Production of Gelatin Hydrolysate with Antioxidant Activity from Fish Skin Using Protease from *Bacillus amyloliquefaciens*

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ชื่อวิทยานิพนธ์ การผลิตเจลาตินไฮโดรไลเสตที่มีฤทธิ์ต้านอนุมูลอิสระจากหนังปลาโดยใช้โปรติเอสจาก *Bacillus amyloliquefaciens*

ผู้เขียน นาย สามารถ สายอุต

สาขาวิชา วิทยาศาสตร์และเทคโนโลยีอาหาร

ปีการศึกษา 2556

บทคัดย่อ

การแยกเชื้อจุลินทรีย์มากกว่า 500 ไอโซเลตจากแพปลาในจังหวัดสงขลา พบแบคทีเรีย 55 ชนิดที่มีกิจกรรมการย่อยเจลาติน จากการวิเคราะห์ลำดับจีน 16S rRNA พบว่าสายพันธุ์จำนวน 3 สายพันธุ์ (K12, O02, S13) สามารถจำแนกเป็น *Bacillus cereus* ด้วยความคล้ายคลึง 99.8% ส่วนอีก 3 สายพันธุ์ (D10, G02 และ H11) สามารถจำแนกเป็น *B. amyloliquefaciens* ด้วยความคล้ายคลึง 99.7% เมื่อตกตะกอนเอนไซม์ย่อยเจลาตินที่ผลิตจากเชื้อ D10, G02 และ H11 ด้วยแอมโมเนียมซัลเฟต และผ่านการไดอะไลซีส พบว่าเอนไซม์มีความบริสุทธิ์เพิ่มขึ้น 19-34 เท่า เอนไซม์มีกิจกรรมสูงสุดที่อุณหภูมิ 50 องศาเซลเซียส และพีเอช 7.5 เมื่อใช้เจลาตินจากหนังปลาเป็นสับสเตรท เอนไซม์ย่อยเจลาตินเป็นชนิดเมทัลโลและซีรีน เอนไซม์ย่อยเจลาตินที่สกัดได้สามารถย่อยเจลาตินได้ใกล้เคียงกับอัลคาเลสทางการค้า แต่เอนไซม์ที่สกัดได้ให้การย่อยสลายที่สูงกว่าภายใน 60 นาทีแรก ดังนั้นเอนไซม์ย่อยเจลาตินจาก *B. amyloliquefaciens* ที่คัดเลือกสามารถใช้ในการผลิตไฮโดรไลเสตจากเจลาตินหนังปลา

จากการศึกษาพบว่า *B. amyloliquefaciens* H11 สามารถผลิตโปรตีเอสชนิด extracellular ซึ่งสามารถย่อยเจลาติน เมื่อศึกษาสภาวะการเลี้ยงต่อการผลิตเอนไซม์ย่อยเจลาตินจาก *B. amyloliquefaciens* H11 โดยใช้ Placket-Burman design และ response surface methodology โดยคัดเลือกตัวแปรที่มีอิทธิพลได้แก่ ความเร็วในการเขย่า ระยะเวลาการเลี้ยง และความเข้มข้นของเจลาติน เพื่อศึกษาสภาวะที่เหมาะสม พบว่าการเพิ่มความเร็วในการเขย่าและความเข้มข้นของเจลาตินมีผลเพิ่มการผลิตเอนไซม์ย่อยเจลาติน โดยปัจจัยทั้งสองมีอิทธิพลร่วมและมีผลต่อการผลิตเอนไซม์ การผลิตเอนไซม์ในอาหารเลี้ยงเชื้อหลัก (basal medium) ได้สูงสุด (2,801 ยูนิต/มิลลิลิตร) เมื่อใช้ความเร็วในการเขย่าเท่ากับ 234 รอบต่อนาที ความเข้มข้นเจลาตินเท่ากับ 8.36 กรัม/ลิตร และใช้ระยะเวลาเลี้ยงเชื้อเท่ากับ 31 ชั่วโมง แบบจำลองที่ทำนายสามารถใช้ได้ดีกับการทดลองจริง (2,731 ± 101 ยูนิต/มิลลิลิตร) โดยสามารถให้ผลผลิตเพิ่มขึ้น 14 เท่าเมื่อเทียบกับการเลี้ยงในสภาวะปกติ

(212 ยูนิต/มิลลิลิตร) ดังนั้นการเลี้ยง *B. amyloliquefaciens* H11 ภายใต้สภาวะที่เหมาะสมสามารถเพิ่มการผลิตเอนไซม์ย่อยเจลาตินได้อย่างมีประสิทธิภาพ

เมื่อทำบริสุทธิ์เอนไซม์ย่อยเจลาตินชนิด extracellular จาก *B. amyloliquefaciens* H11 โดยเจลฟิวเตรชันด้วยคอลัมน์ Sephacryl S-200 และโครมาโทกราฟีโดยการแลกเปลี่ยนอิออนด้วย DEAE-cellulose พบว่าได้ผลผลิต ร้อยละ 35% และมีกิจกรรมจำเพาะเพิ่มขึ้น 14 เท่า เอนไซม์มีน้ำหนักโมเลกุลเท่ากับ 21 กิโลดาลตัน เอนไซม์มีกิจกรรมสูงสุดเมื่อใช้เจลาตินจากหมูเป็นสับสเตรตที่อุณหภูมิ 50 องศาเซลเซียส และพีเอช 8.0 กิจกรรมของเอนไซม์เพิ่มขึ้นในสภาวะที่มี Ca^{2+} และ Mg^{2+} เอนไซม์มีความทนทานต่อสารลดแรงตึงผิว สารออกซิไดซิ่ง และตัวทำละลาย เอนไซม์ที่ผ่านการทำบริสุทธิ์มีความจำเพาะกับเจลาตินจากหนังปลาวัว ดังนั้นเอนไซม์ที่ผ่านการทำบริสุทธิ์จาก *B. amyloliquefaciens* H11 มีศักยภาพในการผลิตเปปไทด์ที่มีฤทธิ์ทางชีวภาพจากเจลาติน

เมื่อศึกษากิจกรรมการต้านออกซิเดชันของเจลาตินไฮโดรไลเสตจากหนังปลาวัวซึ่งเตรียมโดยโปรตีเอสชนิด extracellular จาก *B. amyloliquefaciens* H11 (GH-H11) ซึ่งมีระดับการย่อยสลาย (DHs) ต่างกันเปรียบเทียบกับไฮโดรไลเสตที่เตรียมด้วยอัลคาเลส (GH-Al) พบว่ากิจกรรมต้านออกซิเดชันของไฮโดรไลเสตที่ผลิตจากโปรติเอสทั้งสองเพิ่มขึ้นเมื่อ DH เพิ่มขึ้น ($P < 0.05$) เมื่อ DH เท่ากับ 20-40% GH-H11 มีกิจกรรมการจับอนุมูล ABTS และมีค่า FRAP สูงกว่า GH-Al ($P < 0.05$) แต่ไม่มีความแตกต่างของกิจกรรมการจับโลหะ ($P > 0.05$) ไฮโดรไลเสตทั้งสองที่ระดับความเข้มข้น 100 และ 1000 ppm สามารถยับยั้งการเกิดออกซิเดชันของไขมันในระบบจำลองเลซิติน-ลิโปโซม โดยขึ้นกับความเข้มข้นที่ใช้ เมื่อทดสอบในระบบการย่อยอาหารจำลอง พบว่า กิจกรรมการต้านออกซิเดชันของเจลาตินไฮโดรไลเสตไม่ได้รับผลกระทบจากเปปซิน ส่วนแพนครีเอตินส่งผลให้กิจกรรมการต้านออกซิเดชันเพิ่มขึ้น เปปไทด์ที่มีฤทธิ์ต้านออกซิเดชันใน GH-H11 และ GH-Al มีน้ำหนักโมเลกุลประมาณ 750 และ 3600 ดาลตันตามลำดับ

เมื่อแยกเปปไทด์จากเจลาตินไฮโดรไลเสตที่เตรียมด้วยโปรติเอสจาก *B. amyloliquefaciens* H11 ด้วยคอลัมน์ Sephadex G25 พบว่าเปปไทด์ที่มีน้ำหนักโมเลกุลน้อยมีฤทธิ์ยับยั้งเอนไซม์ ACE ได้ดีกว่าเปปไทด์ที่มีน้ำหนักโมเลกุลสูง และเมื่อทำการแยกเปปไทด์ต่อด้วย RP-HPLC และตรวจวิเคราะห์ลำดับกรดอะมิโนของเปปไทด์ พบว่าเปปไทด์ที่มีฤทธิ์ยับยั้ง ACE ประกอบด้วย AAGAPGGAR, ASGGPAGAR, GPVGHKG, LGASPGR, VVGPGA, DGGPAGVR, RPGGPPGSPG และ AGDVHPSM จากลำดับกรดอะมิโนของเปปไทด์แสดงให้เห็นการมีกรดอะมิโนที่ไม่ชอบน้ำที่ตำแหน่งปลายสายของเปปไทด์

เมื่อเตรียมเจลาตินไฮโดรไลเสตที่มีฤทธิ์ต้านออกซิเดชันโดยใช้โปรตีเอสจาก *B. amyloliquefaciens* H11 และใช้ระยะเวลาในการย่อยต่างกัน พบว่า ปริมาณหมู่แอลฟาอะมิโนและกิจกรรมต้านออกซิเดชันเพิ่มขึ้นเมื่อระยะเวลาในการย่อยนานขึ้น ($P < 0.05$) เมื่อทำแห้งเจลาตินไฮโดรไลเสตที่เตรียมโดยใช้เวลาในการย่อยสลายนาน 3 ชั่วโมง (GH-3H) โดยการทำแห้งแบบระเหิดและแบบพ่นฝอย พบว่า ผงแห้งที่ได้จากการทำแห้งแบบพ่นฝอยมีฤทธิ์การต้านออกซิเดชันลดลง เมื่อทดสอบความสามารถในการรีดิวซ์เฟอร์ริก กิจกรรมการจับโลหะ กิจกรรมการจับอนุมูลอิสระ DPPH และ ABTS เจลาตินไฮโดรไลเสตที่ผ่านการทำแห้งแบบพ่นฝอย (GH-3H-SD) มีความขาวมากกว่าและมีกลิ่นคาวและกลิ่นจากการหมักน้อยกว่า ซึ่งสอดคล้องกับสารประกอบที่ให้กลิ่นต่างๆที่ลดลง ยกเว้นโนนานาลซึ่งมีค่าสูงกว่าผงแห้งที่เตรียมโดยการทำแห้งแบบระเหิด ดังนั้นการทำแห้งแบบพ่นฝอยเป็นวิธีการทำแห้งที่ปรับปรุงสีและลดกลิ่นที่ไม่พึงประสงค์ของเจลาตินไฮโดรไลเสต

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ABSTRACT

Fifty-five isolates of bacteria with gelatinolytic activity were screened from over five-hundred isolates obtained from Songkhla fish docks. Based on 16S rRNA gene sequence analysis, three selected strains (K12, O02 and S13) were identified as *B. cereus* with 99.8% similarity, whilst another three strains (D10, G02 and H11) were identified as *B. amyloliquefaciens* with 99.7% similarity. Gelatinolytic enzymes of D10, G02 and H11 strains were precipitated by ammonium sulfate precipitation, followed by dialysis with the increase in purity by 19-34 folds. Their maximal gelatinolytic activities were attained at 50 °C and pH 7.5 when fish gelatin was used as substrate. Metallo- and serine-gelatinolytic enzymes were dominant for all three strains. All gelatinolytic enzymes showed similar hydrolysis towards fish gelatin to commercial Alcalase, but higher hydrolysis was found in the formers within the first 60 min. Therefore, gelatinolytic enzymes from selected *B. amyloliquefaciens* strains could be used for production of fish gelatin hydrolysate.

B. amyloliquefaciens H11 has been proven as a potential producer of extracellular protease with capacity of hydrolyzing gelatin. The cultivation conditions for the enhanced production of gelatinolytic enzyme from a newly isolated *B. amyloliquefaciens* H11 was investigated using Plackett-Burman design and response surface methodology. Three significant variables (agitation speed, cultivation time and fish gelatin concentration) were selected for optimization. Increase in speed of agitation and fish gelatin concentration markedly increased the production of gelatinolytic enzyme. Gelatin concentration and cultivation time showed significant interaction and both variables played the important role in enzyme production. The maximal gelatinolytic enzyme production in the basal medium was 2,801 U/ml under the following optimal condition: agitation speed of 234 rpm, 8.36 g/l of fish gelatin

and 31 h of cultivation. The predicted model fitted well with the experimental results (2,734 ± 101 U/ml). Fourteen-fold increase in yield was achieved, compared with the basal condition (212 U/ml). Thus, cultivation of *B. amyloliquefaciens* H11 under the optimal condition could enhance the production of gelatinolytic enzyme effectively.

Extracellular gelatinolytic enzyme from *B. amyloliquefaciens* H11 was purified by gel filtration chromatography on Sephacryl S-200 and ion exchange chromatography on DEAE-cellulose with 35% recovery and 14 folds increase in specific activity. The molecular weight of the purified enzyme was 21 kDa. The optimum gelatinolytic activity of purified enzyme in presence of porcine gelatin as substrate was reached at 50 °C and pH 8.0. The purified enzyme was activated by Ca^{2+} and Mg^{2+} and resistant to surfactants, oxidizing agents and solvents. The purified enzyme had specificity toward unicorn leatherjacket gelatin as substrate. This study suggests that the purified enzyme from *B. amyloliquefaciens* H11 had the potential application for the production of collagen gelatin-derived bioactive peptides.

Antioxidative activities of gelatin hydrolysates from unicorn leatherjacket skin prepared using extracellular protease of *B. amyloliquefaciens* H11 (GH-H11) with different degree of hydrolysis (DHs) was comparatively studied with those of hydrolysate produced using Alcalase (GH-Al). Antioxidative activities of hydrolysates produced by both proteases increased with increasing DH ($P < 0.05$). With DHs of 20-40%, GH-H11 showed higher ABTS radical scavenging activity and FRAP than GH-Al ($P < 0.05$) but no difference in chelating activity was found ($P > 0.05$). Both hydrolysates (100 and 1000 ppm) could inhibit lipid peroxidation in lecithin liposome system in a dose-dependent manner. *In vitro* simulated gastrointestinal digestion study indicated that the antioxidative activity of gelatin hydrolysate was not affected by pepsin, whilst further hydrolysis by pancreatin enhanced the antioxidative activity. The dominant antioxidative peptides in GH-H11 and GH-Al had molecular weights of approximately 750 and 3600 Da, respectively.

Angiotensin I-converting enzyme (ACE) inhibitory peptides from the gelatin hydrolysate prepared using protease from *B. amyloliquefaciens* H11 were purified using Sephadex G25 column, followed by reversed-phased high performance liquid chromatography (RP-HPLC). Based on MALDI-TOF mass spectrometry, potential ACE inhibitory peptides were identified as AAGAPGGAR, ASGGPAGAR,

GPVGHKG, LGASPGR, VVGPGA, DGGPAGVR, RPGGPPGSPG and AGDVHPSM. The presence of hydrophobic amino acid residues at the terminal was more likely responsible for the high ACE inhibitory activity of the peptides. Thus, gelatin hydrolysate could serve as the source of peptides lowering hypertension.

Gelatin hydrolysates with antioxidative activity produced by protease from *B. amyloliquefaciens* H11 with different hydrolysis times were prepared. Alpha-amino group content and antioxidative activities increased with increasing hydrolysis time ($P < 0.05$). When gelatin hydrolysate prepared with hydrolysis time of 3 h (GH-3H) was subjected to freeze-drying and spray-drying, the powder obtained from spray drying showed the decrease in antioxidant activity as measured by DPPH and ABTS radical scavenging activities, ferric reducing antioxidant power and metal chelating activity. Spray-dried gelatin hydrolysate (GH-3H-SD) showed higher whiteness with lower fishy odour and off-odour associated with fermentation. This was concomitant with decreases in several odourous compounds in the sample, except for nonanal, which was higher in comparison with the powder obtained by freeze-drying. Therefore, spray-drying could be an effective drying method to improve colour and reduce undesirable odour of gelatin hydrolysate.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Antioxidants play an important role in both food systems and human body, in which the oxidative process can be suppressed. In food systems, antioxidants are useful in retarding lipid oxidation and the formation of secondary lipid peroxidation products. Therefore, flavor, texture, and the color of the food products can be maintained during storage. Antioxidative agents henceforth reduce food protein oxidation as well as the interaction of lipid-derived carbonyls with proteins that leads to an alteration of protein functionality (Elias *et al*., 2008). Dietary supplements and functional foods containing antioxidants like α-tocopherol, vitamin C, or plant-derived phytochemicals find a huge demand in the current market. A great deal of attention has been also focus on antioxidative potential of peptides derived from various protein sources. Their possible applications as functional bioactive foods and nutraceuticals have been emphasized.

Fish skin gelatins have been reported as the protential source of biologically active peptides with high antioxidant activity via hydrolysis process. Peptides have been shown to exhibit radical scavenging capacity, metal chelating effects and reducing power or lipid peroxidation inhibition (Zhao *et al*., 2007; Giménez *et al*., 2009; Alemán *et al.*, 2011; Nikoo *et al.*, 2014). The biological properties of peptides are to a large extent influenced by their molecular weight and conformational structure, which are greatly affected by processing conditions. Peptides isolated from gelatin hydrolysate of Alaska pollack skin prepared using two-step hydrolysis with Alcalase and Pronase E, respectively, showed high antioxidant activity (Kim *et al*., 2001). In general, the amino acid composition of the hydrolysates is very similar to that of the parent gelatins (Kim *et al*., 2001; Gómez-Guillén *et al*., 2010). Nevertheless, gelaitn hydrolysate generally has a low degree of hydrolysis (DH), possibly due to the molecular constraint associated with its amino acid sequence/composition. Gelatin with a glycine content of around 33% might not be a preferable substrate for proteases.

Matrix metalloproteinase (MMP) cleaves native triple-helical type I, II and III collagens after Gly in a particular sequence. MMPs belong to a family of structurally related zinc-dependent enzymes that include interstitial collagenases, stromelysins, gelatinases, and membrane-type metalloproteinases (Woessner, 2002). MMPs are produced by all species of plants, animal, and microorganisms (Wakanabe, 2004). Collagenolytic protease from microorganisms has been extensively utilized in various fields because large amounts can be produced within a short period and at a low cost (Barrett *et al*., 2004). With the new frontiers in biotechnology, the spectrum of protease applications has expanded into new fields such as clinical, medicinal and analytical chemistry (Shanmughapriya *et al*., 2008). Thus, proteases from microorganisms, especially MMP, can have a potential for production of gelatin hydrolysate with high DH and enhanced antioxidant activity.

The microorganisms isolated from fish processing plant or fish dock may possess high collagenolytic or gelatinolytic activities. Their enzymes can be used as the novel protease capable of hydrolyzing gelatin, particularly from fish skin. As a consequence, hydrolysate with high bioactivity can be prepared. Furthermore new peptides possessing bioactivity can be produced with the aid of protease from microorganisims isolated from fish docks. As a whole, protease obtained can be comparable to those available in the market; the cost of protease can be reduced and skin can be used efficiently.

1.2 Literature reviews

Protease from microorganisms

The inability of the plant and animal proteases to meet current world demands has led to an increasing interest in microbial proteases. Microorganisms represent an excellent source of enzymes, owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Microbial proteases account for approximately 40% of the total worldwide enzyme sales (Godfrey and West, 1996). Proteases from microbial sources are preferred to those from plant and animal since they possess almost all the characteristics desired for their biotechnological applications (Padmapriya *et al*., 2012).

Most commercial proteases are mainly neutral and alkaline which are produced by organisms belonging to the genus *Bacillus*. Neutral proteases from bacterial are active in a pH range of 5 to 8 and have rather low thermo-stability. Due to their intermediate reaction rate, the enzymes generate less bitterness in hydrolysed proteins than do the animal proteinases and hence are valuable for use in the food industry (Siddalingeshwara *et al*., 2010). Neutrase knonw as a neutral protease is unconscious to the natural plant proteinase inhibitors and is therefore useful in the beer industry. The neutral proteases of bacterial are featured by their high relevance for hydrophobic amino acid group. Their low thermo-stability is expedient for controlling their activity via the production of food hydrolysates with a low degree of hydrolysis. Other neutral proteases belong to the metallo-protease group and need divalent metal ions for their activity. However, others proteses are serine enzyme, which are not induced by metal chelating agents (Godfrey and West, 1996). Alkaline proteases from bacterial are characterized by their high activity at alkaline pH, e.g., pH 10, and their broad substrate specificity. Their optimal temperature is around 60 °C. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry (Kumar and Takagi, 1999).

Proteases are basically disparted into two mainly groups, for example exopeptidases and endopeptidases, base on their active site. Exopeptidases cleavely react the peptide bond provenance to the amino or carboxy terminal site of the substrate, meanwhile endopeptidases cut peptide bonds from the substrate terminal. Depending on the functional group found at the active site, proteases are further categorized into four distinctive groups, for example aspartic proteases, cysteine proteases, metalloproteases, and serine proteases.

Exopeptidases

The exopeptidases act only near the ends of polypeptide chains. Based on their site of action at the N or C terminus, they are classified as amino- and carboxypeptidases, respectively.

Aminopeptidases

Aminopeptidases act at a free N terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide, or a tripeptide. Aminopeptidases occur in a wide variety of microbial species including bacteria and fungi (Watson, 1976). In general, aminopeptidases are intracellular enzymes, but an extracellular aminopeptidase could be produced by *A. oryzae* (Labbe *et al*., 1974). The substrate specificities of the enzymes from bacteria and fungi are distinctly different, yielding the variety of products (Cerny, 1978). Aminopeptidase I from *Escherichia coli* is a large protease (400,000 Da). It has a broad pH optimum of 7.5 to 10.5 and requires Mg^{2+} or Mn^{2+} for optimal activity (De Marco and Dick, 1978). The *Bacillus licheniformis* aminopeptidase has a molecular weight of 34,000 Da with Zn^{2+} in the active site. Its activity is enhanced by Co^{2+} ions. On the other hand, aminopeptidase II from *B. stearothermophilus* is a dimer with a molecular weight of 80,000 to 100,000 Da (Stoll *et al*., 1976) and is activated by Zn^{2+}, Mn^{2+}, or Co^{2+} ions (Godfrey and West, 1996).

Carboxypeptidases

A carboxypeptidase (EC number 3.4.16 - 3.4.18) is a protease enzyme that hydrolyzes (cleaves) a peptide bond at the carboxy-terminal (C-terminal) end of a protein or peptide. Carboxypeptidases can be categorized into three major groups, serine carboxypeptidases, metallocarboxypeptidases, and cysteine carboxypeptidases, depend on the nature of the amino acid residues at the active site of the enzymes (Godfrey and West, 1996). The serine carboxypeptidases purified from *Penicillium* spp., *Saccharomyces* spp., and *Aspergillus* spp. are identical in their substrate specificities. However, they are gently different in some properties such as optimal pH thermal-stability of enzyme, molecular weight, size, effect of some chemical and inhibitors. Metallocarboxypeptidases from *Saccharomyces* spp. (Felix and Brouillet, 1966) and *Pseudomonas* spp. Lu *et al*. (1969) reported that bacterial carboxypeptidase required co-valent metal like Zn^{2+} or Co^{2+} to activate their activity.

Endopeptidases

Endopeptidases are characterized by their preferential action at the peptide bonds in the inner regions of the polypeptide chain away from the N and C termini. The presence of the free amino or carboxyl group has a negative influence on enzyme activity. The endopeptidases are divided into four subgroups based on their catalytic mechanism, serine proteases, aspartic proteases, cysteine proteases, and metalloproteases (Gupta *et al*., 2002).

Serine proteases

Serine proteases are divided by the presence of a serine group at the enzyme active site. They are numerous and widespread among viruses, bacteria, and eukaryotes, suggesting that they are vital to the organisms. Serine proteases are found in the exopeptidase, endopeptidase, oligopeptidase, and omega peptidase groups. Based on their structural similarities, serine proteases have been grouped into 20 families, which have been further, categorized into about six groups (Barett, 1994). The primary structures of the members from four classes, e.g., chymotrypsin, subtilisin, carboxypeptidase C, and *Escherichia* D-Ala-D-Ala peptidase A are completely non-correlated, recommending that there are at least four separated evolutionary origins for serine proteases. Another interesting feature of the serine proteases is the conservation of glycine residues in the vicinity of the catalytic serine residue to form the motif Gly-Xaa-Ser-Yaa-Gly (Brenner, 1988).

Serine proteases are acknowledged by their irreversible inhibition by 3,4-dichloroiso-coumarin (3,4-DCI), diisopropylfluorophosphate (DFP), phenylmethyl sulfonylfluoride (PMSF) and tosyl-l-lysine chloromethyl ketone (TLCK). Some serine proteases are incommodious by thiol reagents such as *p*-chloromercuribenzoate (PCMB) due to the presence of a cysteine residue near the active site (Godfrey and West, 1996). Serine proteases are basically active at neutral and alkaline pH, with an optimum pH of 7 to 11. They have specificities of broad substrate including esterolytic and amidase activity. Their molecular weight range is belonging to 18 and 35 kDa. Nevertherless, the serine protease from *Blakeslea trispora* has a molecular weight of 126 kDa (Govind *et al*., 1981). The isoelectric

points of serine proteases are generally between pH 4 and 6. Serine alkaline proteases that are active at highly alkaline pH represent the largest subgroup of serine proteases.

Serine alkaline proteases are produced by several bacteria, molds, yeasts, and fungi. They are inhibited by DFP or a potato protease inhibitor but not by tosyl-l-phenylalanine chloromethyl ketone (TPCK) or TLCK. Their substrate specificity is similar to but less stringent than that of chymotrypsin. They hydrolyze a peptide bond which has tyrosine, phenylalanine, or leucine at the carboxyl side of the splitting bond. The alkaline proteases have optimal pH around pH 10. They also have isoelectric point at pH 9 with the molecular weights in the range of 15 to 30 kDa. Even though alkaline serine proteases are created by some bacteria such as *Arthrobacter*, *Streptomyces*, and *Flavobacterium* spp. (Boguslawski *et al*., 1983), subtilisins produced from *Bacillus* spp. are well known as the best enzyme for protein hydrolysate. Alkaline enzymes from microbial are produced by *S. cerevisiae* (Mizuno and Matsuo, 1984) and filamentous fungi such as *Conidiobolus* spp. (Phadatare *et al*., 1993) and *Aspergillus* and *Neurospora* spp. (Lindberg *et al*., 1981).

Subtilisins of *Bacillus* origin represent the second largest family of serine proteases. There are two different types of alkaline proteases, subtilisin Carlsberg and subtilisin Novo or bacterial protease Nagase (BPN′) (Ballinger *et al*., 1996). *Bacillus licheniformis* can produce Subtilisin Carlsberg enzyme that was discovered in 1947 by Linderstrom, Lang, and Ottesen at the Carlsberg laboratory. Subtilisin Carlsberg is exceedingly used in purgative industrial. About 500 tons of pure enzyme protein was produced recently. Subtilisin BPN′ is less commercially important (Ballinger *et al*., 1996). The molecular weights of both subtilisins are around 27.5 kDa but differ deeply in 58 amino acids from each other. Morever they have homogeneous properties such as an optimal temperature (60 °C) and an optimal pH of 9 to 10. Subtilisin Carlsberg enzymes common property in dispute broad substrate specificity and an active-site triads are made up of Ser_{221}, His_{64} and Asp_{32} (Godfrey and West, 1996). The Carlsberg enzyme has broader substrate specificity and does not base on metal ion like Ca^{2+} for its stability. The subtilisins active-site conformation is well-matched to that of trypsin and chymotrypsin despite the individuality in their whole molecular disposition. *Conidiobolus coronatus* serine alkaline protease found in fungus was exhibited to dominate a manifestly different

structure from subtilisin Carlsberg in spite of their functional similarities (Phadatare *et al*., 1993). Serine proteases from different bacteria are summarized in Table 1.

Table 1. Serine proteases from several bacteria and their characteristics

Bacteria	MW/optimal pH and temperature	References
Bacillus lehensis	39 kDa, pH 12.8 and 50 °C	Joshi and Satyanarayana (2013)
Oceanobacillus iheyensis O.M.A18	30 kDa, pH 8–11 and temperature 30–50 °	Purohit and Singh (2014)
Haloalkaliphilic bacterium Ve_2-20-9_1	37.2 kDa, pH 10 and 50 °C	Raval *et al*. (2014)
Bacillus licheniformis NH1	27.28 kDa, pH 10-11 and 65-70 °C	Hadj-Ali *et al*. (2007)
Cirriformia tentaculata CTSP-1	28.8 kDa, pH 8.5–9.0 and 50–60 °C	Park *et al*. (2013)
Bacillus subtilis DM-04	33.1 kDa, pH 10.0–10.5 and 37–45 °C and	Rai and Mukherjee (2009)
Bacillus circulans DZ100	32 kDa, pH 12.5 and 85 °C	Benkiar *et al*. (2013)
Bacillus sp. AS-S20-I	32.3 kDa, pH 7.4 and 37 °C	Mukherjee *et al*. (2012)
Bacillus mojavensis A21	20 kDa, pH 8.0–11.0 and 60 °C	Haddar *et al*. (2010)
Bacillus subtilis P13	31 kDa, pH 7.2 and 37 °C	Pillai *et al*. (2011)

Aspartic proteases

Aspartic proteases are a family of protease enzymes that use an aspartate residue for catalysis of their peptide substrates. In general, they have two shrilly conserved aspartates in the active site and are optimally active at acidic pH. Almost all known aspartyl proteases are inhibited by pepstatin (Barett, 1995). Most aspartic proteases are maximal activity at low pH (pH 3 to 4) and have isoelectric points in the range of pH 3 to 4.5. The molecular weight of aspartic proteases are in the range of 30 to 45 kDa. The members of the pepsin family have two lobes structure with the active-site cleft constitutive between the lobes (Sielecki *et al*., 1991). The active-site aspartic acid residue is bestead within the motif Asp-Xaa-Gly, that Xaa could be Ser or Thr. The aspartic proteases are inhibited by pepstatin (Fitzgerald *et al*., 1990). They are also inhibited by diazoketone compounds such as diazoacetyl-DL-norleucine methyl ester (DAN) and 1,2-epoxy-3-(*p*-nitrophenoxy) propane (EPNP) in the attendance of Cu^{2+}. Bactiral aspartic proteases show specificity against aromatic or amino acid residues on both sides of the peptide bond, in which is selfsame to pepsin, but their action is less rigorous than that of pepsin. Microbial acid proteases are also broadly graduated into two classes, (1) pepsin-like enzymes produced by *Aspergillus*, *Neurospora*, *Penicillium*, and *Rhizopus* and (2) rennin-like enzymes produced by *Endothia* and *Mucor* spp. (Godfrey and West, 1996).

Cysteine/thiol proteases

Cysteine proteases initiate in both prokaryotes and eukaryotes. There are over 20 families of cysteine proteases have been characterized. Most cysteine proteases activity bases on a set of two amino acid consisting of cysteine and histidine (Barett, 1994). Basically, thiol proteases are active only in the residence of reducing agents such as HCN or cysteine. Depend on their side chain specificity, they are broadly balance out to four typs: (1) papain-like, (2) trypsin-like with preference for cleavage at the arginine residue, (3) specific to glutamic acid, and (4) others. Papain, also known as papaya proteinase I, is a cysteine protease (EC 3.4.22.2) enzyme present in papaya (*Carica papaya*). Even though cysteine proteases have neutral pH optima, some of them, for example, lysosomal proteases are highly active at low pH.

They are susceptible to sulfhydryl agents such as PCMB but are unaffected by DFP and metal-chelating agents. Clostripain, produced by the anaerobic bacterium *Clostridium histolyticum*, exhibits a stringent specificity for arginyl residues at the carboxyl side of the splitting bond and differs from papain in its obligate requirement for calcium (Tanaka *et al*., 2011)., In addition, cysteine protease produced by *Streptococcus* spp. called Streptopain shows a broader specificity, including oxidized insulin B chain and other synthetic substrates. Clostripain has an isoelectric point of pH 4.9 and a molecular mass of 50 kDa, whereas the isoelectric point and molecular weight of streptopain are pH 8.4 and 32 kDa, respectively (Manabe *et al*, 2010).

Metalloproteases

A metalloproteinase, or metalloprotease (MMP), is any protease enzyme whose catalytic mechanism involves a metal. An example of this would be meltrin which plays a significant role in the fusion of muscle cells during embryo development, in a process known as myogenesis. Most MMP require zinc, but some use cobal MMP are the most diverse of the catalytic types of proteases (Barett, 1995). There are now at least 25 obvious vertebrate MMPs (Table 2 and Figure 1). Base on ability to cleave substrates, they can be categorized into four main groups: collagenases, gelatinases, stromelysins and membranetype (MT) MMPs. They are characterized by the requirement for a divalent metal ion for their activity. Proteases are from a diversity of origins such as collagenases from higher organisms, hemorrhagic toxins from snake venoms, and thermolysin from bacteria (Hibbs *et al*., 1985). About 30 families of metalloproteases have been recognized, of which 17 contain only endopeptidases, 12 contain only exopeptidases, and 1 (M3) contains both endo- and exopeptidases. Families of metalloproteases have been divided into different class rest on the amino acid nature that completes the metal-binding site; e.g., class MA has the sequence HEXXH-E and class MB corresponds to the motif HEXXH-H. In one of the groups, the metal atom binds at a motif other than the usual motif (Wakanabe, 2003).

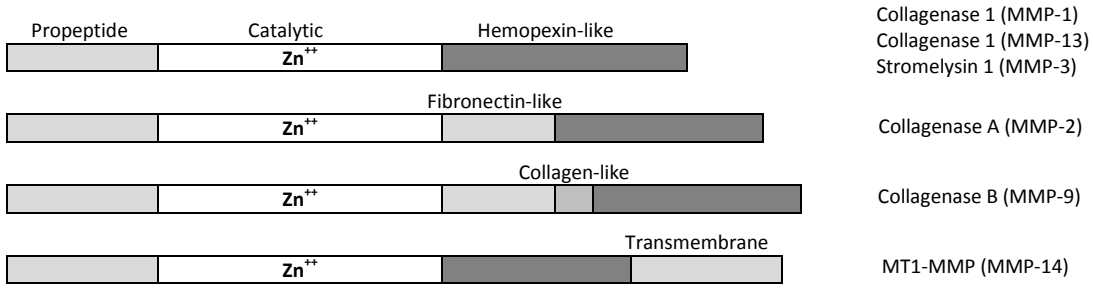

Figure 1. Domain structure of MMPs

Source: Brinckerhoff *et al*. (2004)

Assumed the specificity of their action, MMPs can be categorized into four types, (1) neutral, (2) alkaline, (3) *Myxobacter* I, and (4) *Myxobacter* II. The neutral proteases enzyme shows specificity for hydrophobic amino acids, meanwhile the alkaline enzyme dominate a very extensive specificity (Brinckerhoff *et al*., 2004). *Myxobacter* protease I is specific for diminutive amino acid residues on either part of the cleavage bond, while protease II enzyme is exclusive for residue of lysine on the amino side of the peptide bond. All of them are inhibited by metal chelating agents such as EDTA or EGTA but not by sulfhydryl agents or DFP (Godfrey and West, 1996).

Thermolysin, a neutral protease, is the most thoroughly characterized member of class MA. Like all bacterial extracellular proteases, thermolysin is synthesized as a pre-proenzyme consisting of a signal peptide 28 amino acids long, a pro-peptide 204 amino acids long and the mature enzyme itself 316 amino acids in length. Histidine residues from the HEXXH motif serve as Zn ligands, and Glu has a catalytic function (Weaver *et al*., 1977). *Bacillus stearothermophilus* can produce hermolysin enzyme that is a single peptide without disulfide bridges with 34 kDa of molecular weight. It has an elemental zine atom inserted in a cleft formed between two folded lobes of the protein and four calcium atoms that notify some factors enhancing protein thermostability. Thermolysin knew as a abundantly stable enzyme, with a half-life at 80 °C for 60 min (Godfrey and West, 1996).

Table 2. Matrix metalloproteinases (MMPs).

Enzyme	MMP No.	E.C. No.	Matrix substrates
Collagenases			
Interstitial collagenase-1	MMP-1	3.4.24.7	Collagens I, II, III, VII, X, gelatins, aggrecan, entactin
Neutrophil collagenase-1	MMP-8	3.4.24.34	Collagens I, II, III, aggrecan, link protein
Collagenase-3 (Rodent intersititial collagenase)	MMP-13		Collagens I, II, III
MT1-MMP	MMP-14		Collagens I, II, III, fibronectin
Gelatinases			
Gelatinases A (72 kDa gelatinase, type IV collagenase)	MMP-2	3.4.24.24	Gelatins, collagens I, IV, V, VII, X, XI, bibronectin, laminin, elastin, aggrecan, vitronectin
Gelatinases B (97 kDa gelatinase, type V collagenase)	MMP-9	3.4.24.35	Gelatins, collagens IV, V, XIV, elastin, aggrecan, vitronectin
Stromelysins			
Stromelysins-1 (Transin, proteoglycanase, CAP)	MMP-3	3.4.24.17	Collagens III, IV, IX, gelatins, aggrecan, fibronectin, laminin
Stromelysins-2 (Transin 2)	MMP-10	3.4.24.22	Aggrecan, fibronectin, collagen IV
Membrane-type MMPs			
MT2-MMP	MMP-15		Collagens I, II, III, fibronectin
MT3-MMP	MMP-16		Collagens III, gelatins, fibronectin
MT4-MMP	MMP-17		Not known
MT5-MMP	MMP-24		Gelatins
MT6-MMP	MMP-25		Gelatins
Others			
Matrilysin	MMP-7		Collagen IV, gelatins, aggrecan, fibronectin, elastin, laminin
Stromelysin-3	MMP-11		Collagen IV, gelatins, aggrecan, fibronectin, elastin, laminin
Metalloelastase	MMP-12		Elastin

Source: Brinckerhoff *et al*. (2004); Woessner (2002); Watanabe (2004)

Collagen/gelatin hydrolyzing proteases

Collagenases are enzymes that break the peptide bonds in collagen. They assist in destroying extracellular structures in the pathogenesis of bacteria such as *Clostridium hystolyticum* as a component of toxic products. After that it was descend to be procreated by other microorganisms like aerobic bacterium such as *Achromobacter iophagus* as well as fungi kigdom (Jung *et al*., 1999). The action of collagenase is extremely specific that it cut the bond only on collagen and gelatin and not on any of the other routine protein substrates. Elastase generated by *Pseudomonas aeruginosa* is another significant member of the neutral MMPs family (Barrett *et al*., 2004).

The alkaline MMPs produced by *Pseudomonas aeruginosa* and *Serratia* spp. are function in the pH 7 to 9 with molecular weight of 48 to 60 kDa. *Myxobacter* protease I also has a optimum pH of 9.0 with a molecular weight of 14 kDa. It also can lyse *Arthrobacter crystellopoites* cell walls meanwhile protease II unable to lyse the cells membrane of bacteria. Matrix metalloproteases play a important role in the disconnection of the extracellular matrix during tissue morphogenesis, differentiation, and wound healing and may be useful in the treatment of diseases such as cancer and arthritis (Browner *et al*., 1995).

Maximum collagenolytic activity from *Bacillus* sp. was observed at pH 8.2 in a 0.05 M Tris-HCl buffer (Okamoto *et al*., 2001; Lima *et al*., 2009). The collagenase produced by thermophilic *Bacillus* sp. strain NTAP-1 had the maximum collagenolytic activity at pH 8.0. A retention of more than 60% of the maximum activity was found between pH 5.0 and 9.0, and no activity was detected at pH 3.0 and 4.0 (Nakayama *et al*., 2000). Maximum collagenolytic activity from *Chrysosporium keratinophilum* was reported at pH 8.6 (Dozie *et al*., 1994). Petrova *et al*. (2006) studied the production of collagenolytic enzymes by *Streptomyces* sp. and discovered two collagenases that showed maximum collagenolytic activities at pH 7.5. More than 50% of the maximum activity was detected for these enzymes between pH 6.5 and 9.0. The collagenase was most active at 45 °C and a reduction in activity was observed at higher temperature and inactivated at 80 °C (Lima *et al*., 2009).

Table 3. Bacterial collagenolytic proteases with well-characterized molecular properties

Bacteria	Type	Localization	Molecular mass (kDa)	**References**
Clostridium histolyticum	Metalloprotease	Extracellular	116(ColH)	Yoshihara *et al*. (1994)
			116(ColG)	Matsushita *et al*. (1998)
Clostridium perfringens	Metalloprotease	Extracellular	120 (ColA)	Matsushita *et al*. (1994)
Vibrio alginolyticus	Metalloprotease	Extracellular	82	Takeuchi *et al*. (1992)
Vibrio vulnificus	Metalloprotease	Extracellular	45	Miyoshi *et al*. (1998)
Achromobacter lyticus	Serine protease	Extracellular	50	Ohara *et al*. (1989)
Geobacillus collagenovorans	Serine protease	Extracellular or cell-associated	105×2	Okamoto *et al*. (2001)
Alicyclobacillus sendaiensis	Serine protease	Extracellular	37	Tsuruoka *et al*. (2003)
Porphyromonas gingivalis	Thiol protease	Cell-associated	94	Lawson *et al*. (1992)
	Thiol protease	Cell-associated	37.8 x 2	Kato *et al*. (1992)
	Serine protease	Extracellular	55	Bedi *et al*. (1994)
Bacillus cereus	Metalloprotease	Extracellular	105	Lund *et al*. (1999)
	Metalloprotease	Extracellular	87	Makinen and Makinene (1987)
Treponema denticola	Serine protease	n.d.	67	Sorsa *et al*. (1995)
Streptococcus gordnii	Serine protease	Extracellular or cell-associated	98	Juarez *et al*. (1999)

Note**:** n.d. Not determined

Kaminishi *et al*. (1986) purified and characterized a collagenase from *C. albicans*, which had a optimum collagenolytic activity at 45 °C, and approximately 90% of the enzyme activity was deceased after incubated at 60 °C, and then no activity was obtained at the temperature of 70 °C. Collagenolytic proteases are probably disorganized with collagenases, in which collagenases are directly hydrolyze collagen molecules with unique specificity. Due to the diversity of the collagen structure, it is exceedingly cumbersome to individualize actual collagenases from other collagenolytic proteases and from gelatinases that basically hydrolyze only gelatin or a denatured collagen (Harrington, 1996).

From most the collagenolytic proteases, matrix metalloproteinases from mammalian have been investigated in more specification than collagenolytic proteases from microorganism. Among bacterial collagenolytic proteases, metalloproteases are the most regularly occurring, whereas the number of serine proteases and other proteases is rather minor (Wakanabe, 2004). Collagenolytic serine-carboxy protease produced by *Alicyclobacillus sendaiensis* strain NTAP-1 displays distinctive cleavage sites in collagen (Tsuruoka *et al*., 2003). Among bacterial collagenolytic proteases, collagen-binding segments have been shown the identity in the structures of collagenases ColG and ColH from *C. histolyticum* (Matsushita *et al*., 1998) and collagenase ColA from *C. perfringens* (Matsushita *et al*., 1994). Collagenase enzyme produced by *Vibrio alginolyticus* (Takeuchi *et al*., 1992) and *Achromobacter lyticus* enzyme I also distinguished the ordinary denominator (Ohara *et al*., 1989). However, the sequences of mammalian and bacterial collagen-binding territory are thoroughly distinct from one another (Yoshihara *et al*., 1994).

Collagenolytic proteases have a higher molecular weight than those of other enzyme (Table 3). The molecular masses of collagenolytic proteases from *C. histolyticum*, *C. perfringens*, *V. alginolyticus*, and *Bacillus cereus* are irregularly hulking, paralleled with those of some metalloproteases (Gonzales and Robert-Baudouy 1996). Moreover, the collagenolytic serine proteases produced by *Geobacillus collagenovorans* and *Streptococcus gordnii* have unattached subunits with much higher molecular weights around 105 kDa and 98 kDa, respectively (Okamoto *et al*., 2001). However, this result opposite to the molecular weight from serine proteases produces by bacteria that basically district between 18 and 35 kDa

(Gupta *et al*., 2002). The huge molecular weight of collagenolytic enzyme might be a resemblance commenting a complicated form of development for the pathogenic bacteria existence.

Production of proteases from microorganisms

Proteases from microorganisms have been extensively utilized in various fields because they can be produced at a large amount within a short time and at a low cost (Barrett *et al*., 2004). With the new frontiers in biotechnology, the spectrum of protease applications has expanded into new fields such as clinical, medicinal and analytical chemistry. These organisms should be provided with optimal growth conditions to increase enzyme production. Some of these microbes have been found to be sources of solvent-stable enzymes (Gupta and Khare, 2006; Rahman *et al*., 2007). The culture conditions that promote protease production were found to be significantly different from the culture conditions promoting cell growth (Shanmughapriya *et al*., 2008). In the industrial production of proteases, technical media usually employed contain very high concentrations of complex carbohydrates, proteins, and other media components (Rahman *et al*., 2007). With a view to develop an economically feasible technology, the improvement in the yields of proteases and optimization of the fermentation medium and production conditions have been conducted (Shanmughapriya *et al*., 2008).

Microbial proteases are generally produced by submerged fermentation, in which solid state fermentation processes have been exploited to a lesser extent (George, 1995). To carrying out some business process, the optimization of medium composition is handled to maintain equilibrium between the various medium constitutives, thus minimizing the amount of unutilized constitutives at the end of protease production. Many research efforts have been directed generally toward: evaluation of the effect of various carbon and nitrogenous nutrients as cost-proficient substrates on the enzymes yield; exigency of divalent metal ions in the fermentation medium; and optimization of environmental and fermentation parameters such as pH, temperature, aeration, and agitation (Gupta and Khare, 2006). Each organism or strain has its own special conditions for maximum enzyme production. Extracellular protease produced by microorganisms is substantially

influenced not only by medium components including carbon source, nitrogen source, and trace elements, but also by culture conditions including pH, temperature, orbital agitation speed, and inoculum size (Lima *et al*., 2009).

Nitrogen source

For most microorganisms, both inorganic and organic nitrogens are metabolized to produce amino acids, nucleic acids, proteins, and cell wall components. The protease comprises 15.6% nitrogen (Kole *et al*., 1988) and its production is dependent on the availability of both carbon and nitrogen sources in the medium (Readdy *et al*., 2008). Although complex nitrogen sources are usually used for protease production, the requirement for a specific nitrogen supplement differs from organism to organism (Rahman *et al*., 2007).

Protease production at low level was reported with the use of inorganic nitrogen sources in the production medium (Lima *et al*., 2009). Enzyme synthesis was found to be repressed by rapidly metabolizable nitrogen sources such as amino acids or ammonium ion concentrations in the medium (Giesecke *et al*., 1991). However, an increase in protease production by the addition of ammonium sulphate and potassium nitrate was reported by Sinha and Satyanarayana (1991). Substitution of sodium nitrate in the basal medium with ammonium nitrate increased protease production. The replacement of soybean flour with ammonium sulphate in a fed-batch process proved cost-effective, and resulted in the elimination of unpleasant odours (Kumar and takagi, 1999). Patel *et al*. (2005) and Chauhan and Gupta (2004) reported that casamino acid was proved to be the best organic nitrogen source which acted as inducer for enzyme production from *Bacillus* sp. In a marine microorganism, the proteolytic activity was induced by casamino acid (Daatsellar and Harder, 1974).

Carbon source

Carbon is another factor affecting the production of proteases by microorganisms. A reduction in protease production from *Bacillus* sp. RKY3 due to catabolite repression by glucose was reported by Readdy *et al*. (2008). In commercial practice, high carbohydrate concentrations repressed enzyme production. Therefore,

carbohydrate was added either continuously or in aliquots throughout the fermentation to supplement the exhausted component and keep the volume limited, thereby reducing the power requirements (Lima *et al*., 2009).

Increasing yields of proteases were obtained when different sugars such as lactose, maltose, sucrose and fructose were used (Pillai *et al*., 2011). However, a repression in enzyme synthesis was observed, in the presence of these ingredients at high concentrations. Whey, a waste byproduct of the dairy industry containing mainly lactose and salts was demonstrated as a potential substrate for protease production by *Aureobasidium pullulans* (Donaghy and McKay, 1993). Various organic acids, such as acetic acid, methyl acetate and citric acid or sodium citrate have been demonstrated to increase production of proteases from an alkalophilic *Bacillus* isolate (Kumar *et al*., 1997; Takii *et al*., 1990). Microbial protease production is heavily dependent on the availability of both carbon and nitrogen sources within the medium (Moon and Parulekar, 1991; Chu *et al*., 1992).

Metal ion

Divalent metal ions such as calcium, cobalt, copper, boron, iron, magnesium, manganese, and molybdenum are required in the fermentation medium for optimum production of alkaline proteases. However, the requirement for specific metal ions depends on the source of enzyme (Kumar *et al*., 1999). The use of $AgNO_3$ at a concentration of 0.05 mg/100 ml or $ZnSO_4$ at a concentration of 125 mg/100 ml resulted in an increase in protease production by *Rhizopus oryzae* (Banerjee and Bhattacharyya, 1992)

Potassium phosphate has been used as a source of phosphate, responsible for buffering the medium. Phosphate at the concentration of 2 g/l was found optimal for protease production by *Bacillus firmus* (Moon and Parulekar, 1991). However, an excessive concentration showed an inhibition in cell growth and repression in protease production. When the phosphate concentration was more than 4 g/l, the precipitation of medium during autoclaving was observed (Moon and Parulekar, 1993). This problem, however, could be overcome by the supplementation of the disodium salt of EDTA in the medium (Kumar and Takagi, 1999).

Dozie *et al*. (1994) reported that Ba^{2+}, Co^{+}, and Fe^{2+} caused the augmentation of collagenolytic activity in *Chrysosporium keratinophilum*. The positive effects of divalent metal ions like Ca^{2+}, Mg^{2+} and Mn^{2+} are attributed to their role in the maintenance of active enzyme conformation and also to the stabilization of enzyme-substrate complexes (Hernández *et al*., 1993). Dozie *et al*. (1994) reported that Ba^{2+}, Co^{+}, and Fe^{2+} resulted in the augmentation of keratinolytic activity in *Chrysosporium keratinophilum*. Pillai *et al*. (2011) reported that Ba^{2+} and Ca^{2+} had positive effect in single parametric studies and in Plackett-Burman (PB). Fe^{2+} had a positive effect in single parametric studies, but showed a negative effect in Plackett-Burman design.

pH

The important characteristic of most microorganisms is their strong dependence on the extracellular pH for cell growth and enzyme production. To increase protease yields, the pH of the medium must be maintained at a certain level throughout the fermentation period (Moon and Parulekar, 1991). The culture pH also strongly affects many enzymatic processes and transportation of various components across the cell membrane. When ammonium ions were used, the medium turned acidic, while it turned alkaline when organic nitrogen, such as amino acids or peptides were consumed. The decline in the pH may also be due to production of acidic products (Moon and Parulekar, 1993). In view of a close relationship between protease synthesis and the utilization of nitrogenous compounds, pH variations during fermentation may indicate kinetic information about the protease production, such as the start and end of the protease production period.

Hübner *et al*. (1993) produced alkaline serine protease subtilisin Carlsberg from *Bacillus licheniformis* on complex medium in a stirred tank reactor at pH 6.8 and van Putten *et al*. (1996) produced subtilisin protease from *B. licheniformis* by on-line process monitoring, at pH 7.2. Çalık *et al*. (1998; 2000) produced serine alkaline protease from *B. licheniformis* at pH 7.25. Frankena *et al*. (1986) conducted protease production from *B. licheniformis*, at pH 7.0, and Wright *et al*. (1992) produced protease from *Bacillus brevis* strain 47 at pH 7.5. Interactions between the metabolic reactions and genetic regulatory mechanisms, and product and by-product

formations in the bioprocess for the protease production are dependent on pH conditions and enable introduction of well-defined perturbations (Çalık *et al*., 2002).

Temperature

Temperature is another critical parameter that has to be controlled and varied from organism to organism. Many investigators have studied the correlation between protease secretions with temperature but this depends on the type of organism and culture conditions (Al-Shehri *et al*., 2004; Chi *et al*., 2007; Camila *et al*., 2007). Temperature affects all physiological activities in a living cell and it is an important environmental factor to control the growth, microbial activities, normal functioning of enzyme, the nutritional requirement of the cell and subsequently its composition (Van Demark and Batzing, 1987). The mechanism of temperature control for enzyme production is not well understood. The optimum temperature values reported for protease production are given in Table 3.

Aeration and agitation

Aeration and agitation are implemented in most fermentation process. The word ‘aerobe’ refers to the kind of microorganism that needs molecular for growth and metabolism. Aerobic is the condition of living organisms surviving only in the presence of molecular oxygen. Aerobic bacteria require oxygen for growth and can be incubated to be grown in atmospheric air. During fermentation, the aeration rate indirectly indicates the dissolved oxygen level in the fermentation broth. Different dissolved oxygen profiles can be obtained by various aeration rates, variations in the agitation speed of the bioreactor, or use of oxygen-rich or oxygen-deficient gas phase (appropriate air-oxygen or air-nitrogen mixtures) as the oxygen source (Takami *et al*., 1989). The variation in the agitation speed influences the extent of mixing in the shaking flasks or the bioreactor and will also affect the nutrient availability (Michalik *et al*., 1995). Chi *et al*. (2007) performed an optimization study of medium and cultivation conditions for protease production by the yeast *Aureobasidium pullulans* and observed that the agitation speed influenced the protease production.

Table 4. Optimum temperature values for maximum protease production.

Bacteria	Optimum temperature (°C)	References
Penicillum griseofulvin	28	Dixit and Verma, 1993
Bacillus sp.	30	Fujiwara and Yamamoto, 1987
Streptomyces diastaticus	30	Chaphalkar and Dey, 1994
Aspergillus flavus	32	Malathi and Chakraborty, 1991
Bacillus sp. Y	35	Shimogaki *et al*., 1991
B. licheniformis	36	Mao *et al*., 1992
Bacillus sp. AH-101	36	Takami *et al*., 1989
B. alcalophilus subsp. *halodurans* KP1239	37	Takii *et al*., 1990
B. firmus	37	Moon and Parulekar, 1991
Salinicoccus alkaliphiles sp. Nov.	37	Zhang *et al*., 2002
Bacillus sp. Ve1	37	Patel *et al*., 2005
B. licheniformis	39.5	Hübner *et al*., 1993
Bacillus sp. strain B189	40	Fujiwara *et al*., 1993
Aspergillus pullulans	45	Chi *et al*., 2007
B. lichenformis	45	Sen and Satyanarayana, 1993
B. licheniformis	50	Al-Shehri *et al*., 2004
Bacillus sp	50	Camila *et al*., 2007
Thermoactinomycetes sp. HS682	52	Tsuchiya *et al*., 1992
B. stearothermophilus AP-4	55	Dhandapani and Vijayaragavan, 1994
B. stearothermophilus F1	60	Rahman *et al*., 1994

Optimum yields of collagenolytic protease are produced at 200 rpm for *B. subtilis* ATCC 14416 (Michalik *et al*., 1995) and *B. licheniformis* (Sen and Satyanarayana, 1993). *Bacillus* sp. B21-2 produced the increasing proteases when agitated at 600 rpm and aerated at 0.5 volume per volume per min (Fujiwara and Yamamoto, 1987). Similarly, *Bacillus firmus* exhibited maximum enzyme yields at an aeration rate of 7.0 l/min and an agitation rate of 360 rpm. However, lowering the aeration rate to 0.1 l/min caused a drastic reduction in the protease yields (Moon and Parulekar, 1991). This indicates that a reduction in oxygen supply is an important limiting factor for growth as well as protease synthesis.

Protein hydrolysate

Protein hydrolysates can be prepared by chemical and enzymatic hydrolysis. Gernerlly, enzymatic method has gained increasing attention due to the milder process and specificity in hydrolysis. As a consequence, the desireable peptide, particularly with bioactivities, can be prepared. Various food protein sources including fish, milk, egg, soybean, wheat and zein have been exploited to produce antioxidative protein hydrolysates and peptides (Table 4). Fish and other seafood sources have particularly gained much interest as potential antioxidative peptide sources, mainly due to the abundance of raw materials in the form of processing discards and underutilized species. An array of biological activities for fish protein hydrolysates such as antihypertensive, immunomodulatory, neuroactive, antimicrobial, mineral and hormonal regulating properties has been reported (Bernet *et al*., 2000; Jung *et al*., 2005; Liu *et al*., 2008; Murray and Fitzgerald, 2007; Nikoo *et al*., 2014).

The processes commonly used for the production and processing of protein hydrolysates and the separation of peptides are summarized in Figure 4. Different proteases, both endogenous and exogenous, have been used in making protein hydrolysates and peptides. Use of exogenous enzymes is preferred in most cases over the autolytic process (i.e., use of endogenous enzymes present in the food source itself), due to the shorter time required to obtain the designated degree of hydrolysis. More consistent molecular weight profiles and peptide composition can be obtained.

Suitable enzyme and appropriate hydrolysis conditions such as temperature and pH for the optimal activity of enzyme, as well as the control of hydrolysis time, are crucial for obtaining protein hydrolysates with desirable functional and bioactive properties (Samaranayaka and Li-Chan, 2011). The crude protein hydrolysate may be further processed, e.g. seiving through ultrafiltration membranes, in order to obtain a more uniform product with the desired range of molecular mass (Pihlanto-Leppala and Korhonen, 2003). In large-scale production of hydrolysates, membrane technology may also be coupled with enzymatic hydrolysis in a continuous process, thereby reducing the cost by eliminating the need for heat or pH adjustment to inactivate the enzymes at the end of hydrolysis (Guerard, 2008). Low molecular mass membrane cut-offs are useful for concentrating antioxidative peptides from the higher molecular mass components remaining, including undigested polypeptide chains and enzymes. Other techniques such as nanofiltration, ion-exchange membranes, or column chromatographic methods can be used in further concentrating and purifying antioxidative peptides (Pihlanto-Leppala and Korhonen, 2003).

Type of protease and hydrolysis contains are the crucial parameters determining bioactivities of protein hydrolysates. Industrial food-grade proteinases such as Alcalase®, Flavourzyme®, and Protamex® derived from microorganisms, as well as enzymes from plant (e.g. papain) and animal sources (e.g., pepsin and trypsin), have been widely used in producing antioxidative peptides, particularly from various fish (Table 4). Nevertheless, the use of fish enzymes has also been reported, especially for the production of various antioxidative fish protein hydrolysates. Depending on the raw material, endogenous enzymes such as trypsin, chymotrypsin, pepsin, other enzymes of the viscera and digestive tract, as well as lysosomal proteases or catheptic enzymes in fish or other invertebrate muscle cells may contribute to the breakdown of proteins during autolysis (Kristinsson and Rasco, 2000).

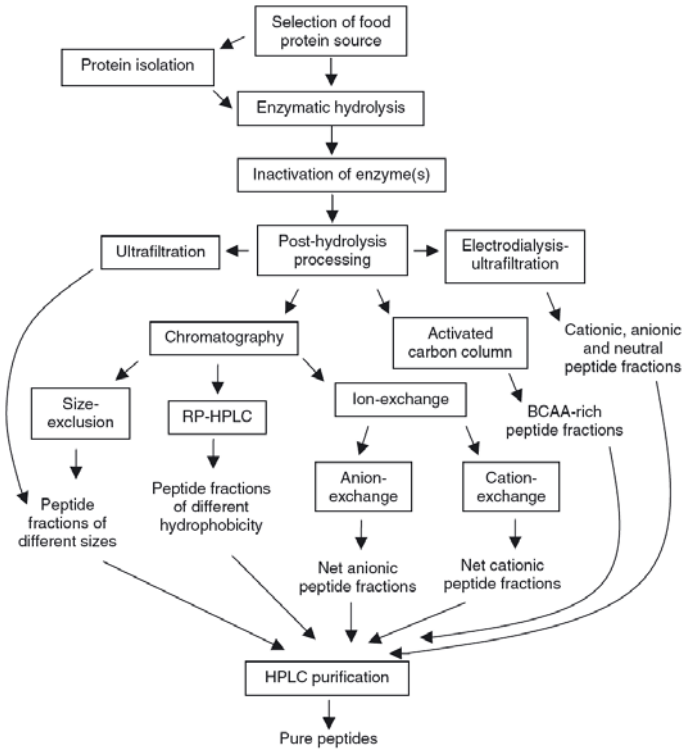

Figure 2. Production and processing of food protein-derived bioactive peptides.
Source: Udenigwe and Aluko (2012)

Peptides with antioxidative properties could be prepared from Alaska pollack (*Theragra chalcogramma*) and yellowfin sole (*Limanda aspera*) frame proteins by using a crude enzyme mixture from mackerel intestine (Je *et al*., 2005; Jun *et al*., 2004). Cathepsin L-like proteases present in parasitized Pacific hake (*Merluccius productus*) muscle was successfully used to produce fish protein hydrolysates with antioxidative properties (Samaranayaka and Li-Chan, 2008; Samaranayaka *et al*., 2010).

Several studies reported that the antioxidative activity of protein hydrolysates and isolated peptides prepared from hoki skin gelatin, marine bluemussel, tuna back bone, and Pacific hake fish fillet was superior to that of α-tocopherol and, in some cases, similar or higher in activity to that of commonly used synthetic antioxidants such as BHA and BHT (Je *et al*., 2007; Mendis *et al*., 2005b; Samaranayaka and Li-Chan, 2011). Synergistic effects of some antioxidative peptides with tocopherols in food and model systems have also been reported (Jun *et al*., 2004; Kim *et al*., 2001).

Table 5. Fish protein hydrolysates and peptides with antioxidative activity.

Sources	*In vitro* methods used for measuring antioxidant capacity	Enzymes used	Antioxidant peptides	References
Hold (*Johnius belengerii*) frame protein	O_2^{-},·OH, DPPH and peroxyl radical scavenging capacity(ESR), LAPS, protection against *t*-butylhydroperoxide-induced cytotoxicity on human embryonic lung fibroblasts and free-radical-induced DNA damage	Pepsin, Alcalase, α-chymotrypsin, Neutrase, papain, trypsin	ESTVPERTHPACPOFN	Kim *et al*., (2007)
Round scad (*Decapterus maruadsi*) muscle	DPPH radical scavenging capacity. reducing power, ferrous ion chelation	Alcalase and Flavourzyme	ND*	Thiansilakul *et al*., (2007)
Grass carp musde	·OH radical scavenging capacity, LAPS	Papain, bovine pancreatin 6.0, Bramelam, Neutrase, Alcalase	PSKYEPFV	Ren *et al*., (2008)
Alaska Pollack skin	DPPH radical scavenging capacity. reducing power	Neutrase, Flavourzyme Alcalase and Protamex		Jia *et al*., (2010)
Tuna (*Thunnus obesus*) dark muscle by-product	DPPH radical scavenging capacity, LAPS	Orientase, Protease XXII	LPTSEAAKY PMOYMVT	Hsu (2010)

Table 5. Fish protein hydrolysates and peptides with antioxidative activity. (Cont.)

Sources	*In vitro* methods used for measuring antioxidant capacity	Enzymes used	Antioxidant peptides	References
Loach (*Misgumus anguillicaudatus*)	·OH and DPPH radical scavenging capacity Cu^{2+} ion chelating activity, LAPS	Papain	PSYV	You *et al*., (2010)
Tuna cooking juice	DPPH radical scavenging capacity	Protease XXIII	PSHDAHPE, SHDAHPE VDHOHPE, PKAVHE PAGY, PHHADS, VOYP	Jao and Ko (2002)
Mackerel (*Scomber austriasicus*) fillet	DPPH assay, reducing power, LAPS	Autlysis Protease N	ND	Wu *et al*., (2003)

ND: Not determined

Three antioxidative peptides from tuna cooking juice hydrolysates prepared by Orientase were isolated by Hsu *et al*. (2009). These three peptides comprised 4–10 amino acids and were identified as Pro-Val-Ser-His-Asp-His-Ala-Pro-Glu-Tyr (1305 Da), Pro-Ser-Asp-His-Asp-His-Glu (938 Da), and Val-His-Asp-Tyr (584 Da). Hsu (2010) identified two antioxidative peptides from tuna dark muscle by-product using Orientase and protease XXIII. Those included Leu-Pro-Thr-Ser-Glu-Ala-Ala-Lys-Tyr (978 Da) and Pro-Met-Asp-Tyr-Met-Val-Thr (756 Da). Bougatef *et al*. (2010) reported seven antioxidative peptides including Leu-His-Tyr, Leu-Ala-Arg-Leu, Gly-Gly-Glu, Gly-Ala-His, Gly-Ala-Trp-Ala, Pro-His-Tyr-Leu and Gly-Ala-Leu-Ala-Ala-His from hydrolysate from sardinelle (*Sardinella aurita*) industrial wastes using crude enzyme extract from sardine (*Sardina pilchardus*). The first tripeptide displayed the highest DPPH radical scavenging activity (63 ± 1.57% at 150 μg/ml) and the presence of His and Tyr in the sequence could contribute significantly to the antioxidant activity of the peptides.

Apart from antioxidation activity, a with range of bioactivities including O_2^-,·OH, DPPH and peroxyl radical scavenging, reducing power capacity, ferrous ion chelation, and Cu^{2+} ion chelating activity, have been reported for fish hydrolysates from various sources (Jao and Ko, 2002; Wu *et al*., 2003; Kim *et al*., (2007); Thiansilakul *et al*., 2007; Ren *et al*., 2008; Jia *et al*., 2010; Hsu 2010; You *et al*.,2010)

Gelatin hydrolysate

Enzymatic process

Gelatin and collagen-derived hydrolysates and peptides are generally obtained by enzymatic proteolysis. A number of commercial proteases have been used for the production of gelatin hydrolysates and peptides. Those include trypsin, chymotrypsin, pepsin, Alcalase, Properase E, Pronase, collagenase, bromelain and papain (Lin and Li; 2006; Mendis *et al*., 2005a; Yang *et al*., 2008). Besides commercial proteases, enzymatic extracts from fish viscera have been used to obtain hydrolysates containing bioactive peptides from gelatin of fish skin and bones (Jung *et al*., 2005; Phanturat *et al*., 2010). Protease specificity affects size, amount, free

amino acid composition and, peptides and their amino acid sequences, which in turn influences the biological activity of the hydrolysates (Wu *et al*., 2003). Alcalase has been used widely for production of protein hydrolysate because of its broad specificity as well as the high degree of hydrolysis that can be achieved in a relatively short time under moderate conditions (Benkajul and Morrissey, 1997; Diniz and Martin, 1996). This enzyme showed an extensive proteolytic activity toward skin gelatin from Alaska pollack, squid *Todarodes pacificus* and giant squid. Gelatin hydrolysate prepared using different proteases from various sources are shown in Table 5.

Collagen and gelatin hydrolysates, mainly obtained from mammalian sources, have long been used in pharmaceutical and dietary supplements (Benito-Ruiz *et al*., 2009; Zuckley *et al*., 2004). Fish skin collagen hydrolysates have been reported to affect lipid absorption and metabolism in rats (Saito *et al*., 2009). Apart from the lipid-lowering effect, chicken bone collagen hydrolysates have been shown to reduce proinflammatory cytokine production in mice (Zhang *et al*., 2010).

Bone mineral density in osteoporotic rats and joint disease in dogs were improved by ingesting chicken and porcine gelatin and collagen hydrolysates (Beynen *et al*., 2010; Watanabe-Kamiyama *et al*., 2010). In contrast, bovine collagen hydrolysate consumption did not produce any effects on bone metabolism as measured by biochemical indices of bone remodelling in postmenopausal women (Cúneo *et al*., 2010).

Bioactivities

Protein hydrolysates are sources of biologically active peptides (Friedman 1996; Gildberg and Stenberg, 2001). Numerous physiological functions such as antihypertensive, immunomodulating, antithrombotic, antioxidative, anticancer, and antimicrobial activities have been reported for protein hydrolysates (Clare and Swaisgood, 2000). Moreover, protein hydrolysates have been applied in the nutritional management of individuals who cannot digest whole/intact protein. Hydrolysates rich in low molecular weight peptides, especially di- and tri-peptides with as little as possible free amino acids, have been shown to have more dietary uses due to their high nutritional and therapeutic values (Bhaskar *et al*., 2007).

Table 6. Antioxidant peptides derived from collagenous sources

Sources	Characteristic	Preparation	Activity	References
Fish skin gelatin (*Alaska Pollack*)	Gly-Glu-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly	Serial digestion (Alcalase, Pronase E, collagenase)	Inhibition of lipid peroxidation Increase of cell viability exposed to *t*-BHP	Kim *et al*. (2001)
Squid skin gelatin (*Dosidicus gigas*)	Phe-Asp-Ser-Gly-Pro-Ala-Gly-Val-Leu Asn-Gly-Pro-Leu-Gln-Ala-Gly-Gln-Pro-Gly-Glu-Arg	Trypsin	Radical scavenging Increase of cell viability exposed to *t*-BHP	Mendis *et al*. (2005a)
Squid tunic gelatin (*Dosidicus gigas*)	Gly-Pro-Leu-Gly-Leu-Leu-Gly-Phe-Leu-Gly-Pro-Leu-Gly-Leu-Ser	Alcalase	Radical scavenging Ferric reducing power	Alemán *et al*. (2011)
Fish skin gelatin (*Jonius belengerii*)	His-Gly-Pro-Leu-Gly-Pro-Leu	Trypsin	Radical scavenging Inhibition of lipid peroxidation Increase of antioxidative enzyme levels in hepatoma cells	Mendis *et al*. (2005b)

Table 6. Antioxidant peptides derived from collagenous sources (Cont.)

Sources	Characteristic	Preparation	Activity	References
Bovine skin gelatin	Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly	Pronase E	Inhibition of lipid peroxidation Increase of cell viability exposed to *t*-BHP	Kim *et al*. (2001)
Tuna backbone	Val-Lys-Ala-Gly-Phe-Ala-Trp-Thr-Ala-Asn-Gln-Gln-Leu-Ser	Pepsin	Radical scavenging Inhibition of lipid peroxidation	Je *et al*. (2007)
Porcine skin collagen	Gln-Gly-Ala-Arg	Mixture of proteases (bovine pancreas, *Streptomyces* and *Bacillus* spp)	Radical scavenging	Li *et al*. (2007)
Amur sturgeon skin gelatin	Pro–Ala–Gly–Tyr	Alcalase	DPPH, ABTS and hydroxyl radicals scavenging activity	Nikoo *et al*. (2014)

Antimicrobial properties

Antimicrobial properties of hydrolysates or peptides from collagen or gelatin have been reported. Gómez-Guillén *et al*. (2010) reported antimicrobial activity in peptide fractions from tuna and squid skin gelatins within a range of 1-10 kDa and < 1 kDa. The hydrolysates were tested using the agar diffusion assay against 18 strains of bacteria (both Gram-positive and negative). *Lactobacillus acidophilus*, *Bifidobacterium animalis* subp. *Lactis*, *Shewanella putrefaciens* and *Photobacterium phosphoreum* were found to be the most susceptible species. The reduced molecular weight in the peptide fractions, better exposure of the amino acid residues, their charges, as well as structure acquisition, were suggested as factors facilitating the interaction with bacterial membranes. A higher antimicrobial activity against both Gram-positive and Gram-negative bacteria was found for those dipeptides having a more pronounced cationic character (Molinero, 1988).

The hydrophobic character of amino acids allows peptides to enter the bacterial membrane and the positive charge of peptide would initiate the peptide interaction with the negatively charged bacteria surface (Wieprecht *et al*., 1997). On the other hand, the differences existing in membrane composition have implications in the mode of action and the specificity of the antibacterial compounds (Floris *et al*., 2003). Patrzykat and Douglas (2005) reported that the degree of lipopolysaccharide (LPS) binding is neither directly nor inversely proportional to peptide activity. Following the rupture of the outer membrane, peptide activity would depend on its ability to interact with the bacterial cytoplasmic membranes. Thus, both the sequence and concentration of the peptide determine the mode of actions (Gómez-Guillén *et al*., 2011).

Antioxidative activity

Antioxidative hydrolysates and peptides from skin gelatin of different fish species, such as Alaska pollack (Kim *et al*., 2001), hoki (*Johnius belengerri*) (Mendis *et al*., 2005a), cobia (*Rachycentron canadum*) (Yang *et al*., 2008) and sole (Giménez *et al*., 2009), as well as from several squid species, such as giant squid (*Dosidicus gigas*) (Giménez *et al*., 2009), Jumbo flying squid (*Dosidicus eschrichitii*

Streenstrup) (Lin and Li, 2006) or squid (*Todarodes pacificus*) (Nam *et al*., 2008) have been prepared and characterzied. Antioxidantive peptides isolated from fish skin gelatin hydrolysate are shown in Table 5.

In general, all 20 amino acids found in proteins can interact with free radicals if the energy of the free radical is high (e.g., hydroxyl radicals) (Elias *et al*., 2008). The most reactive amino acids include the nucleophilic sulfur-containing amino acids Cys and Met, the aromatic amino acids Trp, Tyr, and Phe, and the imidazole-containing amino acid His. However, free amino acids are not generally found to be effective as antioxidants in food and biological systems. Extensive proteolysis of food proteins in fact has been reported to result in decreased antioxidative activity (Rival *et al*., 2001). The higher antioxidative activity of peptides compared to free amino acids is attributed to the unique chemical and physical properties conferred by their amino acid sequences, especially the stability of the resultant peptide radicals that do not initiate or propagate further oxidative reactions (Elias *et al*., 2008).

Gelatin peptides could inhibit lipid peroxidation more efficiently than antioxidative peptides derived from many other protein sources (Kim *et al.*, 2001). Scavenging of free radical species is an important mechanism by which antioxidant peptides enhance cell viability against oxidation-induced cell death. Kim *et al*. (2001) reported that a peptide isolated from Alaska pollack skin gelatin was able to protect rat liver cells from oxidant injury induced by organic hydroperoxide *t*-BHP. Two purified peptides from squid skin gelatin exhibited a dose-dependent cell viability enhancement effect when their ability to overcome *t*-BHP-induced cytotoxicity was tested in human lung fibroblasts (Mendis *et al*., 2005a). Moreover, peptides isolated from hoki skin gelatin were capable of enhancing the expression of antioxidative enzymes such as glutathione peroxidase, catalase and superoxide dismutase in human hepatoma cells (Mendis *et al*., 2005b). Gelatin hydrolysate from blacktip shark skin with 40%DH could act as an antioxidant in oil-in-water emulsion and cooked comminuted pork system (Kittiphattanabawon *et al*., 2012). In addition, the gelatin hydrolysate (10–40%DH) at a concentration of 0.5 g/l inhibited LDL cholesterol oxidation by 8.32–39.21% and effectively inhibited hydroxyl and peroxyl radical-induced DNA scission (Kittiphattanabawon *et al*., 2013). Nile tilapia skin gelatin

hydrolysate (identified to be Gly-Pro-Met (303.38 Da)) exhibited radical-scavenging activity (IC_{50} = 2.09 mg/ml) (Wang *et al*., 2014). The peptide Pro–Ala–Gly–Tyr (PAGT) was isolated from Amur sturgeon skin gelatin showed scavenging activity against DPPH, ABTS, hydroxyl radicals and prevented lipid oxidation in minced fish (Nikoo *et al*., 2014).

Antioxidative properties of peptides are related to their amino acid composition, structure and hydrophobicity (Gómez-Guillén *et al*., 2011). Hydrophobic amino acid residues like Val or Leu can increase the presence of the peptides at the water-lipid interface and therefore facilitate their access to scavenge free radicals generated at the lipid phase (Ranathunga *et al*., 2006). Although the structure–activity relationship of antioxidative His-containing peptides has not been well defined yet, the activity could be attributed to hydrogen donating ability, lipid peroxy radical trapping, and/or the metal ion-chelating ability of the imidazole group (Chan and Decker, 1994). The amino acid composition of collagen and gelatin hydrolysates is very similar to that of the parent proteins, being rich in residues of Gly, Ala, Pro, Hyp, Glx and Asx, but poor in Met, Cys, His and Tyr (Alemán *et al*., 2011; Gómez-Guillén *et al*.; 2010). Dávalos *et al*. (2004) reported that Trp, Tyr and Met showed the highest antioxidant activity, followed by Cys, His and Phe. Kim *et al*. (2001) isolated two peptides containing 13 and 16 amino acid residues, respectively from Alaska pollack skin, both of which contained a Gly residue at the C-terminus and the repeating motif Gly-Pro-Hyp. Li *et al*. (2007) identified the peptide which exhibited the highest antioxidant activity from porcine skin collagen hydrolysates as Gln-Gly-Ala-Arg. A peptide Asn-Gly-Pro-Leu-Gln-Ala-Gly-Gln-Pro-Gly-Glu-Arg, was purified from squid skin gelatin hydrolysate and showed free radical quenching capacity (Mendis *et al*., 2005b). Furthermore, the antioxidant activity of collagen and gelatin peptides has been linked to the high content of hydrophobic amino acids, which could increase their solubility in lipids and therefore enhance their antioxidative activity (Kim *et al*., 2001). Rajapakse *et al*. (2005) found that fish skin gelatin peptides showed higher antioxidant activity than peptides from meat protein, probably because of the higher percentage of Gly and Pro.

Peptide conformation has also been claimed to influence antioxidant capacity, showing both synergistic and antagonistic effects (Hernández-Ledesma

et al., 2005). Alcalase gelatin-derived hydrolysate showed higher antioxidant activity than those prepared from collagenase, pepsin, trypsin, chymotrypsin, papain or neutrase (Alemán *et al*., 2011; Qian *et al*., 2008). Moreover, antioxidant activity is strongly related to peptide molecular weight as demonstrated by Gómez-Guillén *et al*. (2010) who found antioxidant activity in all the peptide fractions from squid skin hydrolysate, but the activity was higher in the fractions with lower-molecular weight. Similarly, the peptide fraction from cobia skin hydrolysate, with molecular mass values below 700Da, showed the highest radical scavenging activity, being approximately 20% higher than that of the non-fractionated hydrolysate (Yang *et al*., 2008).

ACE inhibitory activity

Angiotensin-I converting enzyme (ACE) plays an important role in the regulation of blood pressure and hypertension because ACE catalyzes the conversion of angiotensin I to angiotensin II and degrades bradykinin (Murray and FitzGerald, 2007). Synthetic inhibitors of ACE such as, Captopril Enalapril and Fosinopril, are often used to treat hypertension and other cardio-related diseases. However, these compounds can cause adverse side effects (Atkinson and Robertson, 1979). Therefore, increasing interest in natural products, especially from dietary protein that are perceived as being safer than synthetic inhibitors have been gained. For ACE-inhibitory activity, binding to ACE is strongly influenced by the C-terminal peptide sequence. Although the structure-activity relationship of food derived ACE-inhibitory peptides has not yet been fully established, ACE prefers inhibitors containing hydrophobic amino acid residues at each of the C-terminal positions (Murray and FitzGerald, 2007). Recently, ACE inhibitory peptides derived from marine sources have been documented extensively, including salmon chum muscle (One *et al*., 2005), shark meat (Wu *et al*., 2008), oyster protein (Wang *et al*., 2008), Alaska pollack skin (Byun and Kim, 2001), tuna frame protein (Lee *et al*., 2010) and cod skin (Himaya *et al*., 2012).

Peptide from squid gelatin hydrolyzed with Alcalase contained Leu (Gly-Pro-Leu-Gly-Leu-Leu-Gly-Phe-Leu-Gly-Pro-Leu-Gly-Leu-Ser). The peptide showed antioxidant and ACE-inhibitory activity (Alemán *et al*., 2011). In addition,

the hydrolysate from squid skin gelatin had *in vitro* ACE inhibitory activity in which IC_{50} value was 0.33 mg/ml (Lin *et al*., 2012). After the oral administration of this sample, the systolic blood pressure and diastolic blood pressure of resting heart rate (RHR) decreased, indicating that hysterosalpingoscintigraphy III (HSSG-III) had an intense effect on the reduction of blood pressure *in vivo* (Lin *et al*., 2012). Pacific cod skin was effectively bioconverted into a potent ACE inhibitory peptide, (IC_{50} = 35.7 μM) Leu-Leu-Met-Leu-Asp-Asn-Asp-Leu-Pro-Pro (1301 Da) as well as effectively protects cellular macromolecules from reactive oxygen species (ROS) mediated damage (Himaya *et al*., 2012). A decapeptide (Gly-Arg-Gly-Ser-Val-Pro-Ala-Hyp-Gly-Pro) with potent ACE-inhibitory capacity (IC_{50} = 47.78 μM) was obtained from squid skin hydrolysate (Alemán *et al*., 2013).

Applications of gelatin hydrolysate

Collagen and gelatin-derived peptides in the area of food science and technology have dealt with their antioxidant and antihypertensive/ACE inhibitory activity (Chen *et al*., 2007). These peptides have repeated unique Gly-Pro-Hyp sequences in their structure, and the observed antioxidative and antihypertensive properties have presumably been associated with this unique amino acid composition (Kim and Mendis, 2006). Moreover, collagen and gelatin-derived peptides exhibited numerous other bioactivities, namely antimicrobial activity, mineral binding capacity, the lipid-lowering effect, immunomodulatory activity and beneficial effects on skin, bone or joint health (Gómez-Guillén *et al*., 2010; Hou *et al*., 2009; Zhang, *et al*., 2010).

Functional foods and nutraceuticals

Food-derived peptides have been well established and several functional foods and natural health products are already available in the market (Hartmann and Meisel, 2007). Peptidic antioxidants could have an impact on reducing oxidative stress as well as the risk of various degenerative diseases such as cancer, cardiovascular disease, inflammatory diseases, etc. associated with oxidative stress (Samaranayaka and Li-Chan, 2011). Rajapakse *et al*. (2005) reported that the radical

scavenging peptide HFGDPFH derived from fermented mussel sauce could enhance the viability of oxidation induced cultured human lung fibroblast cells by 76%. Seacure® is a commercially available fermented fish product made by controlled yeast fermentation of Pacific hake, which is claimed to be beneficial for a variety of gut conditions. A pilot human clinical trial using Seacure® pointed out that it could reduce the degree of small intestinal damage caused by the non-steroidal anti-inflammatory drug, indomethacin (Marchbank *et al*., 2008).

Gelatin hydrolysate from shark skin exhibited good ACE-inhibitory activity and antioxidant potential in biological systems. Gelatin hydrolysate with DH of 40% prevented human LDL cholesterol oxidation and lowered peroxyl and hydroxyl radical-induced supercoiled plasmid DNA strand scission (Kittiphattanabawon *et al*., 2013). Therefore, it might serve as a potential functional food ingredient for health promotion.

Cosmeceuticals

Cosmeceuticals are topical products that are designed to improve the appearance of skin by various mechanisms of action (Lupo and Cole, 2007). Topical antioxidants from different food sources are presently being marketed to prevent aging and UV-induced skin damage, as well as to treat wrinkles and erythema due to inflammation (Allemann and Baumann, 2008). Li *et al*. (2007) reported that Pacific cod skin gelatin polypeptides were useful for enhancing the activities of antioxidant enzymes. This led to a protective effect of the collagen fibres below the epidermis and protects skin against the UV-induced skin damage. Kato *et al*. (2011) reported that fish collagen can be used as a functional food material which enhances the resistance to UVA in skin.

Peptides are used in different skin care products to perform various functions, apart from antioxidative action, such as modulation of cell proliferation, cell migration, inflammation, angiogenesis, melanogenesis, and protein synthesis and regulation (Zhang and Falla, 2009). Tripeptide glycyl-L-histidyl-L-lysine-copper (Cu-GHK) complex is used to deliver copper into cells, which is a cofactor for the antioxidant enzyme, superoxide dismutase, as well as for the collagen and elastin synthesis, that help in the wound healing process (Canapp *et al*., 2003; Choi and

Berson, 2006). L-carnosine (*β*-alanylhistidine) and related peptidomimetics like N-acetylcarnosine and carcinine (*β*-alanylhistamine) are considered as antioxidants that are useful to protect the skin from oxidative attacks (Babizhayev, 2006; Stvolinsky *et al*., 2010).

It is widely known that the digestion of collagen proteins or hydrolysates *in vivo* will generate peptides that may be useful for organic biosynthetic processes (Cúneo *et al*., 2010). Several functional properties of enzymatic collagen hydrolysates have been investigated in osteoarthritis and suggest a beneficial stimulating effect with an increased synthesis of extracellular matrix macromolecules produced by chondrocytes (Bello and Oesser, 2006). Thus, the consumption of collagen hydrolysates might be beneficial for bone health, apart from skin health.

Drying technology for protein hydrolysate

Due to their high moisture and protein content, protein hydrolysates are very perishable and could be converted to powder to improve their shelf-life. Bioactive peptides produced during hydrolysis process may possess bitter taste and high hygroscopicity due to the release of hydrophobic and hygroscopic amino acid residues, which frustrates their direct utilization to food processing (Yang *et al*., 2012). An appropriate processing technology to reduce undersirable odour, the bitterness and hygroscopicity of peptides is required. Several methods have been tried to reduce the odour and bitterness of protein hydrolysate, such as removal of hydrophobic peptides, along with hydrolysis process (Komai *et al*., 2007), reducing bitterness by processing technology (Favaro-Trindade *et al*., 2010) and removing undesirable odour (Yarnpakdee *et al*., 2012).

Several protein hydrolysates were successfully dehydrated using spray drying process to attenuate the bitterness and hygroscopicity of the products (Mendanha *et al*., 2009; Yang *et al*., 2012). In addition, freeze drying can ensure the physicochemical and bioactive stability of peptides, but it usually takes an enormous amount of time, labor and expenses (Wang, 2000).

Due to low molecular weight peptides, protein hydrolysates present low glass-transition temperature (Tg) values and, consequently, high hygroscopicity and thermoplasticity. The addition of carrier before spray drying is necessary.

Kurozawa *et al*. (2009b) evaluated the effect of carrier agent on Tg and physical stability of spray-dried chicken protein hydrolysate. The powder, without carrier agents, presented a low Tg and low critical values for moisture content and water activity, indicating its vulnerability during processing, handling and storage. Tg and critical moisture content values raised when maltodextrin or gum arabic were used as drying agents, showing their contribution to the powder stability (Oliveira *et al*., 2010). He *et al*. (2008) did not found any reduction in ACE inhibition capacity for shrimp hydrolysates after spray drying. Contreras *et al*. (2011) found that two peptides in casein hydrolysates were resistant to the spray drying without loss of ACE inhibition capacity. However, Amighi *et al*. (2013) studied the spray drying of ACE inhibitory enzyme-modified white cheese (EMWC) and found that spray drying decreased the ACE-inhibitory of EMWC significantly. Espejo-Carpio *et al*. (2013) showed that antioxidative and ACE inhibitory activity of goat milk protein hydrolysates decreased significantly due to spray drying. Spray drying of many food products has been studied by several researchers. An overview of some studies is shown in Table 7.

Table 7. Spray drying of miscellaneous food products

Food products	Spray drying conditions	Carrier agent	References
Protein hydrolysate from tilapia	T_{in} = 150 and 180 °C, T_{out} = 76 and 90 °C	Maltodextrin	Abdul-Hamid *et al*. (2002)
Hydrolyzed sweetpotato puree	T_{in} = 150, 190 and 220 °C, T_{out} = 100 °C, T_{feed} = 60 °C, m_{feed} = 2 kg/h, Atomizer speed = 20,000 rpm	Maltodextrin 11DE	Grabowski *et al*. (2006)
Lactose hydrolysed skim milk	T_{in} = 130 °C, T_{out} = 65 °C, T_{feed} = 50 °C	Not used	Shrestha *et al*. (2007)
Protein hydrolysate from chicken breast	T_{in} = 180 °C, T_{out} = 91 – 102 °C, m_{feed} = 0.2 kg/h	Maltodextrin 10DE and gum Arabic	Kurozawa *et al*. (2009b)
Protein hydrolysate from chicken breast	T_{in} = 88 – 136 °C, T_{out} = 91 – 102 °C, V_{feed} = 0.4 - 1.0 l/h	Maltodextrin 10DE	Kurozawa *et al*. (2009a)
Protein hydrolysate from fermented shrimp by-products	T_{in} = 180 °C, T_{out} = 140 °C, T_{feed} = 80 °C. , V_{feed} = 1.0 l/h	Not used	Bueno-Solano et al. (2009)
Shark cartilage gelatin	T_{in} = 127 °C, T_{out} = 100 °C	Not used	Kwak *et al*. (2009)
Casein hydrolysate	T_{in} = 140 °C, T_{out} = 110 °C, T_{feed} = 25 °C V_{feed} = 3.6 l/h, Atomization = 50 l/min	Gelatin and soy protein isolate	Favaro-Trindade *et al*. (2010)
Whey protein hydrolysate	T_{in} = 200 °C, T_{out} = 90 °C, T_{feed} = 25 °C, V_{feed} = 1.0 l/h	Sodium alginate	Ma *et al*. (2014)
Milk protein concentrate hydrolysates	T_{in} = 135 °C, T_{out} = 65 °C, Ultrasound = 20 kHz, 800 W	Not used	Uluko *et al*. (2014)

T_{in} : inlet air temperature; T_{out} : outlet air temperature; T_{feed} : feed temperature; V_{feed} : feed velocity.

1.3 References

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CHAPTER 2

GELATINOLYTIC ENZYMES FROM *BACILLUS AMYLOLIQUEFACIENS* ISOLATED FROM FISH DOCKS: CHARACTERISTICS AND HYDROLYTIC ACTIVITY

2.1 Abstract

Fifty-five bacteria with gelatinolytic activity were screened from over five-hundred isolates obtained from Songkhla fish docks. Based on 16S rRNA gene sequence analysis, three selected strains (K12, O02 and S13) were identified as *Bacillus cereus* with 99.8% similarity, whilst another three stains (D10, G02 and H11) were identified as *B. amyloliquefaciens* with 99.7% similarity. Gelatinolytic enzymes of D10, G02 and H11 strains were precipitated by ammonium sulfate, followed by dialysis with the increase in purity by 19-34 folds. Their maximal gelatinolytic activities were attained at 50 °C and pH 7.5 when fish gelatin was used as substrate. Metallo- and serine-gelatinolytic enzymes were dominant for all three strains. All gelatinolytic enzymes showed similar hydrolysis towards fish gelatin to commercial Alcalase, but higher hydrolysis was found in the formers within the first 60 min. Therefore, gelatinolytic enzymes from selected *B. amyloliquefaciens* strains could be used for production of fish gelatin hydrolysate.

2.2 Introduction

Microbial proteases represent one of the largest classes of industrial enzymes, accounting 40% of the total worldwide sale of enzymes (Rao *et al*., 1998). Microbes have been known as a good source of enzymes having the numerous characteristics. They have broad biochemical diversity with the rapid growth and the limited space is required for cultivation. Although a variety of proteolytic fungi and bacteria have been isolated, only a few of them provide high activity with commercial success. *Bacillus* is extensively exploited for protease production (Priest, 1977). Alkaline proteases from *Bacillus* spp. (Alcalase, Savinase, Esperase, Maxatase, Maxacal, Opticlean, and Optimase) constitute 20% of the world enzyme market (Chauhan and Gupta, 2004).

Several *Bacillus* species can produce extracellular proteases with gelatinolytic activity. Those include *B. licheniformis* (Asdornnithee *et al*., 1995), *B. pumilus* (Wu *et al*., 2010), *B. subtilis* (Nagano and To, 1999), *B. cereus* (Suphatharaprateep *et al*., 2011), and *Bacillus* spp. (Nakayama *et al*., 2000). Some *Bacillus* strains secrete gelatinolytic enzymes that are useful for production of gelatin and collagen-derived hydrolysates and peptides (Rao *et al*., 1998; Gómez-Guillénez *et al*., 2011). Protein hydrolysates including gelatin hydrolysate have been used as an ingredient in drugs, drinks, foods, cosmetics, and healthcare products (Liu *et al*., 2010).

Generally, protease showed the low hydrolytic activity toward collagen and gelatin. However, the microorganisms isolated from fish processing plant or fish dock, where the collagen or gelatins are available, may possess collagenolytic or gelatinolytic activities. Those gelatinolytic enzymes can be used as the novel protease capable of hydrolysing gelatin, particularly from fish origin. Therefore, this investigation aimed to isolated and identify microorganisms with gelatinolytic activity from fish docks and to characterise gelatinolytic enzymes produced from selected strains.

2.3 Materials and Methods

Chemicals and enzymes

2,4,6-Trinitrobenzenesulphonic acid (TNBS), ethylene glycol tetraacetic acid (EGTA), bovine serum albumin (BSA), phenylmethanesulfonyl fluoride (PMSF), Alcalase®, and Tween® 40 were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). 1-(L-*trans*-epoxysuccinyleucylamino)-4-guanidinobutane (E-64), soybean trypsin inhibitor (SBTI), pepstatin A, ethylene diaminetetraacetic acid (EDTA), tris(hydroxymethyl)aminomethane (Tris), trichloroacetic acid (TCA), calcium chloride ($CaCl_2$), and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). All chemicals were of analytical grade. Fish skin gelatin (240 bloom) was purchased from Lapi Gelatine (Milano, Italy).

Isolation and screening for gelatinolytic enzyme producing bacteria from fish docks

Over 500 different bacterial strains were isolated from fish docks of Songkhla, Thailand during March and May, 2012. Samples were collected by swabbing the surfaces of fish, containers and equipment. The collected samples were transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand. Upon arrival, the samples were mixed with 9 ml of sterile diluents containing 0.85% NaCl. Microbes in those samples were isolated using spread plate and streaking methods on nutrient agar (Merck, Darmstadt, Germany). These isolates were initially grown in nutrient broth (NB) for 18 h at 37 °C. Culture broths were centrifuged at 10,000 × *g* and at 4 °C for 15 min using a refrigerated centrifuge (Avanti® J-E, Beckman Coulter, Palo Alto, CA, USA). The supernatants were determined for gelatinolytic activity.

To measure gelatinolytic activity, the supernatants were assayed using fish gelatin as a substrate as per the method of McLaughlin and Weiss (1996). A reaction mixture containing 5 mg of gelatin, 1 ml of 50 mM Tris-HCl (pH 7.0) containing 0.36 mM $CaCl_2$, and 0.1 mL of the supernatant was incubated at 37 °C for 20 min. The reaction was stopped by adding 0.2 mL of 50% trichloroacetic acid. The α-amino acid content in the mixture was determined according to the method of Benjakul and Morrissey (1997). To diluted samples (125 µl), 2.0 mL of 0.2 mM phosphate buffer, pH 8.2 and 1.0 ml of 0.01% TNBS solution was added. The solution was mixed thoroughly and placed in a temperature-controlled water bath (Memmert, Schwabach, Germany) at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 mM sodium sulphite. The mixture was cooled at room temperature for 15 min. The absorbance was read at 420 nm and α-amino acid content was expressed in term of L-leucine. One unit (U) of gelatinolytic activity was defined as the amount of enzyme which released 1 µmole of α-amino acid per min at specified condition. Isolates which showed the high gelatinolytic activity were selected for further study.

Identification of gelatinolytic enzyme producing bacteria

Cultivation and collection of bacteria

The selected strains were cultivated by adding a loopful of pure culture into 5 mL of sterile NB medium and incubated at 37 °C for 18 h. Five hundred microlitre of inoculum (A_{600} = 1.0) was added to 500 ml Erlenmeyer flasks containing 150 ml NB. After incubation for 18 h at 37 °C, the mixtures were subjected to centrifugation at 10,000 × *g* for 15 min at 4 °C. Cells were collected as the pellet, which was further subjected to gene sequencing. The cell-free supernatants were analysed for gelatinolytic activities and protein content.

16S rRNA gene sequencing

DNA was extracted and purified from whole cells using GF-1 bacterial DNA extraction kit (Vivantis Tech., Subang Jaya, Malaysia). 16S rRNA gene sequencing was carried out using the methods reported by Shida *et al*. (1996) and Takagi and other (1993). The 16S rRNA gene was amplified by PCR with 16S-27 F forward primer (5'-AGAGTTTGATCMTGGCTCAG-3') annealed at positions 8-27 and 16S-1488 R reward primer (5'-CGGTTACCTTGTTAGGACTTC ACC-3') annealed at positions 1511-1488 (*Escherichia coli* numbering according to Brosius and other (1978). Amplification reaction was carried out in 50-µl volume, using the AtMax Taq DNA Polymerase (Vivantis Tech., Subang Jaya, Malaysia). Amplification was performed in a Techne TC-512 gradient thermal cycler (Progene, Techne Ltd., Cambridge, UK) with the following cycling program: initial denaturation at 95 ºC for 5 min followed by 30 cycles of 95 ºC for 1 min, annealing at 50 ºC for 30 sec, and extension at 72 ºC for 1 min, and a final extension step at 72 ºC for 5 min. The PCR product was purified using GF-1 PCR Clean-up Kit (Vivantis Tech., Subang Jaya, Malaysia). The double-stranded DNA was sequenced by the use of the following three primers; 16S-27, 16S-421 F (5'-CGGATCGTA AAGCTCTGTTG-3') annealed at positions 402-421 and 16S-1488 R. The PCR products were sequenced with an ABI 3730 xl DNA Analyser (Applied Biosystems, Foster, CA, USA). The 16S rRNA gene sequences (1393-1398 base pair) were aligned along with the selected sequences

obtained from the GenBank/EMBL/DDBJ databases using the program CLUSTAL_X (version 1.81) (Thompson *et al*., 1997). Gaps and ambiguous bases were eliminated from the calculations. The distance matrices for the aligned sequences were calculated by the two-parameter method of Kimura (1980). A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) with a program MEGA (version 4.0) (Tamura *et al*., 2007). The confidence values of individual branches in the phylogenetic tree were determined using the bootstrap analysis of Felsenstein (1985) based on 1000 samplings.

Fractionation and characterization of gelatinolytic enzymes

Ammonium sulphate precipitation

Ammonium sulphate (53.28 g) was added to the cell free supernatant (100 ml) to obtain 80% saturation. The precipitate was collected by centrifugation at 12,000 × *g*, 4 °C, for 30 min. The pellet was resuspended with the minimum volume of 50 mM Tris-HCl buffer containing 0.36 mM $CaCl_2$ (pH 7.0). Then, the suspension was dialysed thoroughly with 40 volumes of the same buffer for desalting. The dialysis buffer was changed for 3 times. The dialysate was used for characterisation of enzymes.

Characterisation of gelatinolytic enzymes

pH and temperature profile

An activity was assayed over the pH range of 3.0-10.0 (50 mM acetate buffer for pHs 3.0-7.0; 50 mM Tris-HCl buffer for pHs 7.5-9.0 and 50 mM carbonate-bicabonate for pH 10.0) at 37 °C for 20 min. For temperature profile study, the activity was determined at different temperatures (30, 40, 45, 50, 55, 60 and 70 °C) for 20 min at the optimal pH.

Inhibitor study

Effects of different protease inhibitors towards the gelatinolytic activity were determined as described by Klomklao *et al*. (2007). Enzymes were incubated

with an equal volume of protease inhibitor solutions to obtain the designated final concentrations (1-2 mM pepstatin A, 1-2 mM E-64, 10-20 mM EGTA, 10-20 mM EDTA, 5-10 mM SBTI, 5-10 mM PMSF). The mixtures were incubated at room temperature (25 °C) for 15 min. The remaining activity was determined at pH 7.5 and 50 °C for 20 min. Percent inhibition was then calculated. The control was conducted in the same manner except that deionised water was used instead of inhibitor.

Hydrolysis towards fish gelatin

Gelatin (3 g) was dissolved with 100 ml of 50 mM Tris-HCl containing 0.36 mM $CaCl_2$ (pH 7.5) and pre-heated in a water bath at 50 °C for 10 min. To initiate reaction, the enzyme from selected strains and Alcalase (10 and 100 U/g gelatin) were added. The reaction was performed at 50 °C up to 240 min with continuous shaking. During incubation, sample (1 ml) was randomly taken and 0.2 ml of 50% trichloroacetic acid was added. Degree of hydrolysis (DH) was determined by the 2,4,6-trinitrobenzene sulfonic acid (TNBS) method as described by Benjakul and Morrissey (1997) and defined as follows:

$$\mathrm{DH} = \frac{(L_t - L_0)}{(L_{max} - L_0)} \times 100$$

where L_t corresponded to the amount of α-amino acid released at time *t*. L_0 was the amount of α-amino acid in original sample. L_{max} was the maximum amount of α-amino acid in sample obtained after acid hydrolysis.

Protein determination

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

Statistical analysis

All experiments were carried out in triplicate. Data were subjected to the analysis of variance (ANOVA) and mean comparisons were performed by Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was carried

out using the Statistical Package for Social Sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA).

2.4 Results and Discussion

Isolation and screening for gelatinolytic enzyme-producing bacteria

Over five-hundred isolates were obtained from 30 samples collected from surfaces of fish, plastic containers and fish processing equipment in the docks of Songkhla. Six isolates showed high hydrolytic activity towards fish gelatin (19 to 46 U/mg protein) (Table 8).

Identification of bacteria producing gelatinolytic enzymes

Based on 16S rDNA sequencing, the strains K12, O02 and S13 were grouped most closely with a cluster containing *Bacillus cereus* with 96.96-97.96% similarity while sequence similarities of strains D10, G02 and H11 and *B. amyloliquefaciens* were 99.76, 99.88, and 99.88%, respectively (Figure 3). *Bacillus cereus* is a spore-forming food-borne pathogen and is therefore not safe for food applications. Since *B. amyloliquefaciens* has been known as non-toxigenic and non-pathogenic, these three strains could be used for production of gelatinolytic enzyme. For morphology, *B. amyloliquefaciens* strain D10 was filamentous flat elevation. Strain H11 was punctiform, flat elevation and curled in filamentous, whereas G02 was circular form and umbonate elevation.

Fractionation of gelatinolytic enzymes

The cell-free supernatants of *B. amyloliquefaciens* (strains D10, G02 and H11) were subjected to ammonium sulphate precipitation, followed by dialysis. Increasing purity was achieved for gelatinolytic enzymes of three strains. The purification folds of 19.4, 33.6 and 28.3 were achieved for enzymes from D10, G02 and H11, respectively (Table 9). The result suggested that some impurities were removed during fractionation and dialysis. Yields of 22.0, 46.6 and 44.6% were obtained for D10, G02 and H11, respectively.

Table 8. Specific activity of selected gelatinolytic enzyme producing bacteria

Strain	Specific activity [1] (U/mg)
K12	46.43 ± 1.21[2]
H11	32.97 ± 1.37
G02	27.54 ± 0.38
S13	26.17 ± 0.31
O02	20.29 ± 0.78
D10	19.04 ± 1.14

[1] Activity was assayed at 37 °C, pH 7.0 using fish gelatin as substrate.

[2] Values are mean ± standard deviation (n = 3).

pH and temperature profiles of gelatinolytic enzymes

Maximum gelatinolytic activity of enzymes produced from three strains, D10, G02 and H11, were observed at pH 7.5 (Figure 4A). This result was in agreement with Petrova *et al*. (2006) and Wu *et al*. (2010) who reported the maximum collagenolytic activities at pH 7.5 for enzymes from *Streptomyces* sp. and *B. pumilus*, respectively. Negligible gelatinolytic activity was observed at pH lower than 3.0. The result suggested that gelatinolytic enzyme more likely underwent denaturation at very acidic pH. At pH 10, the activity remained approximately 54-77%, indicating that enzymes were less susceptible to activity loss at alkaline pH, compared to acidic pH. Okamoto *et al*. (2001) found that a collagenase produced by thermophilic *Bacillus* sp. had the maximum activity at pH 8.0. Generally, collagenases have an optimum pH of 7-8 (Harrington, 1996).

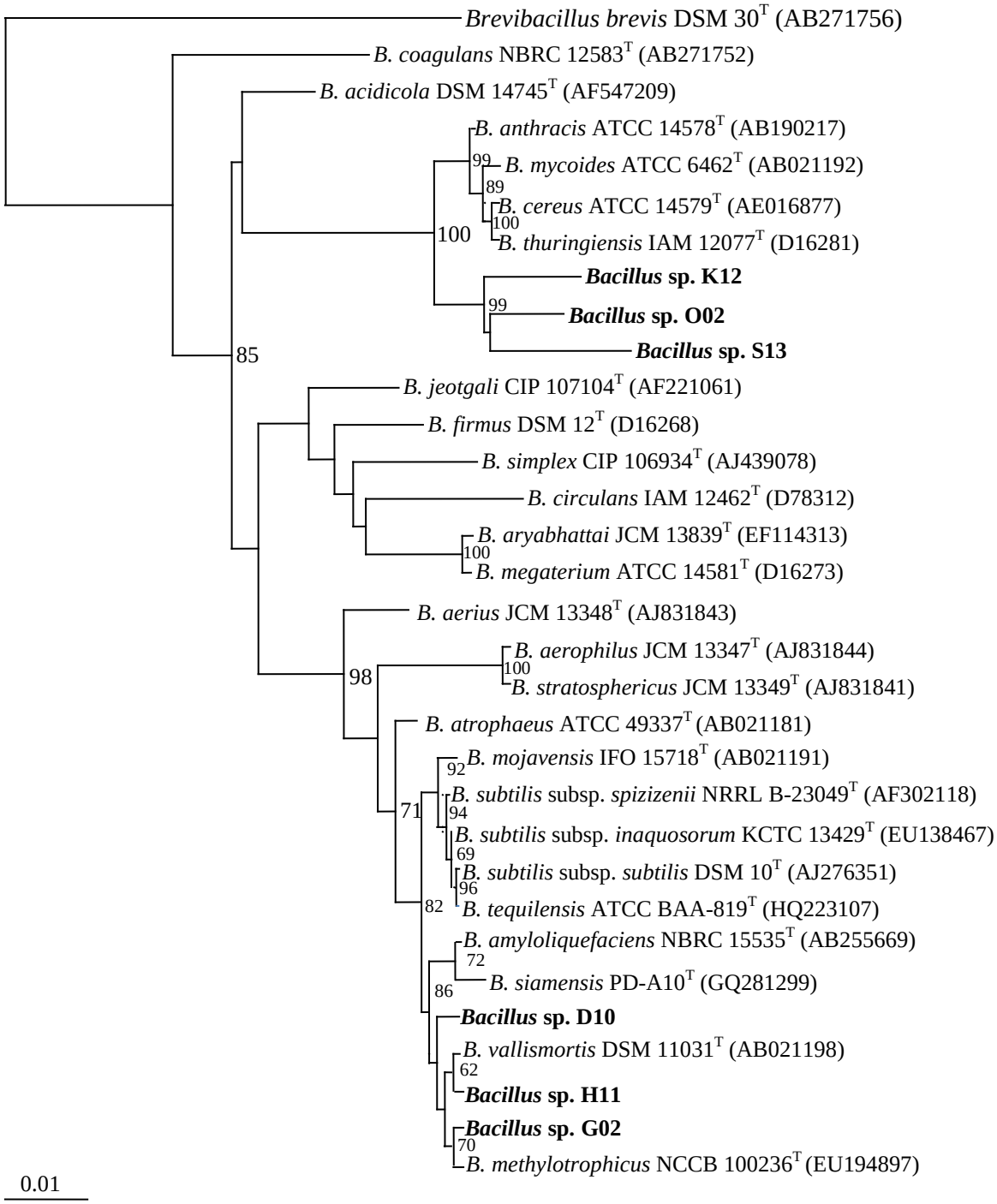

Figure 3. Phylogenetic relationships of strain D10, G02, H11, K12, O02, S13, some *Bacillus* species and related taxa based on 16S rRNA gene sequence analysis.

Table 9. Purification step for gelatinolytic enzymes of three strains of *B. amyloliquefaciens*

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Strain D10					
Crude extract	32,724	307.6	106	100.0	1.0
AS[1]/Dialysis	7,207	3.5	2,065	22.0	19.4
Strain G02					
Crude extract	15,575	353.5	44	100.0	1.0
AS/ Dialysis	7,250	4.9	1,483	46.6	33.6
Strain H11					
Crude extract	39,425	341.9	115	100.0	1.0
AS/ Dialysis	17,579	5.4	3,260	44.6	28.3

[1] AS mean Ammonium sulphate precipitation

Gelatinolytic enzyme from three strains of *B. amyloliquefaciens*, D10, G02 and H11, showed the optimal temperature at 50 °C (Figure 4B). The decreases in activity were observed with increasing temperature. No activity was obtained at 70 °C, mainly due to the complete thermal denaturation of enzyme. Asdornnithee *et al*. (1995) reported that collagenolytic enzyme from *B. licheniformis* N22 was rapidly inactivated at temperatures above 55 °C. Enzyme activity generally depends on the temperatures of the environment (Godfrey and Reichelt, 1983). Nagano and To (1999) reported that collagenase of *B. subtilis* FS-2 had a maximum activity at 50 °C. Maximal collagenolytic activity of *B. cereus* CNA1 was found at 45 °C (Suphatharaprateep *et al*., 2011). Enzymes from some *Bacillus* sp. showed the optimum collagenolytic activity at 60 °C (Nakayama *et al*., 2000).

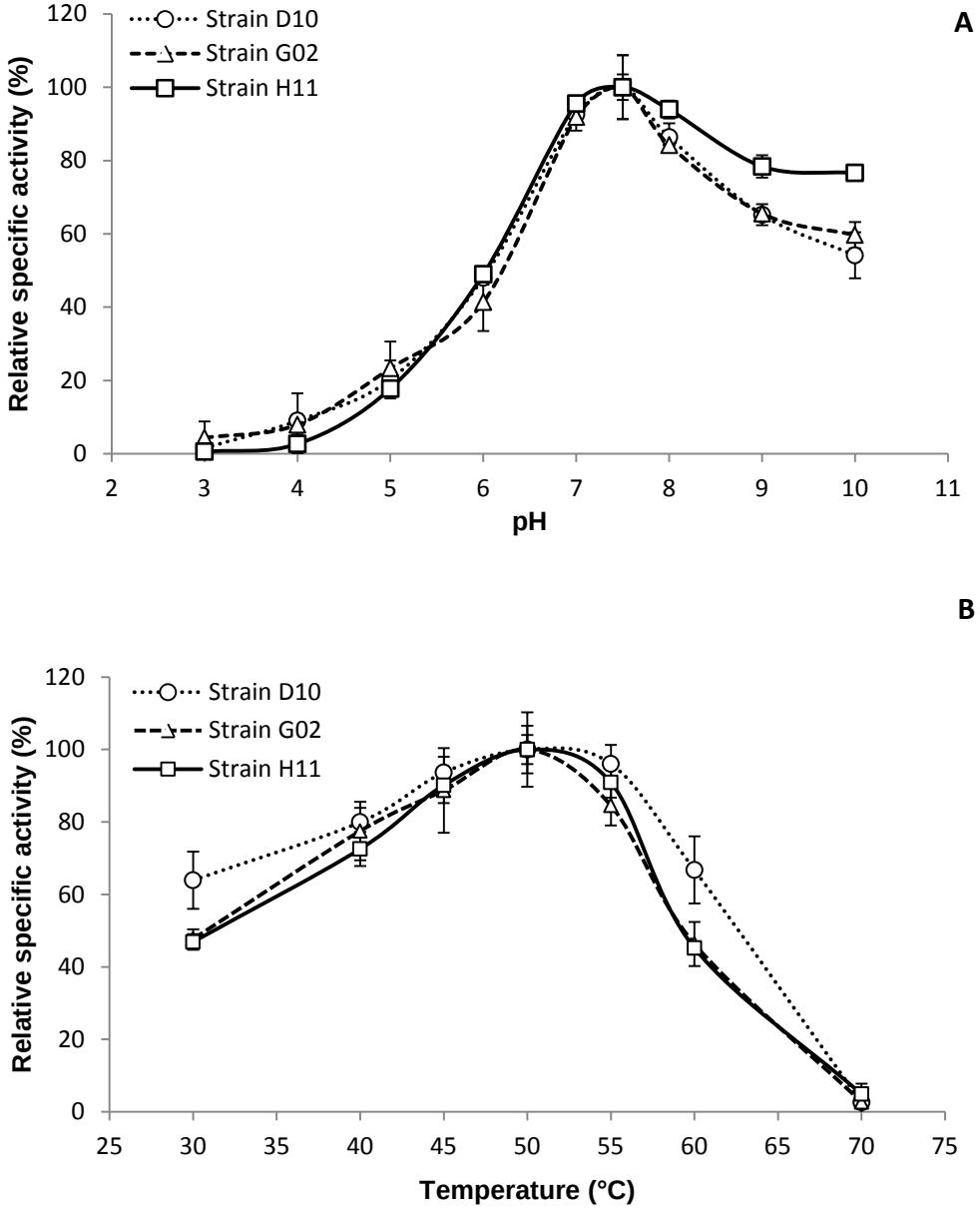

Figure 4. pH (A) and temperature (B) profiles of gelatinolytic enzyme from *B. amyloliquefaciens* strains. For pH profile study, gelatinolytic activity was determined at 37 °C using fish gelatin as substrate. For temperature profile study, the assay was performed at pH 7.5. Bars represent the standard deviation (n = 3).

Effects of various protease inhibitors at different concentrations on gelatinolytic activity of enzymes from D10, G02 and H11 were shown in Table 8. Amongst all protease inhibitors, EDTA (20 mM) exhibited the highest inhibition (74-85%) for gelatinolytic enzymes of all strains, indicating the presence of metallo-enzymes at high levels in all samples. Metalloproteases were dominant in *B. amyloliquefaciens* strains, a family of proteases that shares a zinc-containing motif at the center of the active site (Gonzales and Robert-Baudouy, 1996).

Effect of various protease inhibitors on gelatinolytic activity

Enzymes from G02 and H11 were inhibited by PMSF. However, PMSF also inhibited the activity of enzymes from D10 to some degree. PMSF is a well-known irreversible inhibitor of serine proteases. Nevertheless, SBTI showed no inhibition toward enzymes from all strains. Thus, serine gelatinolytic enzymes were also present in the fractionated enzymes. Cho *et al*. (2011) reported that *B. amyloliquefaciens* FSE-68 mainly secreted two proteases, a neutral metalloprotease and an alkaline serine protease. The most active protease from *B. amyloliquifaciens* was identified to be subtilisin-like serine proteases (Rao *et al*., 1998). However, *B. amyloliquefaciens* DC-4 can produce the fibrinolytic enzyme like subtilisin that is a serine protease, not a metal protease (Peng *et al*., 2003). No inhibition was detected when Pepstatin A or E-64 was used, suggesting the absence of aspartyl- and cysteine gelatinolytic enzymes, respectively. It was noted that EGTA, Ca^{2+} chelator, had the inhibitory activity toward gelatinolytic enzymes from all strains, particularly for D10, when a concentration of 20 mM was used. The result indicated that Ca^{2+} might be necessary for activation of gelatinolytic enzymes. Ca^{2+} might induce structural alteration of enzymes, in fashion, in which they could bind with the substrate more effectively.

Table 10. Effect of various protease inhibitors on inhibition of gelatinolytic enzymes from three strains of *B. amyloliquefaciens***.**

Inhibitors	Concentration (mM)	Inhibition (%)		
		Strain D10	Strain G02	Strain H11
EDTA	20	82.76 ± 11.55[1]	79.13 ± 16.62	73.91 ± 8.12
	10	47.43 ± 16.86	52.85 ± 5.08	37.94 ± 0.74
EGTA	20	55.38 ± 8.75	35.23 ± 13.19	36.37 ± 4.39
	10	22.86 ± 1.06	26.71 ± 8.13	13.54 ± 3.70
PMSF	10	24.57 ± 18.89	54.72 ± 5.68	60.57 ± 1.02
	5	9.54 ± 2.60	41.73 ± 9.99	49.84 ± 1.89
SBTI	10	0	9.53 ± 1.32	0
	5	0	0	0
Pepstatin A	2	0	0	0
	1	0	0	0
E-64	2	0	0	0
	1	0	0	0

[1] Values are mean ± standard deviation (n = 3). Enzyme (200 µl) was mixed with 200 µl single protease inhibitor to obtain the designated final concentration. Residual proteolytic activity was measured using fish gelatin as substrate at pH 7.5 and 50 °C for 20 min.

Hydrolysis of gelatinolytic enzymes towards fish gelatin

When fish gelatin was hydrolysed using gelatinolytic enzymes from D10, G02 and H11 at different levels as a function of hydrolysis time, the rapid hydrolysis was observed within the first 30 min when gelatinolytic enzymes and Alcalase at both levels were used (Figure 5). Thereafter, the slower rate of hydrolysis took place up to 240 min of hydrolysis. At the initial phase, gelatin was hydrolysed rapidly, owing to a large number of peptide bonds. The subsequent decreasing hydrolysis rate was mainly due to a decrease in available hydrolysis sites, enzyme autodigestion and/or product inhibition (Khantaphant and Benjakul, 2008). It was

noted that higher degree of hydrolysis was observed when higher enzyme level (100 U/g gelatin) was used. Generally, the degree of hydrolysis (DH) is the most widely used to indicate the percentage of peptide bonds cleaved (Adler-Nissen, 1979; Guérard *et al.*, 2002). DH generally increased with increasing hydrolysis time (Khantaphant and Benjakul, 2008). At an enzyme level of 100 U/g gelatin, gelatinolytic enzymes from all strains yielded the higher DH than Alcalase within the first 90 min ($P < 0.05$). The difference in DH among different enzymes should be attributed to a different active site of enzyme structure and affinity toward substrate. Thus, the gelatinolytic enzymes from *B. amyloliquefaciens* strains isolated from fish docks could be the potential enzymes in hydrolysing gelatin within the short time.

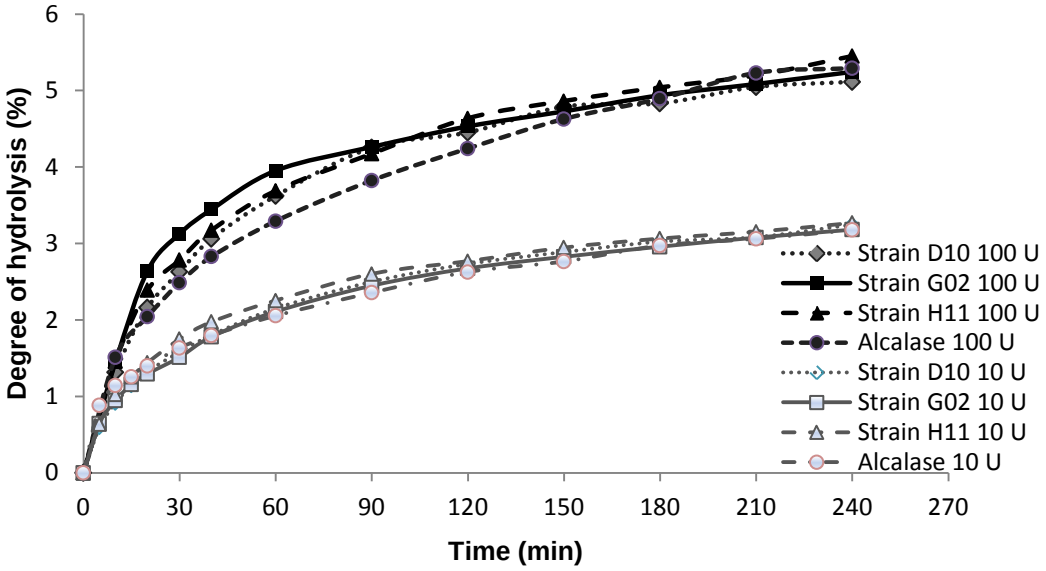

Figure 5. Hydrolysis of fish gelatins by gelatinolytic enzymes from three strains of *B. amyloliquefaciens* in comparison with Alcalase at different levels as a function of time. Hydrolysis was conducted at pH 7.5 and 50 °C.

2.5 Conclusions

Bacillus amyloliquefaciens strains, D10, G02 and H11, were isolated from fish docks in Songkhla. Fractionated gelatinolytic enzymes from three strains had optimal pH and temperature at 7.5 and 50 °C, respectively. The complete loss in activity was observed at 70 ºC. Both serine and metallo-gelatinolytic enzymes were

the major proteases, whilst no cysteine and aspartic proteases were found. The enzymes could hydrolyse fish gelatin in a concentration dependent manner.

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CHAPTER 3

OPTIMIZATION OF GELATINOLYTIC ENZYME PRODUCTION BY *BACILLUS AMYLOLIQUEFACIENS* H11 THROUGH PLACKETT-BURMAN DESIGN AND RESPONSE SURFACE METHODOLOGY

3.1 Abstract

Bacillus amyloliquefaciens H11 has been proven as a potential producer of extracellular protease with capacity of hydrolyzing gelatin. Therefore, the cultivation conditions for the enhanced production of gelatinolytic enzyme from a newly isolated *B. amyloliquefaciens* H11 was investigated using Plackett-Burman design and response surface methodology. Three significant variables (agitation speed, cultivation time and fish gelatin concentration) were selected for optimization. Increase in speed of agitation and fish gelatin concentration markedly increased the production of gelatinolytic enzyme. Gelatin concentration and cultivation time showed significant interaction and both variables played the important role in enzyme production. The maximal gelatinolytic enzyme production in the basal medium was 2,801 U/ml under the following optimal condition: agitation speed of 234 rpm, 8.36 g/l of fish gelatin and 31 h of cultivation. The predicted model fitted well with the experimental results (2,734 ± 101 U/ml). Fourteen-fold increase in yield was achieved, compared with the basal condition (212 U/ml). Thus, cultivation of *B. amyloliquefaciens* H11 under the optimal condition could enhance the production of gelatinolytic enzyme effectively.

3.2 Introduction

Proteases are important enzymes, accounting for > 65% of the total worldwide enzyme sales (Oskouie *et al*., 2008). Proteases have been used for various industrial applications, e.g. dairy, food, pharmaceutical, leather, wool and detergent industries (Johnvesly and Naik 2001). Microbial proteases, especially from *Bacillus* sp., have traditionally held the predominant share of industrial enzyme market (Gupta

et al., 2002) since they possess almost all characteristics desired for biotechnological applications.

Generally, microbial proteases have been produced by fermentation under controlled conditions. It is well known that extracellular protease production by microorganisms is greatly influenced by the medium compositions, especially carbon and nitrogen sources. Other factors such as temperature, pH, incubation time, agitation and inoculum density also play a role in protease production (Johnvesly and Naik, 2001). An approximately 30-40% of the production cost for industrial enzyme is due to the media. Hence, optimization of medium composition is vital (Kirk *et al*., 2002). Optimization of medium compositions by the traditional "one-factor-at-a-time" strategy is the most frequently used in biotechnology (Haaland, 1989). However, this strategy is extremely time consuming and expensive when a large number of variables need to be considered. Additionally, this method is unable to detect true optimal conditions as a result of the interactions among different production factors.

Several statistical approaches involving Plackett-Burman designs and response surface methodology (RSM) have created a potential for optimization of enzyme production (Reddy *et al*., 2008). The Plackett-Burman design has been used for the screening of the main factors from a large number of variables, and this information can be retained in further optimization (Haddar *et al*., 2010). RSM is a collection of statistical techniques that is useful for designing experiments, building models, evaluating the effects of different factors and searching for optimal conditions of studied factors for desirable responses (Haddar *et al*., 2010).

Collagen and gelatin derived peptide has gained increasing interest as the functional foods or drink as well as healthcare products (Watanabe, 2004). Gelatin hydrolysate can be prepared by several commercial proteases. To enhance the hydrolysis toward gelatin, gelatinolytic proteases, which preferably hydrolyze gelatin, should be used to gain the peptides with enhanced bioactivity (Watanabe, 2004). Most of gelatinolytic enzymes are metalloprotease, capable of hydrolyzing the peptide bonds between Y and Gly-X of collagen and gelatin structure (repeated Gly-X-Y unit) (Watanabe, 2004). Gelatinolytic proteases are produced by several bacteria such as *Clostridium histolyticum* (Matsushita *et al*. 1999), *Geobacillus collagenovorans* (Okamoto *et al*., 2001) and *Alicyclobacillus sendaiensis* strain NTAP-1 (Tsuruoka *et*

al., 2003). *Bacillus amyloliquefaciens* H11 has been recently identified as a potential producer of extracellular serine-metallo proteases, which effectively hydrolysed gelatin (Sai-Ut *et al*., 2013). The production of enzyme by this strain can be optimized to obtain the higher yield with lower production cost. Nevertheless, cultivation conditions determining the production of gelatinolytic enzyme by this strain has not been studied. Thus, this investigation aimed to implement the statistical approaches for optimization of culture conditions to maximize the production of gelatinolytic enzyme from *B. amyloliquefaciens* H11.

3.3 Materials and Methods

Chemicals and media

2,4,6-Trinitrobenzenesulphonic acid (TNBS) was purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Tris (hydroxymethyl) aminomethane (Tris), and calcium chloride ($CaCl_2$) were obtained from Merck (Darmstadt, Germany). Fish skin gelatin with bloom strength of 230-250 g was purchased from Lapi Gelatine S.p.A. (Milano, Italy). Peptone and nutrient broth were produced from HiMedia Laboratory (Mumbai, India). Other chemicals were of analytical grade.

Microorganism and culture maintenance

The microorganism with gelatinolytic activity used in this study was isolated from fish dock (Sai-Ut *et al*., 2013). It was identified as *Bacillus amyoliquefaciens* H11 by 16S rRNA gene sequencing. A bacterium was kept frozen at -40 °C in nutrient broth [digest of animal tissue (5 g/l), NaCl (5 g/l), beef extract (1.5 g/l), and yeast extract (1.5 g/l) (NB)] supplemented with 20% (v/v) glycerol. The bacterium was sub-cultured twice in NB at 37 °C for 24 h before use as an inoculum.

Inoculum preparation and gelatinolytic enzyme production

Seed culture was prepared by transferring 0.5 ml of inoculum ($A_{600\ nm}$ = 2.00) into a 250 ml-Erlenmeyer flask containing 50 ml of NB. The cultures were

grown at 37 °C with continuous shaking at 150 rpm for 18 h to obtain a seed culture with an absorbance at 600 nm (A_{600}) of 2.0.

To produce gelatinolytic enzyme, seed culture was inoculated into a 500 ml-Erlenmeyer flask containing 150 ml of production medium. The compositions of production medium and growth conditions were designated as per experimental design (Table 11 and 13). Peptone broth (5 g/l) was used as the basal medium for preliminary study of gelatinolytic enzyme production. After cultivation, the cells were removed by centrifugation at 10,000 × *g* and at 4 °C for 10 min using Allegra™ 25R centrifuge (Beckman Coulter, Palo Alto, CA, USA). The cell-free supernatant was analyzed for gelatinolytic activity.

Assay for gelatinolytic activity

To measure gelatinolytic activity, the supernatant was assayed using fish gelatin as a substrate as per the method of McLaughlin and Weiss (1996). A reaction mixture was 50 mM Tris-HCl (pH 7.5) containing 0.36 mM $CaCl_2$, and 5 mg/mL of fish gelatin. Reaction mixture was incubated at 37 °C for 30 min. To initiate reaction, 0.1 ml of supernatant was added. Reaction was stopped by submerging the reaction mixture in water bath at 90 °C for 10 min (Memmert, Schwabach, Germany). The α-amino acid content in the mixture was determined according to the method of Benjakul and Morrissey (1997). To diluted samples (125 µl), 2.0 ml of 0.20 M phosphate buffer, pH 8.2 and 1.0 ml of 0.01% (v/v) TNBS solution were added. The solution was mixed thoroughly and placed in a temperature-controlled water bath at 50 °C for 15 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulphite. The mixtures were cooled at room temperature for 15 min. The absorbance was read at 420 nm and α-amino acid content was expressed in terms of L-leucine. One unit (U) of gelatinolytic activity was defined as the amount of enzyme which released 1 µmol of α-amino acid per min under the specified condition.

Selection of significant variables by Plackett–Burman design

Impact of several variables including nitrogen sources and cultivation parameters were studied using the Plackett-Burman design for 12 runs and 7 two-level factors. The Minitab v.15 statistical package (Minitab Inc., State College, PA, USA) was used to analysis of data. Based on the Plackett-Burman factorial design, two levels were tested for each factor: −1 for a low level and +1 for a high level (Table 11). The variables were as follows: (1) peptone concentration, (2) fish gelatin concentration, (3) initial pH, (4) inoculum size, (5) cultivation temperature, (6) cultivation time and (7) speed of agitation. The experimental design with the name, symbol code, and actual level of the variables is shown in Table 10. The principal effects of each variable on gelatinolytic activity were estimated as the difference between averages of measurements made at the higher level and at the lower level.

Effect of cultivation temperature on gelatinolytic enzyme production

To study the effect of the cultivation temperature on the production of gelatinolytic enzyme, 500 µl inoculum of culture was transferred into 50 ml of the basal media (5 g/l peptone) containing 5 g/l fish gelatin pH, 7.5) and incubated at 30, 32.5, 35, 37.5 and 40 °C for 24 and 48 h. After cell removal by centrifugation (10,000 × g and at 4 °C for 10 min), gelatinolytic activity of cell-free supernatant was determined as previously described. The gelatinolytic activity at different temperatures was reported, relative to the highest activity obtained.

Optimization by response surface methodology

Response surface methodology (RSM), using a central composite design (CCD), was adopted for further optimization of gelatinolytic enzyme production. The significant variables studied included fish gelatin concentration cultivation time and speed of agitation, in which each was assessed at five coded levels (Table 13). A total of 20 experiments were conducted. The central values of all variables were coded as zero. The minimum and maximum ranges of the variables were used, and the full experimental plan with regard to their values in actual and coded form was provided in Table 13. The response values (Y) in each trial were the

average of the triplicates. The experimental data were fitted to a second-order polynomial model and the regression coefficients were obtained by multiple linear regression.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} {X_i}^2 + \sum_{i=1}^{3} \sum_{j=2}^{3} \beta_{ij} X_i X_j$$

where Y is the predicted response (gelatinolytic activity), X_i, X_j the coded forms of the input variables, β_0 is the model constant, β_i is the linear coefficient, β_{ii}, is the quadratic coefficient, β_{ij}, is the interaction coefficient.

Table 11. Experimental variable at different levels, estimated effect, regression coefficient and corresponding *t* and *P*-values for gelatinolytic enzyme production by *B. amyloliquefaciens* H11 in seven variable Plackett-Burman design experiments.

No.	Variables	Levels		Estimate	Coefficient	*t*-value	*P*-value
		−1 level	+1 level				
X_1	Peptone (g/l)	1	5	-123.7	-61.8	-1.61	0.184
X_2	Gelatin (g/l)	5	10	373.3	186.6	4.85	0.008
X_3	Initial pH	7	9	136.5	68.2	1.77	0.151
X_4	Inoculum size (%, v/v)	1	5	-181.6	-90.8	-2.36	0.078
X_5	Cultivation temperature (°C)	30	40	-227.8	-113.9	-2.96	0.042
X_6	Cultivation time (h)	24	48	490.2	245.1	6.37	0.003
X_7	Agitation speed (rpm)	0	200	589.7	294.9	7.66	0.002

The fitted polynomial equation was then expressed in the form of three-dimensional surface plots, in order to illustrate the relationship between gelatinolytic enzyme production and the experimental variables used. The response surface plots were developed using the Statistica Kernel Release 7.0.61.0 EN (StatSoft Inc., Tulsa, OK, USA) for Windows. The point optimization method was employed in order to optimize the level of each variable for maximum response. The combination of different optimized variables, which yielded the maximum response, was used to produce the gelatinolytic enzyme to verify the validity of model. The Minitab v.15 statistical package was used for the experimental design.

Table 12. Plackett-Burman design for screening important variables for gelatinolytic enzyme production by *B. amyloliqueficiens* H11.

Runs	X_1	X_2	X_3	X_4	X_5	X_6	X_7	U/ml
5	5	10	7	5	40	24	200	617.32 ± 53.48
1	1	10	9	1	30	24	200	810.29 ± 122.31
12	1	5	7	1	30	24	0	214.12 ± 46.26
8	1	5	9	5	40	24	200	563.45 ± 42.79
4	1	10	9	5	30	48	0	437.12 ± 214.06
9	1	5	7	5	40	48	0	288.70 ± 82.46
3	5	5	9	1	40	24	0	313.87 ± 59.10
7	5	5	9	5	30	48	200	1538.48 ± 302.75
2	5	10	7	5	30	24	0	230.85 ± 36.77
10	1	10	7	1	40	48	200	787.16 ± 138.83
6	5	10	9	1	40	48	0	966.80 ± 269.90
11	5	5	7	1	30	48	200	1673.04 ± 308.56

Table 13. Independent variables and their coded and actual values used for optimization of gelatinolytic enzyme production by *B. amyloliquefaciens* H11.

Independents variables	Units	Symbol	Code levels				
			−α	−1	0	+1	+α
Fish gelatin concentration	g/l	X_2	1.64	3.00	5.00	7.00	8.36
Cultivation time	h	X_6	15.8	24.0	36.0	48.0	56.2
Agitation speed	rpm	X_7	66	100	150	200	234

Growth kinetics and protease production

Growth and enzyme secretion were monitored during cultivation. *B. amyloliquefaciens* H11 culture was inoculated in the basal medium under the optimized condition (8.36 g/l fish gelatin, agitation speed of 234 rpm, 37.5 °C). Culture sample was withdrawn aseptically every 6 h. Cell growth was monitored by measuring the absorbance at 600 nm and the supernatant obtained after centrifugation of the culture medium at 9,000g for 10 min (4 °C) was used for gelatinolytic activity assay.

3.4 Results and discussion

Screening of significant variables using Plackett-Burman design

Seven variables were analyzed for their impact on the production of gelatinolytic enzyme by *B. amyloliquefaciens* sp. H11 using a Plackett-Burman design (Table 11). The design matrix for the screening of significant variables for enzyme production and the corresponding responses are shown in Table 12. The adequacy of the model was calculated, and the variables exhibiting statistically significant effects were screened using the Student's *t*-test for ANOVA (Table 15). Factors with *P*-value lower than 0.05 were considered to have significant effects on the production of gelatinolytic enzyme, and were therefore selected for further optimization studies. Speed of agitation, with a *P*-value of 0.002, was considered as the most significant

factor, followed by cultivation time (0.003), fish gelatin concentration (0.008), and cultivation temperature (0.042), respectively. Among all significant variables, the cultivation temperature exerted a negative effect (*t*-value of -2.96), whereas other variables showed the positive effects on gelatinolytic enzyme production. Plackett-Burman design experiments on production of gelatinolytic enzyme from *B. amyloliquefaciens* sp. H11 indicated that the most important parameters were speed of agitation, fish gelatin concentration and cultivation time. Oxygen is a universal component of cells. However, procaryotes display a wide range of responses to molecular oxygen (Dworkin *et al*., 2006). In the presence of gelatin, *B. amyloliquefaciens* H11 produced gelatinolytic enzyme to a higher extent, plausibly due to their enzyme induction, allowing bacteria to synthesize more gelatinolytic enzyme (Deutscher, 2008). Liu *et al*. (2010) reported that initial pH, glycerol, and $MgSO_4 \cdot 7H_2O$ were the most significant variables affecting protease productivity from *Bacillus sphaericus* DS11. Pillai *et al*. (2011) showed that soya bean meal and minerals ($BaCl_2$, KH_2PO_4 and $CaCl_2$) had an enhancing effect on protease production by *B. subtilis* P13.

Hence those three factors were considered as the independent variables and their effects on gelatinolytic enzyme production were further studied using a central composite design (CCD) of RSM. For non-significant variables, their fixed conditions for RSM (5 g/l peptone, pH 8, and 1% (v/v) inoculum size) were used. Most of culture media containing 5 g/l peptone with pH 8 have been used for culturing *Bacillus* sp. (Montville and Schaffner, 2003).

Effect of the cultivation temperature

Effect of different cultivation temperatures on the production of gelatinolytic enzyme was determined as shown in Figure 6. Cultivation condition included 1% (v/v) inoculum in basal medium (5 g/l peptone pH 7.4) containing 5 g/l fish gelatin for 24 h and 48 h. The highest gelatinolytic activity was obtained at 37.5 °C ($P < 0.05$) for both cultivation times.

Table 14. Experimental designs used in RSM studies using three independent variables, observed and predicted values of gelatinolytic enzyme production by *B. amyloliquefaciens* H11.

Trials	X_2	X_6	X_7	Gelatinolytic activity (U/ml)	
				Observed[a]	Predicted
1	3.00	24.0	100	1136 ± 142	1023
2	3.00	24.0	200	1843 ± 177	1881
3	3.00	48.0	100	600 ± 129	638
4	3.00	48.0	200	1148 ± 150	1179
5	7.00	24.0	100	1994 ± 145	1905
6	7.00	24.0	200	2568 ± 111	2472
7	7.00	48.0	100	2127 ± 46	2031
8	7.00	48.0	200	2225 ± 61	2280
9	5.00	36.0	66	1247 ± 139	1373
10	5.00	36.0	234	2350 ± 106	2304
11	5.00	15.8	150	1669 ± 53	1795
12	5.00	56.2	150	1357 ± 23	1311
13	1.64	36.0	150	851 ± 123	825
14	8.36	36.0	150	2388 ± 145	2493
15	5.00	36.0	150	1823 ± 93	1802
16	5.00	36.0	150	1887 ± 81	1802
17	5.00	36.0	150	1744 ± 67	1802
18	5.00	36.0	150	1858 ± 97	1802
19	5.00	36.0	150	1808 ± 166	1802
20	5.00	36.0	150	1705 ± 60	1802

[a] The observed values of gelatinolytic activity were the mean values of triplicates.

Table 15. Analysis of variance (ANOVA) for the parameters of response surface methodology fitted to second-order polynomial equation for gelatinolytic enzyme production by *B. amyloliqueficiens* H11.

Source of variation	DF	SS	MS	*F*- value	*P*- value
Regression	9	5057587	561954	47.92	0.000
Linear	3	4688269	1562756	133.25	0.000
Square	3	146233	48744	4.16	0.037
Interaction	3	223085	74362	6.34	0.011
Residual Error	10	117276	11728		
Lack-of-Fit	5	93814	18763	4.00	0.077
Pure Error	5	23462	4692		
Total	19	5174863			

R^2 = 0.9773; Adj-R^2 = 0.9569; Pred-R^2 = 0.8521; DF, degree of freedom; SS, sum of squares; MS, mean square.

The result suggested that an optimum temperature of 37.5 °C might promote cell growth as well as enzyme synthesis. At higher temperature (40°C), the decrease in activity was observed, especially for 48 h. This might be associated with the lower cell growth at this temperature, thereby decreasing enzyme production. Temperature dependence of protein synthesis of microbial cells may be related with the changes in transportation of amino acids and the alteration of soluble components involved in protein synthesis (Raki, 1991). It has been reported that most of *Bacillus* strains were of mesophilic type with optimal temperature of 30-37 °C. Our finding was in accordance with several earlier reports for *Bacillus* sp., *B. brevis (*Banerjee *et al*., 1999*), Bacillus* sp. strain CA15 (Uyar *et al*., 2011), *B. licheniformis* ATCC 21415 (Mabrouk *et al*., 1999), *Bacillus* sp. TSG437 (Puri *et al*., 2002), and *Bacillus* strains I18, L18 and L21 (Genckal and Tari, 2006). Thus, cultivation temperature of 37.5 °C was used for optimization of gelatinolytic enzyme production using RSM.

Optimization of significant variables using response surface methodology

Based on CCD experiment, the effects of three independent variables, including speed of agitation, fish gelatin concentration and cultivation time on gelatinolytic enzyme production are shown in Table 14. The predicted and observed responses were reported. The results obtained from CCD were then analyzed by standard analysis of variance (ANOVA), and the quadratic regression equation was applied for prediction of gelatinolytic enzyme production. Based on the full quadratic model application, it appeared that the speed of agitation × cultivation time (*P*-value = 0.065) and speed of agitation × fish gelatin concentration (*P*-value = 0.086) interaction as well as quadratic effect for speed of agitation (*P*-value = 0.659) and fish gelatin concentration (*P*-value = 0.108) could be eliminated from the model because the coefficient for interaction was not different from 0 (H_0: $\beta_{ij} = 0$). Even though the speed of agitation showed no interaction with fish gelatin concentration and cultivation time, the linear effect for speed of agitation was still considered as an important factor in this model ($P < 0.05$). After using the designed experimental data and eliminating some terms, the polynomial model for gelatinolytic enzyme produced (Y) was regressed by only considering the significant terms ($P < 0.05$) as shown in the following equation:

$$Y = 1802.01 + 495.87X_2 - 143.95X_6 + 276.91X_7 + 127.52X_2X_6$$

where Y is the predicted gelatinolytic enzyme yield, X_2 is fish gelatin concentration, X_6 is cultivation time, and X_7 is speed of agitation.

For interaction of those variables, there was the interaction only between gelatin concentration and cultivation time ($P < 0.05$). This interaction was constructed by plotting both variables together (data not shown). The interaction of gelatin concentration and cultivation time indicated the importance of those factors for gelatinolytic enzyme production. The maximum gelatinolytic enzyme production was achieved (~2800 U/ml) at the high gelatin concentration (~8 g/l) with the cultivation time of 30-32 h. The increasing gelatin concentration caused a slight shift of cultivation time to higher value, in which gelatinolytic enzyme production increased. The result suggested that higher gelatin concentration might provide the

sufficient nutrient for bacteria to produce enzyme and this might be associated with a slightly extended cultivation time.

The regression equation obtained from analysis of variance (ANOVA) with the R^2 value (multiple correlation coefficients) of 0.9773 revealed that the model could explain 97.73% variation in the response. The "adjusted R^2" and "predicted R^2" values were 0.9569 and 0.8521, respectively. R^2 values were close to 1.0 and all three factors were positive and close to each other, indicating the good statistical model (Myers *et al*. 2004). The *P*-values of the model (< 0.0001) and non-significant lack of fit (0.077) also suggested that the obtained experimental data showed a good fit with the model. ANOVA analysis also confirmed a satisfactory adjustment of the reduced quadratic model to the experimental data.

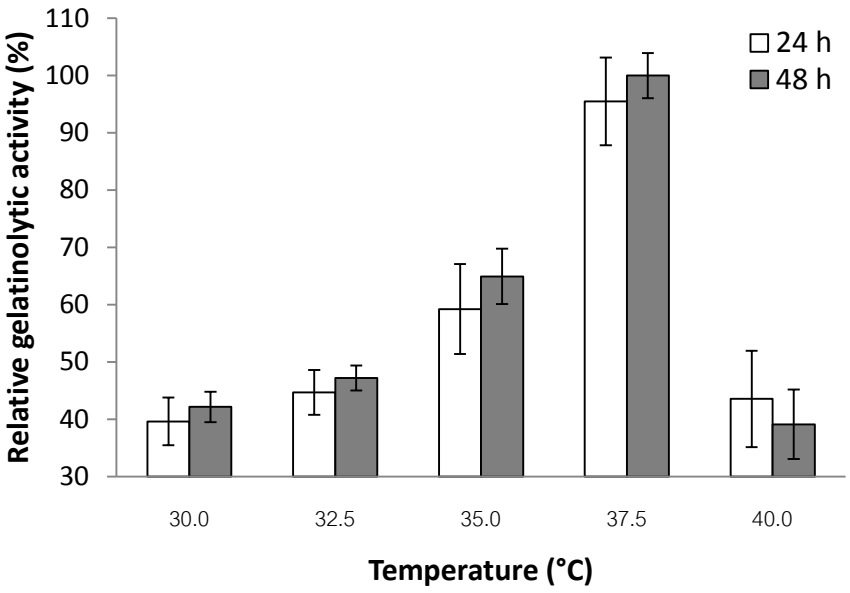

Figure 6. Effect of cultivation temperatures on gelatinolytic enzyme production by *B. amyloliquefaciens* H11 (1%, v/v inoculum in the basal medium containing 5 g/l fish gelatin). Activity was expressed, relative to the highest gelatinolytic activity obtained at 37.5 °C for 24 and 48 h. Bars represented the standard deviation (n = 3).

The three-dimensional response surface graphs were plotted to illustrate the interaction of the cultivation parameters and the optimum level of tested

components on gelatinolytic activity. From the contour plots, general quadratic surface types of all response surface plots were response surface rising ridge. The relationship between cultivation time and agitation speed was curvilinear to nearly linear when moving from the lower to the higher level (Figure 7A). It was noted that the relationship between fish gelatin concentration and cultivation time was curvilinear in a large portion of the middle levels (Figure 7B).

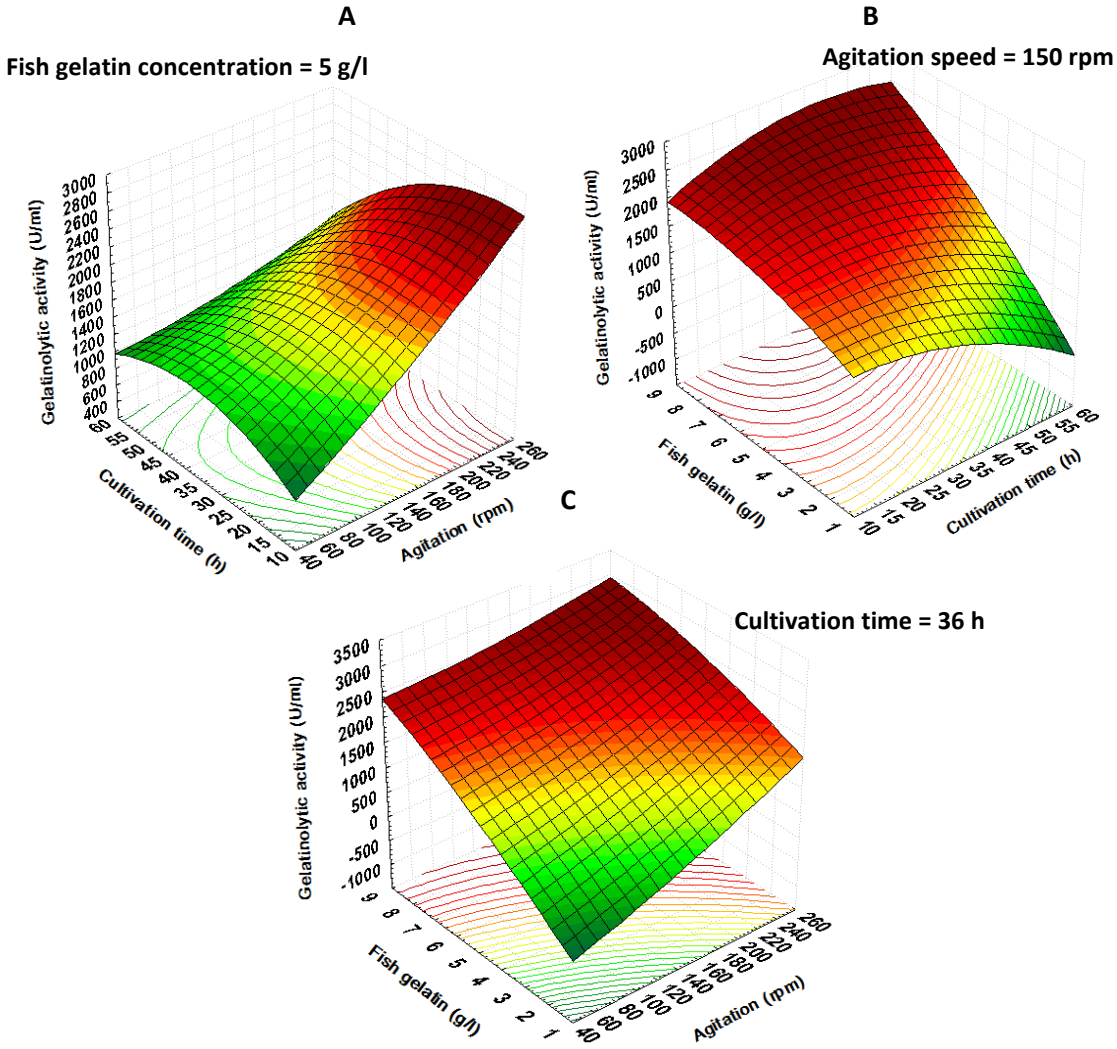

Figure 7. Response surface plots for gelatinolytic enzyme production by *Bacillus amyloliquefaciens* H11. The interaction between (A) cultivation time and agitation speed, (B) fish gelatin concentration and cultivation time, and (C) fish gelatin concentration and agitation speed.

An increase in agitation speed tended to increase gelatinolytic activity, more likely due to higher oxygen supply and nutrient transfer rate. At higher speed, cell growth could be more promoted (Genckal and Tari, 2006), thereby producing more enzyme. It was obvious from the given data that the strain required high agitation speed for enzyme synthesis, probably due to the requirement for oxygen. The present results supported the previous findings (Gupta *et al*., 2002; Joo *et al*., 2002; Joo and Chang, 2005; Reddy *et al*., 2008; Rai and Mukherjee, 2010; Liu *et al*., 2010).

The response varied as a function of fish gelatin concentration showed a response surface rising ridge (Figure 7B). The model predicted the increase in gelatinolytic activity with increasing fish gelatin concentration. These results clearly indicated that fish gelatin was a major factor influencing gelatinolytic enzyme production by *B. amyloliauefaciens* H11. Due to limitation of agitation speed and gel formation of fish gelatin at high concentration (greater than 10 g/l), the factor levels located on the top of the ridge (speed of 234 rpm and concentration of 8.36 g/l fish gelatin) were selected. As shown in Figure 7C, it was apparent that gelatinolytic enzyme production increased steadily when speed of agitation and fish gelatin concentration increased. With increasing cultivation time, gelatinolytic enzyme production increased and reached the plateau at some period of time. Thereafter, no appreciable change in gelatinolytic activity was observed, even with longer cultivation time (Figure 7B). The decrease in gelatinolytic activity with the extended cultivation time could be because of autolysis of enzymes. A broad cultivation time ranging from 24 to 120 h were used for production of protease by *Bacillus* strains (Mabrouk *et al*., 1999; Beg and Gupta, 2003; Chu *et al*., 1992). Maximal protease production within 16-92 h was reported for *Bacillus horikoshii (*Joo *et al*., 2002*), Bacillus licheniformis* NCIM-2042 (Potumarthi *et al*., 2007), and *Bacillus* sp. RGR-14 (Chauhan and Gupta, 2004), respectively.

Thus, the optimum conditions for gelatinolytic enzyme production with the real practice were proposed with the following condition: speed of agitations at 234 rpm, 8.36 g/l of fish gelatin and 31.7 h of cultivation time. The maximal gelatinolytic activity of 2801 U/ml was predicted by the model.

Validation of the experimental model

The validation of the statistical model and regression equation was conducted by cultivation *B. amyloliauefaciens* H11 in the basal medium containing 8.36 g/l fish gelatin by shaking at 234 rpm for 31.7 h. Under this optimized condition, the observed experimental value was 2734 ± 101 U/ml. The close relationship between the predicted (2801 U/ml) and experimental response value from the validation experiment revealed the validity and acceptability of the statistical model for the optimization of the agitation speed, cultivation time and fish gelatin concentration. Therefore, the effective economization of gelatinolytic enzyme production by *B. amyloliquefaciens* H11 could be achieved and further implemented for larger-scale production.

Growth kinetics and gelatinolytic protease production

Growth kinetics of *B. amyloliquefaciens* H11 were studied in the basal medium with optimal culture condition (Figure 8) and gelatinolytic protease production was monitored. The growth was exponential up to 24 h, followed by the stationary phase. Protease secretion was found to be growth dependent and reached a maximum at 30 h, corresponding to stationary phase (Figure 8). A maximum enzyme activity of 2,446 U/ml was observed at 30 h. The result was coincident with the predicted value and the model was proven to be adequate. Production of protease from *Bacillus* sp. has been reported to be related with growth. Satinder *et al*. (2007) and Porto *et al*. (2007) reported that *Bacillus* sp. protease secretion was partially stimulated under the optimal growth conditions. Patel *et al*. (2005) reported that *Bacillus* sp. produced extracellular proteases during stationary phase.

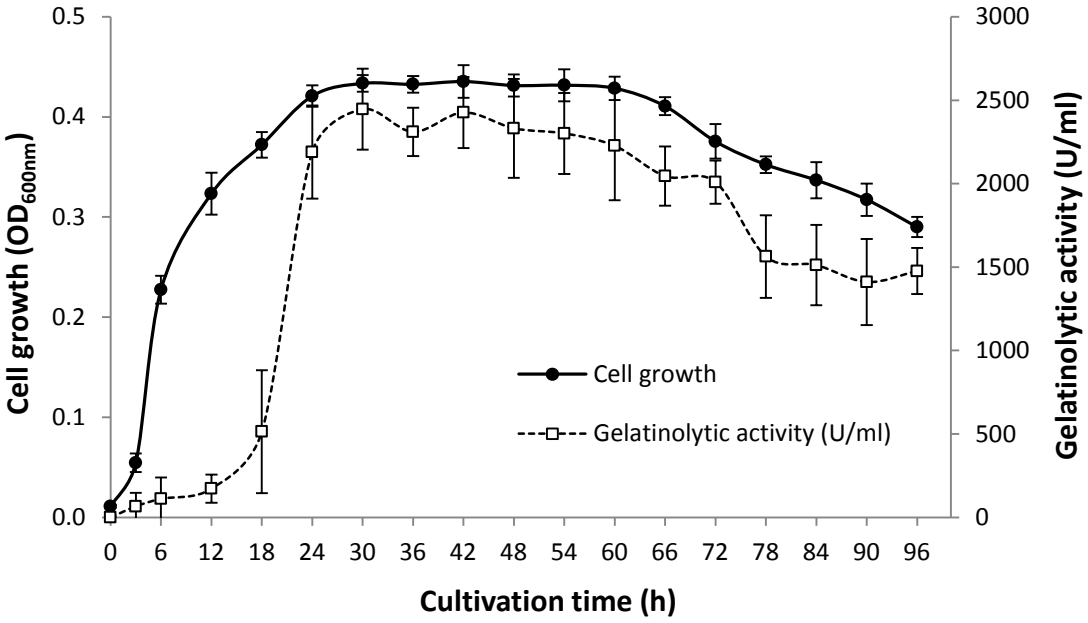

Figure 8. Growth and gelatinolytic protease production of *B. amyloliquefaciens* H11 in the basal medium under the optimal condition. Bars represent the standard deviation (n = 3).

3.5 Conclusions

The optimization of gelatinolytic enzyme production by *B. amyloliquefaciens* H11 was achieved by statistical models and experimental design. Speed of agitation and fish gelatin concentration had the strong influence in gelationolytic enzyme production. The optimal condition for maximal gelatinolytic enzyme production was cultivation of *B. amyloliquefaciens* H11 in the basal medium containing 8.36 g/l fish gelatin with continuous shaking at 234 rpm for 31 h at 37.5 °C.

3.6 References

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CHAPTER 4

PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR GELATINOLYTIC PROTEASE FROM NEWLY ISOLATED *BACILLUS AMYLOLIQUEFACIENS* H11

4.1 Abstract

Extracellular gelatinolytic enzyme from *Bacillus amyloliquefaciens* H11 was purified by gel filtration chromatography on Sephacryl S-200 and ion exchange chromatography on DEAE-cellulose with 35% yield and 14-fold increase in purity. The molecular weight of the purified enzyme was 21 kDa. The optimum gelatinolytic activities of purified enzyme toward porcine gelatin were found at 50 °C and pH 8.0. The inhibitor study revealed that the purified enzyme was a metallo-serine protease. The purified enzyme was activated by Ca^{2+} and Mg^{2+} and was resistant to surfactant, oxidizing agent and solvents. Among all gelatins, that from unicorn leatherjacket skin was more preferable for hydrolysis by the purified enzyme, in comparison with porcine and tilapia counterparts. Thus, the enzyme from *B. amyloliquefaciens* H11 could be used as a potential protease for production of gelatin hydrolysate.

4.2 Introduction

Collagen and gelatin-derived bioactive peptides have been reported to possess various bioactivity and physiological functions, including angiotensin-I-converting enzyme (ACE) inhibition (Byun and Kim, 2001), antioxidant (Alemán *et al.*, 2011), and antimicrobial (Gómez-Guillén *et al.*, 2010) activities, etc. Enzymatic hydrolysis has been widely used for preparation of bioactive peptides from proteinaceous substrate (Kim and Wijesekara, 2010). Several proteases have been used for hydrolysis of gelatin, in which active peptides are generated (Giménez *et al.*, 2009; Alemán *et al.*, 2011). Those include Alcalase, collagenase, trypsin (Alemán *et al.*, 2011), Properase E (Lin and Li; 2006), trypsin, α-chymotrypsin, and pepsin (Mendis *et al.*, 2005). Apart from commercial proteases, enzymatic extracts from fish

viscera have been used to obtain hydrolysates containing bioactive peptides from gelatin of fish skin (Phanturat *et al*., 2010). Metalloproteases, frequently found in bacteria, mostly hydrolyze peptide bonds of residue Y and Gly-X of gelatin (Watanabe, 2004). Metallo- and serine proteases from bacteria such as *Clostridium histolyticum* (Matsushita *et al*., 1999), *Geobacillus collagenovorans* (Okamoto *et al*., 2001) and *Alicyclobacillus sendaiensis* strain NTAP-1 (Tsuruoka *et al*., 2003) have been extensively studied.

Recently, *B. amyloliquefaciens* H11 was isolated and it produced gelatinolytic proteases, capable of hydrolyzing gelatin (Sai-Ut *et al*., 2013). Production of gelatinolytic enzyme by this strain could be achieved by cultivation under the following optimal condition: agitation speed of 234 rpm, 8.36 g/L of fish gelatin and 31 h of cultivation (Sai-Ut et al., 2014a). Furthermore, gelatin hydrolysate prepared using protease from *B. amyloliquefaciens* H11 showed higher antioxidative activity than that prepared by commercial protease (Sai-Ut et al., 2014b). However, no information on molecular properties and kinetics of gelatinolytic enzyme from this strain has been reported. The objective of this study was to purify and characterize gelatinolytic enzymes from *B. amyloliquefaciens* H11.

4.3 Materials and Methods

Chemicals

Phenylmethylsulfonyl fluoride (PMSF) and gelatin (from porcine skin) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Peptone and nutrient broth were obtained from HiMedia Laboratory (Mumbai, India). 1-(L-*trans*-Epoxysuccinyleucylamino)-4-guanidinobutane (E-64), pepstatin A, calcium chloride ($CaCl_2$), ethylenediaminetetraacetic acid (EDTA), tris (hydroxymethyl) aminomethane (Tris) and Folin-Ciocalteu's phenol reagent were procured from Merck (Darmstadt, Germany). Tilapia skin gelatin was purchased from Lapi Gelatine (Milano, Italy). 2,4,6-trinitrobenzenesulphonic acid (TNBS) and L-leucine were obtained from Wako Pure Chemicals Ltd. (Tokyo, Japan). All chemicals were of analytical grade.

Production of gelatinolytic enzyme from *B. amyloliquefaciens* H11

B. amyloliquefaciens H11 was isolated from fish dock (Sai-Ut *et al*., 2013). A bacterium was kept frozen at -40 °C in nutrient broth (NB) supplemented with 20% (v/v) glycerol. The bacterium was sub-cultured twice in NB at 37 °C for 18 h before use.

B. amyloliquefaciencs H11 was cultured in 0.5% peptone medium containing 0.8% fish gelatin at 37.5 °C with continuous shaking at 234 rpm for 36 h. The culture was centrifuged at 9,000 × *g* for 15 min at 4°C, and the supernatant containing protease was then subjected to freeze-drying using a freeze dryer (Scanvac, Coolsafe, Lynge, Denmark). The powder termed 'crude enzyme powder' obtained was subjected to purification.

Enzyme purification

The extracellular protease of *B. amyloliquefaciencs* H11 was purified by combination of Sephacryl S-200 chromatography and DEAE- Sepharose ion exchange chromatography under controlling temperature at 4 °C. Briefly, crude enzyme powder (0.35 g) was dissolved in 10 ml of 10 mM Tris–HCl (pH 8.0) containing 1 mM $CaCl_2$, (buffer A) and loaded onto a Sephacryl S 200 column (4.5 × 65 cm) equilibrated with the buffer A. Fraction showing the highest gelatinolytic activity was collected and lyophilized. Lyophilized fraction was dissolved in water then dialyzed before loaded onto a DEAE-Sepharose column. The column was washed with a linear gradient of 0-1.0 M NaCl at a flow rate of 5 ml/tube. The highest gelatinolytic activity fraction was pooled and lyophilized then dialyzed in water overnight. Protein concentration was monitored by the absorbance of 280 nm.

Enzyme assay

Gelatinolytic activity was determined by measuring the release of α-amino acid from hydrolysis of porcine gelatin. To assay the activity, the enzyme (100 µl) was added to a preincubated (50 °C) reaction mixture (1 % porcine in 50 mM Tris-HCl (pH 7.5) containing 0.1 mM $CaCl_2$). After being incubated at 50 °C for

15 min, the reaction was stopped by submerging the reaction mixture in boiling water for 15 min. The mixture was determined for α-amino acid content according to the method of Benjakul and Morrissey (1997).

To appropriately diluted reaction mixture (50 µl), 1 ml of 100 mM Tris-HCl buffer (pH 8.2) was added, followed by adding 0.5 ml of 0.01% TNBS solution. The solution was mixed thoroughly and placed in a temperature-controlled water bath at 50 °C for 30 min in the dark. The reaction was terminated by adding 1 ml of cold 0.1 M sodium sulfite. The absorbance was read at 420 nm and α-amino acids content was expressed in terms of L-leucine. One unit (U) of gelatinolytic activity was defined as the amount of enzyme which released 1 µmol of L-leucine per min under the specified condition.

Polyacrylamide gel electrophoresis and activity staining

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970). The samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS, 20% glycerol and 10% BME) and boiled for 3 min. The samples (20 µg protein) were loaded onto the polyacrylamide gel (12.25% running and 4% stacking gel) and subjected to electrophoresis at a constant current of 15 mA per gel, using a Mini-Protean II cell apparatus (Atto Co., Tokyo, Japan). After separation, the proteins were stained with colloidal coomassie stain (0.08% Coomassie Brilliant Blue G-250, 1.6% ortho-phosphoric acid, 8% ammonium sulfate and 20% methanol) on a rotary shaker overnight and destained the gel with several changes of 1 % acetic acid until the background was transparent.

Native-PAGE was performed using 12.25 % separating gels in a similar manner, except that the sample was not heated. SDS and reducing agent were omitted.

For activity staining, the enzyme was separated in regular SDS–PAGE containing 0.5 % porcine gelatin in the separating gel (12.25%). After electrophoresis, the gel was rinsed twice with cold 0.25% Triton X 100 and incubated in 10 mM Tris–HCl, pH 8.0 containing 1 mM $CaCl_2$ at 50 °C for 2 h. The gel was rinsed using cold distilled water prior to staining and destaining, as described above.

Effects of pH and temperature on gelatinolytic activity

For temperature profile, gelatinolytic activity was determined using porcine gelatin as substrate at temperature range of 20-80 °C at pH 7.5. The optimum pH of gelatinolytic enzyme was determined in 0.1 M acetate buffers (pH 4.0-6.0) and 0.1 M Tris-HCl buffers (pH 7.0-10.0) at 50 °C. For thermal stability, the enzyme was incubated at different temperatures (40, 50 and 60 °C) for 0.5-3.0 h in a temperature controlled water bath. Thereafter, the heat treated samples were rapidly cooled in an iced bath. The residual gelatinolytic activity was assayed under the optimal condition as previously described. The effect of pH on enzyme stability was evaluated by measuring the residual gelatinolytic activity after incubation at pH 7, 8 and 9 for 0.5-3.0 h at 4 °C.

Effects of protease inhibitors, metal ions and chemicals on gelatinolytic activity

Different inhibitors including EDTA, E-64, pepstatin A, PMSF, TLCK and TPCK were used. Each inhibitor was mixed with the enzyme to obtain the final concentrations of 1.0 and 5.0 mM (E-64, pepstatin A, TLCK and TPCK.) or 5.0 and 10.0 mM (EDTA and PMSF). To determine the effect of cation metal ions, the following metal ions at the final concentrations of 0.1 and 1.0 mM (Ca^{2+}, Co^{2+}, Hg^{2+}, Mg^{2+} and Zn^{2+}) were added. Various chemicals including SDS, Triton X-100, H_2O_2, ethanol and methanol at the final concentration of 1 or 10% (v/v) were used. The mixtures were incubated at 4 °C for 1 h. The residual gelatinolytic activity was determined under the optimal condition. The control was prepared in the same manner except that deionized water was used instead of the chemicals.

Enzyme kinetics

The kinetic constants for the gelatinolytic activity were determined using porcine, unicorn leatherjacket skin gelatin and tilapia skin gelatin as substrates, at different substrate concentrations (1-20 mg/ml). The hydrolysis was conducted at pH 8.0 and 50 °C. The kinetic parameters, including the maximal velocity (V_{max}) and Michaelis–Menten constant (K_m), were evaluated using a Lineweaver–Burk double-reciprocal plot. Catalytic constant (k_{cat}) was calculated from the following

equation: $k_{cat} = V_{max}/[E]$, where [*E*] is molar concentration of enzyme calculated, based on the molecular weight determined by SDS–PAGE and protein concentration.

Statistical analysis

All experiments were carried out in triplicate. Data were subjected to the analysis of variance (ANOVA) and mean comparisons were performed using Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA).

4.4 Results and Discussion

Purification of gelatinolytic enzymes

Purification of the extracellular protease of *B. amyloliqufaciens* H11 with gelatinolytic activity was carried out using Sephacryl S-200 column and DEAE-cellulose ion-exchange column, respectively. The summary of the purification steps is shown in Table 16. Protein synthesis and secretion in prokaryotes is considerably less complex than in eukaryotes (Soole *et al*., 1992). In the present study, A_{280} was used to monitor the peak of protease during each purification step. Fractions containing proteases from *Bacillus* sp. were pooled based on A_{280} (Etezad *et al*., 2009; Asoodeh *et al*., 2010; Annamalai *et al*., 2014).

After being subjected to Sephacryl S-200 column, fractions from different A_{280} peaks were pooled and the gelatinolytic activity was assayed (Figure 8A). Among different pooled fractions, the fraction No. 2 showed the highest gelatinolytic activity (2.58 U/ml). Sephacryl S-200 column was used as the first column to separate the proteins with different sizes (Skoog, 2006). Pooled fraction No. 2 had the specific activity of 0.52 U/mg protein. This pooled fraction had the purification fold of 5. The selected fraction was further purified by DEAE-cellulose column. The proteins bound on the column were eluted by a linear NaCl gradient of 0.0–1.0 M and the gelatinolytic activity of each fraction pooled based on A_{280} was assayed. Those pooled fractions were tested for gelatinolytic activities. Pooled fractions No. 1, 2 and 3 had the gelatinolytic activity of 1.55, 1.07 and 0.91 U/ml,

respectively. Among all fractions, pooled fraction No. 1 eluted using NaCl with concentration around 0.2-0.3 M showed the highest gelatinolytic activity. After being chromatographed using DEAE-cellulose column, the purification fold of 14 was achieved. The selected pooled fraction had the specific activity of 1.36 U/mg protein.

Table 16. Summary of the purification of extracellular protease from *B. amyloliquefaciens* H11.

Purification steps	Crude enzyme	Sephacryl S-200	DEAE-cellulose
Recovery (ml)	45	40	37
Enzyme activity (U/ml)	3.68 ± 0.78	2.58 ± 0.31	1.55 ± 0.15
Total activity (U)	165.59	103.04	57.19
Protein content (mg/ml)	37.00 ± 6.65	4.93 ± 1.45	1.13 ± 0.70
Specific activity (U/mg)	0.10	0.52	1.36
Yield (%)	100	62	35
Purification fold	1	5	14

After being subjected to DEAE-cellulose column, the enzyme was successfully purified to homogeneity as shown by the single band on SDS-PAGE and active staining. Based on SDS-PAGE, the purified enzyme had the molecular weight (MW) of 21 kDa (Figure 10B). Extracellular protease from different microorganisms showed various molecular weights. In the present study, protease produced by *B. amyloliquefaciens* H11 had MW in the range reported previously for protease from *Bacillus* sp. e.g. *B. amyloliquefaciens* CD-4 with MW of 28 kDa, *Bacillus subtilis* AP-MSU 6 with MW of 18.3 kDa (Maruthiah *et al*., 2013), *Bacillus mojavensis* A21 with MW of 20 kDa (Haddar *et al*., 2009b), *Bacillus cereus* SIU1 with MW of 22 kDa (Singh *et al*., 2012), *Bacillus cereus* with MW of 28 kDa (Doddapaneni *et al*., 2009), and *Bacillus megaterium* with MW of 28 kDa (Asker *et al*., 2013).

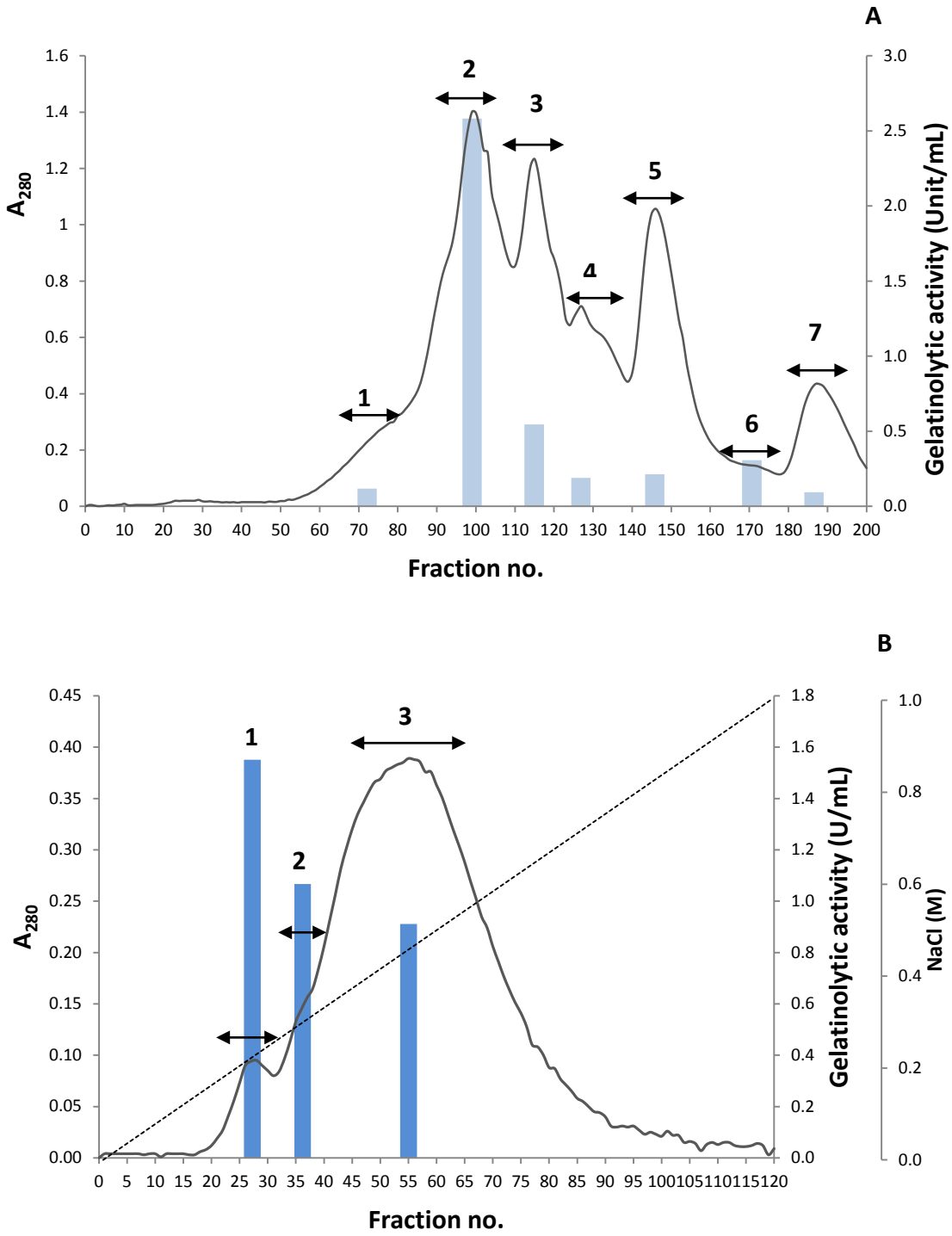

Figure 9. Elution profile and gelatinolytic activity of extracellular protease from *B. amyloliquefaciens H11* using Sephacryl S-200 column (A) and DEAE-cellulose column (B). ↔ represents the pooled fraction based on A_{280}.

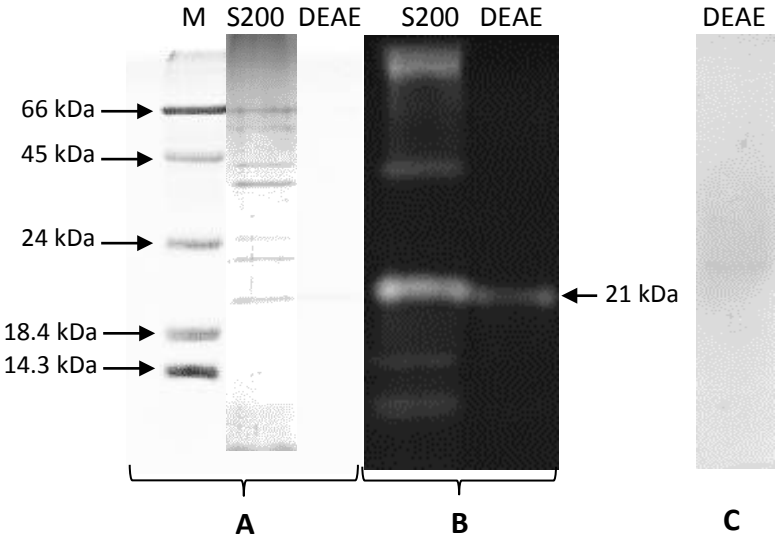

Figure 10. SDS-PAGE (A), active staining (B) and native-PAGE (C) of the purified gelatinolytic enzyme from *B. amyloliquefaciens* H11. M: marker; S200: enzyme purified by Sephacryl S200 column; DEAE: enzyme purified by DEAE-cellulose column**.**

Temperature profile and stability of gelatinolytic enzyme

The purified gelatinolytic protease from *B. amyloliquefaciens* H11 showed different activities when various temperatures were used (Figure 11A). The maximum gelatinolytic activity was observed at 50 °C ($P < 0.05$). At temperature above 50 °C, the activity continuously decreased. At 80 °C, the relative activity of 7.3% was obtained. This indicated the thermal denaturation of enzyme, in which the conformational change was induced at high temperature. Protease from *B. licheniformis* (Sareen and Mishra, 2008) and *B. megaterium* (Asker *et al*., 2013) exhibited the maximum activity at 50 °C, while an alkaline protease from *B. thermoruber* had an optimum activity at 45 °C (Abusham *et al*., 2009). Extracellular proteases from *Bacillus* sp. generally have an optimal temperature in the range of 40-60 °C (Joshi and Satyanarayana, 2013; Sinha and Khare, 2013).

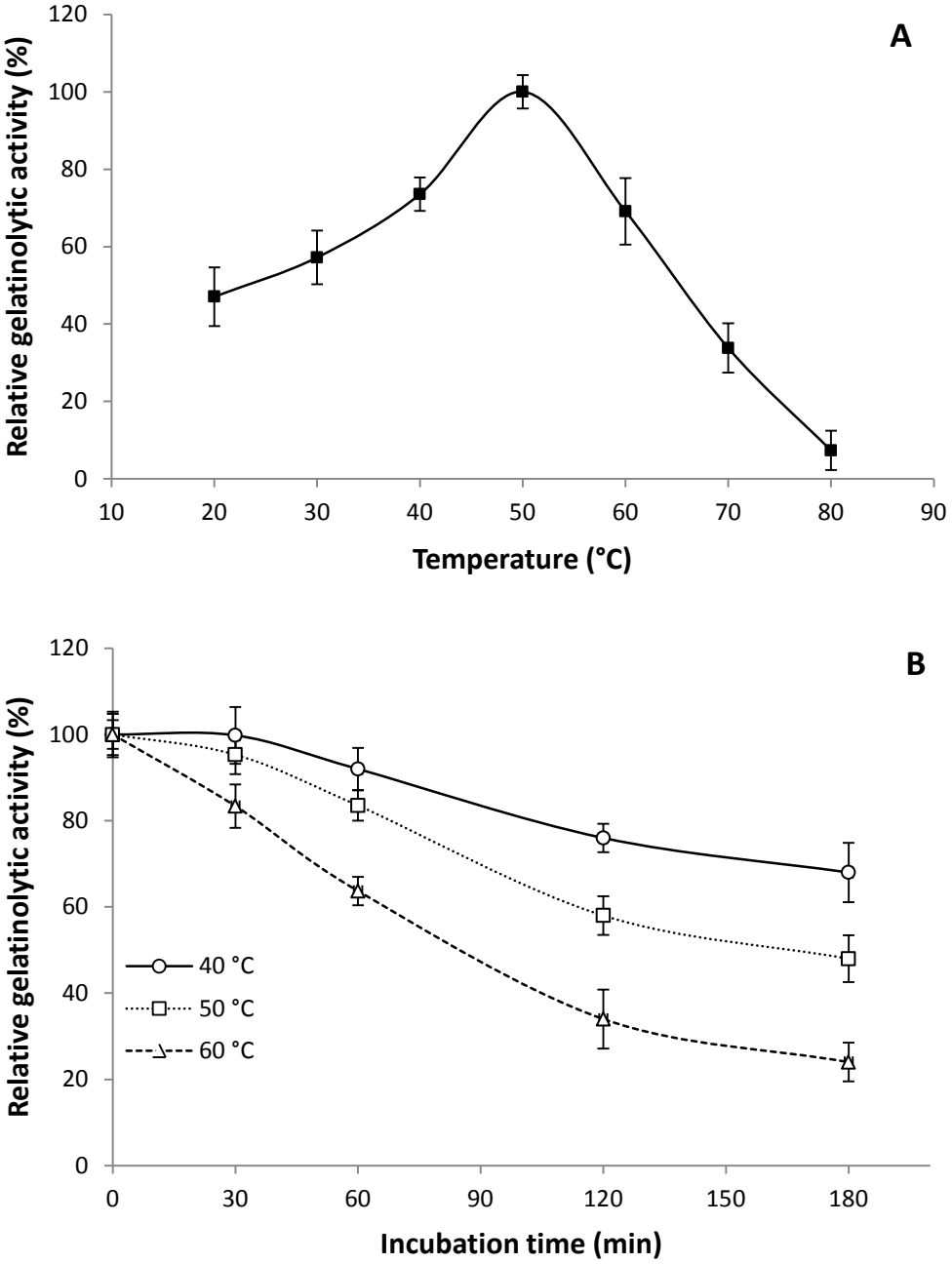

Figure 11. Effects of temperature on activity and stability of purified gelatinolytic enzyme from *B. amyloliquefaciens* H11.

Thermal stability of purified enzyme was tested at 40, 50 and 60 °C for various times (Fig. 11B). Within the first 30 min, enzyme incubated at 40 °C had no change in activity, while a slight decrease in activity was observed after incubation at

50 and 60 °C for 30 min. In general, the activity gradually decreased with increasing incubation time up to 180 min ($P < 0.05$). The relative activities of 68% and 48% were obtained after 180 min of incubation at 40 and 50 °C. After incubation at 60 °C for 180 min, the activity of 24% was retained. In the temperature range of 40-60 °C, which was close to the optimal temperature, the loss in activity was obtained. This might be due to the accumulated energy, which was able to induce the conformational change of enzyme. This led to the loss in activity. Joo *et al*. (2003) reported that protease from *B. clausii* had optimal temperature of 65 °C and was stable in the temperature range of 30–80 °C. Extracellular protease from *B. subtilis* showed high stability in the temperature range of 40–55 °C (Abusham *et al*., 2009). Moreover, protease from *B. cereus* had the optimum temperature of 60 °C with thermal stability at 40–70 °C (Doddapaneni *et al*., 2009). Thermal stability of microbial enzyme is an important property for industrial application. Therefore, gelatinolytic enzyme from *B. amyloliquefaciens* H11 could be maximally used in the range of optimal temperature. However, the decrease in activity might occur with the extended time of reaction.

pH profile and stability of gelatinolytic enzyme

Gelatinolytic activities are affected by various pH values at 50 °C. For the pH study, the gelatinolytic activity is shown in Figure 12A. The optimal pH was noticeable at 8.0 ($P < 0.05$). A slight decrease in activity was found in the alkaline pH range. It was noticed that the decrease in activity was more pronounced at acidic pH range. A strong acid or basic condition could induce the denaturation of enzyme, thereby resulting in loss of enzyme activity. The ionic or electric charge on the amino acids located at active site could be governed by pH, in which enzyme structure could be affected at varying degree (Neil *et al*., 1999). In addition, the ability of the substrate to donate or receive an H atom is affected by pHs (Breaker, 2000). Generally, most proteases from *Bacillus* sp. have the activity at alkaline pH (Joo *et al*., 2003; Adinarayana *et al*., 2003; Sareen and Mishra, 2008; Haddar *et al*., 2009a; Deng *et al*., 2010; Jellouli *et al*., 2011).

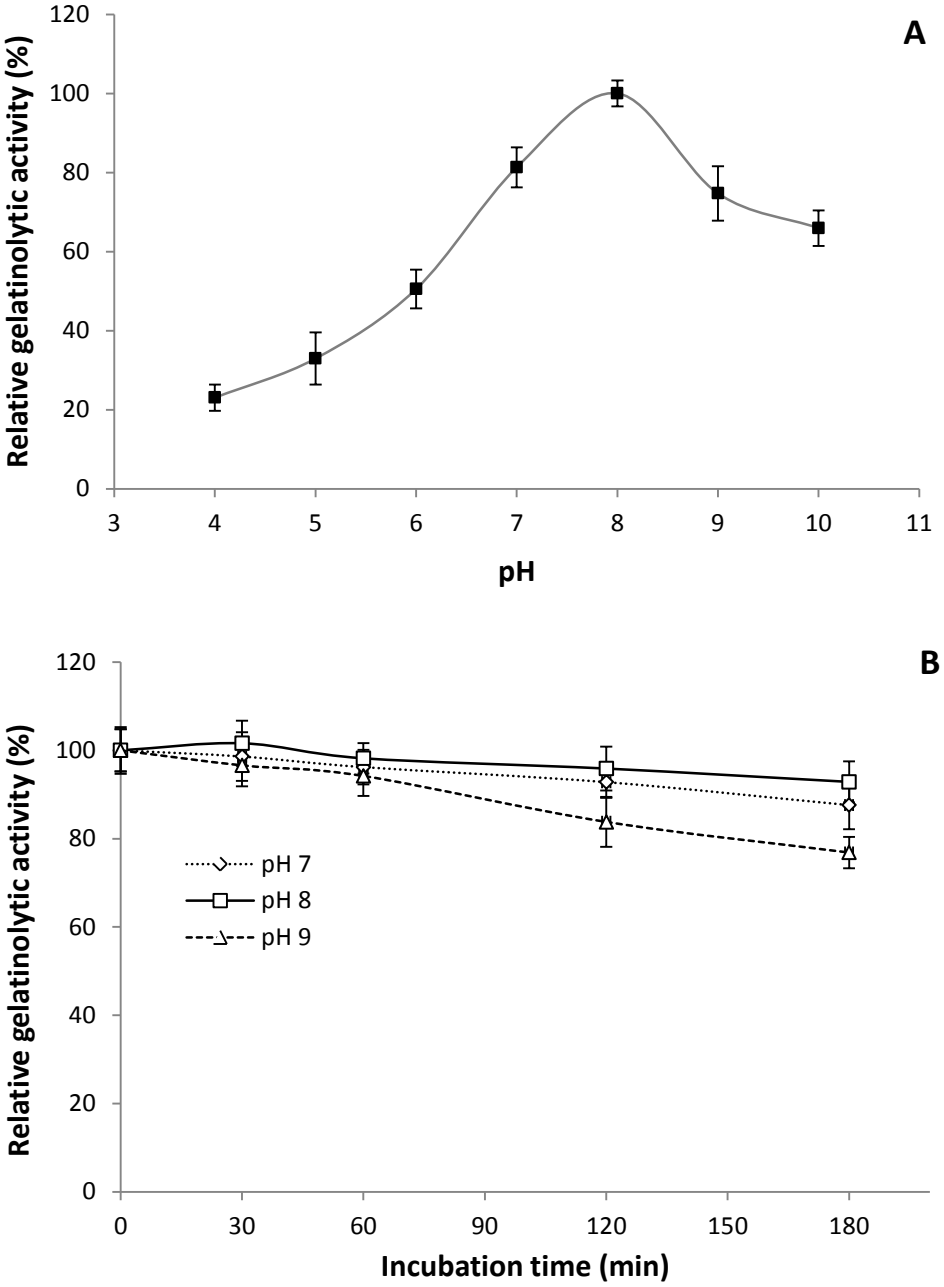

Figure 12. Effects of pH on activity and stability of purified gelatinolytic enzyme from *B. amyloliquefaciens* H11.

The stability in the pH range of 7.0-9.0 as a function of time is shown in Figure 12B. The result revealed that the purified enzyme was stable in alkaline condition. At pH 7.0-8.0, only slight decrease in activity was noticeable when the

exposure time increased. However, at higher pH (pH 9), the higher decrease in activity was obtained. After 180 min of incubation, the relative activities of 87.6, 92.7 and 76.7%, were obtained when being subjected to pH 7.0, 8.0 and 9.0, respectively. Protease from *B. clausii* I-52 had the maximum activity at pH 12.0 and very stable in the pH range of 4.0–12.0 (Joo *et al*., 2003). Protease from *B. subtilis* with the highest activity at pH 10.0 was stable in the pH range of 8.0-11.0 (Adinarayana *et al*., 2003). The pH optima and stability vary with different enzymes as well as different bacteria strains. Protease from *B. licheniformis* showed the activity in broad pH range of 4.0–12.0, which was very stable at pH 10.0 (Sareen and Mishra, 2008).

Effects of inhibitors on gelatinolytic activity

The effects of various protease inhibitors on gelatinolytic activity of purified enzyme are presented in Table 17. Purified enzyme was markedly inhibited by EDTA. Furthermore, the decrease in activity was found in the presence of PMSF. Metalloproteases are commonly deactivated by the addition of EDTA that binds the metal ions, required for enzyme activity (Rawlings and Barrett 1995). EDTA forms strong complexes with Mn (II), Cu (II), Fe (III), Pb (II) and Co (III) (Holleman and Wiberg, 2001). In addition, it is well known that PMSF inhibit serine enzyme by covalently modifying a catalytic serine residue in enzyme's active site of enzyme (Asker *et al*., 2013). In general, the higher inhibition was noticeable with increasing concentrations of protease inhibitors (EDTA and PMSF). The result suggested that purified enzyme was metallo-serine protease. No inhibition was observed when other inhibitors such as E-64, pepstatin A, TLCK and TPCK were used. Similar extracellular metallo- or serine proteases were produced from *B. subtilis* AP-MSU 6 (Maruthiah *et al*., 2013), *Bacillus* sp. B001 (Deng *et al*., 2010), *B. mojavensis* A21 (Haddar *et al*., 2009b), and *Bacillus* sp. (Zambare, 2010). Thus, metal chelator must be avoided to maintain the activity of enzyme.

Table 17. Effect of various protease inhibitors on activity of purified gelatinolytic enzyme

Inhibitor	Concentration (mM)	Relative activity (%)
Control	-	100.0 ± 8.2d
E-64	1	102.3 ± 5.6d
	5	101.0 ± 3.2d
EDTA	5	19.4 ± 7.3a
	10	11.1 ± 8.6a
PMSF	5	76.0 ± 6.4c
	10	52.3 ± 5.6b
Pepstatin A	1	102 ± 12.4d
	5	99.7 ± 3.7d
TLCK	1	98.9 ± 12.4d
	5	102.5 ± 6.1d
TPCK	1	98.8 ± 4.9d
	5	101.7 ± 11.7d

Values are mean ± standard deviation (n = 3). Different letters in the same column denote significant differences ($P < 0.05$).

Effects of metal ions on gelatinolytic activity

The impact of cation metal ions at concentrations of 0.1 and 1.0 mM on the activity of purified enzyme is shown in Table 18. Among all ions tested, $CaCl_2$ effectively enhanced the gelatinolytic activity. Additionally, $MgCl_2$ was also found to activate the enzyme to some extent. The activation efficacy of both ions was in a dose-dependent manner. These results strongly suggested that Ca^{2+} was necessary for the gelatinolytic activity. Ca^{2+} plausibly induced a conformational alteration of enzyme, in the way which favored the substrate-recognition site complex. As a result, hydrogen bonding to the substrate took place easily (Bajorath *et al*., 1988). Ca^{2+} and Mg^{2+} were reported to enhance the protease activity of enzyme from *B. mojavensis* (Beg and Gupta, 2003), *B. thermantarcticus* M1 (Dipasquale *et al*., 2008), *B.*

mojavensis A21 (Haddar *et al*., 2009a) and *B. megaterium* (Asker *et al*., 2013). Furthermore, a serine protease from *B. licheniformis* RSP-09-37 had the increased protease activity in the presence of Ca^{2+} and Mg^{2+} ions, but Zn^{2+} had inhibitory effects toward the activity (Sareen and Mishra, 2008). Serine protease from *Bacillus* sp. SSR1 had the enhanced protease activity to 237.2% and 113.4% when Fe^{2+} and Cu^{2+} were present, respectively (Singh *et al*., 2001). However, Hg^{2+} had no significant stimulatory effect on enzyme activity. On the other hand, Co^{2+} and Zn^{2+} could inhibit the activity to some extent. The inhibition was in a dose-dependent manner. These two cations might alter the enzyme configuration, in the fashion which enzyme lost its activity. Similarly, inhibitory effects of Co^{2+} and Zn^{2+} on alkaline serine protease from *B. lehensis* (Joshi and Satyanarayana, 2013) and alkaline protease from *B. alveayuensis* CAS 5 (Annamalai *et al*., 2013) have been reported, respectively.

Table 18. Effects of metal ions on activity of purified gelatinolytic enzyme

Metal ions	Concentration (mM)	Relative activity (%)
Control	-	100.0 ± 7.5e
$CaCl_2$	0.1	139.6 ± 6.2h
	1.0	152.4 ± 5.8i
$CoCl_2$	0.1	57.6 ± 4.6c
	1.0	44.9 ± 6.8b
$HgCl_2$	0.1	97.4 ± 4.9d
	1.0	99.4 ± 4.4de
$MgCl_2$	0.1	107.5 ± 4.7f
	1.0	114.9 ± 6.8g
$ZnCl_2$	0.1	53.6 ± 5.9c
	1.0	36.6 ± 3.7a

Values are mean ± standard deviation (n = 3). Different letters in the same column denote significant differences ($P < 0.05$).

Effects of some chemicals and solvents on gelatinolytic activity

The effect of surfactants, oxidizing agent and organic solvents on gelatinolytic activity is shown in Figure 13. Purified enzyme showed the relative gelatinolytic activity of 88.8% in the presence of 10% of SDS. The activity of 111.4% was obtained in the presence of Triton X-100. Triton X-100 is considered mild surfactants as they break protein-lipid, lipid-lipid associations, but not protein-protein interactions, and most of them do not denature proteins (Johnson, 2013). Triton X-100 was reported to enhance activities of enzyme from *Bacillus* sp. B001 (Deng *et al*., 2010), *B. clausii* (Joo *et al*., 2003) and *B. subtilis* (Rai and Mukherjee, 2010). When SDS was used, the gelatinolytic activity decreased slightly. SDS is a very effective surfactant, which can disrupt non-covalent bonds within and between proteins. This resulted in the loss of their native conformation and function (Johnson, 2013). Protease from *B. mojavensis* A21 (Haddar *et al*., 2009a) and *B. alveayuensis* CAS 5 (Annamalai *et al*., 2013) had the decreased enzyme activity in the presence of SDS. Therefore, the purified enzyme was stable in the presence of non-ionic surfactants (Triton X-100) rather than anionic surfactants (SDS).

The effect of organic solvents on purified enzyme activity is depicted in Figure 13. Methanol and ethanol had a slight inhibitory effect on gelatinolytic activity. Enzymes are usually inactivated by the addition of organic solvents (Annamalai *et al*., 2013). Moreover, the enzyme was influenced by oxidizing agent (H_2O_2), in which the gelatinolytic activity was decreased to 93.4%. Hydrogen peroxide is proved to be a truly oxidant against amino acids (Wagner *et al*., 2007). Free radicals formed directly oxidize the protein backbone, resulting in the inhibitory effects on the enzyme activity (Finnegan *et al*., 2010). Overall, the enzyme had relative gelatinolytic activity more than 90% in the presence of various solvents at a concentration level of 10%.

Kinetic of gelatinolytic enzyme

The purified enzyme was able to cleave various gelatin substrates, including porcine, tilapia and unicorn leatherjacket gelatin with different rates (Table 19). The result suggested the differences in specificity in hydrolysis of substrate by

the enzyme. The Michaelis–Menten parameters for the cleavage of porcine, tilapia and unicorn leatherjacket gelatin as substrates by the purified enzymes were varied. The lowest K_m value was found when unicorn leatherjacket gelatin was the substrate. The result indicated that a small amount of substrate was needed to saturate the enzyme, indicating a high affinity for substrate. Similar K_{cat} values (turnover number) were noticeable when porcine and unicorn leatherjacket gelatins were used as substrates (6.52 and 6.62 s^{-1}, respectively). However, K_{cat} value of tilapia gelatin was higher than that of other substrates, indicating that the purified enzyme was more favorable to turned over molecules of tilapia gelatin.

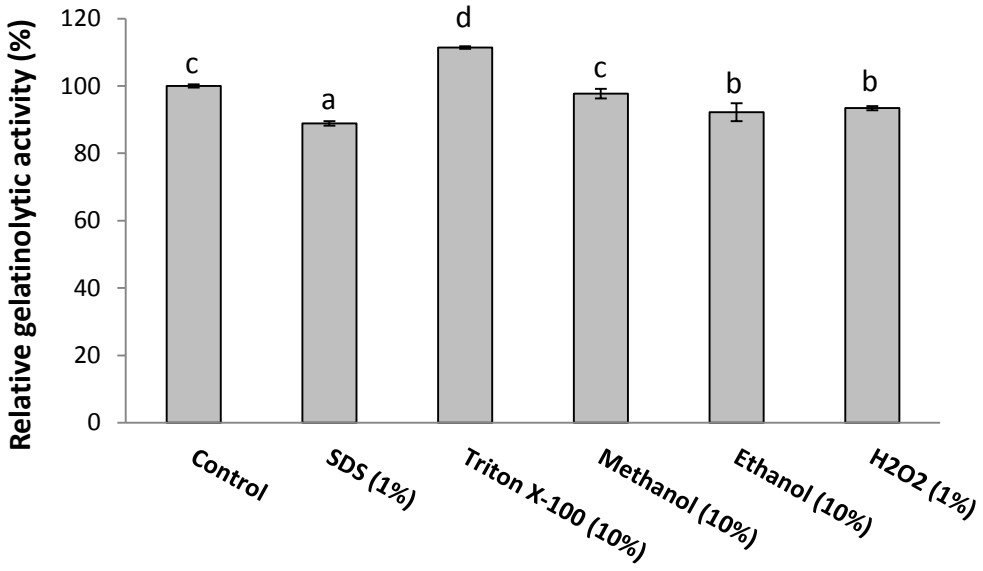

Figure 13. Effect of some chemicals and solvents on activity of purified gelatinolytic enzyme from *B. amyloliquefaciens* H11. Bars represent the standard derivation (n = 3). Different letters on the bars indicate the significant differences ($P < 0.05$).

K_{cat}/K_m is often used as a specificity constant to compare the relative rates of reaction toward substrates, when each is catalytically transformed by an enzyme (Eisenthal *et al.*, 2007). K_{cat}/K_m ratios were shown to vary, depending on substrate. K_{cat}/K_m ratio of unicorn leatherjacket gelatin (1.72 mg/ml·s) was higher than that of other substrates. K_{cat} was much larger than K_m, indicating that a greater proportion of the substrate was converted into product. Thus, unicorn leatherjacket

gelatin was the most specific substrate for purified enzyme due to its high affinity and catalytic efficiency. Several factors affect the rate at which enzymatic reactions proceed such as temperature, pH, enzyme concentration, substrate concentration, and the presence of any inhibitors or activators. Purified enzymes exhibit stereochemical specificity, and therefore distort substrate to conformation of transition state and the enzyme also change and alter affinity of other binding site (Nigh, 1976). Different substrates generally bind to the active site of enzyme differently, leading to conformational change of enzyme in various fashions. As a result, varying hydrolysis was observed among different substrates.

Table 19. Kinetic parameters of purified gelatinolytic enzyme in hydrolysis of various gelatins

Substrates	K_m **(mg/ml)**	V_{max} **(mM/s)**	K_{cat} **(1/s)**	K_{cat}/K_m **(mg/ml·s)**
Porcine gelatin	7.69c	0.007a	6.52a	0.85a
Tilapia gelatin	13.47b	0.012b	11.29b	0.84a
Unicorn leatherjacket gelatin	3.85a	0.007a	6.62a	1.72b

Different letters in the same column denote significant differences ($P < 0.05$).

4.5 Conclusion

Gelatinolytic enzyme from *Bacillus amyloliquefaciens* H11, which was metallo-serine protease, was purified to homogeneity with the yield of 35% and 14 purification fold. The enzyme showed the maximum gelatinolytic activity at 50 °C and pH 8. Calcium or magnesium ions could activate enzyme. The enzyme was quite resistant to surfactants, solvent and oxidizing agent. Enzyme had higher substrate specificity toward unicorn leatherjacket gelatin, compared with other gelatins.

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CHAPTER 5

ANTIOXIDANT ACTIVITY OF GELATIN HYDROLYSATE PRODUCED FROM FISH SKIN GELATIN USING EXTRACELLULAR PROTEASE FROM *BACILLUS AMYLOLIQUEFACIENS* H11

5.1 Abstract

Antioxidant activities of gelatin hydrolysates from unicorn leatherjacket skin prepared using extracellular protease from *Bacillus amyloliquefaciens* H11 (GH-H11) with different degrees of hydrolysis (DHs) were comparatively studied with those of hydrolysate produced using Alcalase (GH-Al). Antioxidative activities of hydrolysates produced by both proteases increased with increasing DH ($P < 0.05$). With DHs of 20-40%, GH-H11 showed higher ABTS radical scavenging activity and FRAP than GH-Al ($P < 0.05$) but no differences in chelating activity were found ($P > 0.05$). Both hydrolysates (100 and 1000 ppm) could inhibit lipid peroxidation in lecithin liposome system in a dose-dependent manner. *In vitro* simulated gastrointestinal digestion study indicated that the antioxidative activity of gelatin hydrolysate was not affected by pepsin, whilst further hydrolysis by pancreatin enhanced the antioxidative activity. The dominant antioxidative peptides in GH-H11 and GH-Al had molecular weights of approximately 750 and 3600 Da, respectively.

5.2 Introduction

A growing interest in antioxidative peptide from natural sources, especially dietary proteins, has been gained for consumers and industries. Gelatin hydrolysate has been known as the potential source of biologically active peptides with high antioxidative activity via hydrolysis process (Kittiphattanabawon *et al*., 2012). Additionally, a number of peptides have shown a potential to act as inhibitors of angiotensin I converting enzyme (Kim *et al*., 2001a) and have chemotactic activity to fibroblast (Postlethwaite *et al*., 1978), and peripheral blood neutrophil (Laskin *et al*., 1986), etc. Fish gelatin derived hydrolysate can be used as a functional food material which enhances the resistance to ultra violet A (UVA) in skin (Kato *et al*.,

2011). Some gelatin-derived peptides, such as Asp-Gly-Glu-Ala stimulate osteoblast-related gene expression of bone marrow cells (Mizuno and Kuboki, 2001), and Gly-Pro-Hyp is suggested to be involved in platelet aggregation (Knight *et al*., 1999).

Basically, antioxidant gelatin-derived peptides have been prepared by various commercial microbial proteases such as Alcalase, Properase E, Neutrase, Flavozymes and Protamex (Kim *et al*., 2001b; Lin and Li, 2006; Giménez *et al*., 2009b; Šližytė *et al*., 2009; Jia *et al*., 2010). Alcalase has been widely used for production of hydrolysate with bioactivities, owing to the superior hydrolytic activity to other proteases such as collagenase, pepsin, trypsin, chymotrypsin, papain or neutrase (Aleman *et al*., 2011; Qian *et al*., 2008), thus Alcalase was used to compare with protease from *Bacillus amyloliquefaciens* H11. Alcalase has generally a broad spectrum for hydrolyzing peptide bonds in several proteins (Watanabe, 2004). However, the peptides generated via its cleavage of gelatin might not have the maximized bioactivity. As a consequence, the protease with high capacity of hydrolyzing peptides and yielding the active peptides with desirable bioactivity is still needed. Recently, *Bacillus amyloliquefaciens* H11 has been identified as a potential producer of extracellular serine-metallo proteases, which effectively hydrolyzed gelatin (Sai-Ut *et al*., 2013). With its high specificity and efficacy, the extracellular proteases from *B. amyloliquefaciens* H11, can serve the increasing need for production of gelatin hydrolysate with increased bioactivity. Nevertheless, no information regarding gelatin hydrolysate prepared using the extracellular proteases from this strain in comparison with the widely used commercial enzyme, Alcalase, has been reported.

This study aimed to comparatively determine antioxidant activities in various *in vitro* and model systems as well as gastrointestinal digestion model system of fish gelatin hydrolysates produced with the aid of protease from *B. amyloliquefaciens* H11 and Alcalase.

5.3 Materials and Methods

Chemicals

2,4,6-Trinitrobenzenesulphonic acid (TNBS), 2,2′-azinobis(3-ethyl benzothiazoline-6-sulphonic acid) (ABTS), 2,4,6-tripyridyltriazine (TPTZ), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulphonic acid sodium salt (ferrozine), ethylenediaminetetraacetic acid (EDTA), trolox, pepsin from porcine gastric mucosa (EC 3.4.23.1) (683 U mg^{-1}), and pancreatin from porcine pancreas (EC Number 232-468-9) were purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada). Trichloroacetic acid (TCA), calcium chloride ($CaCl_2$), Tris (hydroxymethyl) aminomethane and Folin-Ciocalteu's phenol reagent were procured from Merck (Darmstadt, Germany). Alcalase® 2.4L FG (25%) was obtained from novozymes (Bagsvaerd, Denmark). Fish skin gelatin with bloom strength of 230-250 g was purchased from Lapi Gelatine S.p.A. (Milano, Italy). Low molecular weight marker was obtained from GE healthcare UK Limited (Buckinghamshire, UK). All chemicals were of analytical grade.

Microorganism and culture maintenance

Bacillus amyoliquefaciens H11 isolated from fish dock by Sai-Ut *et al*. (2013) was used. Bacterium was kept frozen at -40 °C in nutrient broth (NB) supplemented with 20% glycerol. The bacterium was sub-cultured twice in NB at 37 °C for 24 h before use.

Inoculum preparation and production of gelatinolytic enzyme

Seed culture was prepared by transferring 50 µl of bacterium suspension into a 250 ml-Erlenmeyer flask containing 50 ml of nutrient broth (NB). The cultures were grown at 37 °C with continuous shaking at 150 rpm for 18 h to obtain a seed culture with an absorbance at 600 nm (A_{600}) of 2.0.

To produce gelatinolytic enzyme, seed culture was inoculated into a 1000 ml-Erlenmeyer flask containing 500 ml of production medium (pH 8) containing 5 g/l peptone and 0.836% unicorn leatherjacket skin gelatin. The mixture was

continuously shaken at 234 rpm for 31 h at 37.5 °C in an incubator shaker (VS-8480SR-L, LMS, Korea). After cultivation, the cells were removed by centrifugation at 10,000 × *g* and at 4 °C for 10 min using Allegra™ 25R centrifuge (Beckman Coulter, Palo Alto, CA, USA).

To precipitate gelatinolytic enzyme in the supernatant, ammonium sulfate (266.4 g) was added to cell free supernatant (500 ml) to obtain 80% saturation. The precipitate was collected via centrifugation at 12,000 × *g* at 4 °C for 30 min. The pellet was resuspended in a minimum volume of 50 mM Tris-HCl buffer containing 0.36 mM $CaCl_2$ (pH 7.5). The suspension was then dialyzed thoroughly with 40 volumes of the same buffer for desalting. The dialysis buffer was changed for 3 times. The dialysate was freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The obtained powder referred to as "crude enzyme" with activity of 106.4 U/mg was placed in brown bottle and stored at -40 °C until use.

Assay of protease activity

Both proteases from *B. amyloliquefaciens* H11 and Alcalase were assayed as described by Sai-Ut *et al*. (2013) using commercial fish gelatin as a substrate. A reaction mixture was 50 mM Tris-HCl (pH 7.5) containing 0.36 mM $CaCl_2$, and 2% of fish gelatin. Reaction mixture was incubated at 37 °C for 15 min. To initiate reaction, 0.1 ml of enzyme solution was added into 1 ml of reaction mixture. Reaction was stopped by submerging the reaction mixture in water bath at 90 °C for 15 min (Memmert, Schwabach, Germany). One unit (U) of gelatinolytic activity was defined as the amount of enzyme which released 1 µmol of α-amino acid per min under the specified condition.

Comparative study on gelatin hydrolysis between protease from *B. amyloliquefaciens* H11 and Alcalase

One gram of unicorn leatherjacket skin gelatin extracted following the method of Kaewruang *et al*. (2013) was dissolved with 100 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 0.36 mM $CaCl_2$. The reaction was initiated by adding 1 U of protease from *B. amyloliquefaciens* H11 and Alcalase. The mixtures were shaken at

50 °C for 5, 10, 15, 20, 30, 40, 60, 90, 120, 150 and 180 min. At designated time, the reaction was stopped by immersing the reaction mixture at 90 °C for 15 min and the supernatant was determined for α-amino acids content.

The α-amino acids content in the mixture was determined according to the method of Benjakul and Morrissey (1997). To diluted samples (125 µl), 2.0 ml of 0.20 M phosphate buffer, pH 8.2 and 1.0 ml of 0.01% TNBS solution were added. The solution was mixed thoroughly and placed in a temperature-controlled water bath at 50 °C for 15 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulfite. The mixtures were cooled at room temperature for 15 min. The absorbance was read at 420 nm and α-amino acids content was expressed in terms of L-leucine.

Production of gelatin hydrolysate with different degrees of hydrolysis (DH)

Unicorn leatherjacket skin gelatin (4 g) was dissolved in distilled water. The pH of mixture was adjusted to 7.5 with 1 M NaOH. The volume of solution was made up to 50 ml by distilled water previously adjusted to pH 7.5 to obtain a protein concentration of 2% as determined by the Lowry method (Lowry *et al*., 1951). The hydrolysis reaction was started by the addition of the crude enzyme from *B. amyoliquefaciens* H11 and Alcalase at the amounts of 0.35, 1.75, 5.79 and 19.18 g/100 g protein and 0.19, 0.97, 4.88 and 24.56 g/100 g protein, respectively, which were calculated from the plot between log (enzyme concentration) and DH to obtain DH of 10%, 20%, 30% and 40%, respectively (Benjakul and Morrissey, 1997). After 1 h of hydrolysis at 50 °C, the enzyme was inactivated by heating at 90 °C for 15 min in a temperature controlled water bath (model W350, Memmert, Schwabach, Germany). The mixture was then centrifuged at 5000 × *g* at room temperature for 10 min. The supernatant was freeze-dried using a freeze-dryer. The obtained powder referred to as “gelatin hydrolysate (GH)” was placed in polyethylene bag and stored at -20 °C. Hydrolysate with different DHs name “GH-H11” and “GH-Al” produced by protease from *B. amyloliquefaciens* and Alcalase, respectively, were subjected to analysis.

Determination of antioxidative activities

Hydrolysates were dissolved in distilled water to obtain the concentration of 1%. The solution was determined for antioxidative activity using different assays.

ABTS radical scavenging activity

The ABTS radical scavenging activity was determined as described by Binsan *et al*. (2008). A standard curve of trolox ranging from 0 to 500 μM was prepared. The activity was expressed as μmol Trolox equivalents (TE)/g sample.

Ferric reducing antioxidant power (FRAP)

FRAP was determined according to the method of Benzie and Strain (1996). The standard curve was prepared using Trolox ranging from 0 to 500 μM. The activity was expressed as μmol trolox equivalents (TE)/g sample.

Chelating activity of ferrous ion

Ferrous ion chelating activity was measured by the method of Thiansilakul *et al*. (2007a). EDTA with the concentration range of 0-50 μM was used as the standard. The Fe^{2+} chelating activity was expressed as μmol EDTA equivalents (EE)/g sample.

Prevention effect of gelatin hydrolysate in lecithin liposome system

Lecithin was suspended in doubly deionized water at a concentration of 8 g/l by sonication with a sonicator (model Transsonic 460/H, Elma, Singen, Germany). Gelatin hydrolysate samples (3 ml) were added to the lecithin liposome system to obtain the final concentrations of 100 and 1000 ppm. The liposome suspension was then sonicated for 2 min. To initiate the assay, 50 μl of cupric acetate (0.15 μM) was added. The mixture was shaken at 120 rpm using a shaker (Unimax 1010, Heidolph, Schwabach, Germany) at 37 °C in the dark. The control and system containing 100 ppm Trolox were also prepared. Samples were taken every 6 h for

determining the conjugated diene content (CD), peroxide value (PV) and thiobarbituric acid reactive substances (TBARS).

Measurement of conjugated diene

Conjugated diene content was measured as per the method of Frankel *et al*. (1997). Liposome sample (0.1 ml) was dissolved in methanol (5.0 ml). The absorbance of the solution obtained was read at 234 nm using the UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan).

Determination of peroxide value (PV)

PV was determined as per the method of Wu *et al*. (2003). 50 µl of the liposome were added with 2.35 ml of 75% ethanol, 50 µl of 30% ammonium thiocyanate and 50 µl of 20 mM ferrous chloride solution in 35% HCl. After 3 min, absorbance of the coloured solution at 500 nm was measured using a spectrophotometer. The standard curve was prepared using cumene hydroperoxide as a standard. PV was expressed as g cumene hydroperoxide/l liposome.

Determination of thiobarbituric acid reactive substances (TBARS)

TRARS value was determined according to the method of Lee and Hendricks (1997). Liposome sample (0.5 ml) was mixed with 2.5 ml of TBARS solution (0.375% thiobarbituric acid, 15% TCA and 0.25 M HCl). The mixtures were mixed well and placed in the boiling water to develop the pink color for 10 min. The mixture was cooled using the running tap water, followed by centrifugation at 5000 × *g* for 15 min. The absorbance of supernatant was read at 532 nm using a UV-1601 spectrophotometer. The standard curve was prepared in the same manner using malondialdehyde (MDA) as a standard. TBARS was expressed as g MDA/l liposome.

Stability of gelatin hydrolysate in gastrointestinal (GI) tract model systems

Gelatin hydrolysate (40% DH) was used for stability studies. To simulate the *in vivo* digestion, the sequential reaction, which mimics the conditions of the digestive organ, was used according to the method of Cinq-Mars *et al*. (2008) with

a slight modification. The pH of the fraction with antioxidative activity containing 3 mg protein (15 ml) was adjusted to 2.0 with 6 M HCl. Pepsin was then added (E/S 1:35 w/w), and the mixture was incubated with continuous shaking (W350, Memmert, Schwabach, Germany) for 1 h at 37 °C. The pH was then adjusted to 6.8 with 0.9 M $NaHCO_3$ solution and further to pH 8.2 with 3 M NaOH. Thereafter, pancreatin was added (E/S 1:25 w/w), and the mixture was further incubated with continuous shaking for 3 h at 37 °C. To terminate the digestion, the solution was submerged in boiling water for 10 min. Then, the GI digest was cooled to room temperature and centrifuged at 5000 × *g* for 15 min. Finally, the pH of the supernatant was adjusted to 7.0. Chelating activity and ABTS radical scavenging activity were determined. The residual activity was expressed, relative to that without any treatment.

Fractionation of antioxidative peptides from gelatin hydrolysate

Gelatin hydrolysates prepared using *B. amyloliquefaciens* H11 protease or Alcalase with 40% DH were subjected to gel filtration chromatography. 5 mL of hydrolysates (concentration of 500 mg/ml) were loaded onto a Sephadex G-25 column (2.5 × 50 cm) (17-0032-01, GE Healthcare Bio-Science AB, Uppsala, Sweden). After being loaded, the elution was performed using a low pressure chromatography system (Bio-Rad Laboratories, Hercules, CA, USA). The elution was done with 50 mM sodium phosphate buffer (pH 7.0) at a flow rate of 0.5 ml/min. The 3-ml fractions were collected and their absorbance was read at 220 and 280 nm. Blue dextran (2,000,000 Da) was used for void volume measuremet. Insulin chain B (3,496 Da), vitamin B_{12} (1,355 Da), glycine-tyrosine (238 Da) and tyrosine (181 Da) were used as the molecular weight standards. The fractions were subjected to analysis for ABTS radical scavenging activity. Molecular weight of fraction with ABTS radical scavenging activity was estimated from the plot between available partition coefficient and the logarithm of the molecular weight of the protein standards.

Statistical analysis

All experiments were carried out in triplicate using three different lots of samples. Data were subjected to the analysis of variance (ANOVA) and mean

comparisons were performed using Duncan's multiple range test (Steel and Torrie, 1980). For pair comparison, *t*-test was used. Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA).

5.4 Results and Discussion

Hydrolysis of gelatin by protease from *B. amyloliquefaciens* H11 and Alcalase

Hydrolysis of gelatin from skin of unicorn leatherjacket by protease from *B. amyloliquefaciens* H11 and Alcalase as monitored by α-amino acids released during 180 min is shown in Figure 14. The α-amino acids content increased with increasing hydrolysis time ($P < 0.05$). A rapid hydrolysis rate of both enzymes was obtained within the first 30 min. Thereafter the rate of hydrolysis subsequently decreased. At the initial phase, unicorn leatherjacket skin gelatin was hydrolyzed rapidly, owing to a large number of peptide bonds available. The decreased hydrolysis rate observed in the later stage was mainly due to a decrease in available substrate, enzyme autodigestion and product inhibition (Khantaphant and Benjakul, 2008). After 10 min of hydrolysis, protease from *B. amyloliquefaciens* H11 yielded a higher α-amino acid content than did Alcalase ($P < 0.05$). The result indicated that gelatin from unicorn leatherjacket skin served as the better substrate for protease from *B. amyloliquefaciens* H11 rather than Alcalase. *B. amyloliquefaciens* H11 was cultured in the medium containing unicorn leatherjacket skin gelatin. This might cause the gelatin from unicorn leatherjacket skin to be more preferable substrate. Similar hydrolytic curve was reported for squid skin gelatin when Alcalase was used (Giménez *et al*., 2009a). Generally, the rate of enzymic cleavage of peptide bond controls the overall rate of hydrolysis (Benjakul and Morrissey, 1997). Thus, protease from *B. amyloliquefaciens* H11 could be the alternative potential protease for hydrolyzing fish gelatin for production of bioactive peptides.

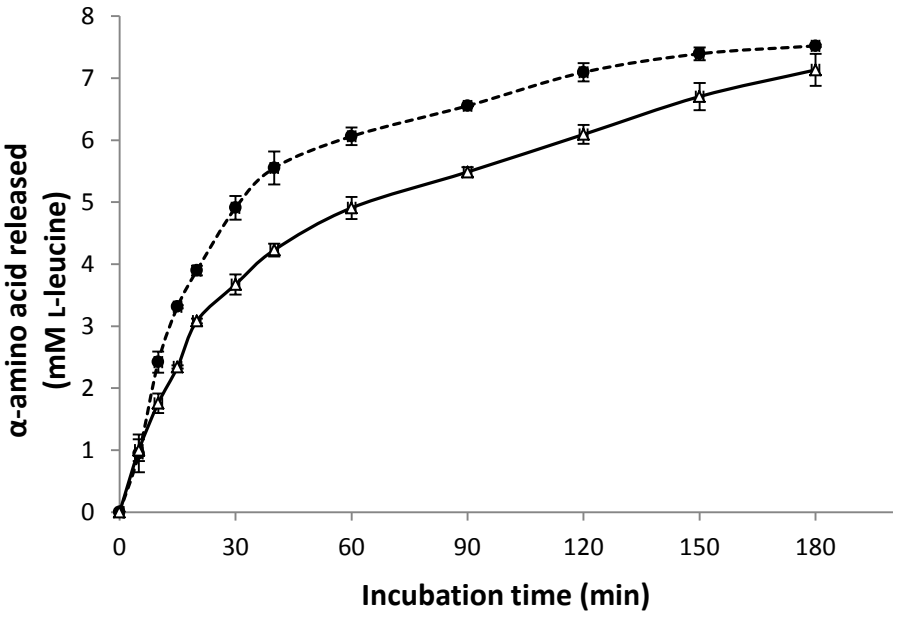

Figure 14. Hydrolysis of gelatin from unicorn leatherjacket skin by protease from *B. amyloliquefaciens* H11 (●) and Alcalase (Δ) as a function of time. Hydrolysis was conducted using enzyme at a level of 1 U/g gelatin at 50 ºC pH 7.5. Bars represent standard deviation (n = 3).

Antioxidative activity of gelatin hydrolysates with different DHs produced by protease from *B. amyloliquefaciens* H11 and Alcalase

ABTS radical scavenging activity

ABTS radical scavenging activities of gelatin hydrolysate produced by protease from *B. amyloliquefaciens* H11 (GH-H11) and gelatin hydrolysate prepared by Alcalase (GH-Al) with different DHs are depicted in Figure 15. As the DH increased, ABTS radical scavenging activities increased ($P < 0.05$). Both hydrolysates with 40% DH exhibited the highest activity in which the activities of 66 and 48 μmol TE/g solid were obtained for GH-H11 and GH-Al, respectively. It was noticed that there were no differences in ABTS radical scavenging activity between two hydrolystes with DH of 10% ($P > 0.05$). With DH of 20-40%, GH-H11 exhibited higher activity than did GH-Al ($P < 0.05$). Phanturat *et al*. (2010) reported that gelatin hydrolysates from bigeye snapper skin with DH ranging from 5% to 25% prepared

using Alcalase had the increased ABTS scavenging activity with increasing DH. The result suggested that more antioxidative peptides were produced when the gelatin was more cleaved. Active antioxidative peptides were more produced when DH increased. Generally, all hydrolysates contained peptides or proteins which were hydrogen donors and could react with the radicals to convert them to more stable products, thereby terminating the radical chain reaction (Kittiphattanabawon *et al*., 2012). ABTS radical assay is an excellent tool for determining the antioxidative activity, in which ABTS radical scavenging activity is based on the ability of antioxidants to donate a hydrogen atom or an electron to stabilize radical by converting them to the non-radical species (Binsan *et al*., 2008).

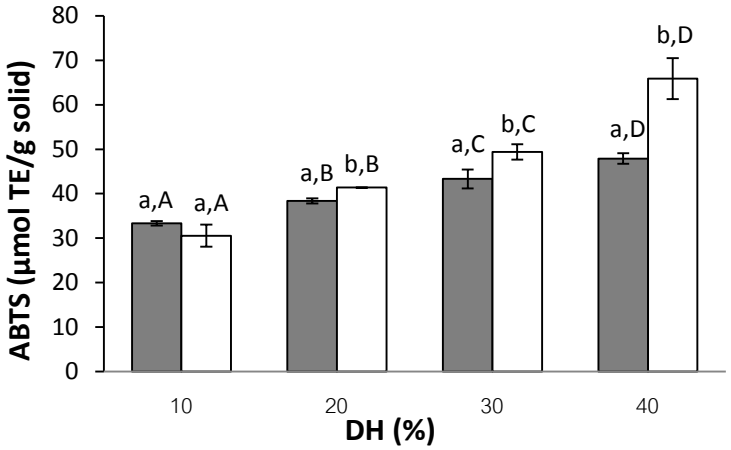

Figure 15. ABTS radical scavenging activity of gelatin hydrolysates from unicorn leatherjacket skin prepared using protease from *Bacillus* H11 (□) and Alcalase (■) with different DHs. Bars represent standard deviation (n = 3). Lowercase letters on the bars within the same DH indicate significant difference (P < 0.05)**.** Uppercase letters on the bars within the same enzyme used indicate significant differences (P < 0.05).

Ferric reducing antioxidant power (FRAP)

FRAP of GH-H11 and GH-Al with various DHs is shown in Figure 16. Both hydrolysates had the increases in FRAP when DH increased (P < 0.05). Similar

result was observed to those of ABTS radical scavenging activity. With DH above 10%, GH-H11 showed the higher FRAP (7.2-9.5 µmol TE/g solid), compared with GH-Al ($P < 0.05$). Different FRAP between both hydrolysates might result from the existing differences in enzyme specificity towards gelatin as substrates (Bayram *et al*., 2008). Proteases from *B. amyloliquefaciens* H11 more likely hydrolyzed gelatin at the different positions of peptide chains, leading to the different peptides with various antioxidative activities. The greater reducing power of hydrolysate indicated that hydrolysates could donate the electron to the free radicals, leading to the prevention or retardation of propagation (Klompong *et al*., 2008).

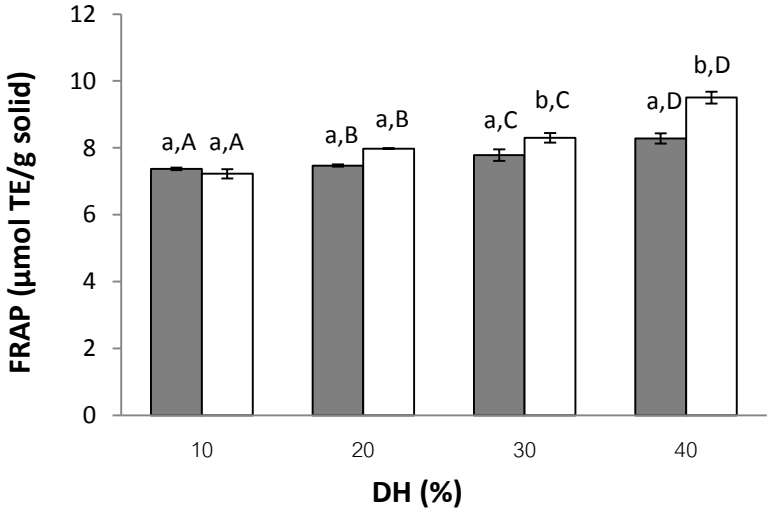

Figure 16. Ferric reducing antioxidant power activity of gelatin hydrolysates from unicorn leatherjacket skin prepared using protease from *Bacillus* H11 (□) and Alcalase (■) with different DHs. Bars represent standard deviation ($n = 3$). Lowercase letters on the bars within the same DH indicate significant difference ($P < 0.05$)**.** Uppercase letters on the bars within the same enzyme used indicate significant differences ($P < 0.05$).

Increases in reducing power of hydrolysate with increasing DH have been reported in gelatin hydrolysate from bigeye snapper prepared using Alcalase, Neutrase and pyloric caeca extract (Thiansilakul *et al*., 2007a), and gelatin

hydrolysate from blacktip shark skin prepared using papaya latex enzyme (Kittiphattanabawon *et al*., 2012). FRAP is generally used to measure the capacity of a substance in reducing TPTZ-Fe(III) complex to TPTZ-Fe(II) complex (Binsan *et al*., 2008). The result suggested that gelatin hydrolysate with higher DH plausibly contained higher amounts of peptides, which donated electrons to free radicals, thereby terminating the chain reaction. DH directly influenced the peptide chain length and the exposure of terminal amino groups of products obtained (Thiansilakul *et al*., 2007b; Klompong *et al*., 2007; Kittiphattanabawon *et al*., 2012). As a result, FRAP of gelatin hydrolysate varied with DH and types of protease used.

Chelating activity of ferrous ion

The ferrous (Fe^{2+}) chelating activity of both gelatin hydrolysates increased when DH increased ($P < 0.05$) (Figure 17). Nevertheless, no differences in chelating activity were found between GH-H11 and GH-Al ($P > 0.05$), when the same DH was tested. Gelatin hydrolysate from black tip shark skin was also reported to possess metal chelating activity (Kittiphattanabawon *et al*., 2012). Cleavage of peptides led to an enhanced metal ion binding capacity, plausibly due to the increased concentration of amino groups such as aromatic residues, Hyp, Pro, Ala or Gly of the N-terminus end of peptides (Mendis *et al*., 2005; Liu *et al*., 2010; Kittiphattanabawon *et al*., 2012). The presence of transition metals in foods is generally a major factor in the promotion of lipid oxidation (Klompong *et al*., 2007). Thus, gelatin hydrolysate with higher %DH could act as a chelating agent, which was able to prevent lipid oxidation.

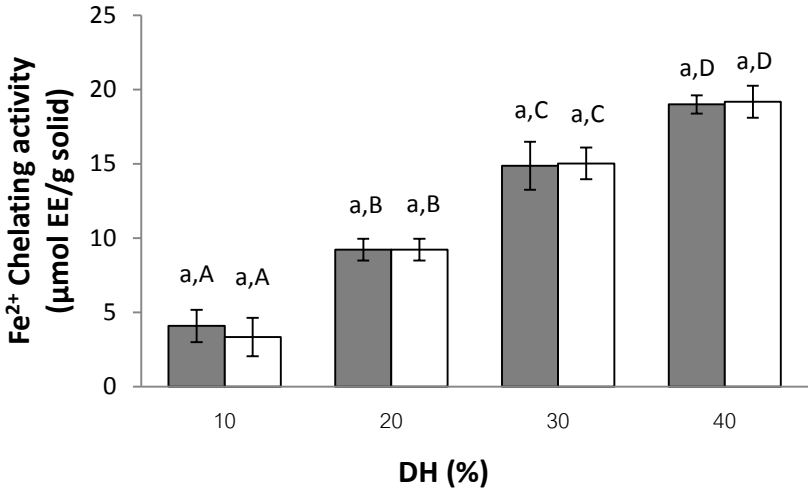

Figure 17. Chelating activity of gelatin hydrolysates from unicorn leatherjacket skin prepared using protease from *Bacillus* H11 (□) and Alcalase (■) with different DHs. Bars represent standard deviation (n = 3). Lowercase letters on the bars within the same DH indicate significant difference ($P < 0.05$)**.** Uppercase letters on the bars within the same enzyme used indicate significant differences ($P < 0.05$).

Antioxidative activity of gelatin hydrolysates in a lecithin liposome system

GH-H11 and GH-Al at levels of 100 and 1000 ppm affected the oxidation of lecithin liposome system differently as indicated by different conjugated dienes (CD), PV and TBARS values (Figure 18, 19 and 20). CD and PV were used to monitor the formation of the primary lipid oxidation products as shown in Figure 17 and 18, respectively. Within the first 18 h of incubation the control showed the higher CD and PV, compared with others ($P < 0.05$). Thereafter, CD and PV of the control decreased up to the end of incubation (48 h). It was noted that the system added with both GH-H11 and GH-Al had the lower CD than the control with the first 18 h ($P < 0.05$). However, the lower PV was found in all samples than the control throughout of 48 h incubation period. Generally, the preventive effect of both hydrolysates was in a dose-dependent manner. Nevertheless, there was no difference in PV between sample containing GH-H11 and GH-Al ($P > 0.05$) at the same concentration used. For the

system containing 100 ppm Trolox, only slight increase was noticeable up to 48 h of incubation, indicating the efficacy in prevention of oxidation by Trolox. The decreases in CD and PV were found in all samples as incubation time increased, plausibly due to the decomposition of hydroperoxide to other compounds. The formation of conjugated dienes occurs at the early stages of lipid oxidation and hydroperoxides are expected to decompose to create the secondary products (Frankel *et al*., 1997). The decrease or reaching of a stagnant level in CD was generally accompanied by an increase in TBARS (Peña-Ramos and Xiong, 2002).

As shown in Figure 20, GH-H11 and GH-Al were able to retard TBARS formation, indicating the ability to inhibit the oxidation of lipids in the liposome system. This was evidence by the lower TBARS of system, compared with the control especially within the first 30 h ($P < 0.05$). In general, the efficiency in retarding lipid oxidation was dependent on the concentration used. For the control, the decrease in TBARS was found after 24 h of incubation, while the systems containing hydrolysates had the decreases in TBARS after 30 h ($P < 0.05$). It was noted that Trolox showed the profound effect in prevention of TBARS formation in the system.

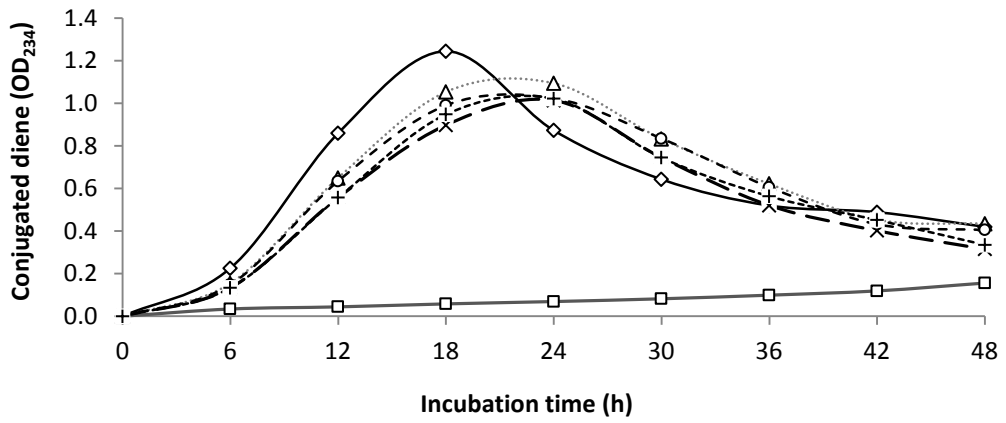

Figure 18. Conjugated dienes (CD) of lecithin liposome system in comparison with the control as affected by gelatin hydrolysate from unicorn leatherjacket skin prepared using protease from *B. amyloliquefaciens* H11 and Alcalase with 40% DH at different levels. Bars represent the standard deviation ($n = 3$). (◊): Control, (□): Trolox 100 ppm, (Δ): GH-H11100 ppm, (×): GH-H11 1000 ppm, (○): GH-Al 100 ppm, and (+): GH-Al 1000 ppm.

In general, liposomes are the appropriate lipid models to evaluate antioxidants for both food and lipoprotein particles containing phospholipids (Frankel *et al*., 1997). Polar portions of peptides might interact with the liposomes of phospholipids, where they function effectively as antioxidant at the interface. In the free radical-mediated lipid peroxidation system, antioxidative activity of peptides or proteins is dependent on molecular size and properties such as hydrophobicity and electron transferring ability of the amino acid residues in the sequence (Qian *et al*., 2008). The results suggested that gelatin hydrolysates could inhibit the early stages of lipid oxidation (formation of CD and PV) as well as retard propagation of the oxidation process (degradation of hydroperoxide to TBARS). Therefore, peptides in unicorn leatherjacket fish skin gelatin hydrolysates, produced either by protease from *B. amyloliquefaciens* H11 or Alcalase, had the antioxidant properties in the liposome system.

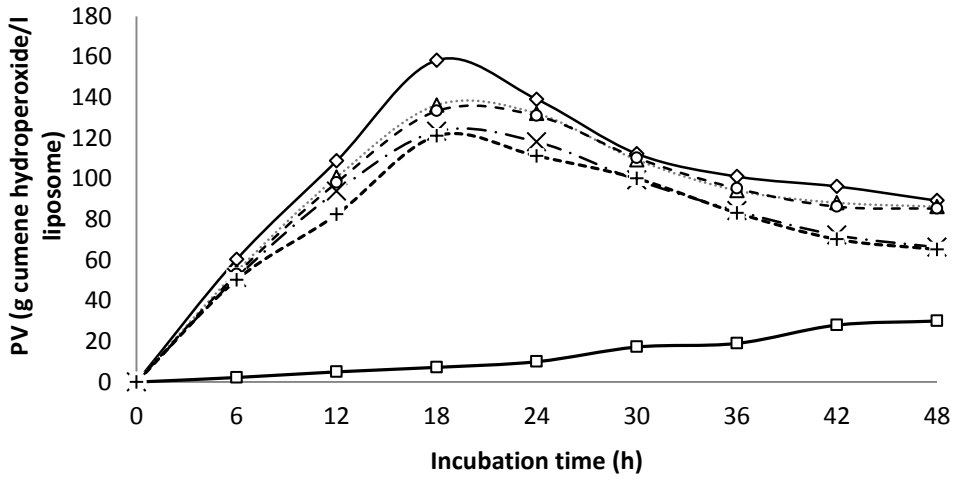

Figure 19. Peroxide value (PV) of lecithin liposome system in comparison with the control as affected by gelatin hydrolysate from unicorn leatherjacket skin prepared using protease from *B. amyloliquefaciens* and Alcalase with 40% DH at different levels. Bars represent the standard deviation (n = 3). (◊): Control, (□): Trolox 100 ppm, (Δ): GH-H11100 ppm, (×): GH-H11 1000 ppm, (○): GH-Al 100 ppm, and (+): GH-Al 1000 ppm.

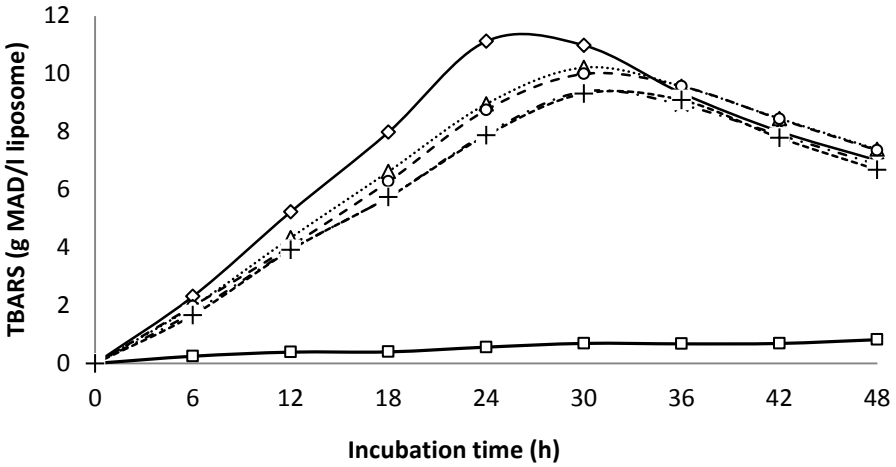

Figure 20. Thiobarbituric acid reactive substances (TBARS) of lecithin liposome system in comparison with the control as affected by gelatin hydrolysate from unicorn leatherjacket skin prepared using protease from *B. amyloliquefaciens* and Alcalase with 40% DH at different levels. Bars represent the standard deviation (n = 3). (◊): Control, (□): Trolox 100 ppm, (Δ): GH-H11100 ppm, (×): GH-H11 1000 ppm, (○): GH-Al 100 ppm, and (+): GH-Al 1000 ppm.

Antioxidative activity of gelatin hydrolysate in *in vitro* pepsin-pancreatin simulated GI system

ABTS radical-scavenging activity and chelating activity of GH-H11 and GH-Al with 40% DH in the simulated GI system are depicted in Figure 21. Both hydrolysates had no changed in ABTS radical scavenging and ferrous chelating activities in the stomach condition (the first 60 min). This might be due to the limitation of cleavage by pepsin because pepsin preferentially cleaves the C-terminal of phenylalanine, leucine and glutamic acid as well as the increase of hydrophobic properties of GI digests after pepsin treatment makes them less likely to react with water-soluble ABTS free radical (You *et al*., 2010). Moreover, Chen and Li (2012) reported that the antioxidant peptides above 3000 Da were more sensitive to the pepsin and low pH during the gastric digestion, meanwhile our antioxidant peptides contained majorly low molecular weight. As a result, no further degradation or

generation of new antioxidant peptides took place. In intestinal simulated system (pancreatin digestion), the sharp increases in both ABTS radical scavenging and ferrous chelating activities were obtained within 30 min ($P < 0.05$). Thereafter, the rate of increase became slightly lower with increasing digestion time up to 240 min ($P < 0.05$). GH-Al showed the higher ABTS radical scavenging activities, but lower ferrous chelating activity, compared with GH-H11 in the duodenal condition ($P < 0.05$). Further digestion with pancreatin caused the dramatic increase in ABTS radical scavenging activity and ferrous chelating activity, suggesting that antioxidative peptides in both hydrolysates were modified by pancreatin digestion. During the intestinal digestion, pancreatin contains many enzymes, including trypsin and additional proteases that might cleave the peptides to some degrees, leading to the release of new potent antioxidative peptides (Megías *et al*., 2009). In addition, pancreatin not only hydrolysed the peptides into smaller pieces, but also produced more free amino acids that enhanced antioxidant peptides with stronger activity were produced during the GI digestion. Since the increase of hydrophilic property of GI digests after pancreatin treatment favours their trapping of the ABTS free radical (You *et al*., 2010). ABTS radical scavenginig activity of protein hydrolysate from loach (You *et al*., 2010), gelatin hydrolysate from blacktip shark skin (Kittiphattanabawon *et al*., 2012), protein hydrolysates from the muscle of ornate threadfin bream (Nalinanon *et al*., 2011) and protein hydrolysates from the muscle of brownstripe red snapper (Khantaphant *et al*., 2011) increased along with GI digests, particularly after pancreatin treatment. Thus, antioxidative activities of both GH-H11 and GH-Al were more likely preserved or enhanced after digestion in the simulated gastrointestinal tract of human body.

Molecular weight distribution of antioxidative peptides

Peptides in GH-H11 and GH-Al were fractionated using Sephadex-G25 gel filtration chromatography (Figure 22). After separation on Sephadex G-25, GH-H11showed the activity peaks at the fraction no. 30 and 42, with MW of 4000 and 750 Da, respectively. However, peptide with MW of 750 Da exhibited the higher ABTS radical scavenging activity, compared with that having MW of 4000 Da. It was noted that peak with MW of 750 Da had high OD_{280}

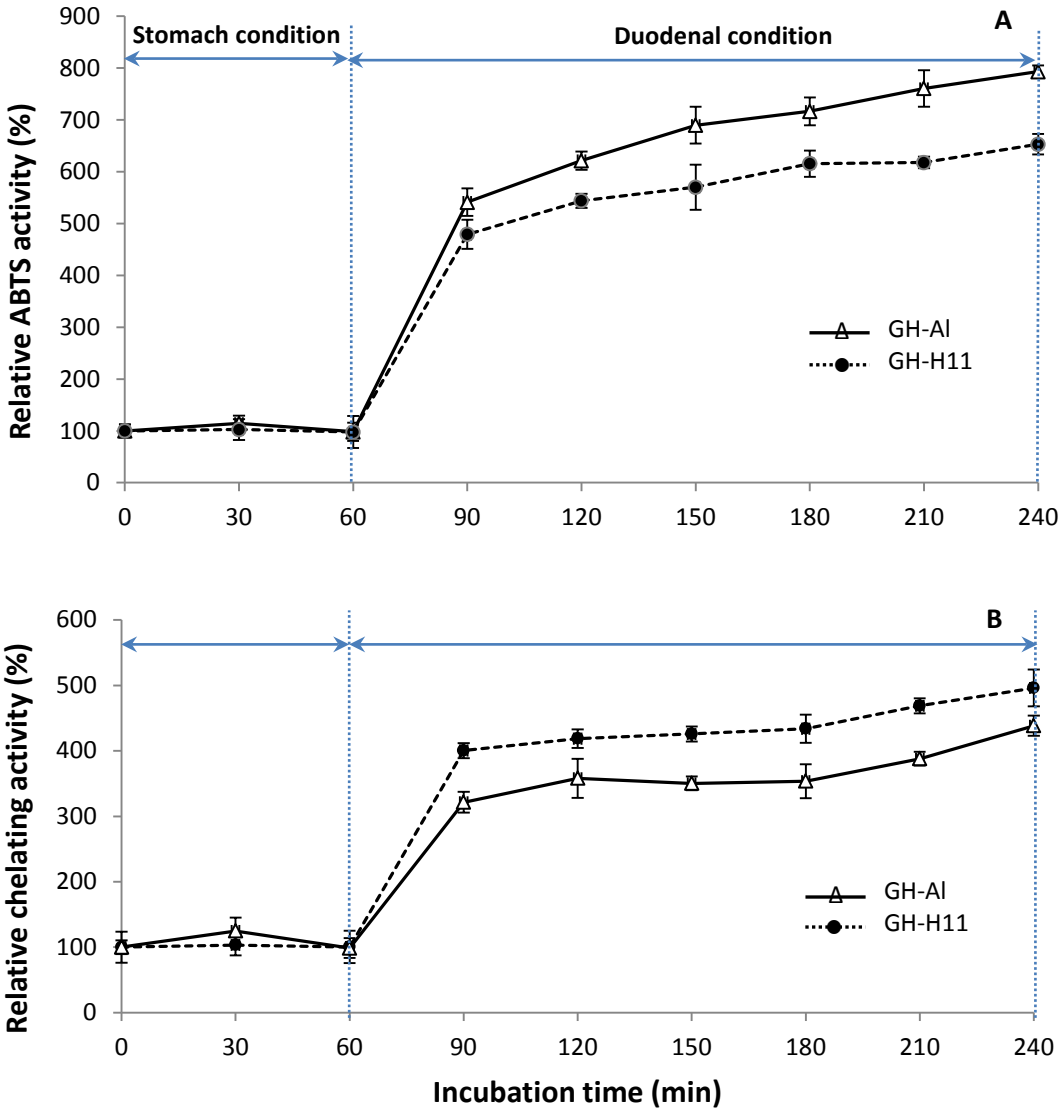

Figure 21. Antioxidative activity of gelatin hydrolysate from unicorn leatherjacket skin prepared by protease from *B. amyloliquefacines* H11 (●) and Alcalase (Δ) in the simulated GI system as monitored by ABTS radical scavenging activity (A) and chelating activity (B) assays. Bars represent standard deviation (n = 3).

It was suggested that those peptides probably contained aromatic amino acids in the peptide chain, e.g. tyrosine, tryptophan and other aromatic compounds (Thiansilakul *et al*., 2007ab). The amino acid composition of collagen and

gelatin hydrolysates is very similar to that of the parent proteins, being rich in residues of glycine, alanine, proline and hydroxyproline, but poor in methionine, cysteine, histidine and tyrosine (Alemán *et al*., 2011). However, Dávalos and other (2004) reported that tryptophan, tyrosine and methionine showed the highest antioxidant activity, followed by Cys, His and Phe. For the fractions of GH-Al, activity peaks were obtained with fraction no. 31, 36 and 45 with MW of 4000, 3600 and 270 Da, respectively. Among all the selected fractions, fraction having MW of 3600 Da showed the highest ABTS radical scavenging activity.

The antioxidative activity of the peptides fractionated from gelatin hydrolysates depended on their amino acid sequences. Numerous studies have reported that antioxidative peptides from gelatin hydrolysates had varying MW, e.g. 1500-4500 Da from Alaska pollack skin gelatin hydrolysates (Kim *et al*., 2001b), 700 Da from cobia gelatin hydrolysates (Yang *et al*., 2008) and 317-645 Da from tilapia skin gelatin hydrolysates (Zhang *et al*., 2012). The result suggested that peptides generated from gelatin of unicorn leatherjacket skin were different when different proteases were used. This more likely determined their antioxidative activity, in which higher antioxidative activity was obtained for GH-H11.

5.5 Conclusion

Production of unicorn leatherjacket skin gelatin hydrolysates could be achieved with the aid of protease from *B. amyloliquefaciens* H11. The hydrolysate showed higher antioxidative activity than that prepared by commercial Alcalase. The activity of peptides was enhanced after digestion in a gastrointestinal tract model system. Active peptides with MW of 750 Da probably contain high proportion of aromatic amino acids. Therefore, gelatin hydrolysate prepared using protease from *B. amyloliquefaciens* H11 might serve as a potential source of natural antioxidant, which can prevent oxidation in both food and biological systems.

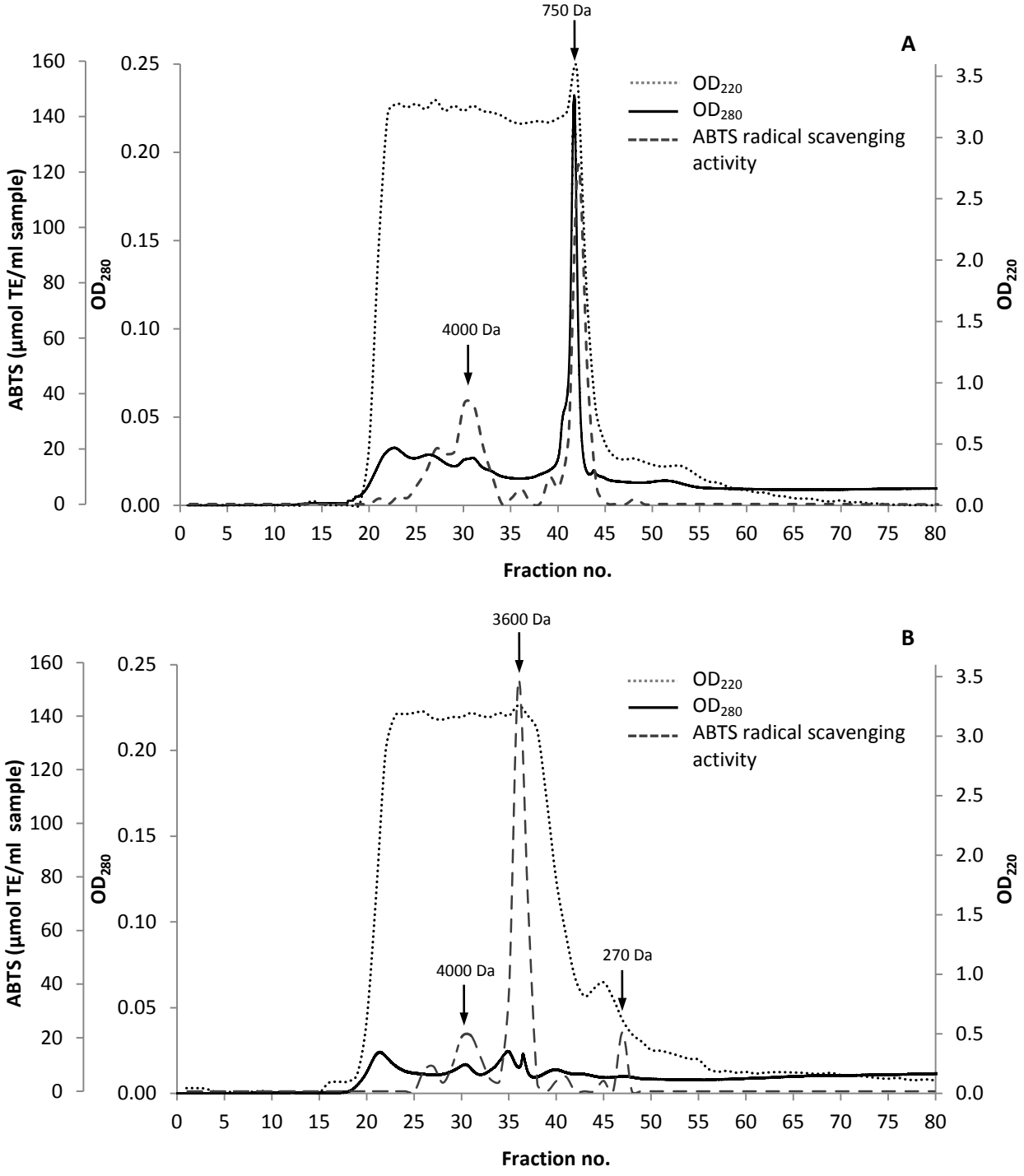

Figure 22. Separation of antioxidative peptides from gelatin hydrolysates (40%DH) prepared using protease from *B. amyloliquefacines* H11 (A) and Alcalase (B) by Sephadex G-25.

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CHAPTER 6

ACE-INHIBITORY ACTIVITY PEPTIDE FROM UNICORN LEATHERJACKET SKIN GELATIN PREPARED USING EXTRACELLULAR PROTEASE FROM *BACILLUS AMYLOLIQUEFACIENS*

6.1 Abstract

Angiotensin I-converting enzyme (ACE) inhibitory peptides from unicorn leatherjacket (*Aluterus monoceros*) skin gelatin hydrolysate prepared using protease from *B. amyloliquefaciens* H11 were identified. The gelatin hydrolysate was fractionated using Sephadex G25 column, followed by reversed-phased high performance liquid chromatography (RP-HPLC). Potential ACE inhibitory peptides were identified as AAGAPGGAR, ASGGPAGAR, GPVGHKG, LGASPGR, VVGPGA, DGGPAGVR, RPGGPPGSPG and AGDVHPSM based on MALDI-TOF mass spectrometry. The presence of hydrophobic amino acid residues at the terminal was more likely responsible for the high ACE inhibitory activity of the peptides. Thus, gelatin hydrolysate could serve as the source of peptides lowering hypertension.

6.2 Introduction

Marine-derived proteins, including collagen and gelatin, are becoming more popular among consumers because of their numerous health beneficial effects (Kim, 2013). Marine bioactive peptides have attracted a great deal of attention due to their potential effects in promoting health and reducing the risk for diseases (Ngo *et al*., 2014). Currently, collagen and gelatin-derived bioactive peptides show the possibility of wide commercialization in the food and pharmaceutical industry. Collagen and gelatin-derived peptides have been shown to possess a variety of biological and physiological functions including antioxidant, antihypertensive/ACE inhibitory, antimicrobial, anticancer, human LDL cholesterol inhibitory, DNA oxidation inhibitory, opioid agonistic, immunomodulatory, prebiotic, mineral binding,

anti-thrombotic and hypocholesterolemic properties (Betoret *et al*., 2011; Kittiphattanabawon *et al*., 2013).

Angiotensin-I converting enzyme (ACE) plays an important role in the regulation of blood pressure and hypertension. ACE catalyzes the conversion of angiotensin I to angiotensin II and degrades bradykinin (Murray and FitzGerald, 2007). Synthetic inhibitors of ACE such as Captopril, Enalapril and Fosinopril, etc. are often used to treat hypertension and other cardio-related diseases. However, these compounds can cause the adverse side effects (Atkinson and Robertson, 1979). Therefore, natural products, especially from dietary proteins, that are perceived as being safer than synthetic inhibitors have gained increasing interest. Peptides containing hydrophobic amino acids at each of the C-terminal positions were effective in inhibiting ACE (Murray and FitzGerald, 2007).

Unicorn leatherjacket (*Aluterus monoceros*), one of important fish species in the Southeast Asia, has been caught more than 7 million tons per year (Kaewruang *et al*., 2013). The fish skin, which is generated as a byproduct of fillet processing, contain about 70-80% of collagen in dry matter (Kaewruang *et al*., 2013). As a consequence, a large amount of skin can be converted into collagen or gelatin, which is further hydrolyzed to potential bioactive peptides.

Microbial proteases have been used widely for production of gelatin hydrolysate with bioactivity. Alcalase is one of most popular microbial proteases used for hydrolysis of marine proteins from resources (Guérard *et al*. 2001). Recently, Sai-Ut *et al*. (2013) reported that microbial protease from *B. amyloliquefaciens* H11 could hydrolyze fish gelatin effectively. Hydrolysate from unicorn leatherjacket skin gelatin had antioxidative activity (Sai-Ut *et al*., 2014). Gelatin hydrolysate has also been reported to have ACE inhibitory activity. However, no information regarding ACE-inhibitory activity of unicorn leatherjacket skin gelatin hydrolysates has been reported. Therefore, the aim of this study was to purify and identify ACE inhibitory peptides from unicorn leatherjacket skin gelatin hydrolysate prepared using protease from *B. amyloliquefaciens* H11.

6.3 Materials and Methods

Materials

Unicorn leatherjacket (*Aluterus monoceros*) skin was obtained from fish dock, Songkhla, Thailand. ACE (from rabbit lung, 0.2 U/mg protein) and a substrate peptide (*N*-Hippuryl-His-Leu) and acetonitrile were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). All reagents were of analytical grade.

Production of gelatinolytic enzyme from *B. amyloliquefaciens* H11

B. amyloliquefaciens H11 was isolated from fish dock (Sai-Ut *et al*., 2013). A bacterium was kept frozen at -40 °C in nutrient broth (NB) supplemented with 20% (v/v) glycerol. The bacterium was sub-cultured twice in NB at 37 °C for 18 h before used.

For production of gelatinolytic enzyme, *B. amyloliquefaciencs* H11 was cultured in 0.5% peptone medium containing 0.8% fish gelatin at 37.5 °C with continuous shaking at 234 rpm for 36 h. The culture was centrifuged at 9,000 × g for 15 min at 4°C, and the supernatant containing protease was then subjected to freeze-drying using a freeze dryer (Scanvac, Coolsafe, Lynge, Denmark). The powder termed ‘crude enzyme powder’ (118.4 U/g powder) obtained was used for production of gelatin hydrolysate.

Preparation of gelatin hydrolysate

Gelatin from the skin of unicorn leatherjacket (*Aluterus monoceros*) was extracted with distilled water at 65 °C for 12 h following the method of Kaewruang *et al*. (2013). The resulting gelatin powder was used for the preparation of gelatin hydrolysate. Gelatin (2% w/v) was hydrolyzed with crude enzymes in the presence of 10 mM $CaCl_2$ at 50 ºC and pH 8 using an enzyme/substrate ratio of 1/20 (w/w). The mixture was incubated for 3 h with continuous shaking and the reaction was then terminated with boiling water for 15 min. The hydrolyzed mixture was centrifuged at 9,000 × *g* for 15 min. The supernatant was collected and lyophilized.

The obtained gelatin hydrolysate powder (≈30%DH) was placed in polyethylene bag and stored at −20 °C until used.

Purification of ACE inhibitory peptides from gelatin hydrolysates

Gelatin hydrolysate powder was dissolved in deionized water to obtain a concentration of 20 mg/ml. The solution was loaded onto a Sephadexl G-25 column (4.5 × 65 cm) equilibrated with distilled water, and then eluted at a flow rate of 0.5 ml/min. The fractions (5 ml) were collected and A_{280} was measured. Each A_{280} peak, from pooled fractions, was assayed for ACE-inhibitory activity. The peak with the highest ACE-inhibitory activity was further separated by RP-HPLC (model SPE-MA10AVP, Shimadzu, Kyoto, Japan) on Atlantis®DC_{18} (20 × 150 mm,) column. The volume injected was 500 µl and the flow rate was 2 ml/min. MilliQ water containing 0.1% trifluoroacetic acid (TFA) and acetonitrile containing 0.1% TFA were used as solvents A and B, respectively. The gradient was 1% B for 5 min, 1–50% B for 45 min, 50% B for 5 min, and 50% to 1% B for 5 min. Elution of peptide was monitored by A_{230}. The A_{230} peaks, from pooled fractions, were collected, lyophilized and tested for ACE-inhibitory activity.

Assay of ACE inhibitory activity

ACE inhibitory activity was determined by the spectrophotometric assay according to the method of Cushman and Cheung (1971) with modifications. The sample solution (15 µl) was mixed with 30 µl of ACE solution (0.2 U/ml). The mixure was pre-incubated at 37 °C for 5 min, and then the mixture was added with 30 µl of substrate (8.3 mM *N*-Hippuryl-His-Leu (HHL) in a 50 mM sodium borate buffer containing 0.5 M NaCl at pH 8.3) at 37 °C for 60 min. After incubation, the reaction was stopped by the addition of 1 M HCl (75 µl) and the resulting hippuric acid was extracted by the addition of 450 µl ethyl acetate. The reaction mixture was centrifuged at 9,000 × *g* for 10 min and then 400 µl of the upper layer was transferred into a test tube. Ethyl acetate was removed by heat evaporation at 60 °C for 3 min. The hippuric acid was redissolved in 1.5 ml of distilled water and the absorbance was

measured at 228 nm using a spectrophotometer. The inhibition activity was calculated using the following equation.

$$\text{ACE inhibitory (\%)} = \frac{(A_a - A_b)}{(A_a - A_c)} \times 100\%$$

where A_a is the absorbance with ACE and HHL without the sample; A_b is the absorbance with ACE, HHL and the sample; and A_c is the experimental blank where no enzyme–substrate reaction is facilitated.

MALDI-TOF MS Analysis

The sample was analyzed by MALDI-TOF MS according to method of (Zhang *et al*., 2012). One microliter was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Then, 0.4 µl of a 3 mg/ml of α-cyano-4-hydroxy-transcinnamic acid matrix (Sigma) in 50% acetonitrile were added to the dried peptide digest spots and allowed again to air-dry at room temperature. MALDI-TOF MS analyses were performed in Voyager PK2 (Applied Biosystems, Framingham, MA) operated in positive reflector mode with an accelerating voltage of 20,000 V. The sequences of interested peptides were also compared with those of the NCBInr database.

6.4 Results and Discussion

Unicorn leatherjacket skin gelatin was hydrolyzed with extracellular protease from *Bacillus amyloliquefaciens* H11 and fractionated by Sephadex G25 column as shown in Figure 23A. The gelatin hydrolysate was separated into ten peaks based on A_{280}. Each A_{280} peak was measured for ACE inhibitory activity. The ACE inhibitory activities of the gelatin hydrolysate peak no. 6 and 7 (G25-6 and G25-7) showed the highest ACE inhibitory activity (75 and 78%, respectively). The result suggested that the peak with low molecular weight (MW) peptides had higher ACE inhibitory activity than those with higher MW. Nevertheless, peptides with very small size might lose their activity. This was evidenced by the low activity of peak no. 8, 9 and 10. Peptide with low MW is believed to exhibit high ACE inhibitory activity (Lin *et al*., 2012; Quirós *et al*., 2007; Miguel *et al*., 2009). Peptides from yellowfin sole frame protein hydrolysates with MW < 5 kDa (Jung and Kim, 2006) and sea

cucumber gelatin hydrolysate with MW < 1 kDa (Zhao *et al*., 2007) showed ACE inhibitory activity. Based on gel filtration (Sephadex G25), peak no. 6 and 7 had MW approximately of 750 and 700 Da, respectively.

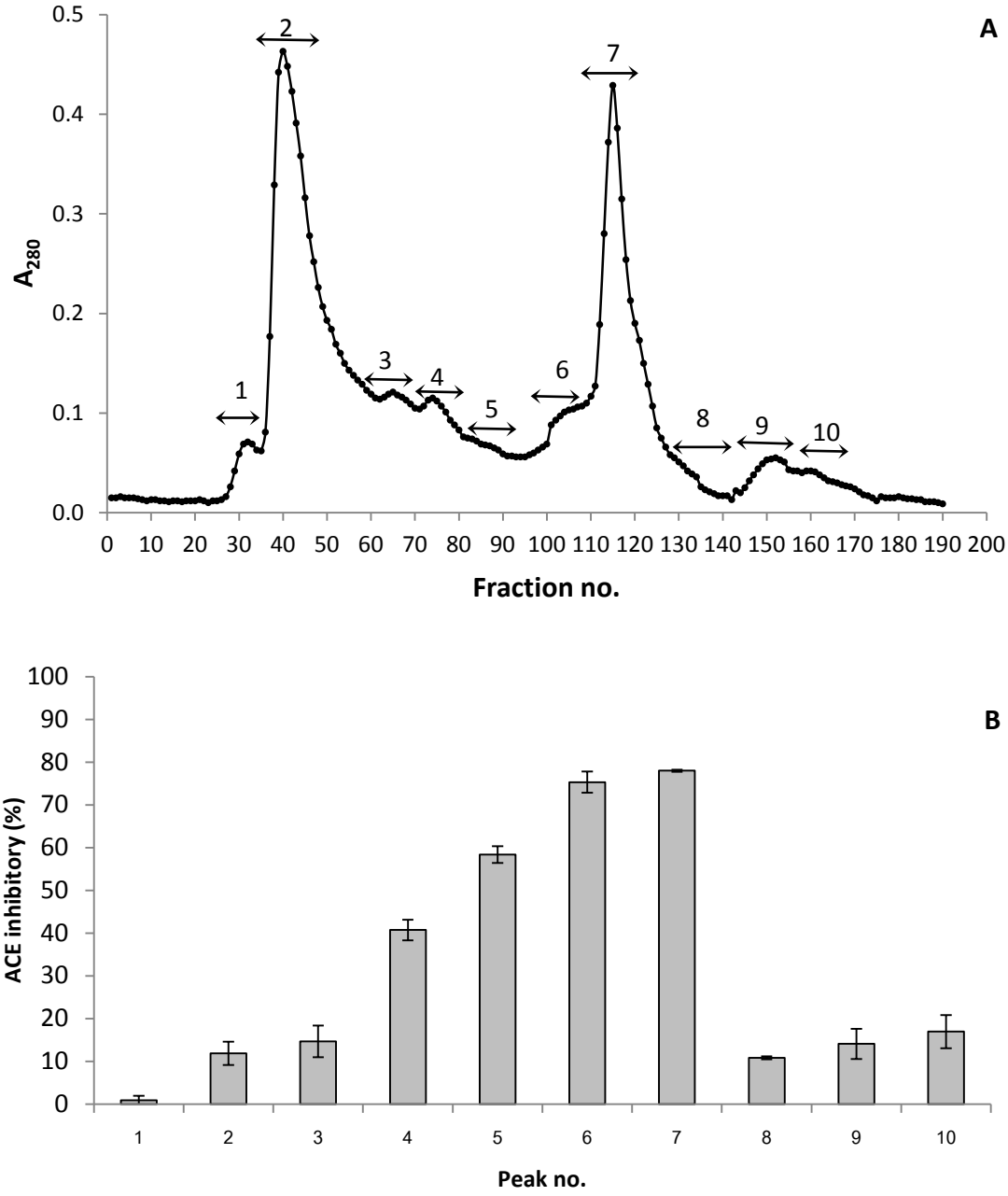

Figure 23. Purification profile of gelatin hydrolysate from unicorn leatherjacket skin prepared using protease from *B. amyloliquefaciens* H11 by Sephadex G25 column (A) and ACE inhibitory activity (B). ↔ represents the pooled fraction based on A_{280}.

When G25-6 and G25-7 peaks were subjected to RP-HPLC, eleven peaks were obtained from G25-6 (Figure 24A) and six-teen peaks were collected from G25-7 (Figure 25A). Peaks were obtained by pooling all fractions based on A_{230}. All peaks were analyzed for their potency in inhibiting ACE (Figure 23B and 24B). Purified peptides from G25-6 had higher ACE inhibitory activity than those from G25-7. This might be due to the differences in peptides, especially in term of amino acid sequences and size distribution. Muguruma *et al*. (2009) suggested that the terminal aromatic amino acid residue plays an important role in the expression of ACE inhibitory activity. Among purified peptides of G25-6, peak no. 5 (G25-6-RP-HPLC-5) showed the highest ACE inhibitory activity and it was identified as Ala-Gly-Asp-Val-His-Pro-Ser-Met based on MS spectrum (Figure 26). It was noted that strong ACE inhibitory activity of the peptide peaks in no. 5 was supported by the presence of hydrophobic amino acids (Ala) at the carboxy terminal and branched chain aliphatic amino acids (Met) at the amino terminal (Cheung and Chushman, 1971; Qian *et al*., 2007). In addition, peak no. 2 with m/z of 499.3 was identified as Val-Val-Gly-Pro-Gly-Ala. Peptides in peak no. 1 with m/z of 727.4 and peak no. 3 with m/z of 728.4 were identified as Ala-Ala-Gly-Ala-Pro-Gly-Gly-Ala-Arg and Asp-Gly-Gly-Pro-Ala-Gly-Val-Arg, respectively. Chen *et al*. (2012) reported that the sequences consisting of a center amino acid residue with low electronic properties and hydrophilicity and bulky side amino acid residues such as alanine, valine and proline have strong ACE-inhibitory activities. The sequences in peak no. 1 with m/z of 651.3 and peak no. 3 with m/z of 878.4 were of homology with those described as collagen chain-like in the NCBInr database. It was noted that these sequences had Gly at every third position, typical for collagen molecule where glycine represents 33–34% of the amino acid residues (Alemán *et al*., 2011). Bioactive peptides derived from protein hydrolysate were mainly affected by the amino acid sequences of peptides. The peptide exhibited typical amino acid sequences that represent antihypertensive characteristics had hydrophobicity at the end of the peptide (Norris and FitzGerald, 2013; Qian *et al*., 2007). Binding sites of peptides with long chain peptides (more than five amino acid residues) were strongly influenced by peptide conformation. ACE inhibitory capacity was more related to tetrapeptide residues at the C-terminal (Meisel *et al*., 2005).

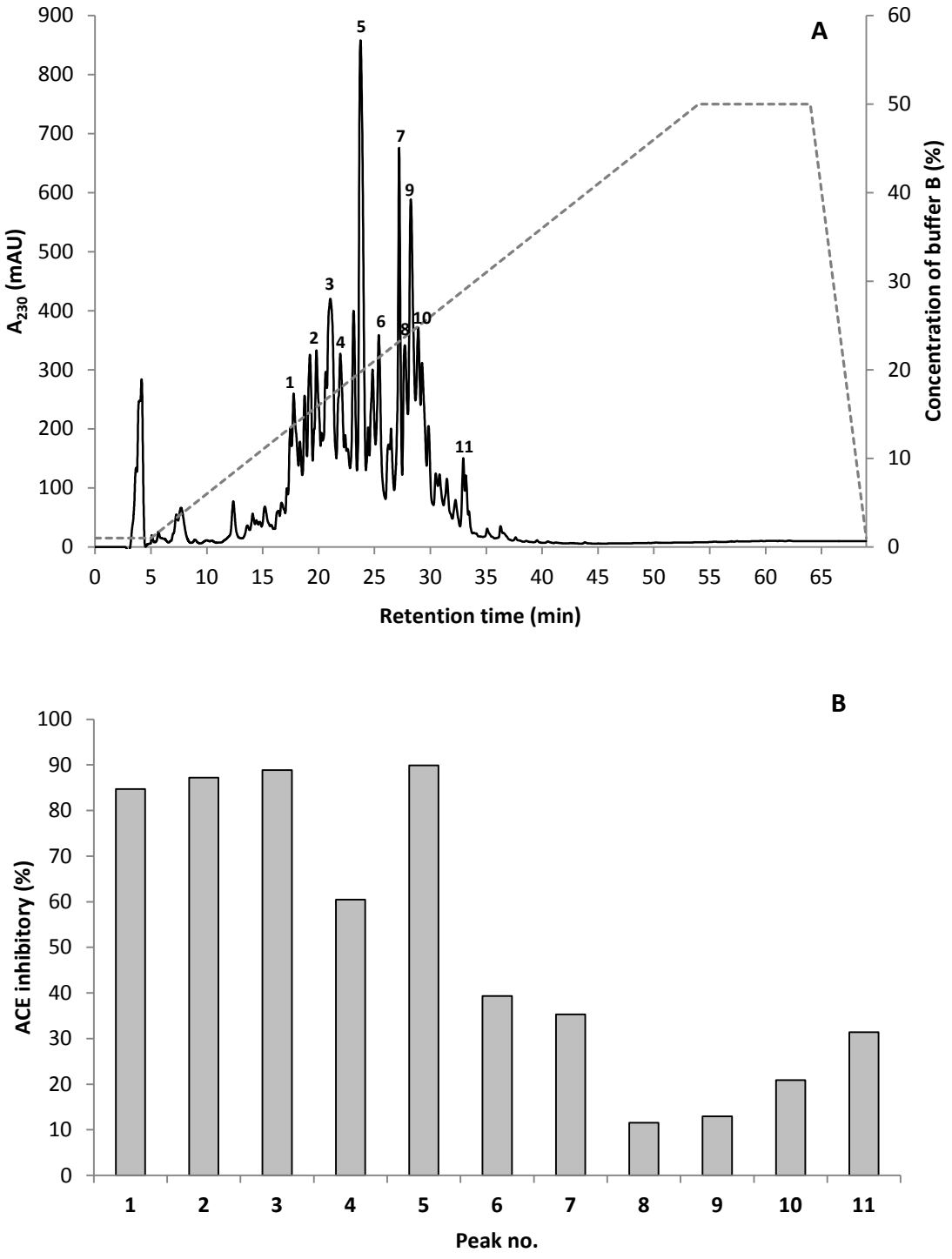

Figure 24. Purification profiles of ACE inhibitory peptide from G25-6 (A) and ACE inhibitory activities (B).

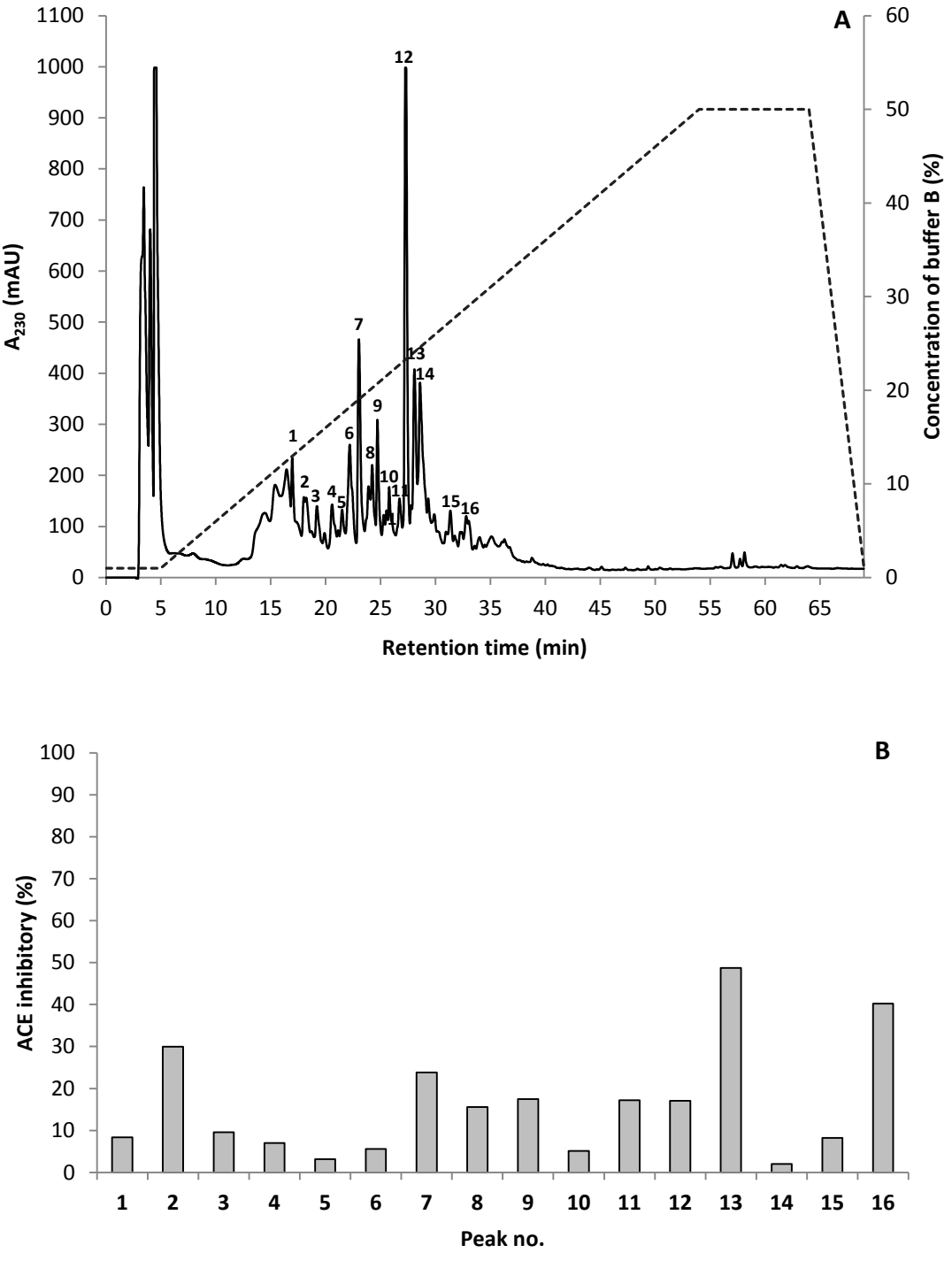

Figure 25. Purification profiles of ACE inhibitory peptide from G25-7 (A) and ACE inhibitory activities (B).

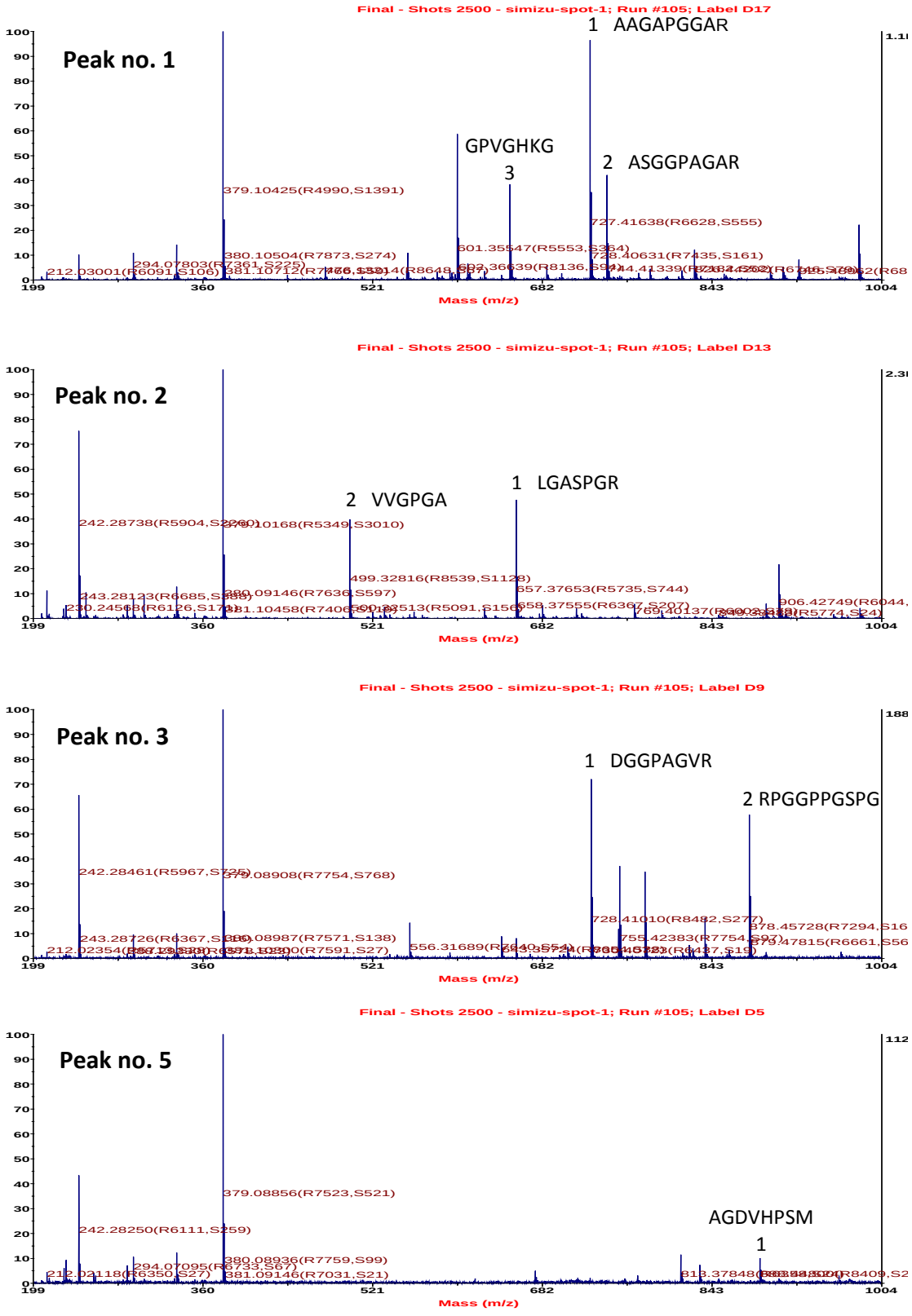

Figure 26. MS spectrum of ACE inhibitory peptide in G25-6-RE-HPLC fraction.

Other sequences of potential peptide analyzed by MALDI-TOF mass spectroscopy are summarized in Table 20. The isolated peptides of all peaks contained at least one hydrophobic amino acid at the carboxy terminal or the amino terminal, which might contribute largely to its ACE inhibitory potential. Structure–activity studies indicated that hydrophobic amino acids at terminus are a feature favoured by ACE, thereby contributing to ACE inhibitory activities (Norris and FitzGerald, 2013). The presence of negatively charged amino acids, such as arginine residues presented in peptides peak no. 1, 2 and 3 at the C-terminal might decrease the inhibition activity of the peptide (Li *et al*., 2004).

Table 20. Amino acid sequences of peptides in G25-6-RE-HPLC-fraction identified using the MALDI-TOF mass spectrometry.

Peak no.	Peptide sequence	Spectrum length (m/z)	Peaks score
1	(1) AAGAPGGAR	727.4	76.26
	(2) ASGGPAGAR	743.4	75.00
	(3) GPVGHKG	651.3	87.55
2	(1) LGASPGR	657.3	73.65
	(2) VVGPGA	499.3	88.34
3	(1) DGGPAGVR	728.4	75.14
	(2) RPGGPPGSPG	878.4	84.69
5	(1) AGDVHPSM	813.3	70.78

Pacific cod skin hydrolysate showed ACE inhibitory activity (IC_{50} = 35.7 μM) and Leu-Leu-Met-Leu-Asp-Asn-Asp-Leu-Pro-Pro (1301 Da) was the active peptide (Himaya *et al*., 2012). A decapeptide (Gly-Arg-Gly-Ser-Val-Pro-Ala-Hyp-Gly-Pro) with potent ACE-inhibitory capacity (IC_{50} = 47.78 μM) was purified from squid skins hydrolysate (Alemán *et al*., 2013). Two peptides, Met-Val-Gly-Ser-Ala-Pro-Gly-Val-Leu (829 Da) and Leu-Gly-Pro-Leu-Gly-His-Gln (720 Da), obtained from skate skin gelatin hydrolysate were responsible for ACE inhibitory activity with IC_{50} values of 3.09 and 4.22 μM, respectively (Ngo *et al*., 2014). The ACE inhibitory effects of gelatin hydrolysates prepared from various sources and the isolation of

several ACE-inhibitory peptides from sea cucumber gelatin hydrolysate (Zhao *et al*., 2007), chicken collagen hydrolysate (Saiga *et al*., 2008) and porcine gelatin hydrolysate (Ichimura *et al*., 2009) have been reported previously. Natural ACE inhibitory peptides are preferred over synthetic drugs as they do not show any harmful side effects (Himaya *et al*., 2012).

6.5 Conclusion

Unicorn leatherjacket skin gelatin hydrolysate produced using extracellular protease from *B. amyloliquefaciens* H11 had ACE inhibitory potential. Peptides with lower molecular weight of gelatin hydrolysate showed higher ACE inhibitory activity. The potential ACE inhibitory peptides were identified as AAGAPGGAR, ASGGPAGAR, GPVGHKG, LGASPGR, VVGPGA, DGGPAGVR, RPGGPPGSPG and AGDVHPSM. Furthermore, the presence of hydrophobic amino acid at the C- and N-terminal more likely contributed to the high ACE inhibitory activity of the gelatin hydrolysate.

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CHAPTER 7

EFFECT OF DRYING METHODS ON ODOUROUS COMPOUNDS AND ANTIOXIDATIVE ACTIVITY OF GELATIN HYDROLYSATE PRODUCED BY PROTEASE FROM *BACILLUS AMYLOLIQUEFACIENS* H11

7.1 Abstract

Gelatin hydrolysates with antioxidative activity produced by protease from *Bacillus amyloliquefaciens* H11 with different hydrolysis times were prepared. Alpha-amino group content and antioxidative activities increased with increasing hydrolysis time ($P < 0.05$). When gelatin hydrolysate prepared with hydrolysis time of 3 h (GH-3H) was subjected to freeze-drying and spray-drying, the powder obtained from spray drying showed the decrease in antioxidant activity as measured by DPPH and ABTS radical scavenging activities, ferric reducing antioxidant power and metal chelating activity. Spray-dried gelatin hydrolysate (GH-3H-SD) showed higher whiteness with lower fishy odour and off-odour associated with fermentation. This was concomitant with the decreases in several odourous compounds in the sample, except for nonanal, which was higher in comparison with the powder obtained by freeze-drying. Thus, spray-drying could be an effective drying method to improve colour and reduce undesirable odour of gelatin hydrolysate.

7.2 Introduction

Gelatin hydrolysates with bioactivities have gained attention for consumers. Enzymatic method using commercial protease has been implemented for production of hydrolysate from gelatin of Alaska pollack (Kim *et al*., 2001), hoki (*Johniusbelengerri*) (Mendis *et al*., 2005), cobia (*Rachycentroncanadum*) (Yang *et al*., 2008), mussel (Silva *et al*., 2012) and sole (Giménez *et al*., 2009). Due to the tedious process for enzyme purification, this leads to the increasing cost of enzymes, owing to the investment of equipment, manpower and some loss of enzyme activity (Binod *et al*., 2013). The use of inoculum of selected microorganism, which is able to

secret gelatinolytic enzyme during fermentation, can be an alternative means for production of gelatin hydrolysate without prior enzyme preparation.

Bacillus amyloliquefaciens secretes a large amount of extracellular serine-protease (Peng *et al*., 2003) which can efficiently hydrolyse fish gelatin (Sai-Ut *et al*., 2013). *Bacillus amyloliquefaciens* did not possess the genes encoding *Bacillus* enterotoxins or the key gene implicated in the synthesis of emetic toxins, or otherwise not to demonstrate phenotypic characteristics of toxin production (EFSA, 2008). Several strains of *B. amyloliquefaciens* are also used for the production of enzymes such as alpha amylase and subtilisin (Xiao *et al*., 2004). Thus, *B. amyloliquefaciens* offers an alternative to be used directly as the potential aid for the production of gelatin hydrolysate with antioxidative activity.

Fishy odour is one of constraint for gelatin hydrolysate, especially from fish skin. Fish skin underwent fishy odour development, especially when fish was stored in ice for a longer time (Sae-leaw *et al*., 2013). Off odour associated with fermentation is another main problem of gelatin hydrolysate when the inoculum is applied directly in the gelatin substrate. This is plausibly due to the presence of some metabolites produced by microorganism. Spray-drying technology can be an approach to remove undesirable odour from gelatin hydrolysate (Reineccius, 2004). During the transformation of liquid feed into dry powder at high temperature, volatile odourous compounds can be eliminated to some degree. Spray-drying has been used for production of casein hydrolysate (Contreras *et al*., 2011), protein hydrolysate from black tilapia (*Oreochromis mossambicus*) (Abdul-Hamid *et al*., 2002), and chicken meat protein hydrolysate (Kurozawa *et al*., 2009). However, the loss of dry powder may occur (Contreras *et al*., 2011). Some negative effects have also been reported for food protein hydrolysates, such as changes in peptide composition as well as reduction of amino acid content (He *et al*., 2008; Zeng *et al*., 2013), which might be associated with the decreased antioxidative activities.

However, drying at high temperature might induce the loss in bioactivity of hydrolysate, particularly antioxidative activity. Nevertheless, there is no information on the characterisations and antioxidative activity of fish gelatin hydrolysate produced by extracellular protease from *Bacillus amyloliquefaciens* H11, isolated from fish dock. This study aimed to investigated odour and antioxidative

activity of gelatin hydrolysate as influenced by drying methods, freeze-drying and spray-drying.

7.3 Materials and Methods

Chemicals

2,4,6-trinitrobenzenesulphonic acid (TNBS) and trolox were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Methanol and ammonium thiocyanate were obtained from Lab-Scan (Bangkok, Thailand). Peptone and nutrient broth were procured from HiMedia Laboratory (Mumbai, India). Calcium chloride ($CaCl_2$), ethylenediaminetetraacetic acid (EDTA), tris(hydroxymethyl)aminomethane (Tris) and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

Microorganism and culture maintenance

Bacterium with gelatinolytic activity was isolated from fish dock and identified as *Bacillus amyloliquefaciens* H11 based on 16S ribosomal RNA gene sequence analysis (Sai-Ut *et al*., 2013). The bacterium was kept frozen at -40 °C in nutrient broth (NB) supplemented with 20% (v/v) glycerol. The bacterium was sub-cultured twice in NB at 37 °C for 18 h before use as an inoculum.

Extraction of gelatin

Gelatin from the skin of unicorn leatherjacket (*Aluterus monoceros*) was extracted with distilled water at 65 °C for 12 h following the method of Kaewruang *et al*. (2014). The resulting gelatin was used for the preparation of gelatin hydrolysate.

Production of fish gelatin hydrolysate with different hydrolysis times

Gelatin (1%, w/v) was dissolved with distilled water containing 0.36 mM $CaCl_2$ and the mixture was adjusted to pH 7.5 using 1 M NaOH. The substrate solution did not contain peptone. The solution was sterilised by autoclave for 15 min

at 121 °C. The obtained solution was added with 2% inoculum. The mixture was incubated at 37.5 °C with continuous shaking at 150 rpm for 24 h. After cultivation, the cells were removed by centrifugation at 9,000 × *g* and at 4 °C for 15 min using Allegra™ 25R centrifuge (Beckman Coulter, Palo Alto, CA, USA). The cell-free supernatant containing extracellular protease was further incubated at 50 ºC, an optimal temperature for protease, in a water bath (model W350, Memmert, Schwabach, Germany) for 0, 1, 2 and 3 h. To inactivate protease, the mixture was subjected to heating at 90 °C for 15 min. The mixture was then centrifuged at 5000 × *g* at room temperature for 10 min. The supernatants referred to as 'gelatin hydrolysate' including GH, GH-1H, GH-2H and GH-3H were sterilised (121 ºC and 100 kPa for 15 min) before analyses.

Drying of gelatin hydrolysate

GH-3H with the highest ABTS radical scavenging activity and metal chelating activity was subjected to freeze-drying or spray-drying. For freeze-drying, gelatin hydrolysate solution was freeze-dried using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lynge, Denmark) at -50 ºC for 48 h. The powder obtained was referred to as 'freeze-dried gelatin hydrolysate' (GH-FD). The spray drying of sample was performed using a spray dryer (SD-06 Basic, North Yorkshire, England) equipped with a spry-drying chamber having the dimensions of 500 mm height and 210 mm diameter and a spray nozzle type of 2-liquid nozzle (0.5 mm in size). A cyclone separator, a hot air blower and an exhaust blower were equipped. The gelatin solution was fed by a peristaltic pump at 485 mL/h into the chamber, atomized by the hot air (air velocity of 2 m/s) from blower in a downward current flow mode, using the following process conditions: inlet temperature of 180 °C, outlet temperature of 80 ± 2 °C and an atomising pressure of 2.8 bars. The resultant powder was blown through the cyclone separator and collected in a container. The powder was referred to as 'spray-dried gelatin hydrolysate' (GH-SD). Both GH-FD and GH-SD with the moisture contents of 3.3 and 6.4%, respectively, were subjected to analyses.

Determination of α-amino group content

The α-amino group content was determined according to the method of Benjakul and Morrissey (1997). To properly diluted hydrolysate samples (125 µl), 2.0 ml of 0.2 M phosphate buffer (pH 8.2) and 1.0 ml of 0.01% TNBS solution were added. The solution was mixed thoroughly and placed in a temperature controlled water bath (Model W350, Memmert, Schwabach, Germany) at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulphite. The mixtures were cooled at room temperature for 15 min. The absorbance was read at 420 nm and α-amino acid content was expressed in terms of L-leucine.

Determination of antioxidative activities

ABTS radical scavenging activity

ABTS radical scavenging activity was determined as described by Binsan *et al*. (2008). A standard curve of trolox ranging from 0 to 500 µM was prepared. The activity was expressed as µmol trolox equivalents (TE)/g sample.

DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described by Binsan *et al*. (2008). A standard curve was prepared using trolox in the range of 0 to 50 µM. The activity was expressed as µmol trolox equivalents (TE)/g sample.

Ferric reducing antioxidant power (FRAP)

FRAP was determined according to the method of Benzie and Strain (1996). The standard curve was prepared using trolox ranging from 0 to 500 µM. The activity was expressed as µmol trolox equivalents (TE)/g sample.

Chelating activity of ferrous ion

Ferrous ion chelating activity was measured by the method of Thiansilakul *et al*. (2007). EDTA with the concentration range of 0 to 50 µM was

used as the standard. Fe^{2+} chelating activity was expressed as μmol EDTA equivalents (EE)/g sample.

Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was assayed according to the method of Wettasinghe and Shahidi (2000). Trolox (0-10 mM) was used as the standard. The hydrogen peroxide scavenging activity was expressed as μmol trolox equivalents (TE)/g sample.

Colour measurement

Gelatin hydrolysate powders were measured for total difference of colour (ΔE^*) and whiteness using a colourimeter (ColourFlex, Hunter Lab Reston, VA, USA). Illuminant C was used as the light source of measurement. CIE L^*, a^* and b^* values were measured. The ΔE^* were calculated as follows:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL^*, Δa^* and Δb^* are the differentials between colour parameter of the samples and the colour parameter of the white standard (L^* = 93.52, a^* = -0.89, b^* = 0.39).

Whiteness was calculated using the following equation:

$$\text{Whiteness} = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}}$$

Sensory evaluation

Sensory evaluation for fishy odour and off-odour associated with fermentation in gelatin hydrolysate powder was conducted by 20 trained panelists (12 female and 8 male) with the ages of 23–36. Prior to the evaluation, the panelists were trained two times a week. Panelists were trained with standards for two sessions using a 15-cm line scale anchored from ‘none’ to ‘extremely strong’ for off-odour associated with fermentation and fishy odour. Unicorn leatherjacket skin gelatin stored at room temperature for two weeks was prepared and used as a reference off-

odour. For working standard, the gelatin was dissolved in distilled water to obtain the concentrations of 0, 0.05, 0.1 and 1.5% (v/v), representing the score of 0, 5, 10 and 15, respectively. The working standard for off-odour associated with fermentation was prepared by dissolving *B. amyloliquefaciens* H11 fermentation broth (containing 0.5% peptone cultured for 36 h) in distilled water to obtain concentration of 0, 33, 67 and 100% (v/v), representing the score of 0, 5, 10 and 15, respectively.

To test the samples, all gelatin hydrolysates (0.5%) dissolved in water or pomegranate juice were placed in a sealable plastic cup and heated at 60 °C in a temperature controlled water bath for 4 min before serving. The panelists were asked to open the sealable cup and sniff the headspace above the samples for determining off-odour associated with fermentation and fishy odour.

Measurement of volatile compounds

The volatile compounds in gelatin hydrolysate were determined using solid-phase micro extraction gas chromatography mass spectrometry (SPME-GCMS) following the method of Iglesias and Medina (2008) with a slight modification.

Extraction of volatile compounds by SPME fibre

To extract volatile compounds, 500 mg of sample was mixed with 4 ml of deionised water and stirred continuously to dissolve sample. The mixture was heated at 60 °C in 20 ml headspace vial with equilibrium time of 10 h. The SPME fibre (50/30 μm DVB/Carboxen™/PDMS StableFlex™) (Supelco, Bellefonte, PA, USA) was conditioned at 270 °C for 15 min before use and then exposed to the headspace. The 20 ml-vial (Agilent Technologies, Palo Alto, CA, USA) containing the sample extract and the volatile compounds were allowed to absorb into the SPME fibre at 60 °C for 1 h. The volatile compounds were then desorbed in the GC injector port for 15 min at 270 °C.

GC–MS analysis

GC–MS analysis was performed in a HP 5890 series II gas chromatography (GC) coupled with HP 5972 mass-selective detector equipped with a

splitless injector and coupled with a quadrupole mass detector (Hewlett Packard, Atlanta, GA, USA) according to method of Yarnpakdee *et al*. (2012). Compounds were separated on a TR-WaxMS column (Hewlett Packard, Atlanta, GA, USA) (30 m × 0.25 mm ID, with film thickness of 0.25 μm). The GC oven temperature program was: 35 °C for 3 min, followed by an increase of 3 °C/min to 70 °C, then an increase of 10 °C/min to 200 °C, and finally an increase of 15 °C/min to a final temperature of 250 °C and holding for 10 min. Helium was employed as a carrier gas with a constant flow of 1 ml/min. The injector was operated in the splitless mode and its temperature was set at 270 °C. Transfer line temperature was maintained at 260 °C. The quadrupole mass spectrometer was operated in the electron ionisation (EI) mode and source temperature was set at 250 °C. Initially, full-scan-mode data were acquired to determine appropriate masses for the later acquisition in scan mode under the following conditions: mass range: 25–500 amu and scan rate: 0.220 s/scan. All analyses were performed with ionisation energy of 70 eV, filament emission current at 150 μA, and the electron multiplier voltage at 500 V.

Analyses of volatile compounds

Identification of volatile compounds in the samples was based on the retention times of individual compounds by consulting ChemStation Library Search (Wiley 275.L). Quantification limits were calculated to a signal-to-noise (S/N) ratio of 10. Repeatability was evaluated by analysing 3 replicates of each sample. The identified volatile compounds were presented in the term of abundance of each identified compound.

Statistical analysis

All experiments were carried out in triplicate. Data were subjected to the analysis of variance (ANOVA) and mean comparisons were performed by using Duncan's multiple range test. For pair comparison, *t*-test was used (Steel and Torrie, 1980). Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA).

7.4 Results and Discussion

Effect of reaction time on hydrolysis and antioxidative activity of gelatin hydrolysate in the presence of protease from *B. amyloliquefaciens* H11

α-amino group content

The α-amino group contents of gelatin and gelatin hydrolysate (GH) prepared by incubation of gelatin solution containing extracellular protease from *B. amyloliquefaciens* H11 for 1-3 h (GH-1H, GH-2H and GH-3H) are presented in Table 21. The α-amino group content in gelatin and GH free of *B. amyloliquefaciens* H11 cells were 683 ± 2 and 2343 ± 42 mmole L-leucine/mg solid, respectively. The α-amino group content of GH with additional hydrolysis under the optimal temperature (50 ºC) increased from 2343 ± 42 to 2542 ± 22 mmole L-leucine/mg solid when the incubation time increased from 1 to 3 h. Amongst all samples, GH with the additional incubation of 3 h (GH-3H) showed the highest α-amino group content, suggesting the enhanced hydrolysis of gelatin by extracellular protease from *B. amyloliquefaciens* H11. It was noted that GH-1H had no difference in α-amino group content, compared with GH. After sterilisation using autoclave, the increases in α-amino group content were observed in all samples ($P < 0.05$). Thermal-pressure process resulted in enhanced hydrolysis of skin gelatin, in which hydrolysates with antioxidative activity were obtained (Yang *et al*., 2008).

Antioxidative activities

ABTS radical scavenging activity and metal chelating activity of all gelatin hydrolysates are shown in Figure 27. Both activities increased as the incubation time of gelatin solution containing extracellular proteases increased from 1 to 3 h ($P < 0.05$). ABTS radical scavenging activity is based on the ability of antioxidants to donate a hydrogen atom or an electron to stabilise radicals by converting it to the non-radical species (Chandrasekara and Shahidi, 2011). *B. amyloliquefaciens* H11 secreted extracellular protease during culturing. When gelatin was incubated in the presence of secreted protease, the peptide bonds were more cleaved, thereby producing a larger number of peptides that could react with free

radicals, thus terminating the radical chain reaction. Similar trend was found for metal chelating activity.

Table 21. α-amino group content of gelatin and gelatin hydrolysate as affected by hydrolysis time in the presence of protease from *B. amyloliquefaciens* H11 and subsequent sterilisation.

Treatment	α-amino group content (mmole L-leucine/mg solid)	
	Non-sterilised	Sterilised
Gelatin	683 ± 2aA	730 ± 14aB
GH	2343 ± 42bA	2408 ± 27bB
GH-1H	2344 ± 37bA	2447 ± 6cB
GH-2H	2391 ± 50cA	2535 ± 27dB
GH-3H	2542 ± 22dA	2641 ± 17eB

Different small letters within the same column indicate significant differences ($P < 0.05$). Different capital letters within the same row indicate significant differences ($P < 0.05$).

Generally the increases in ABTS radical scavenging activity and metal chelating activity were found in sterilised gelatin hydrolysate, compared with non-sterilized gelatin hydrolysate. The results indicated that antioxidative peptides were further produced during high thermal-pressure sterilisation process. This was in accordance with the increase in α-amino group content (Table 14). Yang *et al.* (2003) reported that the retorting (121 °C for 30 min) was able to degrade cobia skin gelatin and produce antioxidant peptides with smaller molecular sizes. Increasing carboxylic groups and amino groups of the peptides after hydrolysis might enhance metal ion binding capacity (Liu *et al.*, 2010). Thus, freeze-dried gelatin hydrolysate with 3 h hydrolysis time (GH-3H-FD) was selected for further study due to its highest in ABTS radical scavenging activity and metal chelating activity.

Effect of drying methods on properties of gelatin hydrolysate

Antioxidative activities

Antioxidative activities of gelatin hydrolysates produced by incubation of gelatin solution containing the secreted extracellular protease from *B. amyloliquefaciens* H11 for 3 h after freeze-drying (GH-3H-FD) or spray-drying (GH-3H-SD) are shown in Table 22. In general, the lower DPPH and ABTS radical scavenging activities were found in GH-3H-SD (10.7 and 80 μmole TE/g) in comparison with those of GH-3H-FD (12.7 and 102 μmole TE/g). The results indicated that antioxidative peptides were partially destroyed during spray-drying at high temperature. In addition, heat sensitive amino acids such as tryptophan and methionine, which acted as strong radical scavenger, might be lost during the excessive heating (Maa *et al*., 2010). Hydrogen peroxide scavenging activity of GH-3H-SD decreased slightly, compared with that of GH-3H-FD ($P < 0.05$). Hydrogen peroxide, a weak oxidising agent, is the precursor for the generation of hydroxyl radical, which is a strong initiator of lipid oxidation (Choe and Min, 2005).

Ferric reducing antioxidant power (FRAP), measuring the capacity of GH-3H-FD and GH-3H-SD in reducing TPTZ-Fe(III) complex to TPTZ-Fe(II) complex, was 2.37 and 2.23 μmol TE/g solid sample, respectively. The result suggested that spray-drying was a major factor in damaging some antioxidative peptides. The ferrous (Fe^{2+}) chelating activity of GH-3H-SD was lower, compared to GH-3H-FD ($P < 0.05$). The low metal chelating ability of GH-3H-SD indicated poorer ability in chelating proxidative metals (Klompong *et al*., 2007; McClements and Decker, 2000). Conformational changes of peptides in gelatin hydrolysate might occur to a high extent during spray-drying process at high temeprature. The changes in composition of free amino acids and/or peptides could affect the antioxidant activity (Thiansilakul *et al*., 2007ab). On the other hand, freeze-drying involves freezing and slow sublimation under vacuum. This process most likely prevented peptides in gelatin hydrolysate from aggregation or conformational change.

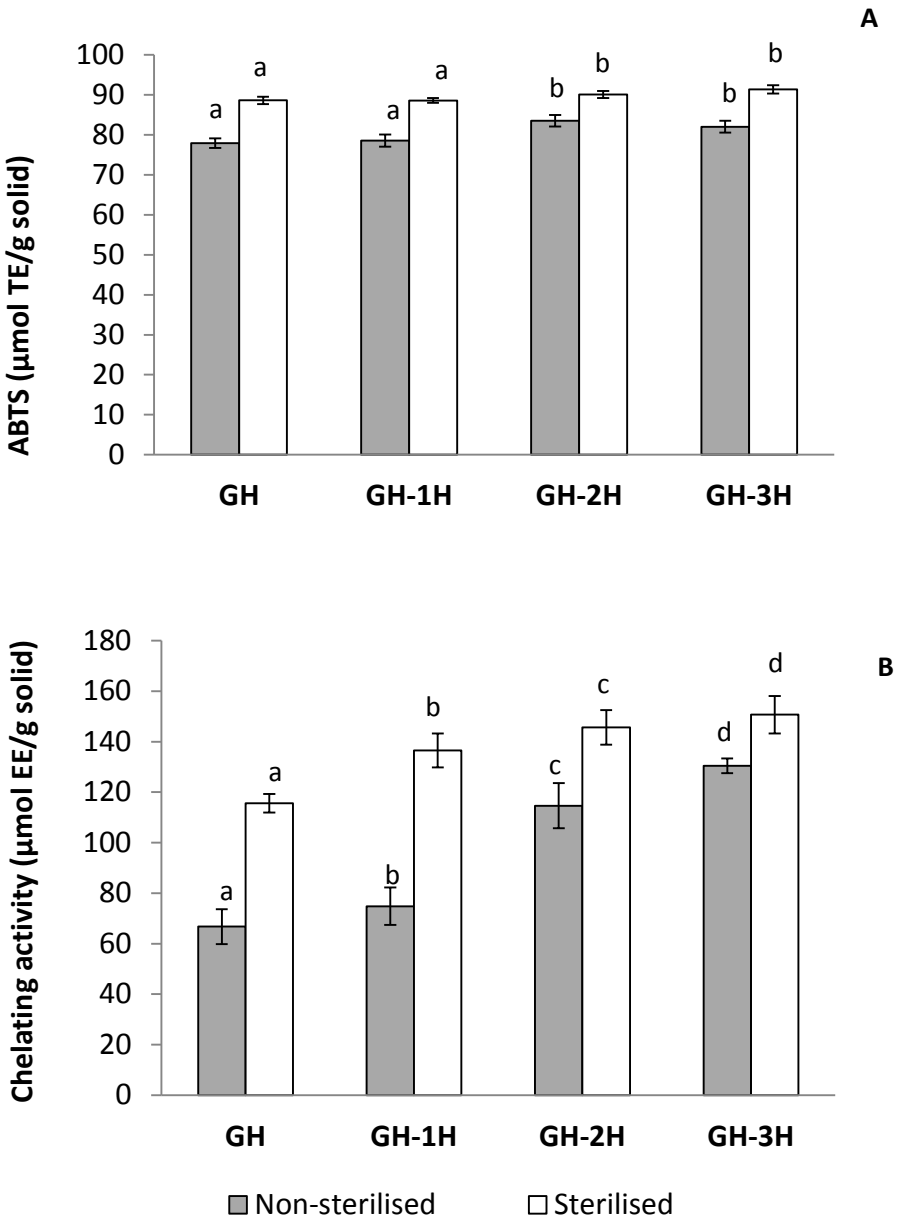

Figure 27. ABTS radical scavenging activity (A) and metal chelating activity (B) of non-sterilised and sterilised gelatin hydrolysates as affected by hydrolysis time in the process of protease from *B. amyloliquefaciens* H11. Bars represent standard deviation (n = 3). Different letters within the same sterilization condition on the bars denote significant differences (P < 0.05).

Table 22. Antioxidative activities of gelatin hydrolysates as affected by drying methods.

Antioxidative activities	GH-3H-FD	GH-3H-SD
DPPH (μmole TE/g solid sample)	12.72 ± 1.00b	10.66 ± 0.05a
ABTS (μmole TE/g solid sample)	102 ± 8b	80 ± 7a
FRAP (μmole TE/g solid sample)	2.37 ± 0.06b	2.23 ± 0.09a
Chelating activity of Fe^{2+} (μmole EE/g solid sample)	2.59 ± 0.11b	0.63 ± 0.13a
H_2O_2 scavenging activity (μmole TE/g solid sample)	383 ± 5b	368 ± 4a

Different small letters within the same row indicate significant differences ($P < 0.05$).

Colour

L^*, a^*, b^*, ΔE^* and whiteness of GH-3H-FD and GH-3H-SD are shown in Table 23. In general, the colour of GH-3H-SD powder was different from that of GH-3H-FD powders ($P < 0.05$). GH-3H-SD powder had higher L^* than GH-3H-FD powder ($P < 0.01$). ΔE^* of powder was therefore impacted by spray-drying ($P < 0.05$). Lower a^* and b^* values and higher hue angle were found in GH-3H-SD in comparison with those of GH-3H-FD. These results indicated that the loss of redness and yellowness of powder was induced by spray drying. Spray-drying process with a high ratio of surface area and volume of feed mixture plausibly caused rapid pigment oxidation (Desobry *et al*., 1997). With low temperature used for freeze-drying, the indigenous pigments could be preserved. Fine powder more likely had the increased light scattering as evidenced by higher whiteness. The result indicated that spray-drying process had a positive effect on improving colour of fish gelatin hydrolysates, in which whiter colour of powder was obtained.

Table 23. L^*, a^*, b^*, ΔE^* and whiteness of fish gelatin hydrolysate powder as affected by drying methods.

Samples	L^*	a^*	b^*	ΔE^*	Whiteness
GH-3H-FD	92.92 ± 0.08a	-0.18 ± 0.18b	12.18 ± 0.14b	14.09 ± 0.11b	85.91 ± 0.11a
GH-3H-SD	96.21 ± 0.02b	-0.42 ± 0.03a	7.36 ± 0.11a	8.29 ± 0.09a	91.71 ± 0.09b

Different small letters within the same column indicate significant differences ($P < 0.05$).

Volatile compounds in fish gelatin hydrolysate

Selected volatile compounds in fish gelatin hydrolysate powder produced by freeze-drying and spray-drying, GH-3H-FD and GH-3H-SD, are presented in Table 24. Several odourous compounds including nonanal, 1-dodecanol, 2,6-di(t-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one and phenol, 2,4-bis (1,1-dimethylethyl) were found in both samples. Dibutyl phthalate or 1,2-Benzenedicarboxylic aciddibutyl ester was the most dominant volatile compound in GH-3H-FD, but the compound was not detected after spray-drying process. Generally, dibutyl phthalate can be found in a variety of products, mainly contaminated from plastic containers (ATSDR, 1989). Dibutyl phthalate in fish ranged from 78 to 200 parts per billion (ppb) (ATSDR, 1990). Thus, decomposition of dibutyl phthalate more likely took place at high temperature used for spray drying. Nonanal was responsible for oxidised oil odour (Fu *et al*., 2009). In GH-3H-SD, nonanal content was increased. Formation of nonanol could be induced during spray-drying. The primary oxidation products, such as hydroperoxide, might undergo decomposition at high extent during spray-drying. 1-Dodecanol is grassy liked odor (Fu *et al*., 2009) and could be found in GH-3H-SD at a lower level, compared with that found in GH-3H-FD. This fatty alcohol is contributed basically to the off-odour in some food stuffs. Amongst ketones, 2-pentadecanone and 3-[4'-(t-butyl) phenyl] furan-2, 5-dione were detected in GH-3H-FD. Ketones could be generated by *B. amyloliquefaciens* during fermentation. Yuan *et al*. (2012) reported that *B.*

amyloliquefaciens NJN-6 could produce ketones (2-undecanone, 2-dodecanone and 2-tridecanone) at high levels. However, those compounds disappeared in GH-3H-SD. Phenol, 2,4-bis(1,1-dimethylethyl) is a precursor to more complex compounds used as antioxidants and light-protection agents (Fiege *et al*., 2002). 2,6-di(t-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one is generally found in oxidised fish skin (Yarnpakdee *et al*., 2012).

Overall, the amounts of volatile compounds generally decreased after spray-drying except nonanal. Fish gelatin hydrolysate exhibited distinctly different profiles of odourous compounds, depending on the drying methods. Thus, the reduction of volatile compounds was achieved by spray-drying.

Table 24. Volatile compounds of fish gelatin hydrolysate powder as affected by drying methods.

Compounds	Abundance ($\times 10^6$)	
	GH-3H-FD	GH-3H-SD
Nonanal	526	1,026
Butyl hydroxy toluene	1,215	ND
1-Dodecanol	1,217	360
2-Pentadecanone	1,255	ND
2,6-di(t-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one	870	235
Isopropyl tetradecanoate	1,320	ND
1,3-Dioxolane, 2-phenyl	618	ND
Phenol, 2,4-bis(1,1-dimethylethyl)	1,263	349
3-[4'-(t-butyl)phenyl]furan-2,5-dione	1,144	ND
Dibutyl phthalate	5,020	ND

ND: not detectable.

Sensory properties of fish gelatin hydrolysate

The sensory properties of GH-3H-FD and GH-3H-SD are shown in Table 25. Several fish protein hydrolysates have been shown to have potential bioactivity (Thiansilakul *et al*., 2007b; Yarnpakdee *et al*., 2012), but fishy odour or off-odour associated with fermentation limit its applications. In general, GH-3H-FD had higher intensity of fishy odour and off-odour associated with fermentation than GH-3H-SD ($P < 0.05$) when water was used as medium. GH-3H-SD dissolved in pomegranate juice showed the lowest score of fishy odour and off-odour associated with fermentation (1.17 and 1.42) (Table 25). These results indicated that the spray drying process was effective in lowering the fishy odour and off-odour associated with fermentation of fish gelatin hydrolysate. Since the pomegranate juice had strong volatile components, ethyl acetate, 2-methyl-1-butanol, furfural, and 3-hexen-1-ol, which contributed to the general aroma with sweet or fruity odour (Vázquez-Araújo *et al*., 2010), this juice could mask those offensive odours of gelatin hydrolysate to some extent. Thus, spray-drying process could remove fishy odour and off-odour associated with fermentation effectively. As a consequence, gelatin hydrolysate could be supplemented to a higher level, particularly in drink with strong and intense odour/flavour.

7.5 Conclusion

Further hydrolysis of gelatin by secreted extracellular protease from *B. amyloliquefaciens* H11 could increase hydrolysis and antioxidative activity. Subsequent sterilisation also enhanced hydrolysis and antioxidative activity. Spray-drying improved the whiteness of hydrolysate and lowered some volatile compounds, thereby improving sensory acceptability. Thus, gelatin hydrolysate produced using protease from *B. amyloliquefaciens* H11 and spray-drying would be an alternative effective process to produce antioxidative gelatin hydrolysate with reduced off-odour.

Table 25. Fishy odour and off-odour associated with fermentation of fish gelatin hydrolysate powder as affected by drying methods.

Samples	Media	Fishy odour	Off-odour associated with fermentation
GH-3H-FD	Distilled water	9.0 ± 2.7aA	8.4 ± 2.1aA
	Pomegranate juice	2.8 ± 2.3cA	3.0 ± 2.1cA
GH-3H-SD	Distilled water	6.7 ± 2.8bB	5.9 ± 2.6bB
	Pomegranate juice	1.3 ± 1.2dB	1.2 ± 1.0dB

Different samll letters within the same column indicate significant differences ($P < 0.05$). Different capital letters within the same column under the same medium indicate significant differences ($P < 0.05$).

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CHAPTER 8

CONCLUSION AND SUGGESTION

8.1 Conclusion

Bacillus amyloliquefaciens strains, D10, G02 and H11, isolated from fish docks in Songkhla, had optimal pH and temperature of 7.5 and 50 °C, respectively. They were serine and metallo-gelatinolytic enzymes, which could hydrolyze fish gelatin in a concentration dependent manner.

The optimization of gelatinolytic enzyme production by *B. amyloliquefaciens* H11 was achieved by statistical models and experimental design. Speed of agitation and fish gelatin concentration had the strong influence in gelationolytic enzyme production. The optimal condition for maximal gelatinolytic enzyme production was cultivation of *B. amyloliquefaciens* H11 in the basal medium containing 8.36 g/l fish gelatin with continuous shaking at 234 rpm for 31 h at 37.5 °C.

Purified enzyme from *B. amyloliquefaciens* H11 showed the maximum gelatinolytic activity at 50 °C and pH 8. The enzyme had calcium or magnesium ion as cofactors and was resistant to surfactants, solvent and oxidizing agent. The enzyme could potentially hydrolyze gelatin, especially from unicorn leatherjacket skin.

Gelatin hydrolysates with antioxidative activity and ACE inhibitory activity could be prepared with the aid of protease from *B. amyloliquefaciens* H11. The activity was in dose-dependent manner.

Spray-drying improved the whiteness of hydrolysate and lowered some offensive volatile compounds, thereby improving sensory acceptability. However, antioxidative activity of hydrolysate was decreased after spray drying.

8.2 Suggestion

1. Reduction of undesirable fermentation odour of crude enzyme produced by *Bacillus amyloliquefaciens* H11 should be investigated.
2. Mechanisms of selected peptides on inhibition of lipid oxidation and hypertension should be further studied.
3. Stability of active peptides toward drying should be improved.

APPENDIX

List of 16S rRNA gene sequencing of bacterial

Specie: *Bacillus amyloliquefaciens*

Strain: H11

Base pair: 1394

Similarity: 99.93% similar to *B. amyloliquefaciens* subsp. plantarum FZB42T

Gene sequence:

CCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAA
GGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAG
CTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGAT
TGGCTTAACCTCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTGTAGCACGTGTGTA
GCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTT
GTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAG
GGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAA
CCATGCACCACCTGTCACTCTGCCCCCGAAGGGGACGTCCTATCTCTAGGATTGT
CAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACAT
GCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCG
TACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAAC
CCCCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCC
TGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCT
TCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCC
ACTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCGGTTGAGC
CGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGAGCCCTTTACGCCCAATA
ATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAG
CCGTGGCTTTCTGGTTAGGTACCGTCAAGGTGCCGCCCTATTTGAACGGCACTTGT
TCTTCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCATCACTCACGCGGCGT
TGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGG
AGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTAC
GCATCGTCGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGTC
CATCTGTAAGTGGTAGCCGAAGCCACCTTTTATGTCTGAACCATGCGGTTCAGAC
AACCATCCGGTATTAGCCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCAGGT
TACCCACGTGTTACTCACCCGTCCGCCGCTAACATCAGGGAGCAAGCTCCCATCT
GTCCGCTCGACTTG

Specie: *Bacillus amyloliquefaciens*

Strain: G02

Base pair: 1393

Similarity: 99.93% similar to *B. amyloliquefaciens* subsp. plantarum FZB42T

Gene sequence:

GTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGT
AACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTA
ATACCGGATGGTTGTCTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTA
CCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACC
AAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACG
AAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGC
TCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTAC
CTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGT
GGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAG
TCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAAC
TTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAG
ATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGA
GGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGT
AAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACG
CATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATT
GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA
GAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTT
CGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGT
TGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTT
GGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTC
AAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACA
AAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCG
GATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATC
AGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCCACCACG
AGAGTTTGTAACACCCGAAGTCGGTGAGGTAA

Specie: *Bacillus amyloliquefaciens*

Strain: D10

Base pair: 1398

Similarity: 99.93% similar to *B. siamensis* KCTC 13613T

99.86% similar to *B. amyloliquefaciens* subsp. plantarum FZB42T

Gene sequence:

CATAAAGGTTACCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGC
GGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACT
AGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAGAACA
GATTTGTGGGATTGGCTTAACCTCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTGT
AGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACC
TTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAA
CTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGA
GCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGGACGTCCTATC
TCTAGGATTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAA
TTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAG
TCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAG
GGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGG
TATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACAGACCAG
AGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACAC
GTGGAATTCCACTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCC
CCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGAGCCCTTT
ACGCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGC
ACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTGCCGCCCTATTTGA
ACGGCACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCATCAC
TCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTG
CCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTC
AGGTCGGCTACGCATCGTCGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATG
CGCCGCGGGTCCATCTGTAAGTGGTAGCCGAAGCCACCTTTTATGTCTGAACCAT
GCGGTTCAAACAACCATCCGGTATTAGCCCCGGTTTCCCGGAGTTATCCCAGTCT
TACAGGCAGGTTACCCACGTGTTACTCACCCGTCCGCCGCTAACATCAGGGAGCA
AGCTCCCATCTGTCCGCT

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