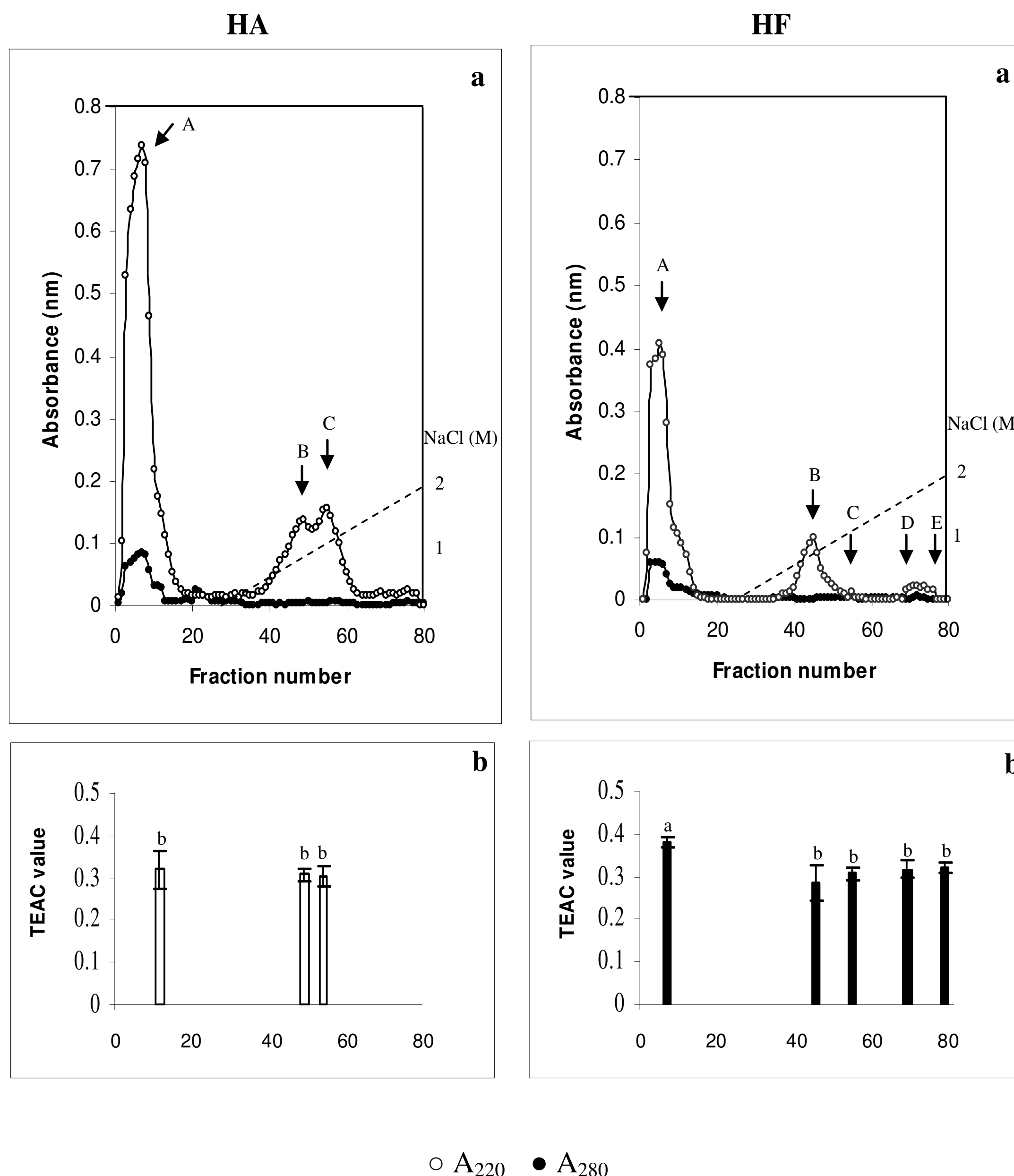


Figure 17. Separation of the selected Sephadex G-50 fraction of hydrolysates prepared using Alcalase (HA) and Flavourzyme (HF) by SP Sephadex C-25 column. A_{220} and A_{280} were monitored (a). Fractions showing antioxidative activity (TEAC value) are indicated by arrow signs (b). Bars represent standard deviations from triplicate determinations. Different letters on the bars of both HA and HF indicate the significant differences ($p<0.05$).

gradient. The major peaks of bound fractions of HA (peaks B, C) were eluted at approximately 1.5 M NaCl, while the major peak of that of HF (peak B) was eluted at approximately 1 M NaCl. The unbound peak (peak A) from HF showed a higher TEAC value than did those of the bound fractions ($p<0.05$) (Figure 17b). Nevertheless, no differences in TEAC value were found between unbound and bound fractions of HA ($p>0.05$). The unbound fractions of HF showed slightly higher TEAC value than did unbound HA counterpart. For bound fractions from both HA and HF, similar antioxidative activity expressed as TEAC value was observed ($p>0.05$). Mendis *et al*. (2005b) and Kim *et al*. (2001) separated antioxidative peptides from gelatin hydrolysates of hoki skin and Alaska pollack skin, respectively, using consecutive chromatographies.

For further separation, the strongest antioxidative fractions (fractions A) from both HA and HF were subjected to a Vydac 218TP54 reversed phase C_{18} HPLC column. Seven and 5 distinct fractions of HA and HF were obtained, respectively (Figure 18a). Fraction A of HA and fraction D of HF showed the highest TEAC value ($p<0.05$) (Figure 18b). However, fraction A of HA possessed a stronger antioxidative activity than that of fraction D of HF ($p<0.05$). Thereafter, fraction A of HA and fraction D of HF were further purified using a Zorbax SB C_{18} reversed phase HPLC column (Figure 19). Fraction A from both HA and HF possessed greater TEAC value than did fraction B ($p<0.05$). Antioxidative activity of protein hydrolysates depended on the proteases used (Jun *et al*., 2004). During hydrolysis, a wide variety of smaller peptides and free amino acids is generated, depending on enzyme specificity. Changes in size, level and composition of amino acids of peptides affect the antioxidative activity (Wu *et al*., 2003). Jun *et al*. (2004) reported that yellowfin sole hydrolysate, prepared using pepsin, had a higher antioxidative activity, than did those produced using other enzymes, such as Alcalase, chymotrypsin, papain, pepsin, Pronase E, Neutrase and trypsin. Basically, TEAC assay is used to determine hydrogen donating ability of antioxidant. $ABTS^{•+}$ is a relatively stable radical and is readily reduced by antioxidant (Miller *et al*., 1993). The result revealed that the yellow stripe trevally hydrolysates potentially contained peptides which were electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction.

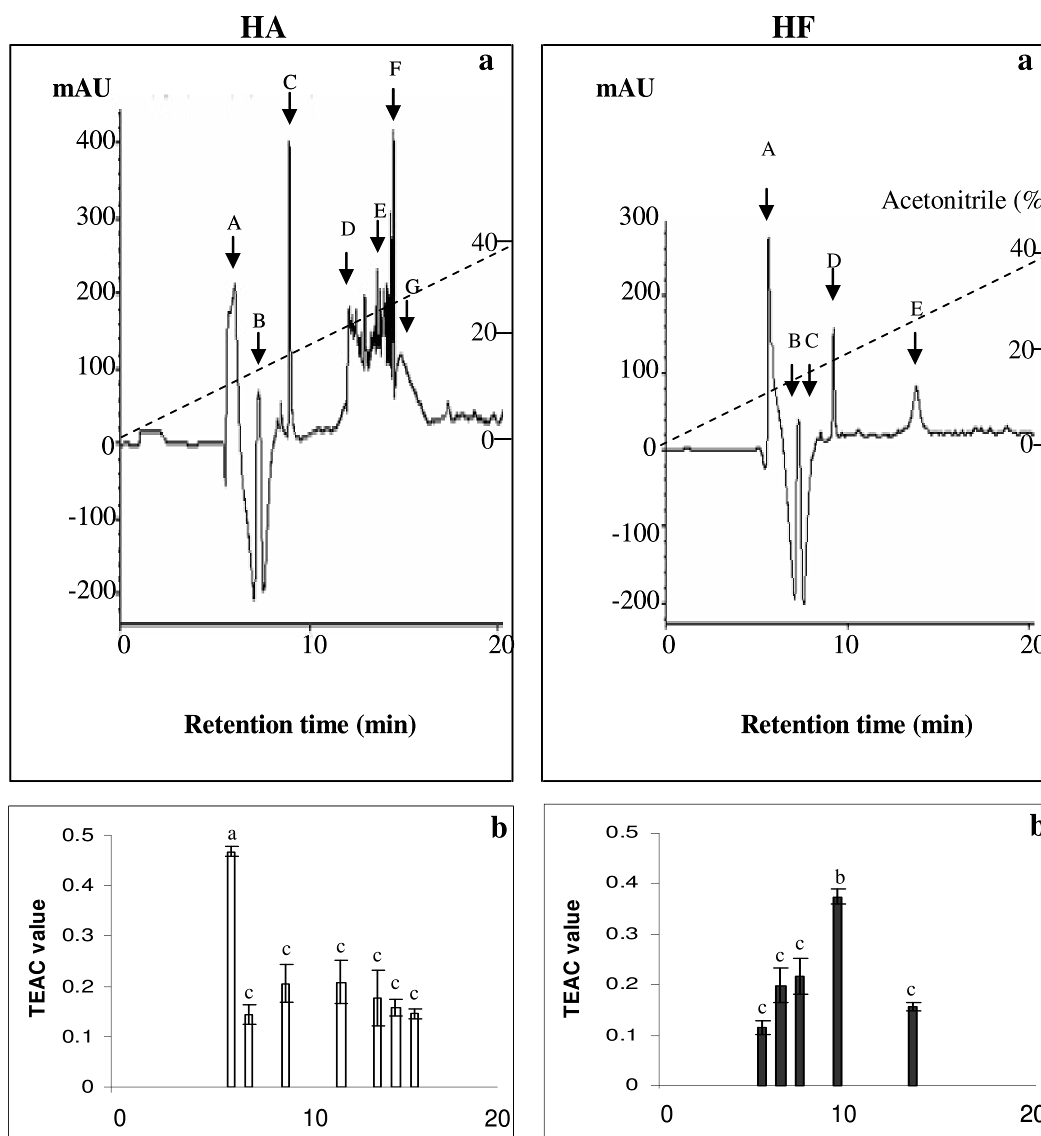


Figure 18. Separation of the selected SP Sephadex C-25 fraction of hydrolysates prepared using Alcalase (HA) and Flavourzyme (HF) by a Vydac 218TP54 reversed phase C_{18} column. A_{210} was monitored (a). Fractions showing antioxidative activity (TEAC value) are indicated by arrow signs (b). Bars represent standard deviations from triplicate determinations. Different letters on the bars of both HA and HF indicate the significant differences ($p<0.05$).

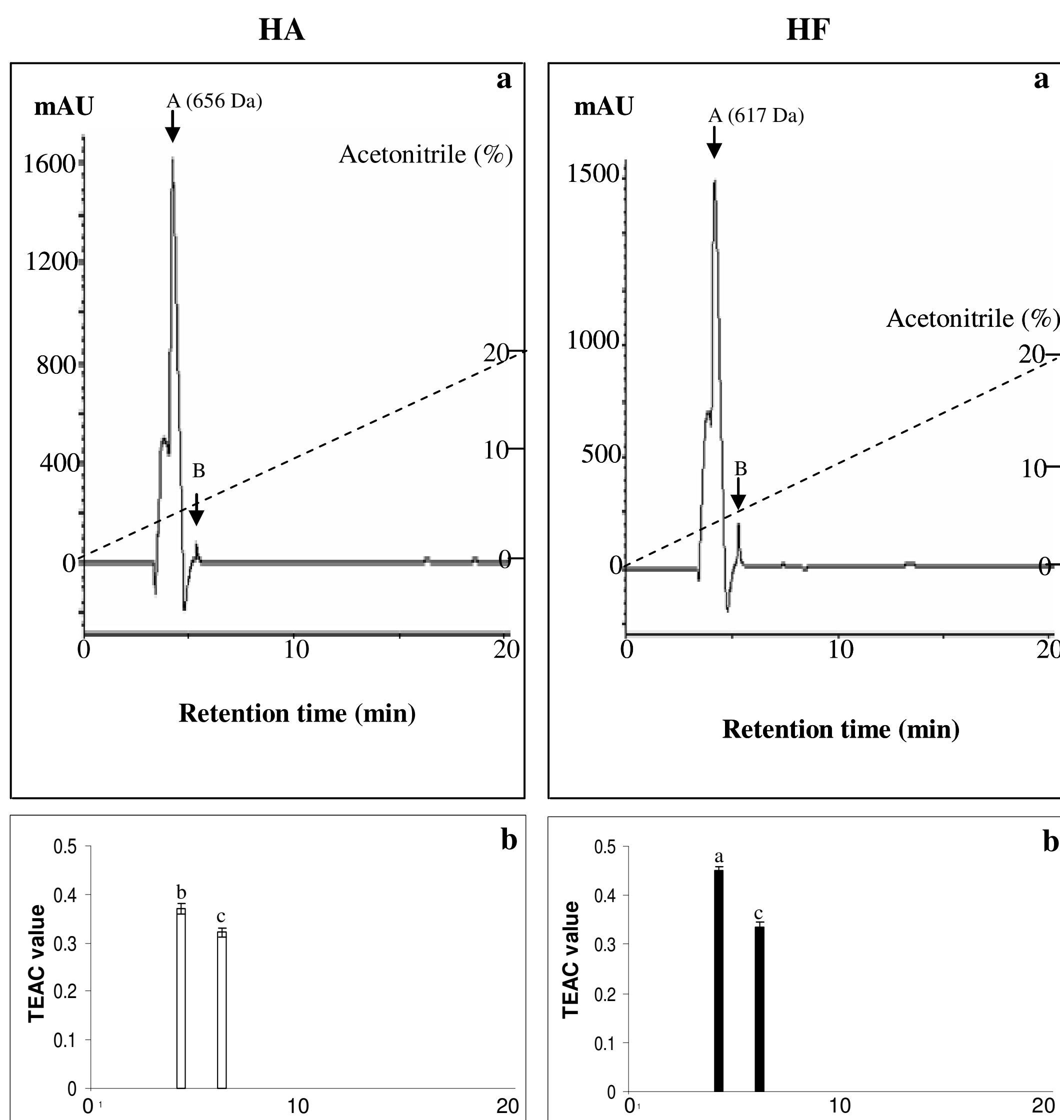


Figure 19. Separation of the selected Vydac 218TP54 reversed phase C_{18} fraction of hydrolysates prepared using Alcalase (HA) and Flavourzyme (HF) by a Zorbax SB C_{18} reversed phase HPLC column. A_{210} was monitored (a). Fractions showing antioxidative activity (TEAC value) are indicated by arrow signs (b). Bars represent standard deviations from triplicate determinations. Different letters on the bars of both HA and HF indicate the significant differences ($p<0.05$). Molecular weights of purified antioxidative peptides from yellow stripe trevally protein hydrolysates were determined by MALDI TOF/TOF mass spectrometer.

The MW of potential antioxidative peptides in fraction A of HA and HF were estimated to be 656 Da and 617 Da, respectively (Figure 19a) using MALDI TOF/TOF mass spectrometer. The lower MW peptides purified by consecutive chromatographies possessed antioxidative activity (Rajapakse *et al*., 2005). Peptides with different MW were found in hydrolysates derived from hoki skin gelatin (797 Da) (Mendis *et al*., 2005b), from jumbo squid skin gelatin (880 and 1242 Da) (Mendis *et al*., 2005a), from the sauce of fermented blue mussel (620 Da) (Jung *et al*., 2005), from giant squid muscle (747 Da and 1307 Da) (Rajapakse *et al*., 2005) and from conger eel (928 Da) (Ranathunga *et al*., 2006). Size of peptides influences their antioxidative activity (Thiansilakul *et al*., 2007; Chen *et al*., 1998; Ranathunga *et al*., 2006).

4.5 Conclusions

Protein hydrolysates from yellow stripe trevally were rich in essential amino acids and possessed antioxidative activity which varied with the type of enzymes used. Hydrolysates could prevent DNA damage in Fenton reaction system. Potential antioxidative peptides of hydrolysates prepared using Alcalase and Flavourzyme with 15% DH were purified and characterized to be 656 and 617 Da, respectively. The purified peptides were capable of donating hydrogen atoms and could be used as natural antioxidants. Nevertheless, the bitterness of protein hydrolysate should be evaluated and the appropriate debittering should be applied in order to obtain a fish protein hydrolysate with the sensorial acceptability for further uses.

CHAPTER 5

USE OF PROTEIN HYDROLYSATE FROM YELLOW STRIPE TREVALLY (*SELAROIDES LEPTOLEPIS*) AS MICROBIAL MEDIA

5.1 Abstract

To investigate the potential use of protein hydrolysate from yellow stripe trevally as a nitrogen source for the growth of different microorganisms, hydrolysates with different degrees of hydrolysis (DH) (5, 15 and 25%) were produced by hydrolyzing fish meat with Alcalase (HA) or Flavourzyme (HF) using pH-stat method. HA and HF were determined for microbial growth, compared with commercial peptone (Bacto Peptone). For bacteria, *Staphylococcus aureus* and *Escherichia coli*, HF with 25% DH (HF_{25}) yielded the highest cell density and specific growth rate (μ_{max}) and the lowest generation time (t_d) ($p<0.05$). For yeasts, *Saccharomyces cerevisiae* and *Candida albicans*, Bacto Peptone yielded higher growth rate than did HA and HF ($p<0.05$), while no differences in μ_{max} and t_d were observed for fungus, *Aspergillus oryzae* ($p>0.05$). The pH of culture broth containing HF_{25} decreased markedly during the first 8 h of cultivation of *S. aureus* and *E. coli* ($p<0.05$). This directly lowered the colony size of *S. aureus* ($p<0.05$). However, buffered culture broth and agar containing HF_{25} rendered the similar growth and colony size of *S. aureus* ($p>0.05$), compared with that containing Bacto Peptone. Scanning electron microscopic study revealed no differences in size and shape of microorganisms cultured in HF_{25} and Bacto Peptone ($p>0.05$). Therefore, production of fish protein hydrolysate for upgrading low market value species as a high value nitrogenous substrate for microbial growth could be achieved.

5.2 Introduction

Fish processing by-products and under-utilized species are commonly recognized as low-value resources with negligible market value. Additionally, their

inappropriate disposal is a major cause for environmental pollution. To tackle these problems, hydrolysis processes have been developed to convert such resources into marketable and acceptable forms. Protein hydrolysates from different fish species such as tuna, cod, salmon and unspecified fish have been used as nitrogenous sources for microbial growth (Dufosse *et al*., 2001). Soluble fish protein hydrolysate from extensive hydrolysis comprises free amino acids and low molecular weight peptides, which can be used as an excellent nitrogen source for microbial growth and thus can be used as microbial peptone (Clausen *et al*., 1985; Jassim *et al*., 1988). The peptides with the molecular weight of 6,500 Da, di-peptides and amino acids from tuna treated with Alcalase served as a suitable nitrogenous source in microbial media (Guerard *et al*., 2001). Recently, protein hydrolysates from yellow stripe trevally have been produced successfully using Alcalase and Flavourzyme (Klompong *et al*., 2007a, b). Degree of hydrolysis (DH) can affect the functional properties and antioxidative activity of resulting protein hydrolysate (Klompong *et al*., 2007a). DH influencing peptide chain length may have the impact on the ease of utilization by microorganisms. Additionally, different peptides produced by different proteases can be used by microorganisms at varying degree. Therefore, the objective of this study was to investigate the potential use of protein hydrolysate from yellow stripe trevally produced by Alcalase or Flavourzyme as a nitrogen source for the growth of different microorganisms including bacteria, yeasts and mold.

5.3 Materials and Methods

5.3.1 Enzymes and cultivation media

Alcalase and Flavourzyme were obtained from Novozymes (Bagsvaerd, Denmark). Bacto Peptone was purchased from Difco Laboratories (Sparks, MD, USA). Nutrient Broth (NB) was procured from Merck (Darmstadt, Germany) and Potato Dextrose Broth (PDB) was obtained from Himedia Laboratories (Mumbai, Maharashtra, India).

5.3.2 Fish sample collection and preparation

Yellow stripe trevally (*Selaroides leptolepis*) caught along the coast of the Andaman Sea with the size of 65g/fish, off-loaded approximately 24-36 h after capture, were obtained from the fishing port in Satul province, Thailand. Fish were placed in ice with the fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 2 h. Upon the arrival, fish were washed and the meat was separated manually. The meat was minced using a mincer with the 0.4 cm diameter holes. The mince was stored in ice until use.

5.3.3 Production of protein hydrolysates from yellow stripe trevally

Mince (60 g) was suspended in 240 ml of distilled water. The mixture was homogenized using a homogenizer (IKA Labortechnik, Selangor, Malaysia) at a speed of 11,000 rpm for 1 min. The homogenate was adjusted to pHs 8.5 and 7.0 and preincubated at 60 or 50°C for 20 min prior to enzymatic hydrolysis using Alcalase and Flavourzyme, respectively. The hydrolytic reaction was started by addition of 0.0076, 0.05 and 0.33% Alcalase (w/w) or 0.26, 1.58, 9.77% Flavourzyme (w/w) based on the protein content of mince (Klompong *et al.*, 2007a). The reaction was conducted as per the pH-stat method (Adler-Nissen, 1986) for 20 min to obtain the DH of 5, 15, 25 % as described by Klompong *et al.* (2007a).

To terminate enzymatic reaction, the reaction mixtures were heated in a water bath at 90°C for 15 min with occasional agitation. The samples were cooled and the pH of samples was then adjusted to 7.0 with 6 M HCl or 1 M HCl. Hydrolysates were centrifuged at 2000g for 10 min using a centrifuge (Sorvall, RC 5B plus, Norwalk, CT, USA). Supernatants obtained were kept in plastic bottles at -20°C until use. Before use, frozen samples were thawed in running water until the temperature reached 0-2°C.

5.3.4 Media formulas

Different broths were prepared for culturing different microorganisms. Nutrient Broth (NB) for bacteria consisted of 3 g beef extract and 5 g peptone. Yeast-Malt Broth (YMB) for yeasts contained 3 g yeast extract, 3 g malt extract, 10 g

dextrose and 5 g peptone. Malt Extract Broth (MEB) for molds comprised 12.75 g maltose, 2.75 g dextrin, 2.35 g glycerol, and 0.78 g peptone. Agar (15 g) was added in NB and MEB to obtain Nutrient Agar (NA) and Malt Extract Agar (MEA), respectively, while 20 g of agar was added in YMB to obtain Yeast-Malt Agar (YMA). The mixtures for each formula were added to distilled water and finally adjusted to 1 l. The solutions obtained were adjusted to pH 6.8±0.2, 6.2±0.2 and 4.7±0.2 for NB, YMB and MEB, respectively, and autoclaved at 121°C for 15 min (Tomy, SS-325, Tokyo, Japan). To study the use of HA or HF as the replacer for commercial peptone, Bacto Peptone was substituted by HA with DHs of 5% (HA_5), 15% (HA_{15}) and 25% (HA_{25}) or HF with DHs of 5% (HF_5), 15% (HF_{15}) and 25% (HF_{25}) at the amount yielding an equivalent level of nitrogen content to Bacto Peptone, determined by Kjeldahl method (AOAC, 2000).

5.3.5 Preparation of microorganisms and inocula

Staphylococcus aureus (*S. aureus*) TISTR 118, *Escherichia coli* (*E. coli*) TISTR 780 and *Saccharomyces cerevisiae* (*S. cerevisiae*) TISTR 5017 were obtained from the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand. *Candida albicans* (*C. albicans*) PSSCMI 7010 and *Aspergillus oryzae* (*A. oryzae*) PSSCMI 3004 were gifted from the Department of Microbiology, Prince of Songkla University, Hat Yai, Thailand. Bacteria were kept on NA slants, whereas yeasts and mold were kept on PDA slants at 4°C until use. To activate microorganisms before culturing, the inocula were prepared. One loopful of each microorganism cultured on NA slants for 15 h for bacteria (*S. aureus* and *E. coli*) and on PDA slant for 24 h for yeasts (*S. cerevisiae* and *C. albicans)* was inoculated in NB and PDB for bacteria and yeasts, respectively. The culture broths were then incubated at 37°C for 15 h for bacteria and 25°C for 24 h for yeasts. Thereafter, 1.5 ml of culture broths were inoculated in 50 ml NB and PDB for bacteria and yeasts, respectively and incubated at 37°C for 15 h for bacteria and 25°C for 24 h for yeasts. The culture broths were shaken at 120 rpm using a shaking incubator (VS-8480SR-L, LMS, Hat Yai, Thailand). Culture broths were adjusted to obtain A_{660} of 0.02 and then subjected to centrifugation at 3,000g (Hettich, Mikro 20, Zentrifugen, Germany) for 20 min at 25°C. Supernatants were discarded and the cells were washed twice by

normal saline (0.85% NaCl solution) to eliminate residual nutrient. For each wash, the suspension was centrifuged at 3,000g for 20 min at 25°C. Supernatants were discarded and the fresh culture media were added to the cell pellet to obtain A_{660} of 0.02. The obtained cell suspension was used as late log phase inocula. For mold, *A. oryzae* was sub-cultured onto PDA slant and incubated at 25°C for 48 h. This process was conducted twice to produce spores. Spore suspension with the concentration of 10^6 spores/ml measured by direct count using a hemacytometer was prepared.

5.3.6 Determination of growth kinetic of microorganisms

Growth rates were determined by submerged cultivation. To 50 ml of tested broths containing HA or HF at different DHs or standard broths containing Bacto Peptone, 1.5 ml of inocula with A_{660} of 0.02 or 10^6 spores/ml was added. NB, YMB and MEB were used for culturing bacteria, yeasts and mold, respectively. Incubation was carried out at 37°C for 24 h for bacteria and at 25°C for 48 h for yeasts with continuous shaking at 120 rpm. The growth of bacteria and yeasts were monitored by turbidity (A_{660}) measurements every 4 h. For mold, dry cell weight (DCW) measurement was used to monitor the growth every 4 h up to 48 h. Briefly, 2 ml of liquid culture was centrifuged at 3,000g for 20 min at 25°C (Hettich, Mikro 20, Zentrifugen, Germany). The cell pellet was resuspended in 2 ml normal saline and centrifuged under the same conditions. Washed cells were dried at 105°C until the constant dry weight was obtained.

Growth curves of each microorganism cultured in different media containing HA or HF (5, 15 and 25% DH) or commercial peptone (Bacto Peptone) were obtained by plotting A_{660} for bacteria and yeasts or DCW for mold against incubation time. Specific growth rate (μ_{max}) and generation time (t_d) were then calculated (Lynch and Poole, 1979) as follows:

$$\mu_{max} = \frac{\ln x_t - \ln x_o}{t_t - t_o}$$

$$t_d = 0.693/\mu$$

where x_o is initial population; x_t is population at instant t; t_o is initial time and t_t is instant time.

Protein hydrolysate with the DH rendering the highest growth rate of all microorganisms was selected for further study.

5.3.7 Measurement of growth and colony size of microorganisms cultured in medium containing protein hydrolysate

To study the effect of HF_{25} on qualitative microbial growths, the colony size was determined. One loopful of each bacterium cultured in NA slants for 15 h and yeast cultured in YMA slant for 24 h was subcultured on NA and YMA containing Bacto Peptone or HF_{25} at equivalent total nitrogen (HF_{25tn}) or total solid (HF_{25ts}) contents to Bacto peptone. The culture was incubated at 37°C for 15 h for bacteria and 25°C for 24 h for yeasts. Thereafter, bacteria and yeasts were inoculated into NB and YMB containing HF_{25tn}, HF_{25ts} or Bacto peptone for bacteria and yeasts, respectively and then incubated at 37°C for 15 h for bacteria and 25°C for 24 h for yeasts with continuous shaking at 120 rpm. Culture broths obtained were adjusted with corresponding culture media to obtain A_{660} of 0.5 in order to obtain a similar amount of cells, since cell density on agar media may affect colony size (Boyd and Hoerl, 1991). For mold, *A. oryzae* was sub-cultured onto MEA slant containing HF_{25tn}, HF_{25ts} or Bacto peptone and incubated at 25°C for 48 h twice to produce spores. Spore suspension was prepared as previously described and adjusted to 10^6spores/ml using sterile normal saline. Serial dilutions of bacteria, yeasts and mold were prepared with sterile normal saline. Thereafter, aliquots of 0.1 ml dilution were pipetted onto corresponding agar plates containing HF_{25tn}, HF_{25ts} or Bacto Peptone and then spread using a sterile spreader. The plates were upside-down incubated at 37°C for 24 h for bacteria and incubated at 25°C for 48 h for yeasts and mold before counting and measuring colony diameter. The colony counts were recorded and the diameters of 20 colonies were measured per plate.

5.3.8 Measurement of growth and colony size of *S. aureus* cultured in medium containing protein hydrolysate supplemented with buffer

To study the effect of buffer to improve the colony size of *S. aureus*, all NB and NA used for culturing *S. aureus* were supplemented with 0.2% dipotassium

hydrogen phosphate (K_2HPO_4) and the pH was adjusted to 6.8±0.2 using 1 M NaOH or HCl. One loopful of *S. aureus* cultured in NA slants for 15 h was subcultured on NA, which was supplemented with 0.2% K_2HPO_4 and contained HF_{25} at equivalent total nitrogen (HF_{25tn}) and total solid (HF_{25ts}) contents to Bacto peptone or NA containing Bacto Peptone and 0.2% K_2HPO_4 (pH 6.8). The culture was incubated at 37°C for 15 h. Thereafter, *S. aureus* was inoculated into NB, which was supplemented with 0.2% K_2HPO_4 and contained HF_{25tn}, HF_{25ts} or Bacto peptone and then incubated at 37°C with continuous shaking at 120 rpm. Culture broths were taken after cultivation for 4, 8 and 12 h, representing the log phase of *S. aureus*. Culture broths obtained were adjusted with NB to obtain A_{660} of 0.5 in order to gain a similar amount of cells. Serial dilutions were prepared with sterile normal saline. Thereafter, aliquots of 0.1 ml dilution were pipetted onto NA, which was supplemented with 0.2% K_2HPO_4 and contained HF_{25tn}, HF_{25ts} or Bacto Peptone and then spread using a sterile spreader. The plates were upside-down incubated at 37°C for 24 h before counting and measuring colony diameter. The colony counts were recorded and the diameters of 20 colonies were measured per plate.

5.3.9 Determination of microbial morphology

Morphology of the late log phase of bacteria and yeasts and the stationary phase of mold cultured in NA and YMA and MEA containing HF_{25ts} or Bacto Peptone were analyzed by a scanning electron microscopy (SEM). Cultured bacteria and yeasts were smeared on slides by a sterile loop. For mold, cultured agar was cut into a piece of 0.5×0.5 cm. All samples were fixed at room temperature in 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 2 h. Fixed samples were rinsed with 0.2 M phosphate buffer (pH 7.2) for 15 min, followed by rinsing with distilled water for 15 min twice at room temperature. The samples were dehydrated in graded series of ethanol (50, 60, 70, 80, 90 and 100 %) and then were mounted on SEM stubs using a double backed cellophane tape. The stub and samples were coated with gold and examined using SEM (JEOL JSM-5800LV, Tokyo, Japan).

5.3.10 Statistical analysis

One-way analysis of variance (ANOVA) was used and mean comparison was performed by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analysis was carried out using SPSS statistic program (Version 11.0) for Windows (SPSS Inc. Chicago, IL, USA).

5.4 Results and Discussion

5.4.1 Effect of protein hydrolysate from yellow stripe trevally prepared using Alcalase or Flavourzyme with different DHs on microbial growth

Growth curves of bacteria, yeasts and mold cultured in the media containing HA or HF, in comparison with those cultured in media with Bacto Peptone are depicted in Figure 20, 21 and 22, respectively. As the cultivation time increased, cell densities of all microorganisms increased, regardless of media used, and reached the plateau differently, depending on the types of microorganism ($p<0.05$). pHs of media were changed differently and varied with microorganisms. μ_{max} and t_d also varied with media as well as types of hydrolysate and DHs used (Table 8).

5.4.1.1 Bacteria

S. aureus is a representative microorganism for Gram positive bacteria and some are Methicillin-resistant *S. aureus* (MRSA), which are responsible for difficult-to-treat infection in humans due to their resistance to a large group of antibiotics called the beta-lactams, which include the penicillins and the cephalosporins (Okuma *et al*., 2002). No changes in cell density of *S. aureus* were found within the first 4 h of cultivation period. Thereafter, the log phase started and reached stationary phase at h 16 (Figure 20a). In log phase, once the metabolic machinery is running, bacterium starts multiplying exponentially (Boyd and Hoerl, 1991). *S. aureus* cultured in media containing HA or HF at various DHs or Bacto peptone started log phase and reached stationary phase synchronously. The media containing HA or HF with 25% DH (HA_{25} and HF_{25}) showed the higher cell density and μ_{max} with a lower t_d than did those with HA or HF at lower DHs ($p<0.05$). At the

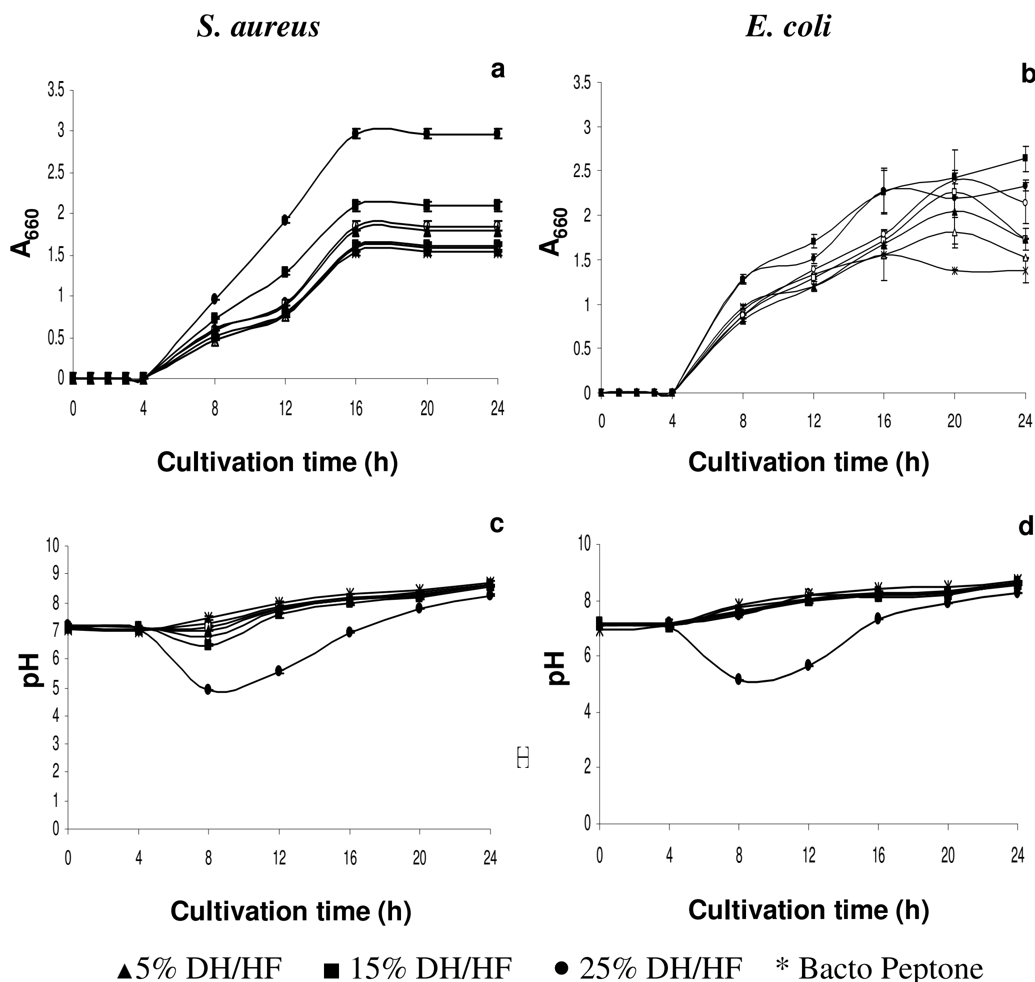


Figure 20. Growth curve (a-b) and pH changes (c-d) of *S. aureus* and *E. coli* during cultivation in NB containing protein hydrolysate from yellow stripe trevally prepared using Alcalase (HA) and Flavourzyme (HF) with different DHs: HA with DH of 5%, 15%, 25%; HF with DH of 5%, 15%, 25%, Bacto Peptone. Bars represent standard deviations from triplicate determinations.

same DH tested, HF yielded the greater cell density and μ_{max} and lower t_d than did HA, manifesting that the types of enzyme used for hydrolysate preparation affected the growth of *S. aureus*. All media containing HA or HF exhibited the higher cell density and μ_{max} with lower t_d than did that containing Bacto peptone except for HA_5.

The pHs of media containing HF started to drop at h 4. This was coincidental with the beginning of log phase. The decrease in pH was more intense at h 8, followed by the gradual increase (Figure 20c). The medium containing HF_{25} showed the greater cell density and μ_{max} with lower t_d than those containing HF_{15}, HF_5, HA_{25}, HA_{15}, Bacto Peptone or HA_5 in descending order. Coincidentally, the medium containing HF_{25} had the lower pH than did others. The result suggested that the extraordinary growth of *S. aureus* occurred in the medium containing HF_{25}.

E. coli is a representative microorganism for Gram negative bacteria and it has been used as the model prokaryote in microbiology molecular and cell biology (Downes and Ito, 2001). *E. coli* maintained the lag phase up to 4 h of cultivation period, followed by the log phase and reached the stationary phase at h 16-24 (Figure 20b). *E. coli* cultured in media containing HA or HF with various DHs or Bacto peptone started the log phase at the same time but reached stationary phase and death phase at different times. Even though the media containing HF_{25} or HF_{15} showed the greatest cell density, that containing HF_{25} possessed the highest μ_{max} and the lowest t_d ($p<0.05$). Generally, the media containing HA or HF with higher DHs exhibited the higher cell density and μ_{max} with lower t_d. The media containing HF yielded the higher cell density and μ_{max} with the lower t_d than did those containing HA with the same DH. Thus, the types of enzyme used to produce HA and HF also affected the growth of *E. coli* cultured in media containing HA or HF. All media containing HA or HF exhibited the higher cell density and μ_{max} with the lower t_d than did media containing Bacto peptone except HA_5. The pHs of all media containing HA or HF with all DHs except HF_{25} increased gradually with increasing cultivation time (Figure 20d). For the medium with HF_{25}, the pH decreased within the first 8 h of cultivation. The pH drop of medium containing HF_{25} was much more intense than was other media. This was concomitant with the highest cell density and μ_{max} and the lowest t_d ($p<0.05$). However, the continuous increase in pH was observed during 8-24 h of cultivation. The increase in pH was associated mostly with the increase in populations.

The differences between cell density, μ_{max}, t_d and pH changes of *S. aureus* and *E. coli* cultured in media containing various protein hydrolysates (HA_5, HA_{15}, HA_{25}, HF_5, HF_{15}, HF_{25}) or Bacto Peptone might be due to the differences in size

of peptide. HA and HF with different DHs contained peptides with different sizes, in which molecular weights lower than 204, 70 and 7 kDa were found with DHs of 5, 15 and 25%, respectively (Klompong *et al*., 2007b). This could contribute to a different biomass production of bacteria. Reductions of the molecular size of hydrolysate appeared when the DH increased (Klompong *et al*., 2007b). The results suggested that the DH of hydrolysates correlated with the maximum cell density, μ_{max}, t_d and pH changes in both cultures. Dufosse *et al*. (2001) also found a correlation between the peptone hydrolysis rate and the biomass production of *E. coli* and *Lactobacillus casei*. De la Broise *et al*. (1998) compared growth kinetics of *E. coli* in media containing fish hydrolysates and casein hydrolysates. With casein hydrolysate, the lag phase was shorter, whereas the growth rate and maximum cell density were higher. Degree of hydrolysis of proteins, amino acid composition and total amino acid content of protein hydrolysates had the impact on the kinetic parameters of microbial cultures (De la Broise *et al*., 1998). Reissbrodt *et al*. (1995) reported that the growth of *E. coli* could be governed by the molecular weight of peptides in casein hydrolysates. Peptides with the molecular weight of 6,500 Da, di-peptides and amino acids from tuna treated with Alcalase were a suitable nitrogenous source in microbial media (Guerard *et al*., 2001). HF_{25} was prepared by hydrolyzing yellow stripe trevally with Flavourzyme and had MW lower than 7 kDa (Klompong *et al*., 2007b). Thus it was a suitable nitrogenous source for culturing bacteria. Peptide in protein hydrolysate is an important source of nutrients (Amezaga and Booth, 1999). During cultivation, amino acids and peptides are taken up from the medium by cells. Free amino acids are incorporated directly without modification or degradation by cells (Batistote *et al*., 2006). It could be concluded that HF_{25} with MW lower than 7 kDa might provide the valuable nutrients, which were readily available for growths of both *S. aureus* and *E. coli.*

The lower growth of bacteria found in the media containing HA or HF with the low DH (5%) might be due to the lower nutritional properties (Tsoraeva and Zhurbenko, 2000) associated with the less availability of larger peptides. The major amino acids of HA and HF with 5% DH were glutamic acid and glutamine (data not shown). Furthermore, some hydrolytic products might show inhibitory activity toward the growth of microorganisms (De la Broise *et al*., 1998). Chen *et al*.

(2003) concluded that arginine, lysine, tryptophan or isoleucine residues enhanced effectiveness against certain bacteria.

Differences in maximum cell densities and growth rates of *S. aureus* and *E. coli* were noticeable when different hydrolysates and DHs were used ($p<0.05$). For all DHs, HA showed the inferior growth stimulation to HF. The result suggested the certain differences in hydrolysate composition such as size of peptides, amino acid composition of peptides, vitamins, fatty acids and inhibitory peptides. Flavourzyme consists of both endo- and exopeptidase activity. The hydrolysis resulted in the release of an N-terminal amino acid of the sequential order. The first hydrolyzed amino acid is preferably leucine, but may be other amino acids including proline (Hammershoj *et al*., 2008). Alcalase has a broad specificity for peptide bonds and a preference for a large uncharged residue (Hammershoj *et al*., 2008). At the same DH, HA and HF might contain different peptides due to the cleavage of peptides at different positions or amino acids, generating various amino acids and peptides. Owing to exopeptidase activity, HF might contain more free amino acids that were readily available for bacterial growth than did HA. Aspmo *et al*. (2005) found that proteolytic enzyme clearly affected the growth performance of microorganisms cultured in media containing protein hydrolysate. Alcalase led to optimum uptake of available amino acid resources because this endoprotease activity is most complementary to the bacterial proteolytic and peptide uptake systems (Aspmo *et al*., 2005). Hydrolysate from octopus wastewater can be used as the culture media (Vazquez *et al*., 2004). The results suggested that HA and HF showed the better efficacy for bacterial growth than did commercial peptone (Bacto Peptone) ($p<0.05$).

In fish muscle, glycogen is the main carbohydrate. After capture, postmortem degradation of glycogen to glucose-6-phosphate and glucose occurs via phosphorolytic pathway and via hydrolytic or amylolytic pathway (Eskin, 1990). This sugar could be consumed by bacteria as a carbon source (Downes and Ito, 2001). After sugar fermentation, organic acids were produced, resulting in the dramatic drop of pH of the medium containing HF_{25} used for culturing *S. aureus* and *E. coli*. The result suggested that medium containing HF_{25} might have the low buffering capacity. pH increases or decreases in culturing system depended on media and buffering capacity (Fernandez-Lopez *et al*., 2008). Protein and free amino acid are major

contributors to pH buffering in the biological system. The pH buffering capacity of proteins resulted from amino acids with basic and acidic side chain. The interactions of cations with functional group in these side chains affect the pH buffering capacity (Salaun *et al.*, 2005). Additionally, variations in the pH buffering capacity of system are also due to the different mineral and protein content associated with varying composition of raw material, different pretreatment and condition of process (Upreti *et al.*, 2006). After 8 h of cultivation, pH of media culturing *S. aureus* and *E. coli* increased gradually. When protein and amino acid were degraded during microbial growth, ammonia, non-protein nitrogen and amino acid catabolism products were accumulated (Nychas *et al.*, 1998; Parente *et al.*, 1994).

5.4.1.2 Yeasts

S. cerevisiae is a species of budding yeast and it is the most useful yeast, especially for baking and brewing. Therefore, it is one of the most intensively studied eukaryotic model microorganisms in molecular and cell biology (Downes and Ito, 2001). The log phase of *S. cerevisiae* started after cultivation for 16 h (Figure 21a). *S. cerevisiae* cultured in media containing HA or HF with various DHs or Bacto peptone reached the plateau after 44 h. Similar growth curves were obtained for *S. cerevisiae* cultured in all media. The medium containing HF_5 showed the greater μ_{max} and the lowest t_d than did those added with HA or HF having other DHs. Nevertheless, the medium containing Bacto Peptone possessed the highest μ_{max} and the lowest t_d ($p<0.05$) and had the greatest cell density during 16-24 h of the cultivation. Thereafter, no differences in cell densities in all media were distinguishably observed. Basically, the media containing of HA or HF with higher DHs exhibited the lower μ_{max} and higher t_d ($p<0.05$).

The pH of all media for culturing *S. cerevisiae* decreased after 12 h, corresponding to the beginning of log phase at h 16 (Figure 21c). As the cell density increased, the pHs slightly decreased in all media. However, a slight increase in pH was noticeable after 24 h. The lower pH of the medium containing Bacto Peptone was more distinguished than other media, likely associated with the highest cell density and μ_{max} and the lowest t_d, particularly during 16-24 h of cultivation ($p<0.05$).

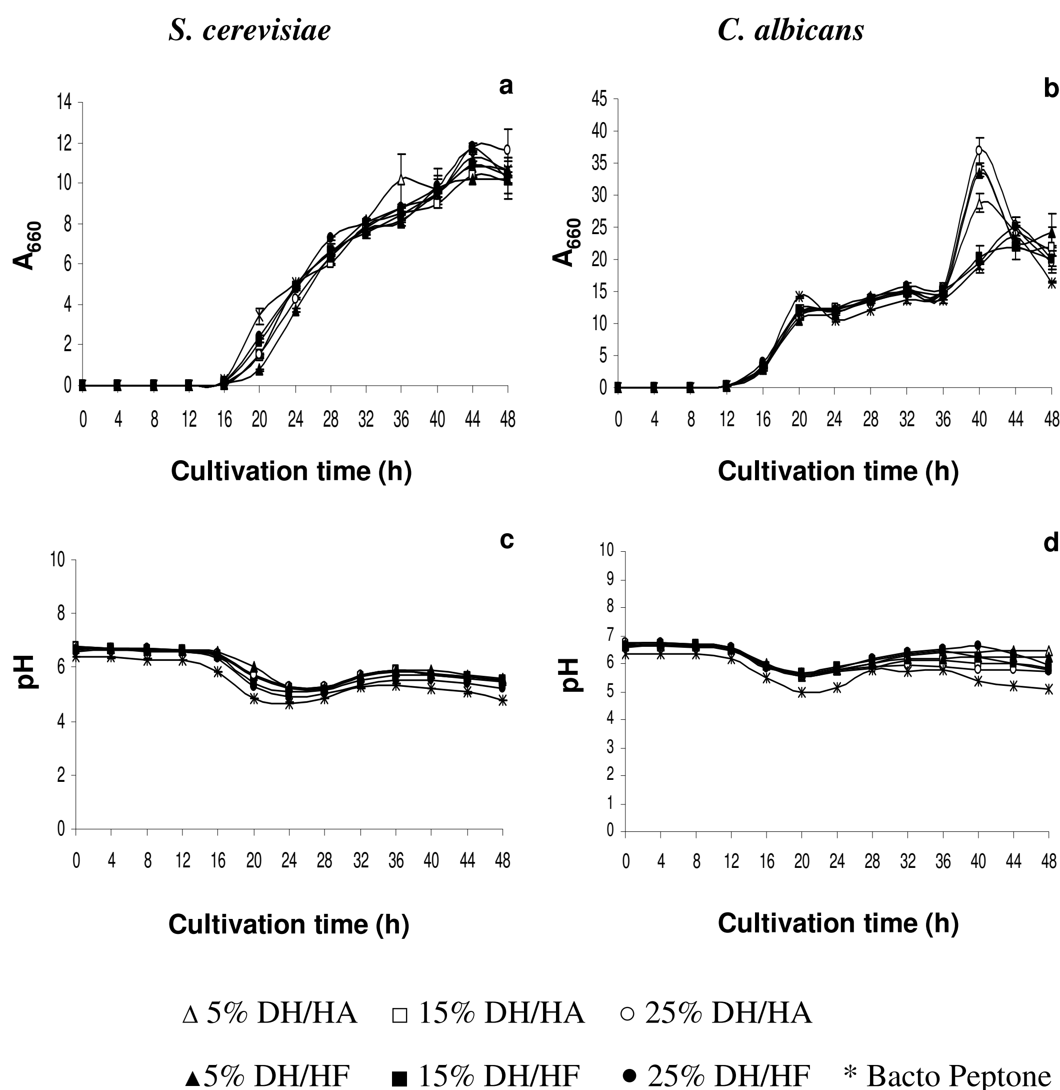


Figure 21. Growth curve (a-b) and pH changes (c-d) of *S. cerevisiae* and *C. albicans* during cultivation in YMB containing protein hydrolysate from yellow stripe trevally prepared using Alcalase (HA) and Flavourzyme (HF) with different DHs: HA with DH of 5%, 15%, 25%; HF with DH of 5%, 15%, 25%, Bacto Peptone. Bars represent standard deviations from triplicate determinations.

C. albicans is among the gut flora, which live in the human mouth and gastrointestinal tract. It is a causative agent to opportunistic oral and genital infections in humans (Downes and Ito, 2001). The log phase of *C. albicans* began after cultivation for 12 h and reached stationary phase at h 20 (Figure 21b). In

stationary phase, microorganisms were competing for dwindling nutrients, booming growth stopped and the number of microorganisms was constant (Boyd and Hoerl, 1991). *C. albicans* cultured in all media containing HA or HF at various DHs or Bacto peptone began log phase synchronously. Nevertheless, the system containing Bacto Peptone possessed the highest μ_{max} and the lowest t_d ($p<0.05$) and showed the greatest cell density during 12-20 h of cultivation. Thereafter, no differences in cell density among the media containing HA or HF were observed ($p>0.05$). Generally, the media added with HA or HF having the higher DHs exhibited the lower μ_{max} and higher t_d ($p<0.05$).

The pHs of all media decreased at h 12. This was associated with the multiplying of cells in the late lag phase found at h 12 (Figure 21d). In general, the pH gradually increased after 24 h of cultivation. The pH of the medium containing Bacto Peptone was lower than those of other media containing HA or HF, particularly during 12-20 h ($p<0.05$). *C. albicans* reached stationary phase within 4 h after the beginning of log phase. After the easily fermentable sugar (dextrose in YMB) was exhausted, *C. albicans* used slow fermenting nutrients (Fernandez-Lopez *et al*., 2008). *C. albicans* expressed the diauxie at h 40-44. Diauxie was found at h 40 for *C. albicans* cultured in media containing HA with 5, 15 and 25% DH and HF with 5% DH and at h 44 when it was cultured in media added with HF having 15 and 25% DH or Bacto peptone. Yeast is able to use a wide variety of compounds as carbon and nitrogen sources (Batistote *et al*., 2006). Sugar fermentations by yeasts were strongly affected by the structural complexity of nitrogen source varying from a single ammonium salt to free amino acid and peptides. Diauxie was observed at low sugar concentration, independent of nitrogen supplementation and the types of sugar (Batistote *et al*., 2006). When the primary carbon sources are absent or present at concentrations low enough to limit growth, others nitrogen source such as amides, amino acids and peptides can be used as a carbon source. The utilization of secondary nitrogen source requires the synthesis of specific catabolic enzymes and permeases (Batistote *et al*., 2006). Batistote *et al*. (2006) reported that the growth of *S. cerevisiae* in medium containing maltose and glucose supplemented with different nitrogen sources exhibited diauxie. Diauxie was found in *S. cerevisiae* of this study. The yeasts initially utilized sugar and after sugar exhaustion, ethanol was used as a carbon

source. At low glucose and maltose concentrations, diauxic growth was observed. Diauxie was not easily observed at high sugar concentration. *C. albicans* reached the death phase at the period of 40-44 h. In death phase, toxic waste products were produced and nutrients were depleted. As a result, the microorganisms began to die (Boyd and Hoerl, 1991).

5.4.1.3 Mold

A. oryzae is an important filamentous fungus for fermentation industries (Downes and Ito, 2001). *A. oryzae* was in the lag phase up to 12 h of cultivation period before the log phase was started (Figure 22a). *A. oryzae* reached stationary phase synchronously for all media at h 32 except for the medium containing Bacto Peptone, which reached plateau at h 28. That might be due to the greater biomass in the medium containing Bacto Peptone during 12-20 h of cultivation ($p<0.05$), leading to the faster beginning of stationary phase. However, no significant differences of μ_{max} and t_d were found among the media containing HA, HF and Bacto Peptone (Table 8). DHs and enzyme types had no impact on μ_{max} and t_d of *A. oryzae* cultured in media containing HA or HF that might be due to the ability of *A. oryzae* in producing extracellular enzymes.

The pHs of all media began to drop at h 8 and decreased continuously up to 48 h of cultivation (Figure 22b). As the dry biomass increased, the pHs decreased in all systems. The medium containing Bacto Peptone exhibited the lowest pH ($p<0.05$), which was concomitant with the greatest growth in this system. The decreased pH observed after growth of *A. Oryzae* is likely to be caused by assimilation of sugars and a metabolic capability (Pitt and Hocking, 1999). In microbial cultures, small peptides and amino acids are often needed as a nitrogen substrate that is useful not only for biomass production, but also for production of a large variety of metabolites including some organic acids in stationary phase (Urdaneta *et al*., 1995). Carbohydrates or sugar in media also could be used as substrates for mold metabolism, resulting in production of organic acids and lowering of pH (Papadima *et al*., 1999).

For all bacteria tested, growth rates and maximum cell densities were higher when HF_{25} was used as a replacer of Bacto Peptone. However, HF_{25} was

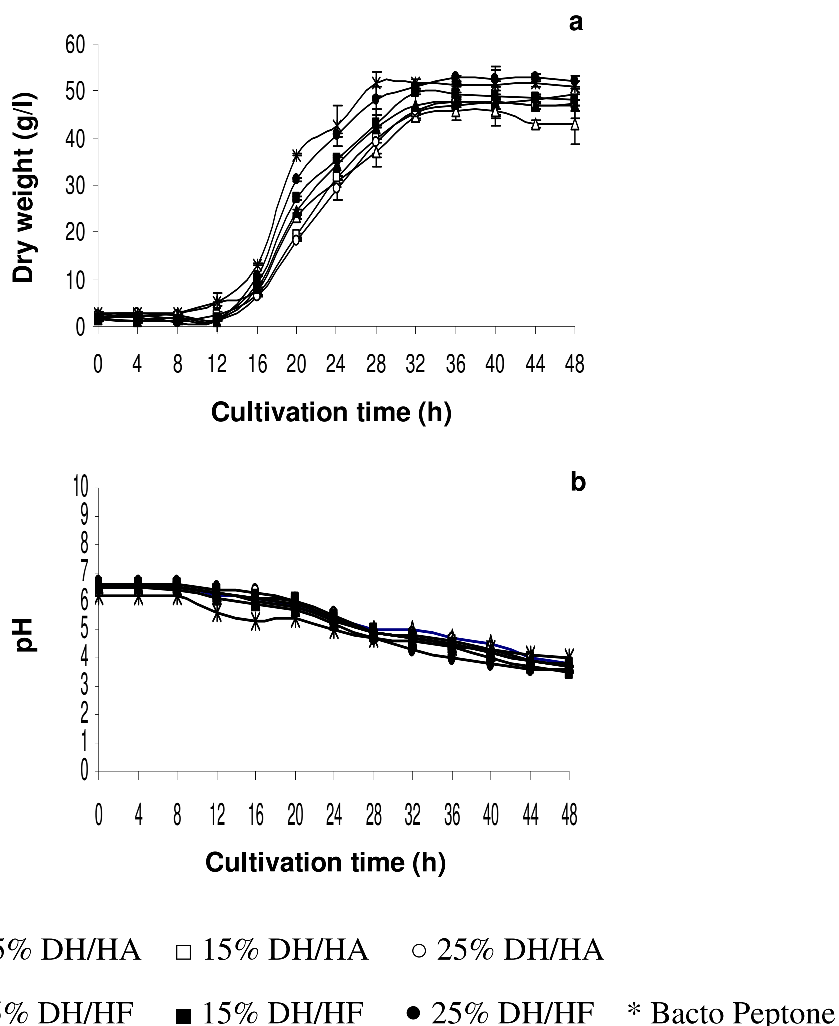


Figure 22. Growth curve (a) and pH changes (b) of *A. oryzae* during cultivation in MEB containing protein hydrolysate from yellow stripe trevally prepared using Alcalase (HA) and Flavourzyme (HF) with different DHs: HA with DH of 5%, 15%, 25%; HF with DH of 5%, 15%, 25%, Bacto Peptone. Bars represent standard deviations from triplicate determinations.

inferior to Bacto Peptone for yeast cultivation. Additionally, no difference was observed between HF_{25} and Bacto Peptone for mold culturing. Each peptone has its own biologic characteristics and could not meet the requirements of all microorganisms during cultivation (Kurbanoglu and Algur, 2002). Performances of peptones for production of cell biomass also exhibited large differences, depending on

the strain tested (Boyd and Hoerl, 1991). Guerard *et al*. (2001) manifested that tuna protein hydrolysate prepared using Alcalase were effectively served as a nitrogenous source in microbial growth media. Clausen *et al*. (1985) prepared an autolysate of fish viscera as the nitrogenous source in a bacterial growth medium. Jassim *et al.* (1988) used fish waste, including heads, bone and fins to prepare media for microorganisms using trypsin. Vecht-Lifshitz *et al*. (1990) reported that industrial fish peptone was an excellent substrate for biomass production in solid and submerged fermentations. Dufosse *et al*. (2001) showed that three peptones from cod, salmon and undetermined fish gave better result than the peptone from casein. It could be inferred that HF_{25} could be used as a source of peptone for microbial growth.

5.4.2 Effect of protein hydrolysate from yellow stripe trevally prepared using Flavourzyme on microbial growth and colony size

Growth and colony size of microorganisms cultured in agar medium containing HF_{25} as a replacer of Bacto Peptone (based on total nitrogen and solid contents) are shown in Table 9.

5.4.2.1 *S. aureus*

Even though the cell density was adjusted to 0.5 of A_{660} to obtain a similar cell density, total viable count of *S. aureus* cultured on NB containing HF_{25tn} was greater than that observed in NB with HF_{25ts} or Bacto Peptone ($p<0.05$). As a consequence, *S. aureus* cultured on NA comprising HF_{25tn} exhibited the smaller colony than that found on NA containing Bacto Peptone and HF_{25ts} ($p<0.05$). The smaller size of colony cultured on medium comprising HF_{25tn} might be affected by the decrease in pH during culturing (Figure 20c).

5.4.2.2 *E. coli*

E. coli cultured in NB comprising HF_{25ts} had the greater number than that found in NB containing HF_{25tn} or Bacto Peptone ($p<0.05$). NB containing HF_{25ts} possibly contained a suitable amount of nitrogen source for the growth of *E. coli* than did NB containing HF_{25tn}. However, no significant difference in colony size

of *E. coli* cultured on NA containing HF_{25tn}, HF_{25ts} and Bacto Peptone was observed ($p>0.05$).

An excessive amount of nitrogen did not increase the microbial growth (Lynch and Poole, 1979). The lower growth of *E. coli* was found in the medium containing HF_{25tn} than that comprising HF_{25ts}. The inhibition might be due to the differences in nutrient composition, unsuitable carbon and nitrogen ratio (C/N ratio) and some toxic materials (Dufosse *et al*., 2001). Kurbanoglu and Algur (2002) found that ram horn hydrolysates at a level of 4% were optimal for bacterial growth and applications higher than 4% had an inhibitory effect on growth.

5.4.2 3 *S. cerevisiae*

A great number of *S. cerevisiae* cultured in YMB comprising Bacto Peptone was found, when compared with that cultured in YMB containing HF_{25tn} or HF_{25ts} ($p<0.05$). YMB comprising Bacto Peptone might contain a suitable peptide size for the growth of *S. cerevisiae* under the condition used. However, a similar colony size was observed among *S. cerevisiae* cultured on YMA containing HF_{25tn}, HF_{25ts} and Bacto Peptone ($p>0.05$).

5.4.2.4 *C. albicans*

No differences in the number and colony size of *C. albicans* cultured on YMA containing HF_{25tn}, HF_{25ts} and Bacto Peptone were observed ($p>0.05$).

5.4.2.5 *A. oryzae*

Similar number and colony size of *A. oryzae* cultured in MEB containing HF_{25tn}, HF_{25ts} or Bacto Peptone was obtained ($p>0.05$). It might be due to the fact that all protein hydrolysates were available substrates as a nitrogen source for *A. oryzae.*

The media containing different amount of nitrogen sources from yellow stripe trevally protein hydrolysate (HF_{25tn}, HF_{25ts}) showed no effect on the quantitative and qualitative growth of *C. albicans* and *A. oryzae.* Differences in growth of *S. aureus, E. coli* and *S. cerevisiae* observed when culturing in HF_{25} and Bacto Peptone could be related to the differences in the amino acid composition of peptides in protein hydrolysates (De la Broise *et al*., 1998). Additionally, amount of

hydrolysate used could exhibit either stimulating or inhibitory effects on microbial growth, depending upon the types of microorganisms. The amounts of nitrogen sources affected the growth of *S. aureus* markedly for both positive and negative aspects. The growth of *S. aureus* in this system was most likely associated with the decrease in pH (Figure 20c). Thus, the use of buffer to maintain the pH of medium could be a promising means to solve the problem about colony size.

5.4.3 Effect of buffer on growth and colony size of *S. aureus*

When 0.2% K_2HPO_4 was supplemented in all media to maintain the pH during microbial cultivation, total viable count of *S. aureus* cultured in the medium containing HF_{25tn} was higher than that found in media containing HF_{25ts} or Bacto Peptone ($p<0.05$) (Table 10). When the cultivation time increased, the growth of *S. aureus* in all media increased throughout the log phase (4-12 h) (Figure 23a). *S. aureus* cultured in NB containing HF_{25tn} exhibited the higher cell density than did NB containing HF_{25ts} or Bacto Peptone, respectively, up to 24 h of cultivation time ($p<0.05$). *S. aureus* started the log phase at h 3 and the stationary phase was observed at h 16. However, the pH of the medium containing HF_{25tn} showed the greater decrease than did the media containing HF_{25ts} or Bacto Peptone ($p<0.05$) (Figure 23b). The more decrease in pH was related with the higher cell density of *S. aureus*. Nevertheless, the decrease in pH of NB supplemented with buffer was not as much as that of NB without buffer (Figure 20c and 23b). Additionally, no difference in colony size of *S. aureus* was observed among the media containing HF_{25tn}, HF_{25ts} and Bacto Peptone supplemented with 0.2 % K_2HPO_4 ($p>0.05$). K_2HPO_4 acts as a potential buffer in culturing system. The result reconfirmed that acidity might affect colony size as previously mentioned (Table 9). In the presence of buffer, *S. aureus* in the medium containing HF_{25tn} showed the higher growth than that cultured in the medium containing HF_{25ts} ($p<0.05$). This might be associated with the higher amount of nitrogen source for the growth. However, in the late log phase at h 12 of cultivation, no difference between the number of *S. aureus* culturing in media containing HF_{25tn} and HF_{25ts} was observed ($p>0.05$). Normally used as inoculants, cells in the late log phase are important. Thus, the medium containing HF_{25ts} was selected for further study.

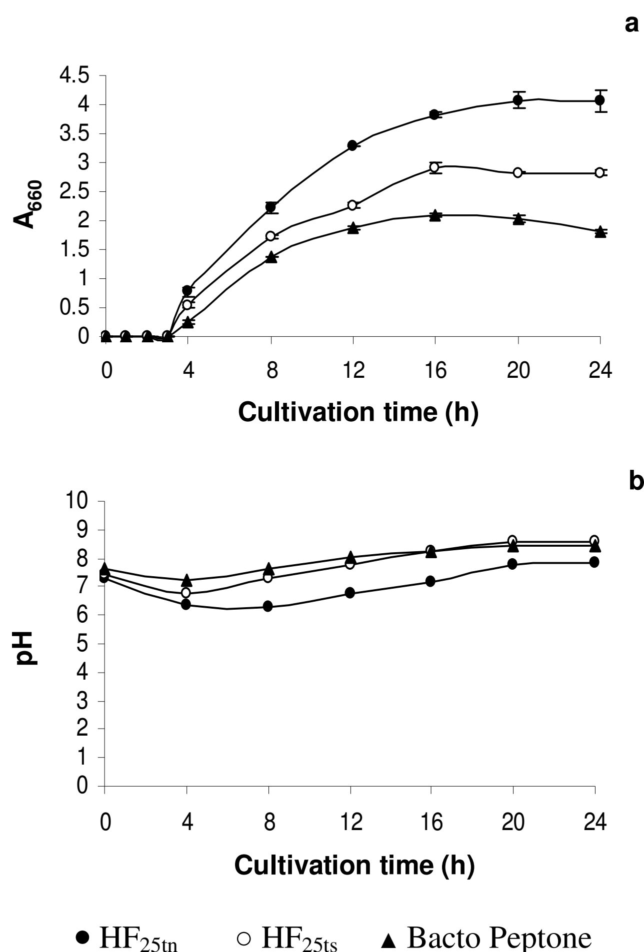


Figure 23. Growth curve (a) and pH changes (b) of *S. aureus* during cultivation in NB containing 0.2% K_2HPO_4 and protein hydrolysate from yellow stripe trevally prepared using Flavourzyme with 25% DH based on total nitrogen content of Bacto Peptone (HF_{25tn}), based on total solid content of Bacto Peptone (HF_{25ts}), compared with Bacto Peptone. Bars represent standard deviations from triplicate determinations.

5.4.4 Effect of protein hydrolysate from yellow stripe trevally on microbial morphology

Microstructures and size of microbial cells are shown in Figure 24 and Table 11. Sizes of cells of all microorganisms cultured on NA containing HF_{25ts} were similar to those of microorganisms cultured on NA with Bacto Peptone ($p>0.05$).

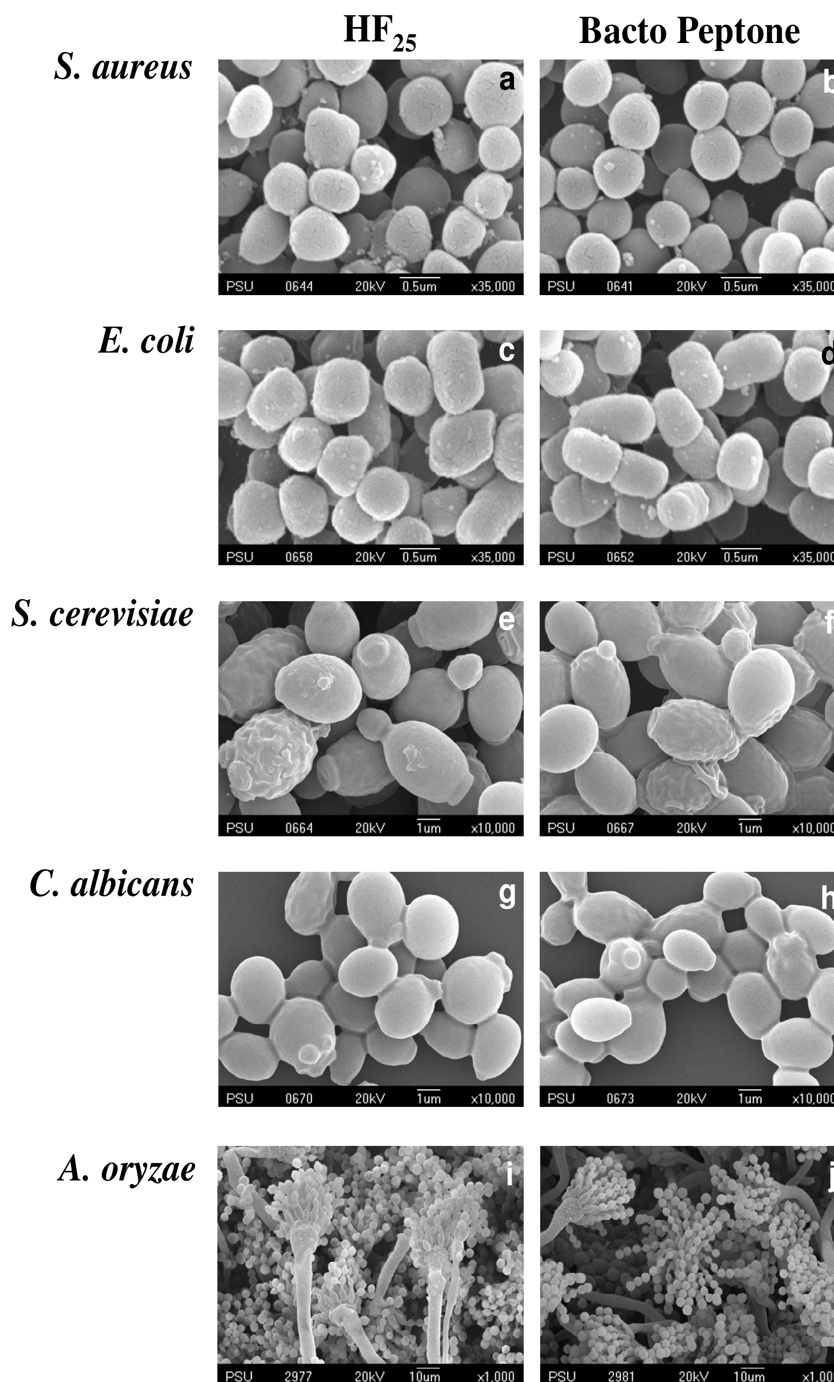


Figure 24. Scanning electron microscopic photographs of *S. aureus, E. coli*, *S. cerevisiae*, *C. albicans* and *A. oryzae* cultured on media containing protein hydrolysate from yellow stripe trevally prepared using Flavourzyme with 25% DH based on total solid content of Bacto Peptone (HF_{25ts}) and those cultured on Bacto Peptone.

Table 11 Size of microorganisms cultured on media containing protein hydrolysate from yellow stripe trevally prepared using Flavourzyme with 25% DH based on total solid contents of Bacto Peptone (HF_{25ts}), compared with Bacto Peptone

Microorganisms	Cell size (µm)	
	HF_{25ts}	Bacto Peptone
S. aureus: diameter	0.67±0.09 ns	0.66±0.05 ns
E. coli: length	0.84±0.12 ns	0.79±0.10 ns
: width	0.58±0.04 ns	0.54±0.03 ns
S. cerevisiae: length	3.68±0.70 ns	3.50±0.57 ns
: width	2.73±0.32 ns	2.70±0.22 ns
C. albicans: length	3.01±0.39 ns	2.81±0.33 ns
: width	2.34±0.31 ns	2.26±0.29 ns
A. oryzae: spore diameter	6.30±0.48 ns	6.01±0.43 ns

Means ± Standard deviations from twenty determinations based on SEM photomicrograph.
ns: non-significant difference (p>0.05).

Cells of *S. aureus* were round. Nevertheless, the cells cultured on NA comprising Bacto Peptone had a more regular spherical shape and finer surface than those cultured on NA containing HF_{25ts} (Figure 24a, b). *E. coli* was short rod in shape. The cells cultured on NA containing HF_{25ts} were similar in length and width to those cultured on NA containing Bacto Peptone (p>0.05) (Figure 24c, d). However, the cells cultured on Bacto Peptone had a more regular shape and smoother surface than that cultured in NA containing HF_{25ts}. Microscopic features of *S. cerevisiae* were unicellular and ellipsoidal in shape and showed the doubling by budding (Figure 24e, f). *C. albicans* were single ellipsoidal cells (Figure. 24g, h). Both *S. cerevisiae* and *C. albicans* cultured on YMA containing HF_{25ts} had a similar shape, in comparison with those cultured on YMA containing Bacto Peptone. For mold, *A. oryzae* cultured on MEA containing HF_{25ts} or Bacto Peptone produced upright conidiophores (Figure 24i, j), which are simple and terminate in a globose or clavate swelling (Tortora *et al.*, 1997). No difference in size of the conidia, which were I-celled and globose in shape were observed on MEA containing HF_{25ts} and Bacto Peptone (p>0.05). However, *A.*

oryzae started producing spore at h 38 and h 40 when cultured on MEA containing HF_{25ts} and Bacto Peptone, respectively (data not shown). The differences in starting production of conidiospores of *A. oryzae* culturing on MEA containing between HF_{25ts} and Bacto Peptone might be governed by peptide composition.

5.5 Conclusions

Efficacy of protein hydrolysate from yellow stripe trevally as microbial nutrient varied with DH, enzyme and amount of hydrolysate used. Microbial kinetics of bacteria, yeasts and mold cultured in media containing protein hydrolysate from yellow stripe trevally produced by Flavourzyme with 25% DH were comparable to those of commercial Bacto Peptone. Therefore, production of fish protein hydrolysate for upgrading low market value species as a high value nitrogenous substrate for microbial growth can be achieved.

CHAPTER 6

CHARACTERISTICS AND THE USE AS CULTURE MEDIA OF PROTEIN HYDROLYSATE FROM YELLOW STRIPE TREVALLY (*SELAROIDES LEPTOLEPIS*)

6.1 Abstract

Characteristics and the use as culture media of protein hydrolysate from yellow stripe trevally (HF_{25}) were determined in comparison with Bacto Peptone. HF_{25} had the higher contents of ash (45.73%), lipid (0.77%) and moisture (4.34%) but lower protein content (42.11%) than did Bacto Peptone ($p<0.05$). HF_{25} powder was slightly darker than Bacto Peptone ($p<0.05$). HF_{25} contained a higher amount of essential amino acids (44.05%) than did Bacto Peptone (19.34%). HF_{25} and Bacto Peptone consisted of several minerals at varying levels and had an excellent solubility over a wide pH range. At water activity greater than 0.75, the much higher moisture sorption was found in HF_{25} ($p<0.05$). HF_{25} showed the higher bacterial productivity ratio than did Bacto Peptone ($p<0.05$). When HF_{25} and commercial Bacto Peptone were used as microbial media to determine microbial load of environmental and food samples and pathogenic bacteria, HF_{25} generally exhibited similar potential in culturing those microorganisms ($p>0.05$).

6.2 Introduction

Fish processing by-products and under-utilized species are commonly recognized as low-value resources with negligible market value. Additionally, their inappropriate disposal is a major cause for environmental pollution. To maximize the use of those resources, hydrolysis processes have been developed to convert such resources into marketable and acceptable forms. Protein hydrolysates from many sources such as fish (De la Broise *et al*., 1998), ram horn (Kurbanoglu and Algur, 2004) and banana peel (Essien, *et al*., 2005) have been produced and used as microbial media and their performances were comparable to that of commercial

peptone (De la Broise *et al*., 1998; Kurbanoglu and Algur, 2004). Protein hydrolysates suitable for culturing microorganisms generally consist of organic and inorganic materials (Kurbanoglu and Algur, 2004). After hydrolysis, protein hydrolysates was rich in low molecular weight peptides and free amino acids, which can be used as an excellent nitrogen source for microbial growth (De la Broise *et al*., 1998). Hydrolysates also contained some minerals such as calcium, sodium, magnesium, potassium, phosphorous, iron, copper, manganese, zinc and sulfur required for microbial growth (Kurbanoglu and Algur, 2004; Essien, *et al*., 2005).

Protein hydrolysate also showed an excellent solubility over a wide pH range and the solubility profiles of capelin protein hydrolysate varied with the type of enzymes used (Shahidi *et al*., 1995). Moisture sorption of spray-dried Antarctic krill protein hydrolysate increased with increasing water activity (a_w) (Chiang, *et al*., 1999). The knowledge of the moisture sorption characteristics is crucial for shelf-life prediction and determination of critical moisture and water activity. Therefore, the deterioration mainly caused by moisture gain during drying, packaging and storage can be controlled (Labuza, 1984). Recently, protein hydrolysates from yellow stripe trevally have been produced successfully from yellow stripe trevally meat (Klompong *et al*., 2007a, b) and can be used as a nitrogen source in culture media. However, the information regarding chemical compositions, properties and the use as enumeration and biochemical test media of this protein hydrolysate was deficient. Therefore, the objective of this investigation was to study the chemical compositions, solubility, moisture sorption isotherm, cultural response of bacteria and the use as enumeration media of protein hydrolysate from yellow stripe trevally in comparison with commercial Bacto Peptone.

6.3 Materials and Methods

6.3.1 Enzyme and Bacto Peptone

Flavourzyme was obtained from Novozymes (Bagsvaerd, Denmark). Bacto Peptone was purchased from Difco Laboratories (Sparks, MD, USA).

6.3.2 Collection and preparation of fish samples

Yellow stripe trevally (*Selaroides leptolepis*) caught along the coast of the Andaman Sea with the size of 65g/fish, off-loaded approximately 24-36 h after capture, were obtained from the fishing port in Satul province, Thailand. Fish were placed in ice with the fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 2 h. Upon the arrival, fish were washed and the meat was separated manually. The meat was minced using a mincer with the 0.4 cm diameter holes. The mince was used for protein hydrolysate preparation.

6.3.3 Production of protein hydrolysate

Protein hydrolysate was prepared as per the method of Klompong *et al.* (2007a). Mince (60 g) was suspended in 240 ml of distilled water. The mixture was homogenized using a homogenizer (IKA Labortechnik, Selangor, Malaysia) at a speed of 11,000 rpm for 1 min. The homogenate was adjusted to pH 7.0 and preincubated at 50°C for 20 min prior to hydrolysis using Flavourzyme. The hydrolytic reaction was started by addition of 9.77% Flavourzyme (w/w) based on the protein content of mince (Klompong *et al.*, 2007a). The reaction was conducted using the pH-stat method (Adler-Nissen, 1986) for 20 min to obtain the degree of hydrolysis (DH) of 25% as described by Klompong *et al.* (2007a)

To terminate enzymatic reaction, the reaction mixture was heated in a water bath at 90°C for 15 min with an occasional agitation. The sample was cooled and the pH of sample was then adjusted to 7.0 with 1 M HCl or 1 M NaOH. Hydrolysate was centrifuged at 2000g for 10 min at 25°C using a centrifuge (Sorvall, RC 5B plus, Norwalk, CT, USA). Supernatant obtained was subjected to spray-drying using a spray dryer (Niro A/S, Gladsaxevej 305 DK-2860, Soeborg, Denmark). The resulting powder referred to as 'HF_{25}' was subjected to analyses in comparison with Bacto Peptone.

6.3.4 Characterization of HF_{25}

6.3.4.1 Proximate analysis

Moisture, protein, fat and ash contents of HF_{25} and Bacto peptone were determined according to the method of AOAC (2000) with the method No. of 950.46, 928.08, 960.39 and 920.153, respectively. Carbohydrate was determined by the phenol-sulfuric method (Dubois *et al.*, 1956).

6.3.4.2 Color measurement

The color of HF_{25} and Bacto Peptone was measured by Colorimeter (Hunter Lab, Color Flex, Reston, VA, USA) and reported in CIE system. L*, a* and b* indicating lightness, redness/greenness and yellowness/blueness, respectively, were recorded.

6.3.4.3 pH measurement

pH of HF_{25} and Bacto Peptone solutions (1% w/v) was measured by a pH meter (Sartorious, PB 10, Goettingen, Germany).

6.3.4.4 Amino acid analysis

HF_{25} and Bacto Peptone were placed in a 6 x 50 mm corning tube, previously heated at 525°C for 6 h. The tubes were placed into a hydrolysis vessel for vapor hydrolysis. To the hydrolysis vessel, 250 µl of 6 M HCl with 0.1% phenol were added. The vessel was flushed 3 times with nitrogen and evacuated for 2 min. The vessel was heated for 24 h at 110±2°C in an oven (Precision Scientific, Thelco Laboratory, Champaign, IL, USA). The tubes were removed, wiped to remove an excess acid, and dried in a speed-vac for 30 min. After the removal of acid, the samples were derivatized according to the vendor's recommendations (Waters Corporation, Milford, MA, USA) using AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) reagent. Thereafter, the samples were transferred to autosampler vials equipped with micro inserts (National Scientific, Rockwood, TN, USA) and heated gently for 10 min. Analysis of samples was performed using a Beckman HPLC (Beckman Coulter, Fullerton, CA, USA) (126 pump, 166 Detector, 507 autosampler, and "System Gold" data system) equipped with a Waters AccQTag

amino acid analysis column. Calibration was performed using an amino acid mixture standard (Pierce, Rockford, IL, USA).

6.3.4.5 Mineral analysis

The contents of calcium (Ca), magnesium (Mg), sodium (Na), potassium (K), phosphorous (P), iron (Fe), lead (Pb), zinc (Zn), cobalt (Co), copper (Cu), molybdenum (Mo) and manganese (Mn) in HF_{25} and Bacto Peptone were determined using the inductively coupled plasma optical emission spectrophotometer (ICP-OES) (Model 4300 DV, Perkin Elmer, Shelton, CT, USA) following the method of AOAC (2000). Solutions of HF_{25} and Bacto Peptone with a concentration of 12 % (w/v) were injected into ICP-OES. Flow rates of argon to plasma, auxiliary and nebulizer were maintained at 15, 0.2, and 0.8 l/min, respectively. Sample flow rate was set at 1.5 ml/min.

6.3.4.6 Determination of solubility

To determine protein solubility, HF_{25} and Bacto Peptone (200 mg) were dispersed in 20 ml of deionized water and pHs of the mixtures were adjusted to 4, 5, 6, 7 and 8 with 1 M HCl or 1 M NaOH. The mixtures were stirred at room temperature for 10 min, followed by centrifugation at 2000g for 15 min. Protein contents in the supernatant were determined by the Biuret method (Robinson and Hodgen, 1940). Total protein contents in the samples were determined after solubilization of the samples in 0.5 M NaOH. Protein solubility was calculated as follows:

$$\text{Solubility (\%)} = \frac{\text{Protein content in supernatant}}{\text{Total protein content in sample}} \times 100$$

6.3.4.7 Determination of moisture sorption isotherm

HF_{25} and Bacto Peptone were conditioned at 25°C in hermetically sealed desiccators containing various saturated salt solutions, including LiCl, $KC_2H_3O_2$, $MgCl_2$, K_2CO_3, $Mg(NO_3)_2$, $NaNO_2$, NaCl, KCl and K_2SO_4, which had the relative humidity of 11.2, 22.7, 32.8, 43.2, 52.9, 64.3, 75.3, 84.3, 97.3%, respectively. The water activity (a_w) of each salt solution was calculated as % Relative humidity

(RH)/100 (Labuza, 1984). Prior to determination, HF_{25} and Bacto Peptone (3 g) were transferred to glass petridishes and placed in desiccators containing different salt solutions. Samples were allowed to reach equilibrium (determined as less than 1% change in the sample mass) for one week at 25°C with the RH specified above. Adsorption isotherms of HF_{25} and Bacto Peptone were determined by plotting % moisture content (dry basis) versus a_w (Labuza, 1984).

6.3.5 Study on the cultural response of different bacteria on HF_{25}

6.3.5.1 Bacteria used

Staphylococcus aureus (*S. aureus*) TISTR 118, *Escherichia coli* (*E. coli*) TISTR 780 *and Salmonella typhi* (*S. typhi*) were obtained from the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand. Pathogenic *S. aureus* and Pathogenic *E. coli* were gifted from Songklanagarind Hospital, Prince of Songkla University, Thailand.

6.3.5.2 Preparation of media

Different media were prepared. Hugh and Leifson's O.F. medium (Basal medium) comprised 2 g HF_{25} or Bacto Peptone, 0.3 g dipotassium hydrogen phosphate, 5 g sodium chloride, 3 g agar and 4 ml of 1.6% bromthymol blue. Tryptone broth consisted of 10 g HF_{25} or Bacto Peptone. Triple Sugar Iron agar (TSI) contained 3 g beef extract, 3 g yeast extract, 15 g HF_{25} or Bacto Peptone, 10 g lactose, 10 g saccharose, 1 g dextrose, 0.2 g ferrous sulfate, 5 g sodium chloride, 0.3 g sodium thiosulfate and 0.024 g phenol red and 12 g agar. Glucose Yeast Extract Broth (GYEB) consisted of 100 g glucose and 10 g yeast extract. Nutrient Broth (NB) contained 3 g beef extract and 5 g Bacto Peptone or HF_{25}. To prepare Nutrient Agar (NA) and Glucose Yeast Extract Agar (GYEA), 15 g and 20 g agar was included in NB and GYEB, respectively. The mixtures were added with distilled water and the final volume was adjusted to 1 l with distilled water. Thereafter, the solution obtained was adjusted to pH 7.1 for Basal medium and to pH 7.4 for TSI and to pH 6.8 for NB, NA, GYEB and GYEA. The mixtures were autoclaved at 121°C for 15 min using an autoclave (Tomy, SS-325, Tokyo, Japan).

6.3.5.3 Cultural response

E. coli was inoculated into Basal medium and Tryptone broth to determine the existence of fermentable carbohydrates and indole production, respectively. *S. typhi* was inoculated into TSI for determining H_2S production. The inoculated media were incubated in an incubator (Memmert, BE 500, CA, USA) at 37°C for 24 h. The interpretation of results was carried out as described by Downes and Ito (2001).

6.3.5.4 Productivity ratio

Productivity ratio was determined according to the modified Miles-Misra method as described by Corry and Atabay (1997). To elucidate the effect of HF_{25} and Bacto Peptone on the bacterial productivity, GYEB and GYEA were used instead of NB and NA since they contained no Bacto Peptone. One loopful of *S. aureus* TISTR 118 and *E. coli* TISTR 780 cultures was subcultured on GYEA and incubated at 37°C for 18 h. Thereafter, bacteria were inoculated into GYEB and then incubated at 37°C for 18 h with a continuous shaking at 120 rpm to obtain stationary phase inocula. Serial dilutions of *S. aureus* and *E. coli* were prepared with sterile 0.85% NaCl solution (normal saline). Thereafter, aliquots of 0.1 ml of appropriate dilution were pipetted onto NA containing HF_{25} or Bacto Peptone and then spread using a sterile spreader. NA containing Bacto Peptone was used as the reference medium. The plates were upside-down incubated at 37°C for 24 h before counting. The colony counts were recorded. Productivity ratio was calculated as follows:

Productivity ratio = count on test medium/count on reference medium

6.3.6 Study on enumeration of microorganisms from different sources

6.3.6.1 Samples collection and preparation

a. Garden soil/swamp water

The garden soil and swamp water were aseptically collected from the garden of the Department of Food Technology, Prince of Songkla University, Thailand, following the method of Downes and Ito (2001). For soil, the sample (50 g) was aseptically weighed and placed in 450 ml of normal saline,

providing a 1:10 dilution as the initial dilution. The mixture was vigorously shaken at 120 rpm for 30 min (Downes and Ito, 2001) using a shaking incubator (VS-8480SR-L, LMS, Hat Yai, Thailand). Preparation of swamp water was performed aseptically by the method of Downes and Ito (2001).

b. Pasteurized milk/ground pork

Pasteurized milk was procured from the Department of Food Technology, Prince of Songkla University. Preparation of the sample was carried out aseptically by the method of Downes and Ito (2001).

Ground pork was purchased from Tesco Lotus supermarket, Hat Yai, Thailand. Sample (25 g) was aseptically weighed and transferred into 225 ml normal saline, providing a 1:10 dilution as the initial dilution. The mixture was mixed well using a stomacher (Seward 400 C, Ontario, Canada) at a normal speed for 3 min (Downes and Ito, 2001).

c. Pathogenic bacteria

One loopful of Pathogenic *S. aureus* and Pathogenic *E. coli* cultures was subcultured on NA slant containing HF_{25} or Bacto Peptone. The culture was incubated at 37°C for 15 h. Thereafter, each bacterium was inoculated into NB containing HF_{25} or Bacto peptone and then incubated at 37°C for 15 h with continuous shaking at 120 rpm using a shaking incubator (VS-8480SR-L, LMS, Hat Yai, Thailand). Culture broths obtained were used as the initial dilution.

6.3.6.2 Microbial enumeration

Serial dilutions of garden soil, swamp water, pasteurized milk, ground pork, pathogenic *S. aureus* and pathogenic *E. coli* samples were diluted with sterile normal saline. Aliquots of 0.1 ml suspension were pipetted onto NA containing HF_{25} or Bacto Peptone and then spread using a sterile spreader. The plates were upside-down incubated at 37°C for 72 h. The colony counts were recorded at h 24, 48 and 72 of incubation period.

6.3.7 Statistical analysis

Data were subjected to Analysis of Variance (ANOVA). Mean comparison was performed using Duncan's multiple range test. For pair comparison,

independence-samples t-test was used (Steel and Torrie, 1980). Statistical analysis was carried out using SPSS statistic program (Version 11.0) for Windows (SPSS Inc. Chicago, IL, USA).

6.4 Results and Discussion

6.4.1 Characteristics of yellow stripe trevally protein hydrolysate

Proximate composition and color of spray-dried yellow stripe trevally protein hydrolysate prepared using Flavourzyme with 25% DH (HF_{25}) and Bacto Peptone is shown in Table 12. HF_{25} contained the lower protein content but the higher ash, fat and moisture contents than did Bacto Peptone ($p<0.05$). HF_{25} powder showed the lower L* value but higher a* value than did Bacto Peptone ($p<0.05$). However, no difference in b* value was observed between HF_{25} and Bacto Peptone ($p>0.05$). The result indicated that HF_{25} had the lower lightness and the slightly higher redness than did Bacto Peptone.

The lower protein content (42.11%) found in HF_{25} was associated with the higher ash content (45.73%). During hydrolysis, pH of hydrolysis system was controlled to obtain the constant value, in which the hydrolysis by Flavourzyme could be maximized (Adler-Nissen, 1986). In the present study, 2 M NaOH was added throughout the hydrolysis process to maintain the pH (Klompong et al., 2007a). Consequently, the salt was formed from the neutralization process as indicated by high ash content. The high ash content (33.2%) was also found in shrimp hull hydrolysate produced by pH-stat method (Stephens *et al.*, 1976). Lipid content of HF_{25} (0.77%) was lowered when compared with that of intact yellow stripe trevally flesh (3.23%) (Klompong *et al.*, 2007a). This might be due to the removal of fat layer after hydrolysis by winterization. Additionally, the muscle cell membranes were prone to round up and formed insoluble vesicles. Thus, the removal of membrane structured lipids during the hydrolysis could be carried out with ease. This may enhance the storage stability of the hydrolysate (Shahidi *et al.*, 1995). The reduction of lipid content after hydrolysis was also reported in protein hydrolysate from round scad (Thiansilakul *et al.*, 2007). The spray-dried HF_{25} contained higher moisture content than did Bacto Peptone, more likely caused by the differences in drying

Table 12 Characteristics of yellow stripe trevally protein hydrolysate (HF_{25}) and Bacto Peptone

Characteristics	HF_{25}	Bacto Peptone	
Compositions (%)			
Protein	42.11±3.80 [b] [#]	85.87±1.37 [a] [¥]	N/A [ψ]
Nitrogen	6.74±0.61 [b]	13.74±0.22 [a]	14.2-17.4
Ash	45.73±0.76 [a]	4.24±0.30 [b]	0.0-6.2
Lipid	0.77±0.11 [a]	0.59±0.09 [b]	N/A
Moisture	4.34±0.01 [a]	2.65±0.32 [b]	0.0-5.0
Carbohydrate	7.19±0.70 [ns]	6.48±0.50 [ns]	6.9
L*	84.90±0.38 [b]	90.45±0.16 [a]	N/A
a*	2.49±0.16 [a]	1.10±0.09 [b]	N/A
b*	16.00±0.22 [ns]	16.20±0.10 [ns]	N/A
pH of 1% solution at 25°C	6.90±0.01 [b]	6.96±0.01 [a]	6.8-7.2

Means ± Standard deviations from triplicate determinations.
[#] Different superscripts in the same row (a and b) indicate the significant difference ($p<0.05$); ns: non-significant difference ($p>0.05$).
[¥] Data from experiment.
[ψ] Data from Certificate of analysis of Bacto Peptone and the Difco Manual (Becton Dickinson and Company, Sparks, MD, USA).
N/A: not applicable.

condition. Moreover, the different amino acids and peptides between HF_{25} and Bacto Peptone might determine the differences in moisture adsorption during handling and storage.

HF_{25} possessed the lower lightness and higher redness than did Bacto Peptone. Flavourzyme used in hydrolysis process might contribute to the dark color of resulting hydrolysate. Additionally, the pigments in the muscle of yellow stripe trevally, a dark fleshed fish, might undergo the oxidation, resulting in dark color of resulting product. Chaijan *et al* (2004) reported that dark fleshed fish such as sardine and mackerel contained a high amount of myoglobin.

When compared with the certificate of analysis of Bacto Peptone, moisture content and pH of HF_{25} were in the range of specification. However, the nitrogen content was lower while carbohydrate and ash contents were higher than the limits.

The differences in proximate composition and color between HF_{25} and Bacto Peptone were probably caused by the differences in raw material, pretreatment, hydrolysis condition (pH, temperature), enzyme type used, and drying process. Shahidi *et al*. (1995) concluded that the composition of protein hydrolysates from capelin hydrolysate varied with the types of enzyme used. Thiansilakul *et al*. (2007) reported that the varying color of protein hydrolysate from round scad depended on the composition of the raw material, enzyme used and hydrolysis conditions.

6.4.2 Amino acid composition of yellow stripe trevally protein hydrolysate

Amino acid compositions of HF_{25} and Bacto Peptone are shown in Table 13. HF_{25} had the different amino acid profiles from Bacto Peptone. Glutamic acid, glutamine, aspartic acid, asparagine, alanine, leucine, glycine and lysine were dominant in HF_{25}. For Bacto Peptone, glycine constituted as the most predominant amino acid. Glycine content in Bacto Peptone used in this present study was higher than that of the certificate of analysis. Furthermore, proline, glutamic acid, glutamine, aspartic acid, asparagine, arginine and alanine were found at high content in Bacto Peptone. HF_{25} had the higher content of most amino acids, except glycine, arginine, alanine and proline. HF_{25} had higher contents of essential amino acid such as histidine, threonine, valine, methionine, lysine, isoleucine, leucine and phenylalanine than did Bacto Peptone. The absence of tryptophan in HF_{25} and Bacto Peptone mainly resulted from degradation during acid hydrolysis prior to amino acid analysis. As a nitrogen source, HF_{25} was abundant in amino acids required for microbial growth. However, the utilization of peptides relied on the ability of cells to transport amino acids or small peptides across plasma membrane (Downes and Ito, 2001).

The differences in amino acid profile between HF_{25} and Bacto Peptone might be caused by the existing differences in raw material, pretreatment, hydrolysis conditions such as pH and temperature of hydrolysis process and enzyme type used. Benjakul and Morrissey (1997) showed that there were some differences in amino composition among Pacific whiting solid waste, muscle and hydrolysate obtained due

Table 13 Amino acid compositions of yellow stripe trevally protein hydrolysate (HF_{25}) and Bacto Peptone

Amino acids (%)	HF_{25}	Bacto Peptone	
Asp+Asn	8.88	7.16 [¥]	5.60 [Ψ]
Ser	5.37	3.40	2.87
Glu+Gln	12.23	11.52	10.21
Gly	8.49	26.40	15.59
His [a]	2.84	0.89	0.58
Arg	3.93	7.78	6.76
Thr [a]	6.44	2.39	1.18
Ala	8.97	9.50	8.67
Pro	3.93	12.96	8.82
Cys/2	1.71	ND	0.20
Tyr	6.43	1.93	0.64
Trp	ND	ND	0.36
Val [a]	3.82	3.29	2.35
Met [a]	2.14	0.60	1.19
Lys [a]	7.95	4.03	3.42
Ile [a]	4.95	1.79	1.45
Leu [a]	8.95	3.71	3.01
Phe [a]	2.96	2.64	1.81

[a] Essential amino acids.
ND: not detectable.
[¥] Data from experiment.
[Ψ] Data from the Difco Manual (Becton Dickinson and Company, Sparks, MD, USA); Asn and Gln were not included.

to the differences in protein compositions of raw material. The levels and compositions of free amino acids and small peptides were changed during hydrolysis, counting on enzyme specificity (Wu *et al*., 2003).

6.4.3 Mineral content of yellow stripe trevally protein hydrolysate

HF_{25} contained quantitatively different minerals from Bacto Peptone as depicted in Table 14. HF_{25} possessed the higher level of all minerals than did Bacto Peptone. However, Fe content in HF_{25} and Bacto Peptone used in this study was lower than that of the certificate of analysis. Generally, both HF_{25} and Bacto Peptone had the low levels of Pb, Co, Mo, and Mn. Na, K, and P were found at high contents, while Mg, Ca, Zn, Fe and Cu constituted at low levels in both HF_{25} and Bacto Peptone. The high content of Na resulted from the formation of NaCl during the production of hydrolysate. Certain transition metals such as Fe, Cu and Co could act as the pro-oxidants in the hydrolysate. However, some minerals are required for microbial growth. Zn, Mo, Cu and Mg act as a cofactor of some enzymes (Edmonds, 1978). Fe is required by all aerobes. Co is a constituent of vitamin B12, while sulfur (S) is a component of some amino acids such as cysteine and methionine (Edmonds, 1978). Na and Cl are the major intra-extracellular cation and anion, respectively. P is involved in energy transfer and is a component of some macromolecules, while K is used for protein synthesis (Edmonds, 1978). Additionally, Ca is a component of membranes and endospores (Edmonds, 1978). Essien *et al*. (2005) found the high amount of K, P, Na and Mg and low level of Fe and Ca in banana peel extract, which was suitable for mold growth and biomass production. Sathivel *et al*. (2003) reported that K, Mg, P, Na, S and Ca were abundant in herring and herring by-product hydrolysates and varied with substrate used. Therefore, HF_{25} was an important source of several ions required for microbial growth.

6.4.4 Solubility of yellow stripe trevally protein hydrolysate

Solubility of HF_{25} and Bacto Peptone in the pH range of 4–8 is depicted in Figure 25. Both HF_{25} and Bacto Peptone were soluble over a wide pH range with the solubility greater than 91%. As the pH increased, the solubility of HF_{25} increased ($p<0.05$), while no differences in solubility were observed in Bacto Peptone over a wide pH range ($p>0.05$). The high solubility of HF_{25} was mainly due to the existence of low molecular weight peptides (lower than 7 kDa) (Klompong *et al*., 2007b). HF_{25} might possess proportionally more polar residues with the ability to form hydrogen

Table 14 Mineral contents of yellow stripe trevally protein hydrolysate (HF_{25}) and Bacto Peptone

Minerals (%)	HF_{25}	Bacto Peptone	
Na	7.050	0.535 [¥]	1.759 [Ψ]
K	0.589	0.200	0.203
Mg	0.064	0.001	0.007
Ca	0.025	0.002	0.008
P	1.740	0.203	0.445
Mo	<0.000008	<0.000008	N/A
Mn	<0.000007	<0.000007	<0.001
Fe	0.000683	0.000158	0.004
Co	<0.000003	<0.000003	<0.001
Pb	<0.000026	<0.000026	<0.001
Cu	0.000192	0.000017	<0.001
Zn	0.002	0.000408	0.001
Cl	N/A	N/A	1.086
S	N/A	N/A	0.410

[¥] Data from experiment.
[Ψ] Data from the Difco Manual (Becton Dickinson and Company, Sparks, MD, USA).
N/A: not applicable.

bonds with water (Gbogouri *et al*., 2004). Additionally, newly exposed ionizable amino and carboxyl groups which increased the hydrophilicity might enhance solubility (Gbogouri *et al*., 2004). Chiang *et al*. (1999) reported that soy protein hydrolysates produced using Alcalase and Flavourzyme were completely soluble over the pH range of 2-9. At pH 4, the lowest solubility of HF_{25} was noticeable ($p<0.05$). Hydrolysates generally show low solubility at their isoelectric points (Klompong *et al*., 2007a). Solubility variations could be attributed to both net charge of peptides that increases as pH moves away from pI and surface hydrophobicity that promotes the aggregation via hydrophobic interaction (Gbogouri *et al*., 2004). The differences in solubility between HF_{25} and Bacto Peptone might be due to the differences in type of raw material, enzyme used, pretreatment condition and hydrolysis process, which resulted in the different size, charge, amino acid sequence and other characteristics of

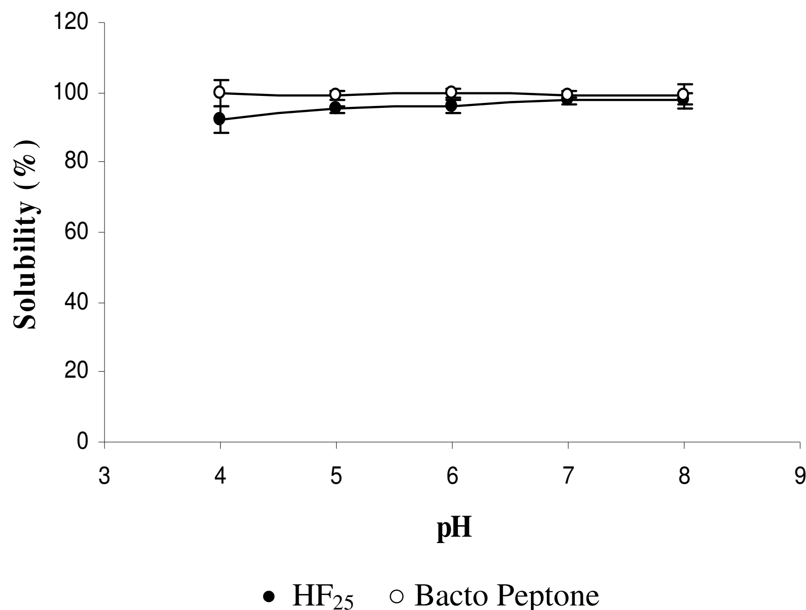


Figure 25. Solubilities of protein hydrolysate from yellow stripe trevally (HF_{25}) and Bacto Peptone as influenced by pHs. Bars represent the standard deviations from triplicate determinations.

hydrolysates. Shahidi *et al.* (1995) concluded that capelin protein hydrolysate prepared by different enzymes showed different solubility profiles at different pH condition owing to the differences in bond specificity of enzymes used. With high solubility over a wide pH range, HF_{25} could be served as readily available nutrient, that could be consumed efficiently by microorganisms cultured in the media with different pHs.

6.4.5 Moisture sorption isotherm of yellow stripe trevally protein hydrolysate

Moisture sorption isotherms of HF_{25} and Bacto Peptone at 25°C are shown in Figure 26. The moisture sorption of HF_{25} and Bacto Peptone increased with increasing a_w ($p<0.05$). Moisture sorption gradually increased when a_w increased up to 0.75. A dramatic increase in moisture sorption was observed at a_w higher than 0.75 ($p<0.05$). No difference between moisture sorption of HF_{25} and Bacto Peptone was observed at the a_w ranging from 0.1 to 0.75 ($p>0.05$). However, at the a_w higher than

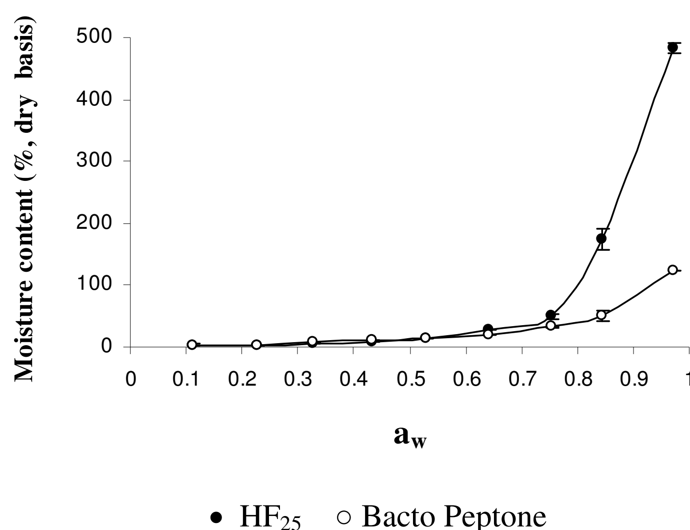


Figure 26. Moisture sorption isotherms of protein hydrolysate from yellow stripe trevally (HF_{25}) and Bacto Peptone. Bars represent the standard deviations from triplicate determinations.

0.75, the higher moisture sorption was observed in HF_{25} in comparison with Bacto Peptone ($p<0.05$). The result indicated that HF_{25} and Bacto Peptone were hygroscopic and HF_{25} was able to bind water more effectively than Bacto Peptone. The hydrophilicity of hydrolysates was most likely associated with a relatively large amount of ionizable polar amino acids such as serine, histidine, threonine, tyrosine and lysine (Table 13). Ionizable polar amino acids can bind almost 3 times as much water as that of non-ionizable polar groups (Chou and Morr, 1979). In native protein, some of these groups are buried and thus are not available for water binding (Chiang *et al.*, 1999). Shahidi *et al.* (1995) found that moisture adsorption of capelin protein hydrolysate with 12% DH increased from 1.56 to 6.23%, as the a_w increased from 0.5 to 0.7. Chiang *et al.* (1999) reported that the water binding capacity of soy protein hydrolysates produced using Alcalase and Flavourzyme increased by 1.8-3.4 times at a_w of 0.6-0.95 as compared to the intact isolated soy protein. Hydrolysates were more effective water binding agents than did the intact isolated soy protein. Protein hydrolysates from Antarctic krill meat and soy were used as a suppressive additive

against the denaturation of myofibrillar protein (Zhang *et al*, 2002) and as a reagent to bind water and improve the texture of foods (Chiang *et al.*, 1999).

The differences in sorption isotherm between HF_{25} and Bacto Peptone might be because of the differences in amino acid composition (Zhang *et al.*, 2002), enzymes used (Chiang *et al.*, 1999), the condition of production and drying process. Apart from the environmental conditions and container permeability, equilibrium moisture sorption isotherm of a product is a key factor governing the rate of moisture uptake by the packed product during storage. Therefore, it would be beneficial, if the moisture sorption isotherm of a product can be predicted at the early stage of product development (Labuza, 1984).

6.4.6 Effect of yellow stripe trevally protein hydrolysate on cultural response of bacteria in comparison with Bacto Peptone

Cultural response of bacteria to HF_{25} and Bacto Peptone is shown in Table 15. HF_{25} contained the fermentable carbohydrates as indicated by the positive result tested by *E. coli*, while Bacto peptone did not contain fermentable carbohydrates. The presence of fermentable carbohydrate might result in the decrease in pH of media when the bacteria, particularly *S. aureus*, were cultured. However, the use of appropriate buffer could tackle this problematic decrease in pH of microbial media (Figure 20). Additionally, to maximize the use as culture media, the fermentable carbohydrates in HF_{25} should be removed properly to avoid such a drawback. HF_{25} and Bacto Peptone also comprised tryptophan and sulfur amino acids as shown by indole and H_2S production, respectively (Tsoraeva and Zhurbenko, 2000). Although the cultural response revealed the presence of tryptophan in both samples, it was not detectable by amino acid analysis (Table 13). This was probably due to the degradation during acid hydrolysis when HF_{25} and Bacto Peptone were subjected to amino acid analysis (Shahidi *et al.*, 1995).

Higher productivity ratio of *S. aureus* and *E. coli* cultured in NA containing HF_{25} was noticeable in comparison with that observed in NA with Bacto Peptone (Table 15). The differences might be associated with the differences in size of peptides, chemical composition, amino acids, minerals and solubility between both

Table 15 Cultural response of bacteria on HF_{25} in comparison with Bacto Peptone

Test	Organisms	Response		
		HF_{25}	Bacto Peptone	
Fermentable carbohydrates	*E. coli*	positive	negative [¥]	negative [Ψ]
Indole production	*E. coli*	positive	positive	positive
H_2S production	*S. typhi*	positive	positive	positive
Productivity ratio	*S. aureus*	1.21	1.00	N/A
	E. coli	1.39	1.00	N/A

[¥] Data from experiment.
[Ψ] Data from Certificate of analysis of Bacto Peptone (Becton Dickinson and Company, Sparks, MD, USA).
N/A: not applicable.

samples. Most modern culture media mostly contain different protein hydrolysates, which are produced from various protein sources with the aid of selected proteases (Tsoraeva and Zhurbenko, 2000).

6.4.7 Effect of yellow stripe trevally protein hydrolysate on microbial enumeration of environmental samples, food samples and pathogenic bacteria

The growth of microorganisms in environmental samples (garden soil and swamp water), food samples (pasteurized milk and ground pork) and pathogenic bacteria (*S. aureus* and *E. coli*) on NA containing HF_{25} or Bacto Peptone is shown in Table 16. When culturing all microorganisms, the similar growth was obtained between NA containing HF_{25} and Bacto Peptone ($p>0.05$). However, the higher growth of *E. coli* was observed when cultured on NA with HF_{25} ($p<0.05$) that was in accordance with the productivity ratio of HF_{25} (Table 15). On the other hand, the higher growth of microorganisms from the pasteurized milk sample was noticeable in NA containing Bacto Peptone ($p<0.05$). HF_{25} and Bacto Peptone might provide some readily available nutrient suitable for pathogenic *E. coli* and microorganisms from pasteurized milk, respectively. Bacto Peptone was produced from beef and might provide microorganisms in milk a familiar nutrient. This resulted in the shorter lag phase, the lower generation time and the higher growth in Bacto Peptone than that

Table 16 Growth of microorganisms from environmental samples, food samples and pathogenic bacteria on NA containing yellow stripe trevally protein hydrolysates (HF_{25}) or Bacto Peptone

Samples	Incubation time (h)	Number (Log CFU/ml)	
		HF_{25}	Bacto Peptone
Garden soil	24	4.18±0.10 [ns #]	4.38±0.28 [ns]
	48	4.38±0.02 [ns]	4.58±0.22 [ns]
	72	4.46±0.24 [ns]	4.60±0.24 [ns]
Swamp water	24	3.25±0.05 [ns]	3.09±0.13 [ns]
	48	4.49±0.14 [ns]	4.53±0.08 [ns]
	72	4.49±0.14 [ns]	4.53±0.08 [ns]
Pasteurized milk	24	3.19±0.14 [b]	3.46±0.01 [a]
	48	3.21±0.13 [b]	3.50±0.01 [a]
	72	3.39±0.02 [b]	3.54±0.01 [a]
Ground pork	24	3.90±0.06 [ns]	3.97±0.03 [ns]
	48	4.07±0.03 [ns]	4.07±0.04 [ns]
	72	4.17±0.01 [ns]	4.17±0.02 [ns]
Pathogenic *S. aureus*	24	6.99±0.02 [ns]	6.99±0.02 [ns]
	48	6.99±0.02 [ns]	6.99±0.02 [ns]
	72	6.99±0.02 [ns]	6.99±0.02 [ns]
Pathogenic *E. coli*	24	7.87±0.01 [a]	7.71±0.06 [b]
	48	7. 87±0.01 [a]	7.71±0.06 [b]
	72	7. 87±0.01 [a]	7.71±0.06 [b]

Means ± Standard deviations from triplicate determinations.
[#] Different superscripts in the same row indicate the significant difference ($p<0.05$); ns: non-significant difference ($p>0.05$).

found in HF_{25}, which was produced from fish meat. It was noted that the number of microorganisms from garden soil culturing on NA containing HF_{25} and Bacto Peptone was quite low. This might be associated with the low nutrients content in soil used for microbial growth.

For garden soil, swamp water, pasteurized milk and ground pork samples, the numbers of microorganisms increased as the incubation time increased from 24 to

72 h. However, no increase in the number of *S. aureus* and *E. coli* was observed on NA containing either HF_{25} or Bacto Peptone after 24 h of incubation ($p>0.05$). In general, the colony size of both *S. aureus* and *E. coli* increased as the incubation time increased (data not shown). For natural samples including garden soil, swamp water, pasteurized milk, and ground pork, they might contain many kinds of bacteria, yeasts and molds having different generation times. Additionally, microorganisms from different sources might take different times to recover and grow under the suitable condition in culture media (Downes and Ito, 2001). For pasteurized milk, microorganisms were subjected to heat treatment in order to eliminate pathogens during pasteurization, resulting in the sublethal injury of some bacteria (Downes and Ito, 2001). The result revealed that HF_{25} did not exhibit inhibitory effects on the microorganisms tested and could serve as a suitable nitrogen source in enumeration media for culturing microorganisms. HF_{25} was rich in both essential organic and inorganic substances such as nitrogen, amino acids and minerals, which are required for microbial growth. Microorganisms need the nitrogen sources, energy sources, mineral salts and some specific growth factor for surviving (Downes and Ito, 2001). Kurbanoglu and Algur (2002) reported that ram horn hydrolysate had an ability to support growth of bacteria in pure cultures and in natural samples such as soil, water, milk and meat. Ram horn hydrolysate medium was also found to be a suitable medium for the enumeration of bacteria from environmental and food samples (Kurbanoglu and Algur, 2004).

6.5 Conclusions

Protein hydrolysate from yellow stripe trevally prepared using Flavourzyme with 25% DH was rich in both organic, particularly protein and inorganic substances required for microbial growth. It had an excellent solubility over a wide pH range, but it was hygroscopic when stored at high relative humidity. Growth of microorganisms cultured in media containing HF_{25} was comparable to that including commercial Bacto Peptone. Therefore, production of fish protein hydrolysate for upgrading low market value species as a high value nitrogen source for microbial growth can be achieved.

CHAPTER 7

STORAGE STABILITY OF PROTEIN HYDROLYSATE FROM YELLOW STRIPE TREVALLY (*SELAROIDES LEPTOLEPIS*) USED AS MICROBIAL MEDIA

7.1 Abstract

Storage stability during storage at room temperature of protein hydrolysate from yellow stripe trevally prepared using Flavourzyme with a degree of hydrolysis of 25% (HF_{25}) was determined in comparison with the commercial Bacto Peptone. During storage of 12 weeks, slight increases in water activity and moisture content of HF_{25} and Bacto Peptone were observed ($p<0.05$). Slight decrease in lightness of HF_{25} and Bacto Peptone was found ($p<0.05$). Generally, browning intensity, A_{294} and fluorescent intensity of HF_{25} and Bacto Peptone increased during storage ($p<0.05$). Both samples had an excellent solubility at pHs 5 and 7 throughout the storage. No change in turbidity of both samples before and after autoclaving was observed ($p>0.05$). Thiobarbituric acid reactive substances in HF_{25} and Bacto Peptone increased ($p<0.05$). The efficacy of HF_{25} as media for *Staphylococcus aureus* and *Escherichia coli* was generally equivalent to that of Bacto Peptone and was quite stable during storage ($p>0.05$).

7.2 Introduction

Hydrolysis processes have been used to convert the proteinaceous materials including by-products from fish processing plant. Protein hydrolysates generally exhibited the improved functional properties associated with the increased solubility. Protein hydrolysates from many sources such as fish (De la Broise *et al*., 1998), ram horn (Kurbanoglu and Algur, 2002) and banana peel (Essien, *et al*., 2005) have been used as microbial media and their performances were comparable to that of commercial peptone (De la Broise *et al*., 1998; Kurbanoglu and Algur, 2002). As

microbial media, protein hydrolysate should be clear or only slightly colored and free from precipitates (Kurbanoglu and Algur, 2002).

Due to its hydrolyticity in nature, the physical and chemical changes take place rapidly when the storage condition is not appropriate. The color of freeze-dried protein hydrolysate from round scad was stable for 6 weeks when storage at 4°C and the changes in color were more pronounced when storage temperature increased. The solubility of round scad protein hydrolysate decreased during 6 weeks of storage due to the aggregation of those peptides (Thiansilakul *et al*., 2007). The available lysine content of dried whey concentrate decreased to the highest extent when stored at water activity (a_w) of 0.41 and 40°C. The loss by 23% was obtained after 3 months of storage (Lindemann-Schneider and Fennema, 1989). Losses in lysine via Maillard browning reaction were influenced more by temperature than by a_w. However, methionine and tryptophan contents were not changed significantly during storage at any of the conditions tested (Lindemann-Schneider and Fennema, 1989). The decrease in lipid content reduced lipid oxidation and enhanced the storage stability of capelin hydrolysate (Shahidi *et al*., 1995).

Recently, protein hydrolysates from yellow stripe trevally have been produced successfully (Klompong *et al*., 2007a, b). Protein hydrolysate from yellow stripe trevally prepared using Flavourzyme with 25% degree of hydrolysis (HF_{25}) can be used as a nitrogen source in culture media and exhibited the comparable performance to Bacto Peptone (Klompong *et al*., 2008). However, the information regarding storage stability of yellow stripe trevally protein hydrolysate has not been reported. Therefore, the objective of this study was to investigate the stability during storage of HF_{25} in comparison with commercial Bacto Peptone.

7.3 Materials and Methods

7.3.1 Enzyme and cultivation medium

Flavourzyme was obtained from Novozymes (Bagsvaerd, Denmark). Bacto Peptone was purchased from Difco Laboratories (Sparks, MD, USA).

7.3.2 Collection and preparation of fish sample

Yellow stripe trevally (*Selaroides leptolepis*) caught along the coast of the Andaman Sea with the size of 65g/fish, off-loaded approximately 24-36 h after capture, were obtained from the fishing port in Satul province, Thailand. Fish were placed in ice with the fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 2 h. Upon the arrival, fish were washed and the meat was separated manually. The meat was minced using a mincer with the 0.4 cm diameter holes. The mince was used for hydrolysate preparation.

7.3.3 Production of protein hydrolysates

Protein hydrolysate was prepared as per the method of Klompong *et al*. (2007a). Mince (60 g) was suspended in 240 ml of distilled water. The mixture was homogenized using a homogenizer (IKA Labortechnik, Selangor, Malaysia) at a speed of 11,000 rpm for 1 min. The homogenate was adjusted to pH 7.0 and preincubated at 50°C for 20 min prior to hydrolysis using Flavourzyme. The hydrolytic reaction was started by addition of 9.77% Flavourzyme (w/w) based on the protein content of mince (Klompong *et al*., 2007a). The reaction was conducted at pH 7.0 using the pH-stat method (Adler-Nissen, 1986) for 20 min to obtain the degree of hydrolysis (DH) of 25% as described by Klompong *et al*. (2007a)

To terminate enzymatic reaction, the reaction mixture was heated in a water bath at 90°C for 15 min with an occasional agitation. The sample was cooled and the pH of sample was then adjusted to 7.0 with 1 M HCl or 1 M NaOH. Hydrolysate was centrifuged at 2000g for 10 min at 25°C using a centrifuge (Sorvall, RC 5B plus, Norwalk, CT, USA). Supernatant obtained was subjected to spray-drying using a spray dryer (Niro A/S, Gladsaxevej 305 DK-2860, Soeborg, Denmark). The resulting powder referred to as 'HF_{25}' (approximately 100g) was placed in airtight plastic container and the lid was closed tightly. Bacto Peptone was kept under the same condition at room temperature. At week 0, 1, 2, 4, 6, 8, 10 and 12, the samples were taken randomly for physicochemical determination and microbiological analyses as microbial medium.

7.3.4 Determination of moisture contents and a_w

Moisture contents of HF_{25} and Bacto Peptone were measured according to the method of AOAC (2000) with the method No. of 950.46. a_w was determined using Water activity measuring system (Novasina, Thermoconstanter, Zurich, Switzerland).

7.3.5 Color measurement

The color of HF_{25} and Bacto Peptone was measured by Colorimeter (Hunter Lab, Color Flex, Reston, VA, USA) and reported in CIE system. L*, a* and b* indicating lightness, redness/greenness and yellowness/blueness, respectively, were recorded.

7.3.6 Browning index measurement

7.3.6.1 Browning intensity

Browning intensity of HF_{25} and Bacto Peptone was measured according to the method of Ajandouz *et al*. (2001). HF_{25} and Bacto Peptone solutions (1%, w/v) were prepared and the absorbance was measured at 420 nm using a spectrophotometer (Shimadzu, UV-1601, Kyoto, Japan).

7.3.6.2 Maillard reaction intermediate product formation

Intermediate products from the Maillard reaction of HF_{25} and Bacto Peptone solutions were monitored. Solutions (1%, w/v) were prepared and the fluorescence intensity was measured at an excitation wavelength of 347 nm and emission wavelength of 415 nm using a spectrofluorophotometer (Shimadzu, RF-1501, Kyoto, Japan) (Morales and Jimennez-Perez, 2001). Additionally, the absorbance at 294 nm was measured and used as an indicator for UV-absorbance intermediate (Morales and Jimennez-Perez, 2001).

7.3.7 pH measurement

pH of HF_{25} and Bacto Peptone solutions (1% (w/v) in distilled water) was measured by a pH meter (Sartorious, PB 10, Goettingen, Germany).

7.3.8 Determination of solubility

To determine protein solubility, HF_{25} and Bacto Peptone (200 mg) were dispersed in 20 ml of deionized water and pHs of the mixtures were adjusted to 5 and 7 with 1 M HCl or 1 M NaOH. The mixtures were stirred at room temperature for 10 min, followed by centrifugation at 2000g for 15 min using a centrifuge (Hettich, Mikro 20, Zentrifugen, Germany). Protein contents in the supernatant were determined by the Biuret method (Robinson and Hodgen, 1940). Total protein contents in the samples were determined after solubilization of the samples in 0.5 M NaOH. Protein solubility was calculated as follows:

$$\text{Solubility (\%)} = \frac{\text{Protein content in supernatant}}{\text{Total protein content in sample}} \times 100$$

7.3.9 Turbidity measurement

Turbidity of HF_{25} and Bacto Peptone solutions (0.5% (w/v) in distilled water) before and after heating at 121°C for 15 min using an autoclave (Tomy, SS-325, Tokyo, Japan) was determined by reading the absorbance at 660 nm using a Spectrophotometer (Shimadzu, UV-1601, Kyoto, Japan).

7.3.10 Determination of 2-thiobarbituric acid reactive substance (TBARS)

TBARS in the samples were determined by the method of Buege and Aust (1978). The solutions of HF_{25} and Bacto Peptone at a concentration of 1 % (w/v) were prepared. To 0.5 ml of sample solution, 4.5 ml of a solution containing 0.0375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 M HCl were added. The mixtures were heated in boiling water for 10 min, followed by cooling with the running water. The absorbance of resulting mixtures was read at 532 nm using a Spectrophotometer (Shimadzu, UV-1601, Kyoto, Japan). TBARS was calculated from the standard curve of molonaldehyde (MDA) with the concentration range of 0-3 mg/kg sample and expressed as mg MDA/kg sample.

7.3.11 Determination of the ability as nutrients for microbial growth

7.3.11.1 Preparation of media containing HF_{25}

Nutrient Broth (NB) and Nutrient Agar (NA) were prepared for culturing bacteria. NB consisted of 3 g beef extract and 5 g HF_{25} or Bacto Peptone. For NA, 15 g agar was included in NB. The mixture was added with distilled water. Final volume was adjusted to 1 l with distilled water. Thereafter, the solution obtained was adjusted to pH 6.8±0.2 and autoclaved at 121°C for 15 min using an autoclave (Tomy, SS-325, Tokyo, Japan).

7.3.11.2 Preparation of bacteria and inocula

Staphylococcus aureus (*S. aureus*) TISTR 118 and *Escherichia coli* (*E. coli*) TISTR 780 were obtained from the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand. Bacteria were kept on NA slants at 4°C until use. To activate bacteria before culturing, inocula were prepared. One loopful of each bacterium cultured on NA slants containing HF_{25} or Bacto Peptone for 15 h was inoculated in NB containing HF_{25} or Bacto Peptone. The culture broths were then incubated at 37°C for 15 h. Thereafter, 1.5 ml of culture broths were inoculated in 50 ml NB containing HF_{25} or Bacto Peptone and incubated at 37°C for 15 h using a shaking incubator (VS-8480SR-L, LMS, Hat Yai, Thailand) at 120 rpm. Culture broths were adjusted to obtain A_{660} of 0.02 with corresponding media. The obtained cell suspension was used as late log phase inocula.

7.3.11.3 Determination of bacterial growth

Growths were determined by submerged cultivation. To 50 ml of NB containing HF_{25} or Bacto Peptone, 1.5 ml of inocula with A_{660} of 0.02 was added. Incubation was carried out at 37°C for 15 h with continuous shaking at 120 rpm using a shaking incubator (VS-8480SR-L, LMS, Hat Yai, Thailand). Culture broths obtained were subjected to the measurements of turbidity (A_{660}) and total viable count. Serial dilutions were prepared from culture broths of *S. aureus* and *E. coli* with sterile normal saline. Thereafter, appropriate dilution (0.1 ml) was pipetted onto NA containing HF_{25} or Bacto Peptone and then spread using a sterile spreader. The plates were upside-down incubated at 37°C for 24 h before counting.

7.3.12 Statistical analysis

Data were subjected to Analysis of Variance (ANOVA). Mean comparison was performed using Duncan's multiple range test. For pair comparison, independence-samples t-test was used (Steel and Torrie, 1980). Statistical analysis was carried out using SPSS statistic program (Version 11.0) for Windows (SPSS Inc. Chicago, IL, USA).

7.4 Results and Discussion

7.4.1 Changes in a_w and moisture content during storage

Changes in a_w and moisture content of spray-dried yellow stripe trevally protein hydrolysate (HF_{25}) and Bacto Peptone during 12 weeks of storage are depicted in Figure 27. Slight increases in a_w and moisture content of both samples were observed throughout the storage ($p<0.05$). At week 0, HF_{25} showed the higher a_w and moisture content than did Bacto Peptone ($p<0.05$). After 12 weeks of storage, HF_{25} and Bacto Peptone had a_w of 0.27 and 0.26, while the moisture contents were 5.49 and 4.10% for HF_{25} and Bacto Peptone, respectively. Changes in physical, chemical or microbiological properties of product can be considered as loss of stability (Labuza, 1984). a_w is one of several important parameters affecting stability of products (Labuza, 1984). a_w is a measure of the free moisture that is available to participate in physical, chemical and biological reactions, while moisture content is the combination of free and bound moisture (Fennema, 1976). a_w plays a role in the microbial stability. Bacteria, molds and yeasts require water for growth and every microorganism has a minimum a_w below which it will not grow (Fennema, 1976). Due to the low level of a_w (<0.70), HF_{25} and Bacto Peptone were most likely stable to microbial spoilage. However, non-enzymatic browning might take place at very low a_w (Fennema, 1976).

During the extended storage, dry and hygroscopic HF_{25} could absorb the moisture in the environment until it reached the equilibrium level. If a_w is above the critical limit for hydrolysate, it will begin to cake, which is unacceptable as microbial media. To lower any change of HF_{25}, the selected packaging exhibiting the excellent water vapor barrier property was required. The transfer of water from an environment

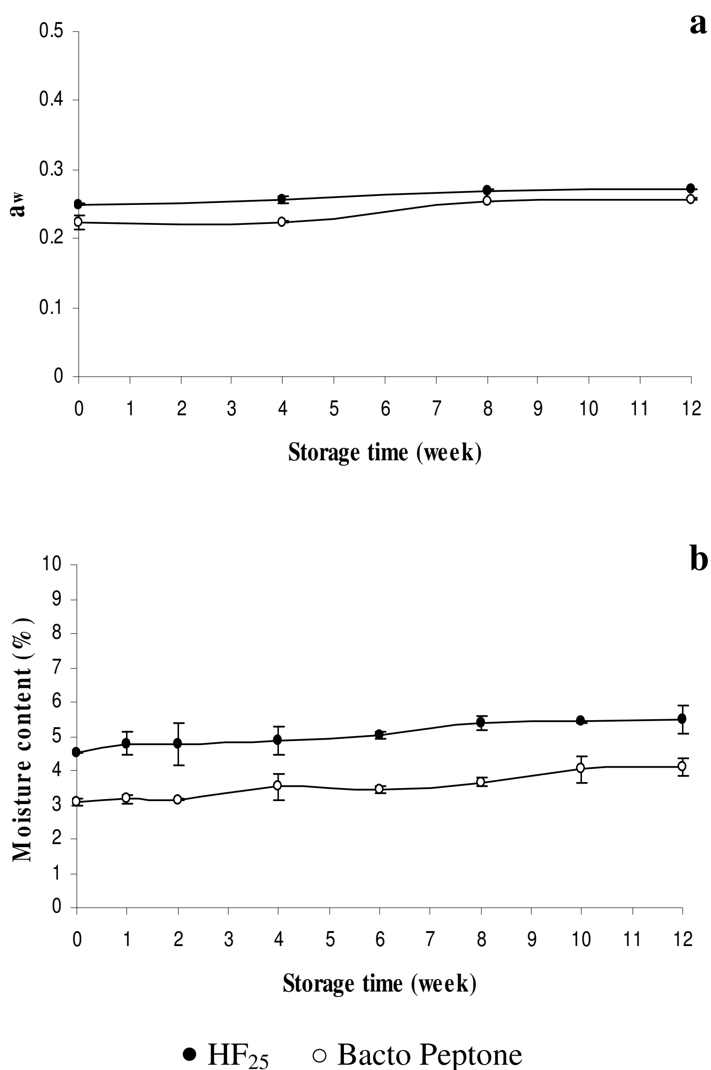


Figure 27. Changes in a_w (a) and moisture content (b) of yellow stripe trevally protein hydrolysate (HF_{25}) and Bacto Peptone during 12 weeks of storage at room temperature. Bars represent the standard deviation from triplicate determinations.

with higher a_w to HF_{25} with a lower a_w caused HF_{25} to reach its critical limit, initiating caking. Storage factors such as temperature can change the rate of water transfer and caking. Lower temperatures slow the water transfer, while higher temperatures speed the water transfer rate (Labuza, 1984). Therefore, a_w could be an important factor determining the chemical and microbiological stability of protein

hydrolysate. Troller (1971) found that at low a_w levels, total number and growth rate of *S. aureus* were diminished and generation times were increased. Additionally, enterotoxin synthesis was extremely sensitive to lowered a_w. Tsoraeva and Zhurbenko (2000) reported that the moisture content of protein hydrolysate should be lower than 7% since the higher levels of moisture content will reduce the stability and shelf-life of protein hydrolysate, causing color changes and falling pH values.

7.4.2 Changes in color during storage

During the storage, the changes in color of HF_{25} and Bacto Peptone are shown in Figure 28. At week 0, HF_{25} had the higher a* value, but lower L* value than did Bacto Peptone ($p<0.05$). However, no difference in b* value was found ($p>0.05$). This suggested that HF_{25} was slightly darker and more reddish. L* value of both HF_{25} and Bacto Peptone slightly decreased after 8 weeks of storage ($p<0.05$). a* and b* values of HF_{25} gradually increased up to 12 weeks of storage ($p<0.05$). Conversely, no significant change in a* and b* values of Bacto Peptone was noticeably observed during storage ($p>0.05$). The result indicated the slight decrease in lightness of HF_{25} and Bacto Peptone after the extended storage. Redness and yellowness of HF_{25} gradually increased during storage ($p<0.05$). The decrease in lightness and the increases in redness and yellowness of HF_{25} during storage might be associated with non-enzymatic browning, known as the Maillard reaction. Maillard reaction is a chemical reaction between simple sugars and amino acids and occurs between a_w of 0.2 and 0.8 (Fennema, 1976). Klompong *et al*. (2008) reported that HF_{25} contained fermentable carbohydrates, while it was not found in Bacto Peptone. Those fermentable carbohydrates might consist of reducing sugars, which underwent Maillard reaction readily under the storage condition. Additionally, the decrease in lightness was probably due to the oxidation of myoglobin and the melanin pigment present in HF_{25} (Benjakul and Morrissey, 1997). The result was in agreement with Hoyle and Merritt (1994) who found that hydrolysates from herring had the decreases in lightness and increases in yellowness, which indicated the darkening, during storage.

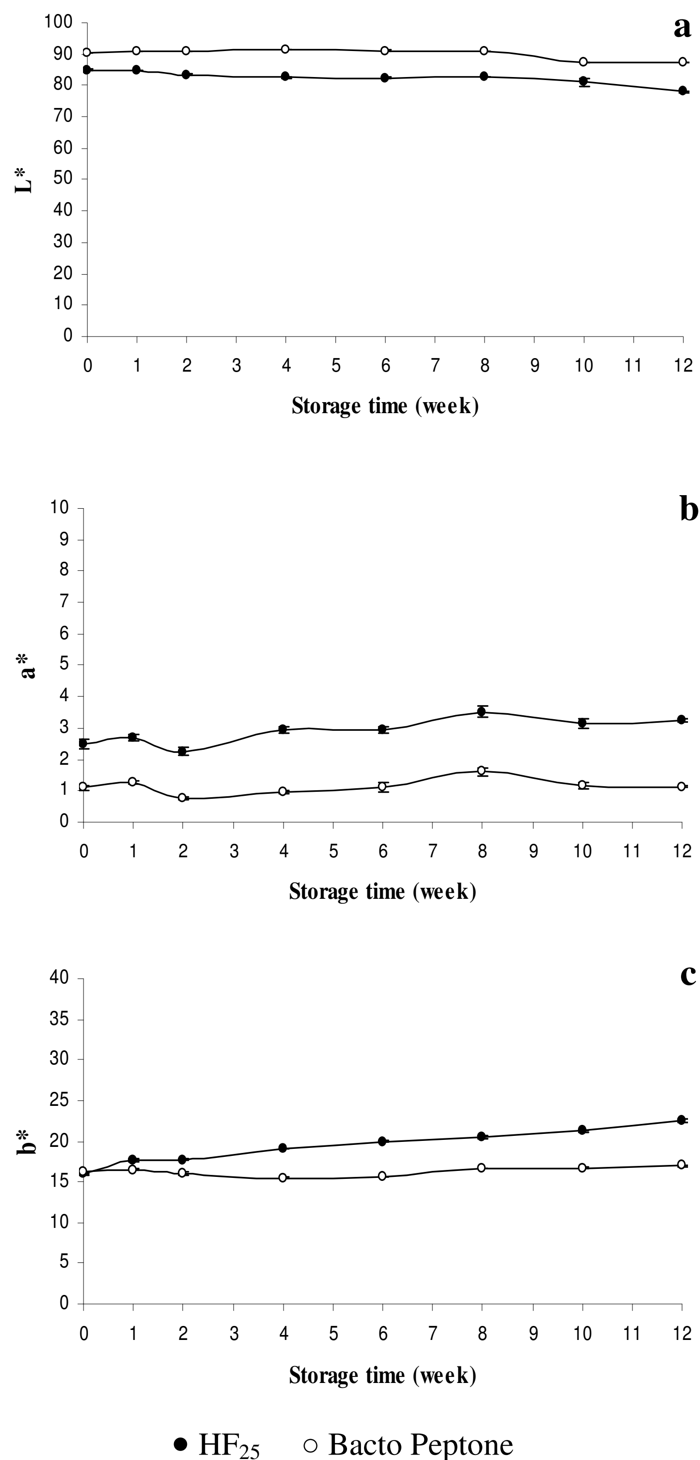


Figure 28. Changes in color L* (a), a* (b) and b* values (c) of yellow stripe trevally protein hydrolysate (HF_{25}) and Bacto Peptone during 12 weeks of storage at room temperature. Bars represent the standard deviation from triplicate determinations.

7.4.3 Changes in browning index during storage

7.4.3.1 Browning intensity

As measured by A_{420}, a slight increase in browning of HF_{25} was observed as the storage time increased ($p<0.05$), while no change was noticeable in Bacto Peptone ($p>0.05$) (Figure 29a). Furthermore, HF_{25} possessed the higher browning intensity than did Bacto Peptone throughout the storage time ($p<0.05$). The increase in browning in HF_{25} might be associated with the presence of reducing sugars or other carbohydrate compounds in HF_{25}. As a consequence, the amino acid-sugar complex could be formed via Maillard reaction. The increase in browning intensity was in accordance with the decrease in L* value and the increases in a* and b* values (Figure 28)

7.4.3.2 UV-absorbance

Continuous increase in A_{294} of HF_{25} was observed within the first 4 weeks of storage ($p<0.05$). Thereafter, A_{294} was quite constant ($p>0.05$). The gradual increase in A_{294} was observed in Bacto Peptone as the storage time increased ($p<0.05$) (Figure 29b). HF_{25} showed the higher A_{294} than did Bacto Peptone throughout the storage ($p<0.05$). A_{294} was used to determine the intermediate compounds of the Maillard reaction (Ajandouz *et al.*, 2001). The result suggested that the formation of an uncolored compound in HF_{25} was more intense than Bacto Peptone.

7.4.3.3 Fluorescence intensity

Fluorescence intensity of HF_{25} and Bacto Peptone increased as the storage time increased ($p<0.05$) (Figure 29c). Bacto Peptone possessed the greater fluorescence intensity than did HF_{25} throughout the storage time ($P<0.05$). Development of fluorescent compounds occurs in the Maillard reaction prior to the generation of brown pigments (Morales *et al.*, 1996). Therefore, the increase in fluorescence intensity suggested the increased formation of fluorescent intermediates for the development of browning. It was noted that the rate of the increase in fluorescence intensity of HF_{25} was slightly greater than Bacto Peptone.

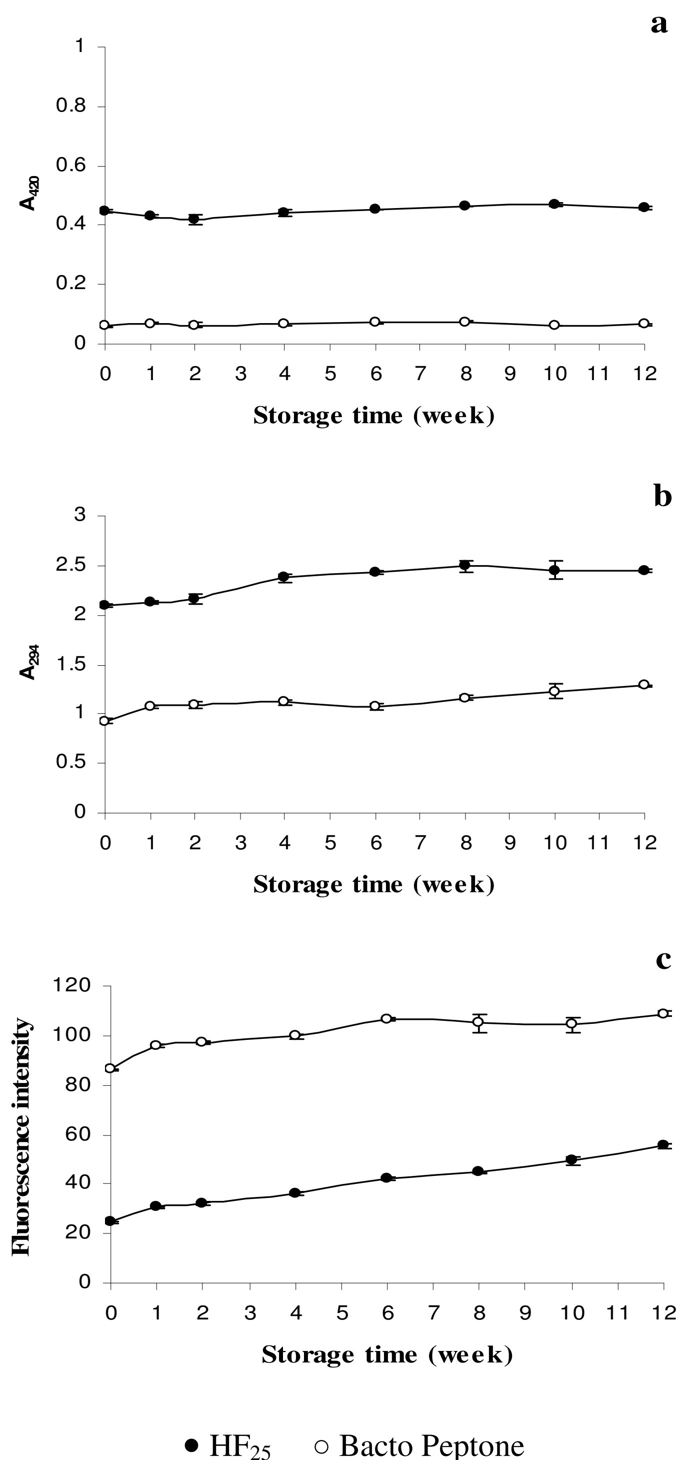


Figure 29. Changes in A_{420} (a), A_{294} (b) and fluorescence intensity (c) of yellow stripe trevally protein hydrolysate (HF_{25}) and Bacto Peptone during 12 weeks of storage at room temperature. Bars represent the standard deviation from triplicate determinations.

The differences in fluorescence intensity and UV-absorbance (A_{294}) of HF_{25} and Bacto Peptone suggested that different types of intermediate products, either fluorescent or non-fluorescent compounds, were formed and underwent the final stage of reaction at different rates. The differences in browning intensity, UV-absorbance and fluorescence intensity between HF_{25} and Bacto Peptone might be due to the differences in amino acid compositions and other substances. Benjakul *et al*. (2005) reported that both concentration and type of sugar influenced the browning of porcine plasma protein-sugar Maillard reaction products and the formation of intermediate compounds was varied, depending on the type of sugar. Hoyle and Merritt (1994) concluded that non-enzymatic browning reaction resulted in the darkening of protein hydrolysate from herring during 3 months of storage.

7.4.4 Changes in pH during storage

pH of HF_{25} and Bacto Peptone ranged from 6.83 to 7.02 (Figure 30). However, no difference in pH between HF_{25} and Bacto Peptone was observed during storage of 12 weeks ($p>0.05$). The pH of both samples was quite stable throughout the storage. In general, media preparation for bacteria requires the pH of 6.8±0.2 (Downes and Ito, 2001). The decrease in pH values of hydrolysate indicated the decrease in stability and shelf-life of protein hydrolysate (Tsoraeva and Zhurbenko, 2000). The changes in pH during storage could be used as a factor determining the stability of protein hydrolysate during storage.

7.4.5 Changes in solubility during storage

Solubility of HF_{25} and Bacto Peptone at pHs 5 and 7 is depicted in Figure 31. Both HF_{25} and Bacto Peptone were soluble at both pHs with the solubility greater than 93%. No difference in solubility between both pHs was observed in HF_{25} and Bacto Peptone ($p>0.05$). The high solubility of HF_{25} was mainly due to the existence of low molecular weight peptides (lower than 7 kDa) (Klompong *et al*., 2007b). Additionally, HF_{25} might possess proportionally more polar residues with the ability to form hydrogen bonds with water (Gbogouri *et al*., 2004). Gildberg *et al*. (1989)

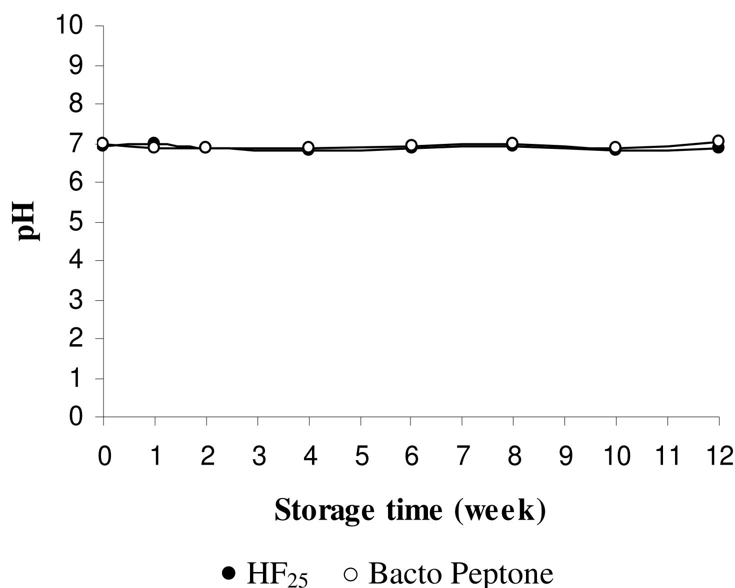


Figure 30. Changes in pH of 1% solution of yellow stripe trevally protein hydrolysate (HF_{25}) and Bacto Peptone during 12 weeks of storage at room temperature. Bars represent the standard deviation from triplicate determinations.

reported that freeze-dried powders of capelin and blue whiting hydrolysates were easily solubilized in distilled water with the aid of heating.

With high solubility at pHs 5 and 7 throughout the storage time, HF_{25} could be served as a readily available nutrient that could be uptaken efficiently by molds, yeasts and bacteria cultured in the media with the pHs of 5 and 7, respectively. Generally, it is a common practice to culture molds at pH approximately 5, whereas the bacteria and yeasts are cultivated at pH approximately 6-7 (Downes and Ito, 2001).

7.4.6 Changes in turbidity during storage

Turbidity before and after autoclaving of HF_{25} and Bacto Peptone stored for different times is shown in Figure 32. As the storage time increased, the turbidity of HF_{25} and Bacto Peptone, either before or after autoclaving, slightly increased. However, no difference in turbidity of both samples before and after

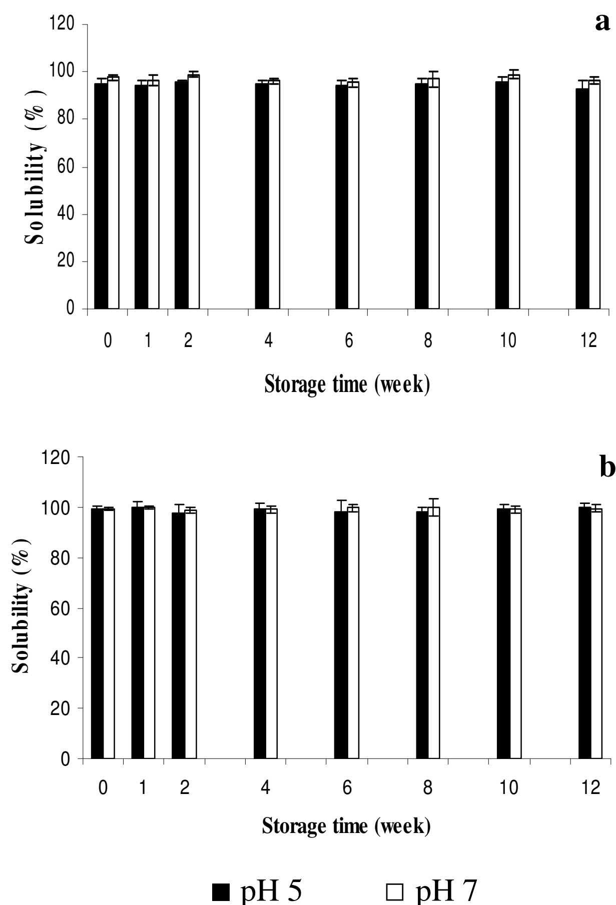
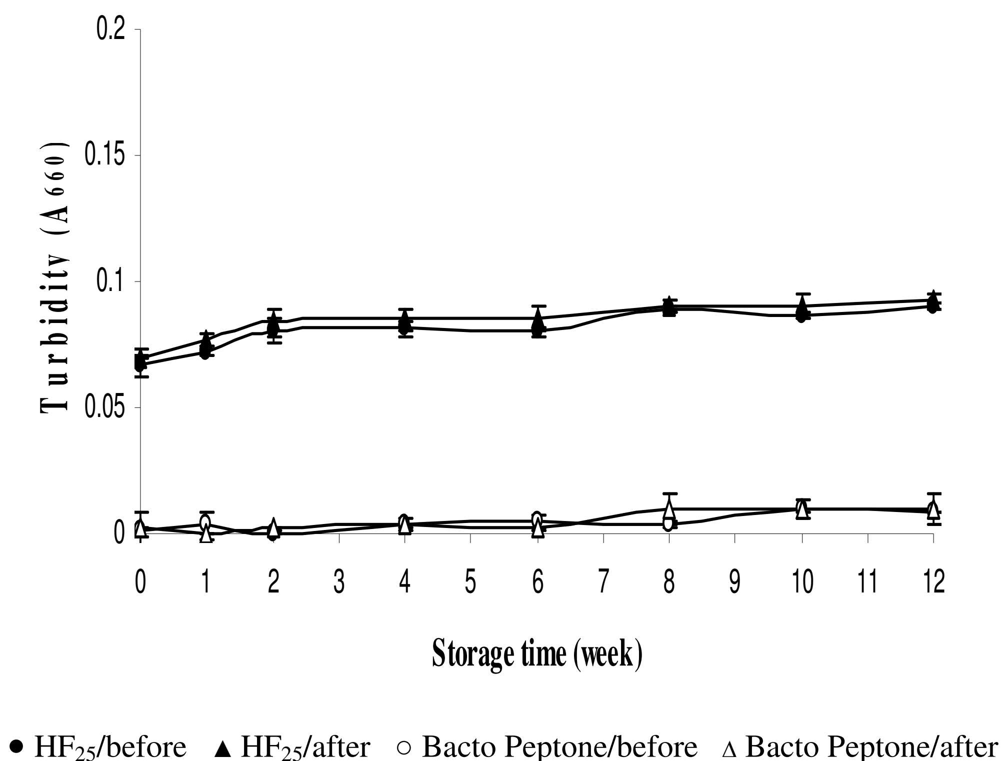


Figure 31. Changes in solubility of yellow stripe trevally protein hydrolysate (HF_{25}) (a) and Bacto Peptone (b) at pH 5 and 7 during 12 weeks of storage at room temperature. Bars represent the standard deviation from triplicate determinations.

autoclaving was observed (p>0.05). HF_{25} contained the small MW peptides, which were not prone to coagulation or precipitation after being subjected to heating at 121°C for15 min. The difference in turbidity between HF_{25} and Bacto Peptone might be associated with the differences in raw material pigments, pretreatment, hydrolysis condition and drying process. Additionally, the higher residual lipid content in HF_{25} (Klompong *et al*., 2008) might result in the higher turbidity, in comparison with Bacto Peptone, which had the lower lipid content. Fish lipid underwent oxidation easily, in which the oxidation products could be formed and acted as protein/peptide cross-linker. The larger aggregate formed probably affected the turbidity of HF_{25} solution.

Gildberg *et al*. (1989) reported that freeze-dried capelin hydrolysate solution was slightly turbid due to some residual lipids.



● HF_{25}/before ▲ HF_{25}/after ○ Bacto Peptone/before △ Bacto Peptone/after

Figure 32. Changes in turbidity of yellow stripe trevally protein hydrolysate (HF_{25}) before and after autoclaving and of Bacto Peptone before and after autoclaving during 12 weeks of storage at room temperature. Bars represent the standard deviation from triplicate determinations.

7.4.7 Changes in TBARS during storage

TBARS values of HF_{25} and Bacto Peptone during storage for 12 weeks are shown in Figure 33. As the storage time increased, TBARS of both HF_{25} and Bacto Peptone increased ($p<0.05$). The increase in TBARS of HF_{25} was higher than that of Bacto Peptone throughout the storage ($p<0.05$). The increase in TBARS of HF_{25} and Bacto Peptone during storage indicated the occurrence of lipid oxidation.

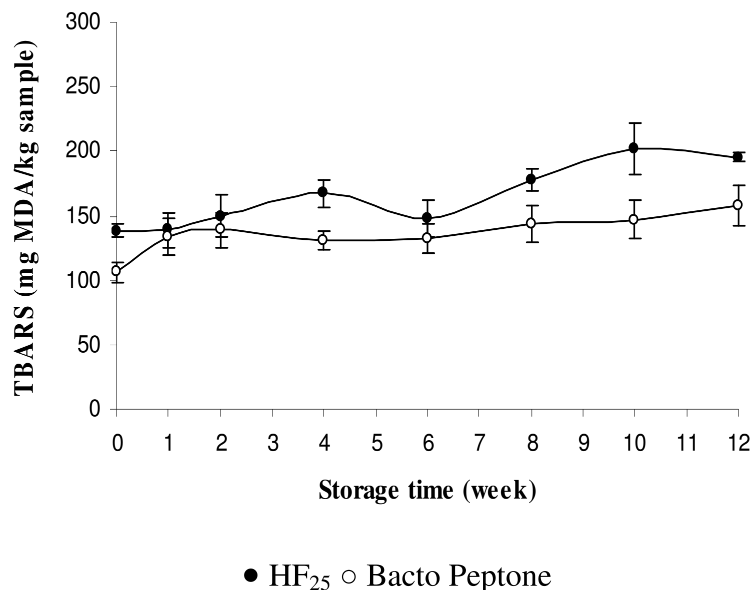


Figure 33. Changes in TBARS of yellow stripe trevally protein hydrolysate (HF_{25}) and Bacto Peptone during 12 weeks of storage at room temperature. Bars represent the standard deviation from triplicate determinations.

Lipid in HF_{25} and Bacto Peptone might be vulnerable to oxidation, resulting in production of TBARS (Pena-Ramos and Xiong, 2003). At low a_w (0.22-0.27), lipid oxidation of HF_{25} and Bacto Peptone could be enhanced since the lipids were more exposed to oxygen at low water level. As a consequence, the oxidation proceeded rapidly in both dried samples. Lipid oxidation during storage occurred relatively rapidly at both low (0.05-0.15) and high (0.5-0.8) a_w (Fennema, 1976).

7.4.8 Changes in efficacy as microbial media during storage

Efficacy of HF_{25} and Bacto Peptone as microbial media during storage is depicted in Figure 34. As measured by A_{660}, a slight decrease in growth of *S. aureus* cultured in NB containing HF_{25} was observed as the storage time increased ($p<0.05$), while no difference was found in that cultured in NB containing Bacto Peptone (Figure 34a) ($p>0.05$). However, NB containing HF_{25} yielded the higher growth of *S. aureus* than did Bacto Peptone within the first 2 weeks of storage ($p<0.05$). Thereafter, no difference was observed ($p>0.05$). The decrease in growth of *S. aureus*

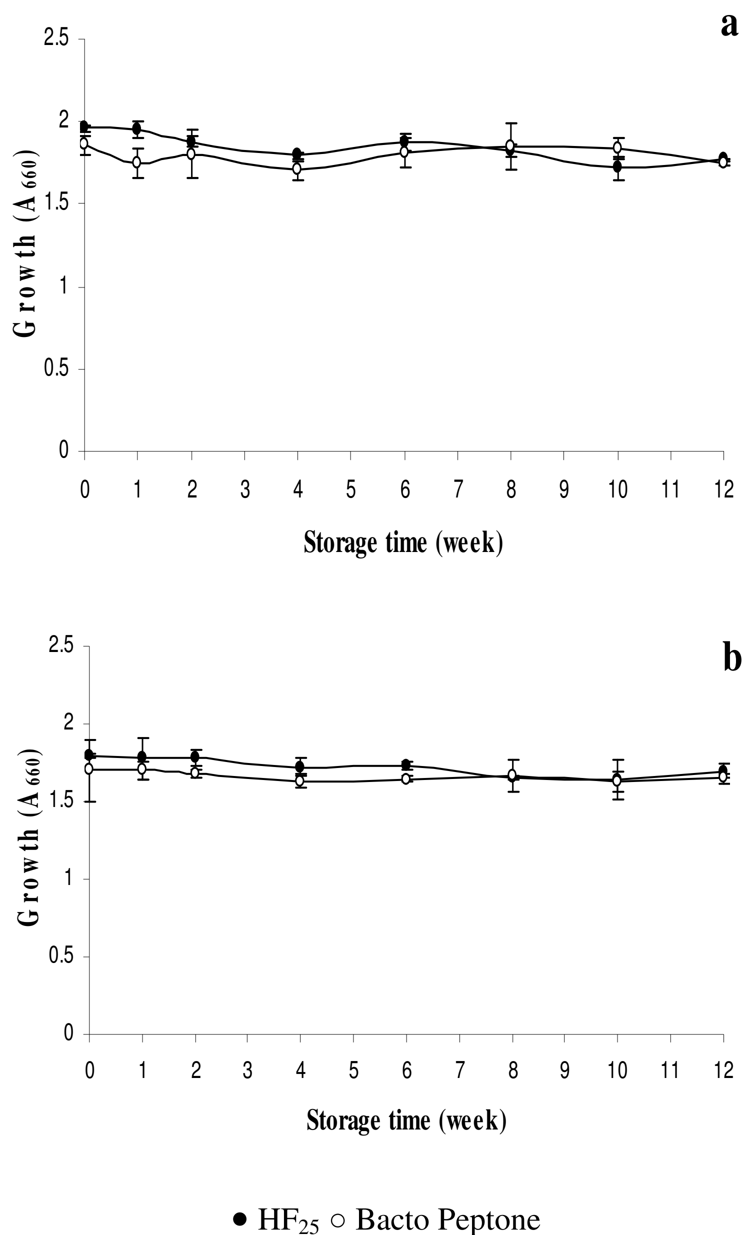


Figure 34. Changes in efficacy of yellow stripe trevally protein hydrolysate (HF_{25}) and Bacto Peptone as microbial media culturing *S. aureus* (a) and *E. coli* (b) for 15 h during 12 weeks of storage at room temperature. Bars represent the standard deviation from triplicate determinations.

during storage might be associated with the loss of some amino acids required for growth of *S. aureus* during storage, possibly via Maillard reaction or lipid oxidation. The rate of Maillard browning was related with the loss in protein nutrition value

(Lindemann-Schneider and Fennema, 1989). Nielsen *et al*. (1985) found that whey proteins stored in the presence of oxidizing lipids had the losses of lysine and extensive methionine oxidation but only minor losses of tryptophan during storage. The losses in nutritive value were due to interaction of protein with oxidized lipids. The decrease in growth of *S. aureus* culturing in HF_{25} during storage was in accordance with the increase in a_w, moisture content, dark color, Maillard reaction and lipid oxidation.

When *E. coli* was cultured in NB containing HF_{25} or Bacto Peptone stored for different times, no different growth was observed throughout the storage (Figure 34b) ($p>0.05$). The growth of *E. coli* culturing in NB containing HF_{25} was higher than that observed in NB containing Bacto Peptone within the first 6 weeks of storage ($p<0.05$). Thereafter, no difference was observed ($p>0.05$). Klompong *et al*. (2008) reported that HF_{25} contained a readily available nitrogen source required for growth of *S. aureus* and *E. coli.* Regardless of storage time, the efficacy of HF_{25} as microbial media was equivalent to Bacto Peptone when tested for the growth of *S. aureus* and *E. coli*.

To elucidate the possible impact of medium turbidity on A_{660} measurement used as an indicator for bacterial growth, total viable count was determined on media containing freshly prepared HF_{25}. The numbers (log CFU/ml) of *S. aureus* cultured on NA containing HF_{25} or Bacto Peptone were 6.99±0.03 and 6.94±0.02, respectively. For *E. coli*, the numbers were 7.80±0.06 and 7.71±0.05, when cultured on NA containing HF_{25} or Bacto Peptone, respectively. The numbers of both organisms cultured on NA containing HF_{25} were greater than those cultured on NA containing Bacto Peptone, which were in accordance with A_{660} measurement (Figure 34). The result suggested that no interference from the medium turbidity was found, when A_{660} measurement was used to determine bacterial growth.

7.5 Conclusions

Protein hydrolysate from yellow stripe trevally prepared using Flavourzyme with 25% DH was quite stable during storage. The efficacy for culturing bacteria was equivalent to commercial Bacto Peptone throughout 12 weeks of storage at room

temperature. Therefore, protein hydrolysate from yellow stripe trevally could be used as alternative microbial media and could be stored for a long time without any loss in bacterial culturing efficacy.

CHAPTER 8

SUMMARY AND FUTURE WORK

8.1 Summary

1. Antioxidative activity of protein hydrolysates from yellow stripe trevally meat varied with DH and enzyme used. Antioxidative activity of protein hydrolysate prepared using Alcalase and Flavourzyme with 15% DH was stable in a wide pH range and to heat treatment up to 90°C. Both protein hydrolysates could retard the lipid oxidation in liposome system effectively, but their efficacy was lower than α-tocopherol. Additionally, hydrolysates could prevent DNA damage in Fenton reaction system. Potential antioxidative peptides of hydrolysates prepared using Alcalase and Flavourzyme with 15% DH were purified and characterized to be 656 and 617 Da. The purified peptides were capable of donating hydrogen atoms. Therefore, yellow stripe trevally protein hydrolysate can be used in food systems as a natural antioxidant.

2. Protein hydrolysates possessed the varying emulsifying and foaming properties, depending on DH and enzyme used. Protein hydrolysates had an excellent solubility over a wide pH range. Thus, they could serve as functional ingredients in food systems.

3. Efficacy of protein hydrolysate from yellow stripe trevally as microbial nutrient varied with DH, enzyme and amount of hydrolysate used. Microbial kinetics of bacteria, yeasts and mold cultured in media containing protein hydrolysate from yellow stripe trevally produced by Flavourzyme with 25% DH were comparable to those of commercial Bacto Peptone. It was rich in both organic, particularly protein and inorganic substances required for microbial growth. Therefore, production of fish protein hydrolysate for upgrading low market value species as a high value nitrogen source for microbial growth can be achieved.

4. Spray-dried protein hydrolysate powder from yellow stripe trevally prepared using Flavourzyme with 25% DH was quite stable during storage. The efficacy for culturing bacteria was equivalent to commercial Bacto Peptone

throughout 12 weeks of storage at room temperature. Therefore, protein hydrolysate from yellow stripe trevally could be used as an alternative microbial medium and could be stored for a long time without any loss in bacterial culturing efficacy.

8.2 Future works

1. The bitterness and sensory property of protein hydrolysates should be evaluated and the appropriate debittering and deodoriztion should be performed in order to obtain fish protein hydrolysate with the sensorial acceptability for further uses, especially for food application.
2. Antioxidative peptides purified from protein hydrolysates should be determined for amino acid sequence, in which the role of amino acid composition and sequence in prevention of lipid oxidation can be elucidated.
3. The formula of microbial media containing protein hydrolysates should be improved to yield the morphology of microorganisms identical to that cultured in media containing commercial peptone.
4. To serve as biochemical test media, fermentable carbohydrates in protein hydrolysates should be eliminated. Lipid should be removed to enhance the storage stability and reduce the turbidity when used as microbial media. Additionally, desalting of protein hydrolysate should be carried out to fit the broad range of applications.

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APPENDIX

The yields of protein hydrolysates produced from yellow stripe trevally muscle using Alcalase and Flavourzyme with different DHs

Protein hydrolysate	DH (%)	Yield (%)	
		Dry matter/mince	Dry matter/whole fish
HA	5	13.04	5.22
	15	20.10	8.04
	25	27.01	10.81
HF	5	12.72	5.09
	15	28.86	11.55
	25	54.43	21.77

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List of Publication and Proceeding

Publications

1. Klompong, V., Benjakul, S., Kantachote, D. and Shahidi, F. 2007. Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. Food Chem. 102: 1317-1327.
2. Klompong, V., Benjakul, S., Kantachote, D., Hayes, K.D. and Shahidi, F. 2007. Comparative study on antioxidative activity of yellow stripe trevally protein hydrolysate produced from Alcalase and Flavourzyme. Int. J. Food Sci. Technol. 43: 1019-1026.
3. Klompong, V., Benjakul, S., Yachai, M., Visessanguan, W., Shahidi, F. and Hayes, K.D. 2008. Amino acid composition, prevention of DNA damage and characteristic of antioxidative peptides from protein hydrolysates from yellow stripe trevally (*Selaroides leptolepis*). J. Food Sci. accepted for publication.

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1. Klompong, V., Benjakul, S., Kantachote, D. and Shahidi, F. 2006. Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. IUFoST 13th World Congress of Food Science and Technology, Nantes, France.
2. Klompong, V., Benjakul, S., Kantachote, D., Hayes, K.D. and Shahidi, F. 2006. Comparative study on antioxidative activity of yellow stripe trevally protein hydrolysate produced from Alcalase and Flavourzyme. 2006 EFFoST Annual Meeting/Total Food 2006, Sustainability of the Agri-Food Chain, The Hague, Netherlands.
3. Klompong, V., Benjakul, S., Hayes, K.D. and Shahidi, F. 2007. Characterization of antioxidative peptides from protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*). 7th International Food Data Conference, Food Composition and Biodiversity, Sao Paulo, Brazil.
4. Klompong, V., Benjakul, S., Shahidi, F. and Hayes, K.D. 2008. Amino acid composition, prevention of DNA damage and characteristic of antioxidative peptides from protein hydrolysates from yellow stripe trevally (*Selaroides leptolepis*). IFT08 Annual Meeting+Food Expo, New Orleans, LA, USA.