A Thesis for the Degree of Doctor of Philosophy

Isolation of Protease-producing Bacteria from the Gut of *Octopus vulgaris* and Characterization of Their Proteases

By

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Octopus vulgaris 의 장관으로부터 분리한 단백질분해효소 생산 균주와 생성된 효소의 특성

Advisor: Prof. Ji-Young Yang

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Abstract

Proteases are widespread in nature and microbes serve as a preferred source of these enzymes. The proteases from microorganisms were widely studied because of their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation. In this study, protease-producing bacteria were isolated and identified from the gut of *Octopus vulgaris*. The optimum condition for protease production of the isolated strains was investigated. The proteases produced from the isolated strains were purified through ammonium sulfate precipitation, and different cation and anion exchange chromatographies. Meanwhile, the properties of the purified proteases were also studied. To study the potential industrial application values of the isolated strains, the strains were incubated with octopus-processing waste to produce octopus scraps hydrolysates. The antioxidative activities of hydrolysates were measured *in vitro* and *vivo*. As a result, two protease high-yield strains named as V-2 and L-2 were successfully isolated from octopus gut. Strain V-2 has 99.2% homology with *Bacillus flexus* 3xWMARB-5, while L-2 strain was identified as *Pseudoalteromonas okeanokoites*. The results show that strain V-2 with fructose as carbon source, peptone as nitrogen source, medium initial pH 8.0, under temperature 30℃, for 3.5 days incubation, could produce the highest activity level of protease. For strain L-2, soluble starch was used as carbon source, peptone was used as nitrogen source, initial medium pH 8.0, culture temperature was 19℃, and 3.5 days were defined to be the optimum

condition. Two purified proteases, named as Fa-2 and F1-1, obtained from strain V-2 and L-2. The purification folds were 2.5 and 1.7, activity recoveries were 12.5% and 7.5%, respectively. The molecular weight of Fa-2 was 61.6 kDa, with optimal temperature of 40℃, optimum pH 9.0 and a good thermal stability. For F1-1, the molecular weight was 61.4 kDa with an optimum temperature at 40℃, and a great thermal stability. Two hydrolysates, labeled as P1 and P2, were obtained from the hydrolysis of the two strains and octopus by-product. The scavenging activities of DPPH• radicals, hydroxyl radicals, and superoxide radicals were studied and P1 showed higher radical scavenging activities than P2. Therefore P1 hydrolysate was applied to the test of antioxidative activity *in vivo*. The D-gal-induced aging model of mice was established and the antioxidant effects of hydrolysate P1 both in serum and liver were determined to have potential ability to attenuate the aging symptoms through its antioxidant properties. These results indicated that, the two strains were firstly isolated from the gut of octopus successfully in China with high antioxidant activities *in vitro* and *vivo*, which have good potential on commercial application. The results also provide a basis for further study.

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Chapter 1

General introduction

1.1. Background

Traditionally, digestion is described as the process by which food in the gastrointestinal tract is split into absorbable simple compounds performed primarily by the digestive enzymes. An understanding of the contribution of endosymbionts to digestion requires information on the relative importance of exogenous (produced by the gastrointestinal endosymbionts) and endogenous (produced by the host) digestive enzymes (Chlments et al., 1997). For the variety of microorganisms fish intestine is appropriate circumstance to survive. The proliferation and secretion of the intestinal flora *in vivo* play an important role in the maintenance of the host normal structure and function of organs.

Fish take food and water through their gills and orals, so the bacteria which may have contaminated with water and food were bound together into the body. These bacteria, from the outside environment, were preserved after a long adaptation period. However, their own physiological and biochemical activities and even the metabolites were changed dramatically with the variety of environment. Eventually the ecological balance was achieved between the fish and the intestinal flora (Calull, 1990; Gatesoupe et al., 1998; Ozaki et al., 1985). Fish intestinal flora can provide nutrients for the fish body to improve immune function and to support digestive system (Kashiwada et al., 1970; Kamei et al., 1985; Rimmer et al., 1987; Das et al., 1991; Sugita et al., 1996). Many gastrointestinal tract microbiotas of aquatic product have been reported to produce a wide range of enzymes such as: amylase, cellulose, lipase, proteases, chitinase and phytase (Ghosh et al., 2002; Kar & Chosh, 2008; Saha et al., 2006; Li et al., 2009; Jiang et al., 2011). Enzyme production capability was found to be different with species of fish and strain, which demonstrated that the enzyme-producing bacteria are widespread in marine fish intestine.

1.2. Protease

Proteases are hydrolytic enzymes that catalyze the total hydrolysis of proteins to amino acids. Although proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications (Chu, 2007). Currently, more than 100 protease species have been crystallized and purified from animals, plants, and microorganisms. Almost all proteases produced by microorganisms are extracellular enzymes. According to different categories, the proteases can be classified as shown in Table 1.

Compared with traditional plant and animal resources, resource of microbial proteases has incomparable advantages (Jiang et al., 2001). With the modern genetic engineering and fermentation technology, industrial microorganisms become a major source of protease preparation.

Significant progress has been made on protease production and application in China. So far, 21 species have been identified and applied to industry (Xu et al., 2001). However, many problems still exist in lack of species and microbial resources. Hence, the research should be focused on finding and developing new sources of microbial protease. In recent years, marine organisms gradually become object of exploitation. Marine biological diversity and uniqueness have been created by the unique marine environment. Enzymes, hormones, and other metabolites with extremely high activities could be developed by low temperature, high pressure, and high salt content of marine. All kinds of marine protease, which are thermophilic (Kakai et al., 1998), psychrophilic (Gerike et al., 1997), piezophilic, halophilic (Lu et al., 2001), or addicted to different pH

Table 1. Enzyme categories and properties of different classification methods.

Classification method	Category	Property
Classified by optimum temperature	Psychrophilic enzyme	Optimum temperature 5 - 10, inactivation temperature: 30
	Mesophilic enzyme	Optimum temperature 0 - 40, inactivation temperature: 50
	Thermophilic enzyme	Optimum temperature 60 - 80
Classified by action mechanism	Serine protease	Optimun pH 7.0 - 11.0, isoelectric point range: 4.0 - 6.0
	Aspartic protease	Optimun pH 3.0 - 4.0, isoelectric point range: 3.0 - 4.5
	Thiol protease	Optimun pH 7.0, thiol protease inhibitor sensitivity
	Metalloprotease	Divalent metal ion in the active site
Classified by optimum pH	Acid protease	Optimun pH 2.0 - 5.0
	Neutral protease	Optimun pH 7.0, stable in the range of 6.0 - 9.0
	Alkaline protease	Optimun pH 9.5 - 10.5, stable in the range of 5.0 - 10.0
Classified by peptide hydrolysis	Endopaptidase	Interal cutting to produce large hydrolysis fragments
	Exopeptidase	Cutting from one end to release one or several amino acid residues

(Bertus et al., 2003), are expected to become a new resource for modern study (Li et al., 2004).

Among those protease-producing bacteria, bacteria belonging to *Bacillus sp.* are by far the most important source of several commercial microbial enzymes (Ferrero et al., 1996; Kumar et al., 1999; Sookkheo et al., 2000; Singh et al., 2001; Gupta et al. 2002; da Silva et al., 2007). Some information is available regarding production of proteases by fish gut bacteria (Table 2).

1.3. Octopus vulgaris

The *octopus valgaris* (common octopus) is one of the most studied octopus species (Fig. 1.). It has prosperous activities above the low-water line during spring, while migrates in the intertidal zone at summer and fall. In winter, it usually dives in the subtidal area with a short distance of wintering migratory habits. The octopus often use its brachiopods crawling on the seabed, and it is also able to have a short distance travel in the bottom of the sea using the reaction force of water spurted by its mouth (Dong, 1988). The octopus valgaris is a common marine resource which is popular and in demand in the market and is an important species for commercial fisheries. The octopus valgaris are widely distributed in the northern and southern sea area of China. The ones, especially in northern part, are large, fleshy, delicious, and rich in protein and amino acids, and their edible portions account for 90% or more. They usually eat on crabs, shellfish, shrimp, and other shellfish. Various enzymes of the octopus can be secreted from intestinal microflora to help digestion system (Ozaki et al., 1985).

The major counties of consuming octopus are Japan, South Korea, followed by Argentina, China, and Morocco. In recent years, China's consumption of octopus has increased significantly. With the rapid economic development and the cheap, high-quality labor market, China has become the world's octopus factory. During the processing, an increasing number of scraps have been

Table 2. Protease-producing bacteria isolated from the digestive tract of fish.

Microorganism	Isolated from	References
Enterobacter spp.; Vibrio spp.; Pseudomonas spp.; Acinetobacter spp.; seromonas spp.	Gray mullet	Hamid etal. (1979)
Strict anaerobes and *Aeromonas hydrophila*	Grass carp	Trust etal. (1979)
N.i*	Grass carp	Das & Tripathi (1991)
Vibrio spp.	Sea bass larvae	Gatesoupe etal. (1997)
Pseudomonas sp.	Arabesque greenling	Hoshino etal. (1997)
Flavobacterium balustinum	Salmon	Morita etal. (1998)
Bacillus cereus	Gray mullet	Esakkiraj etal. (2009)
N.i	Nine different fresh water teleosts	Bairagi etal. (2002a)
Bacillus cereus; B.circulans; B.pumilus	Rohu	Ghosh etal. (2002)
N.i	Roach	Skrodenytė - Arbačiauskienė (2000)
Pseudoalteromonas sp.	Hake	Belchior & Vacca (2006)
Aeromonas spp.; Enterobacteriaceae; Pseudomonas spp.; Flavobacterium spp.; Micrococcus sp.	Roach	Skrodenytė - Arbačiauskienė (2007)
N.i	Rohu and murrel	Kar & Ghosh(2008)
N.i	Seven freshwater teleosts	Mondal etal. (2008)
Bacillus licheniformis; B. subtilis	Bata	Mondal etal. (2010)
Brochothrix sp. and *Brochothrix thermosphacta* isolated from the GI tract of *Atlantic cod* fed fish meal, soybean meal and bioprocessed soybean meal.	Atlantic cod	Askarian etal. (2012b)

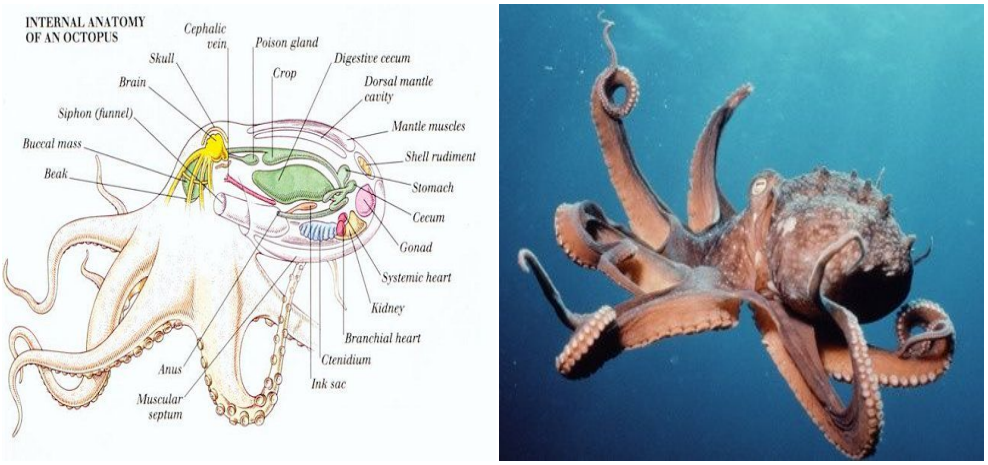


Fig. 1. photograph and schematic representation of *Octopus vulgaris*.

produced, which are mainly octopus offal and about 50% of the total weight of the individual (Zhang et al., 2007). These organs often take a lot of plant places for storage, and finally have to be discarded as waste in the sea. This not only causes pollution of the marine environment, but also increases the financial burden of enterprises. The industry is faced on the need to develop efficient by-product recovery and utilization methods to comply with the pollution control regulations (Choudhury & Bublitz, 1996). These scraps could be made into high value-added products through some suitable methods, so that the problem of environmental pollution can be solved. In the meantime, greater economic benefits shall be created for the company.

1.4. The source of the microbial protease

Proteases are mainly from animals, plants, and microorganisms. The principal sources for animal proteases are the microorganisms in animal offal. Bacteria, mold, yeast, and actinomycete are the major microorganisms that can secrete proteases.

1.4.1. Bacteria

The proteases, including neutral protease and alkaline protease, are mainly produced from *Bacillus* species. The neutral protease can be produced by *Bacillus polymyxa*, *Bacillus megatherium*, and *Bacillus cereus*, etc. (Wang, 2006). The alkaline protease can be secreted by *Bacillus pumilus*, *Bacillus licheniformis*, and *Bacillus alcalophilus*, etc. (Zhao & Gao, 1998). *Bacillus amyloliquefaciens*, *Bacillus subtilis*, and *Bacillus cereus* are the bacteria that can produce neutral and alkaline proteases simultaneously (Qiu et al.,1988; Zheng & Tu, 1993)

1.4.2. Mold

Three kinds of proteases which are neutral, acidic and alkaline proteases can be produced by mold. According to the pH activity curve, the protease can be divided into four types: *Aspergillus oryzae* class, *Aspergillus* class, *Mucor hiemalis* class, and symmetry class (Nasuno, 1971). However, nowadays other molds have been found, which are not in the scope of the above four categories, for instance, mold FU269. Although there is a quite small market for protease produced by mold, a broader range of applications such as, Cheese processing, bread dough improvement, meat tenderizing, and leather industry, etc. has been developed.

1.4.3. Yeast

Yeast, except *candida lipolytica,* mainly produces acidic protease. *Endomycopsis*, *pichia pastoris, Cryptococcus neoformans, candida tropicalis, rhodotorula glutinis,* and *rhodotorula gracitis* are the primary species that produce proteases.

1.4.4. Actinomycete

Both neutral and alkaline proteases can be produced by actinomycete, and the composition of the enzyme is very complex. The same species not only produce one type of protease. For example, *Streptomyces griseus* is able to produce 5 kinds of alkaline proteases. Additionally, proteases with different properties can be secreted at the same time by one actinomycete species, such as, ATCC3464. Lee (2005) has isolated *sp. E79* from actinomycete which could produce alkaline protease with good thermostability and the optimal pH 11 (Lee et al., 1996).

1.5. Isolation and purification of protease

1.5.1. Salting-out

Most enzymes are proteins and water soluble. Salting-out is known as adding

certain amount of neutral salt (such as ammonium sulfate, sodium sulfate, etc.) to precipitate the protein from the protein solution. The difference of precipitation time of proteins depends on the solubility of each protein under certain salt concentration. Therefore, the concentration of salt has to be adjusted to precipitate the target protein, and isolate the protein from other contaminating proteins. In this method, the concentration of salt solution is used to be shown with the degree of saturability. In other words, saturated solution is called 100% saturation. The specific concentration for protein precipitation is subjected to practical experiments.

1.5.2. Ion exchange chromatography

Ion exchange chromatography (IEC) is a process that allows the separation of ions and polar molecules based on their charges. Ion exchangers for protein isolation mainly are carboxymethyl (CM) cellulose and diethylaminoethyl (DEAE) cellulose. The former is a cation exchange resin, while the latter is an anion exchange one.

The binding between proteins and the ion exchangers is relied on electrostatic attraction existed inside the opposite charges. The attraction is associated with the isoelectric point of the sample and the pH of the solution. The separation should be completed by adjusting the pH and ionic strength of the solution. Protein with weak binding force will be eluted firstly.

Protease CGW 23 has been isolated and purified from the fermentation supernatant of *Streptomyces C 23662* using ammonium sulfate precipitation, CM-Sepharose FastFlow and Phenyl-Sepharose FastFlow by Wu (Wu & Wang, 2001). The protease not only has a direct effect of degrading fibrin, but also has an active plasminogen. A fibrinolytic protease has been purified from *Bacillus subtiffsjin 221* by using ion exchange chromatography (Hao et al., 2002).

1.6. Application of protease

It has been reported on the type of microbial protease and commercial applications as early as 1988. Protease is an important industrial enzyme. More than a hundred species of proteases have been produced from microbial organisms, which is productive, widely applied, and with high economic efficiency (Kalisz, 1988).

1.6.1. Application in food and feed

Traditionally, microbial proteases have been applied in many areas in food industries. Alkaline protease is used in the production of hydrolyzed protein, which has high nutritional values on the blood regulation and infant milk formula. After hydrolysis treatment, food products, fortified juices, and soft drinks have been developed with the alkaline proteases (Ward et al., 1985; Neklyudov et al., 2000). For instance, fish protein hydrolyzate, soy protein hydrolyzate, and zein have been produced with high nutritive value and therapeutic effects by the application of alkaline microbial protease in foods (Fujimali et al., 1970; Rebeca et al., 1991; Tanimoto et al., 1991).

Microbial proteases also have a very wide range of application on dairy processing industry, baking industry, soybean processing, and bitterness remover industry. Fish feed can also be produced with used feathers and keratin-containing material by alkaline proteases. For examples, the feed of a high protein content has been obtained from the hydrolysis of feather keratin using proteases isolated from *B. subtilis B72* and *B. licheniformis PWD-1* (Dalev, 1990; Dalev, 1994; Cheng et al., 1995).

1.6.2. Application in detergent industry

Microbial protease has been long used in the detergent industry, because of its efficient removal of protein stains and simple process. Commonly used proteases

include serine protease produced by *B. subilis,* and proteases secreted by *B. lichniformis 2709, B. pumilus 209, B45,* etc. For instance, alkaline proteases of *KP.16* and *KP.43* produced from alkaline *Bacillus KSM.K16* and *Bacillus KSM. KP.43* was demonstrated to have resistant against chemical oxidizer properties which could be used on the preparation of efficient concentrated detergent (Saeki et al., 2007).

1.6.3. Application in leather processing

Chinese leather is rich in resources, and the leather industry is being developed very rapidly. In traditional leather processing, hydrogen sulfide and other chemicals were used, which could cause environmental pollution and have safety hazards. The use of biological enzyme in leather processing has been widely adopted because it is easy to control and has less waste. The alkaline protease produced from *B. subtilis K2* was reported to be applied to the soften stage of leather process (Hameed et al., 1996).

1.6.4. Application in peptides synthesis

Since Bergman reported that the polypeptide synthetic reaction can be catalyzed by two-way enzymolysis (Bergman et al., 1937), protease has been diffusely used in polypeptide hydrolysis and synthesis. There are many incomparable advantages of enzymatic synthesis of peptides such as mild conditions, easy-to-control process, high solubility of non-polar substances, and convertible thermodynamic equilibrium. Surfactant protease has been reported to be applied in the synthesis of novel biocatalysis used in the preparation of hydrophilic organic solvent (Okazake et al., 2000).

1.7. Objectives

During processing, large amounts of offal were produced, which are rich in protein resource. The economic cost is too high if the hydrolysis were taken place

with commercially available enzymes. So far, many studies have been reported on the protease-producing stains obtained from fish intestinal bacteria, but that isolated from octopus is rarely published. In this study, intestinal protease-producing strains were isolated from octopus itself. Morphological and molecular biological identifications were carried out. Finally, the strains were applied to the hydrolysis of octopus scraps to obtain peptides with high antioxidant abilities.

Chapter 2

Isolation and identification of protease-producing bacteria from *Octopus vulgaris* tract and its optimal condition of protease production

2.1. Introduction

Protease is mainly produced from animals, plants, and microorganisms, microbial production of protease accounts for about 60% of total output throughout the world (Zheng & Yuan, 2003). It is widely used in detergent, food processing, pharmaceutical industry, silk industry, and waste utilization etc (Maurer, 1988; Gupta, 2002). Many microorganisms including bacteria, fungi, yeast, and actinomycete could secrete protease.

To our knowledge, the first studies on protease-producing bacteria isolated from the digestive tract of gray mullet and grass carp were carried out by Hamild et al. (1979) and Trust et al. (1979), respectively. In a study isolating bacteria from intestinal contents of Arabesque greenling (*Pleurogrammus azonus*), one of the isolates showed strong proteolytic activity (Hoshino et al., 1997). The isolate was identified to genus *Pseudomonas* and it displayed the highest protease production at 10℃, but the activity decreased with increasing cultivation temperature. Morita et al. (1998) detected protease activity in the culture medium of *Flavobacterium balustinum* isolated from salmon (*Oncorhynchus keta*) intestine. The molecular mass of the protease was 70 kDa, and its isoelectric point was close to pH3.5, and maximal activity towards azocasein was at 40℃ and from pH 7 to 9. Esakkiraj et al. (2009) reported extracellular protease production by *B. cereus* isolated from the intestine of brackish water fish (*Mugil cephalus*) in shake-flask experiment using different preparations of tuna-processing by-product such as raw fish meat, defatted fish meat, alkali hydrolyzate and acid hydrolyzate as nitrogen source. The authors further tested the effect of temperature, pH, different carbon sources and surfactants on protease production by the bacterial strain. Among the tuna preparations tested, defatted fish meat supported the maximum protease production. Its 3% concentration of the same was reported to be optimum for maximizing the protease production. Among the carbon sources,

galactose aided higher protease production than the other tested carbon sources, and a concentration of 1.5% galactose was optimum to enhance the protease production. A protease-producing strain has been isolated from Tuman river of Kashi city in China, which was identified to belong to *Pseudomonas* species through all the physiological and biochemical methods and 16S rRNA sequence identification (Yi & Zhang, 2009).

However, it is rarely reported about any protease-producing intestinal bacteria obtained from octopus. In this study, protease-producing stains were isolated and screened from octopus gut, and identification was also performed. Meanwhile, the condition of enzyme production and the strains properties have been studied, which provide theoretical foundation for the proteases' future industrial application.

2.2. Materials and methods

2.2.1. Materials

Octopus vulgaris purchased from seafood market in Qingdao city was bred for 3 days in laboratory before experiment. The culture temperature was under 10℃, and water has to be changed twice a day.

2.2.2. Reagents and apparatus

Azocasein, casein, acrylamide, coomassie brilliant blue, sodium dodecyl sulfate (SDS), ammonium persulfate (AMPS), tetramethylethylenediamine (TEMED), methylene diacrylamide, trihydroxymethyl aminomethane (Tris), and protein standards used in this chapter were purchased from Sigma, USA. DU-800 Ultraviolet spectrophotometer was from Beckman, USA. TGL-16M Benchtop high speed refrigerated centrifuge was from Xiangyi Lab instr. Dvpt. Co., Ltd., China. DHP-9032 Electro-heating standing-temperature cultivator was from Senko apparatus Co., Ltd, China. Criterion Cell was from BIO-RAD, USA. DYY-

6D Electrophoresis meter was from WoDeLife Sci. Instr. Co., Ltd, China. IS-RDV3 Constant temperature vibrator was from CTI, USA.

2.2.3. Culture medium

In the Isolation of protease-producing strain, two culture mediums, VNSS medium and 2216E medium were prepared. VNSS medium composed by Peptone (1.0 g), yeast extract (0.5 g), glucose (0.5 g), starch (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), Na_2HPO_4 (0.01 g), and agar (15 g) in 1,000 mL NSS (NSS, which consists of, per litre, 17.6 g NaCl, 1.47 g Na,SO_4, 0.08 g $NaHCO_3$, 0.25 g KCl, 0.04 g KBr, 1.87 g $MgCl_2 \cdot 6H_2O$, 0.41 g $CaCl_2 \cdot 2\ H_2O$, 0.008 g $SrCl \cdot 6H_2O$, 0.008 g H_3BO_3, pH 7). 2216E medium was composed by Peptone (0.5 g), yeast extract (0.1 g), Ferric phosphate (0.01 g), and sea water (100 mL). The pH was adjusted to 7.8-8.0 (before sterilization), and both mediums were sterilized under 121℃ for 15 min.

2.3. Methods

2.3.1. Screen of protease producing bacteria from octopus vulgaris gut

2.3.1.1 Sample preparation

The *octopus vulgaris* were dissected. The intestinal tracts were separated from the octopus body and rinsed five times with sterile sea water to remove intestinal debris. The tracts were shredded and grinded with sterile water to obtain bacterial suspension.

2.3.1.2 Preliminary and secondary screening

The bacterial suspension prepared aforehand was diluted 10^{-1} and 10^{-2} times, and it was applied on VNSS medium and 2216E medium. The mediums were sealed and put into the thermostat incubator at 25℃ for 1 day.

The well-growing strains were screened from the mediums, and were picked into casein medium with an inoculation loop. The casein mediums were sealed

and put into incubator at 25℃ for 24 hrs. According to the observation of proteolytic transparent circle around the strains, the protease producing bacteria could be selected.

The well growing colony which has the largest proteolytic transparent circle on the casein medium was applied onto a slant medium with inoculation loop. The slant medium was incubated at 25℃ for 24 hrs. The eugonic colony was selected and inoculated into the liquid mediums (100 mL medium was contained in 250 mL flask), and then was shaked at a speed of 180 rpm under 25℃ with the constant temperature oscillation incubator for 3 days. The protease activity of the bacterial suspension would be measured later.

2.3.2. Measurement of protease activity

Method used for protease activity measurement was Azocasein method (Bezerra et al., 2005). The procedure was described as follows:

One percent of azocasein was dissolved in 0.02 M (pH 7.0) phosphate buffered saline (PBS) as the substrate. Crude enzyme 50 μL was mixed with azocasein buffer thoroughly. The mixture was incubated at water bath oscillator at a speed of 140 rpm, under 37℃ for 1 hr. The reaction was terminated by adding 300 μL of 10% (w/v) trichloroacetic acid to the mixture. The mixture was allowed to stand at room temperature for 15 min, and then was centrifuged at 10,000 rpm for 5 min, 100 μL supernatant was took and mixed with 100 μL of 1 M NaOH. After vortexing, the absorbance was analyzed under 450 nm wavelength to measure enzyme activity.

One unit of enzyme activity is defined as the amount of enzyme which is used to cause 0.001 unit of absorbance change during the hydrolysis of the azocasein per minute. The calculation is described as the below formula:

$$\text{Enzyme activity (U/mL)} = \frac{A}{0.001}\ \frac{400}{100}\cdot\frac{1000}{50}\cdot\frac{1}{60}$$

Where *A* is the sbsorptance detected at 450 nm (U), 400 is the total volume of the reaction (μL), 100 is the amount of the supernatant (μL), 50 is the amount of the added sample (μL), 60 is reaction time (min), 1 U equals to 1 μg Azocasein / min.

2.3.3. Identification of strains

2.3.3.1. Physiological and biochemical identification

The classification and identification were performed based on the morphological, physiological and biochemical characteristics of the bacteria that isolated from octopus gut, referring to "Bergey's Manual Bacterial Identification" and "System Identification"

2.3.3.2. 16S rRNA sequence analysis

2.3.3.2.1. Genomic DNA extraction

The procedure of DNA extraction is different between Gram-negative and Gram-positive bacteria. For Gram-negative bacteria, digestion buffer was fist applied, and later proteinase K was added for bacterial lysis. In the case of Gram-positive bacteria, the cytoderm was removed with lysozyme, and its lysis was taken place using proteinase K to release genomic DNA.

2.3.3.2.2. PCR reaction

A 50 μL PCR reaction by adding template (1 μL), Primer 27F (1 μL), Primer 1492R (1 μL), dNTP (4 μL), Taq polymerase (1 μL), Buffer (5 μL), and H_2O (37 μL) into PCR microcentrifuge tube and mixing gently. The PCR cycle was started with incubation at 98℃ for 5 min. The program for PCR amplification was 35 cycles of [95℃ 35 s, 55℃ 35 s, 72℃ 1 min 30 s]. Sequences of synthesized primers were 27F (5'-AGAGTTTGATCCTGGCTCAG-3' 20bp) and 1492R (5' GGTTACCTTGTTACGACTT 3' 19bp).

Target fragments were collected using DNA gel extraction kit. The purity and concentration of purified DNA were determined again by gel electrophoresis. pEASY-T1 was used as a carrier for T-A cloning, and the ratio between fragment and carrier was 8 : 1. The junctional complex was transferred into *E.coil 110* at 42℃. The strains were finally applied on LB plates containing ampicillin. Positive clones were detected by blue and white screening and colony PCR. The extraction of plasmid DNA was performed with TransGen® plasmid extraction kit. The extracted plasmid DNA was sequenced by Shanghai Sangon Biotech Co., Ltd.

2.3.4. Optimum fermentation conditions

2.3.4.1 Growth curve

Target strains were inoculated into flask shaking medium, and were incubated under 30℃ at a speed of 180r/min. The absorbance of 1 mL bacteria suspension was analyzed at a wavelength of 600 nm every 12 hrs using turbidimetric method. The growth curves of strains were obtained referring to the absorbance.

2.3.4.2. Effect of different carbon sources and nitrogen sources

Different materials which are 0.5% soluble starch, 0.5% glucose, 0.5% sucrose, 0.5% lactose, and 0.5% fructose were added into the fermentation medium to test the optimal carbon source for this fermentation, respectively. Under the condition that other parameters remaining the same, different nitrogen sources, including: 0.5% peptone, 0.5% beef extract, 0.5% ammonium sulfate, 0.5% casein, and 0.5% sodium nitrate, were applied to the fermentation process respectively to detect the best condition.

2.3.4.4. Effect of initial pH, incubation time, and temperature

Based on the optimum parameters detected already, the initial pH was adjusted to 5.0, 6.0, 7.0, 7.5, 8.0, 8.5, 9.0, 10.0, and 11.0 respectively to select the best condition for fermentation. To test the incubation time, the strains were incubated

at 25℃ with the speed of 180 rpm, sampling every 12 hrs under sterile condition. Each strain suspension was centrifuged at 6,000 rpm at 4℃ for 15 min to get the supernatants which would be analyzed to obtain the optimal incubation time. For the incubation temperature, the strains were cultured under different temperatures ranged from 20 -50℃, and the suspension was centrifuged under 4℃ at a speed of 6,000 rpm for 15 min. The supernatants were collected and measured to obtain the effect of incubation temperature.

2.3.4.7. Effect of metal ions and surfactants on protease production

Ca^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, Ba^{2+}, Zn^{2+}, Cu^{2+} ions, surfactants Tween 80, and polyethylene glycol were added into the fermentation medium, respectively to find out the effect of metal ions and surfactants. The protease activity of each supernatant of the fermentation medium was determined after the centrifugation under 4℃ at 6,000 rpm for 15 min.

2.4. Results and discussions

2.4.1. Screening results of protease-producing bacteria from *Octopus vulgaris* gut

It has been rarely reported about the protease-producing bacteria isolated from the visceral of octopus, but there were number of protease secreted by various strains from visceral of other aquatic organisms, includes alcalase, neutrase, protamex, papain, bromelain, actinidin and a plant protease mix, have been studied (Aspmo, Horn, and Eijsink, 2005; Liceaga-Gesualdo and Li-Chan, 1999; Guerard, Guimas, and Binet, 2002; Bhaskar and Mahendrakar, 2008; Ovissipour et al., 2009). Aquatic organisms viscera generated during processing is a potential source of protein that can be used as a raw material for the production of protein hydrolysates, which may have some exceptional properties for various industrial applications (Hathwar, Bijinu, Rai, and Narayan, 2011; Ovissipour et al., 2009).

In the present study, the ability of enzyme production was demonstrated according to the diameter of transparent circle in casein proteolytic medium produced by protease-producing bacteria. The observation of transparent circle could be used to determine protease producing ability of various strains qualitatively. In this experiment, total 6 strains were detected to produce transparent cycles on casein medium. Two strains were from VNSS medium which were named as V1 and V2, and the rest of them were from 2216E medium which were called L-1, L-2, L-3, and L-4. As the results shown in Table 3, the D/d values of V-2 and L-2 were higher than those of the others.

The six strains were inoculated into flask shaking mediums and incubated, respectively. The protease activity from each strain was measured and the results were shown in Fig. 2. The activity produced by stain L-2 was observed to be the highest, followed by V-2, L-3, and L-4. V-1 and L-1 were shown to have lower activity values. The method of using casein as a substrate was widely used on the identification of protease-producing strains. In a study isolating bacteria from intestinal contents of Arabesque greenling (*Pleurogrammus azonus*), one of the isolates showed strong proteolytic activity (Hoshino et al. 1997). Morita et al. (1998) detected protease activity in the culture medium of *Flavobacterium balustinum* isolated from salmon (*Oncorhynchus keta*) intestine with the method used in the present work. Considering both casein proteolytic measurement and protease activity analysis, V-2 and L-2 were selected for further experiments. positive bacterium. The physiological and biochemical identification took place referring to “Bergey’s Manual Bacterial Identification” and “System Identification”. Table 4 presents the results of two strains.

Table 3. Screening results of protease-producing strains from tract of *Octopus vulgaris*

Strain	Diameter of transparent circle(D, mm)	Diameter of colony (d, mm)	*D/d*
V-1	12.9±0.4	6.7±0.3	1.9±0.2
V-2	22.7±0.6	5.4±0.5	4.2±0.1
L-1	8.2±0.2	4.6±0.4	1.8±0.3
L-2	15.8±0.2	4.2±0.2	3.8±0.1
L-3	1.0±0.5	6.3±0.4	0.2±0.1
L-4	7.9±0.9	5.9±0.2	1.3±0.1

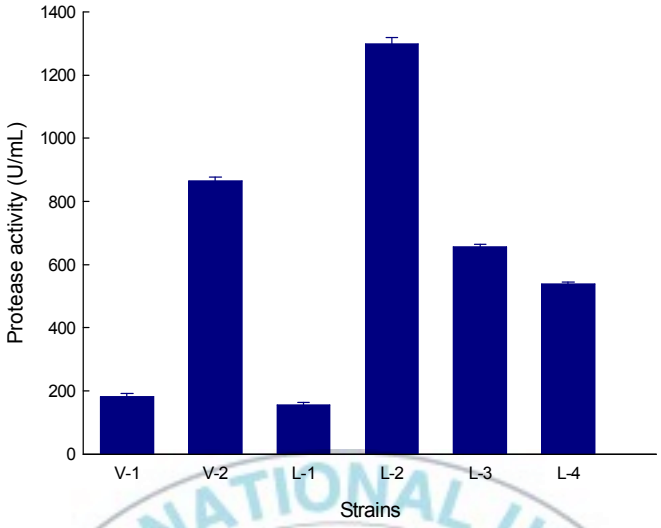


Fig. 2. Protease activities of strains from tract of *Octopus vulgaris*.

Table 4. Results of physiological and biochemical experiments

Identification item	Result		Identification item	Result	
	V-2	L-2		V-2	L-2
Amylohydrolysis	+	-	Glucose test	+	-
Hydrogen sulfide production test	-	+	Fructose test	+	-
Catalase test	+	+	Mannitol test	+	-
Methyl red test	-	-	Gelatin liquefaction	+	-
Voges-proskauer test	-	-	5% NaCl	+	+
Indole test	-	-	7% NaCl	-	+
Nitrate retuction test	-	-	10% NaCl	-	-
Casein hydrolysate	+	-			

2.4.2. 16S rRNA sequence analysis

2.4.2.1. Results of PCR amplification

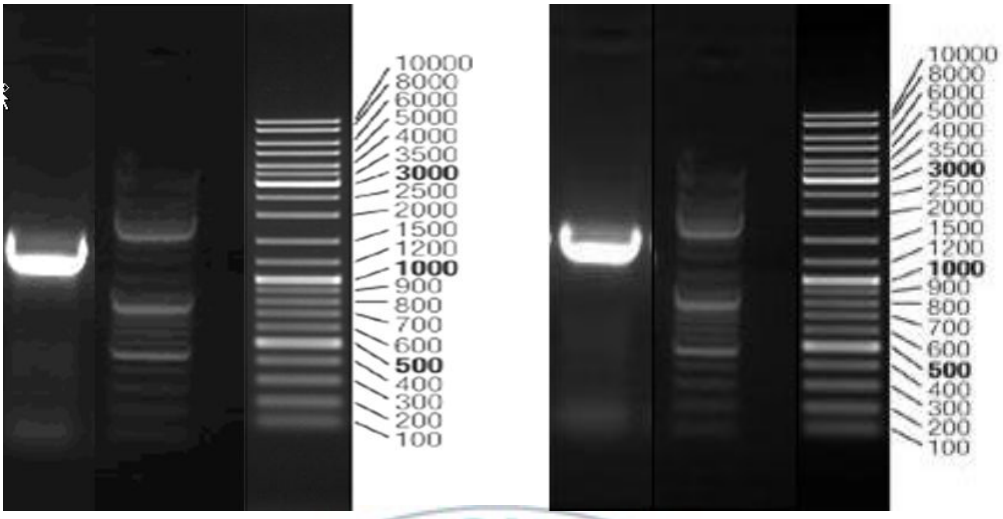
The extracted genomic DNA of the two strains was tested with 1% and 1.5% agarose gel electrophoresis. After PCR amplification, the products were around1500 bp with clear bands which could be used for the determination of strain 16S rRNA sequence (Fig. 3).

2.4.2.2. Phylogenies analysis of V-2 and L-2 strains

Log in NCBI website (www.NCBI.com), landing No. is JQ836666, high similarity strains with V-2 and L-2 strains were obtained through Blast in

GeneBank. Strain sequences were compared with those in GeneBank, and then phylogenetic trees of V-2 and L-2 strains were drawn, shown in Fig. 4 and 5.

The results of phylogenetic trees showed that the V-2 strain belongs to *bacteria, firmicutes, proteobacteria, bacillus mesh, bacillus brance,* and *bacillus.* There was 99.2% homology with *Bacillus flexus* 3xWMARB-5. L-2 strain was identified as *Pseudoalteromonas okeanokoites,* which belongs to *bacteria, firmicutes, bacilli, bacillales, planococcaceae,* and *planomicrobium*. The identified 16S rRNA sequence of strain L-2 showed more than 99% homology with that of both *Planococcus* and *Planomicrobium*. However, phylogenetic analysis revealed that strain L-2 and other *Planomicrobium* strains were located in the same clade, which was separate from the *Planococcus* clade, indicating that strain L-2 belonged to the *Planomicrobium* genus. *Planococcusstrains* have been re-identified as *Planomicrobium* (Jung et al., 2009; Dai et al. 2005; Yoon et al. 2001), indicating that these genera have a high degree of similarity. Several studies have characterized and identified enzyme-producing bacteria from homogenates of the intestinal digesta by culture-based techniques and selective media, followed by conventional morphological and biochemical assays. Saha



(V-2) (L-2)

Fig. 3. PCR products of strain V-2 and L-2 after 16S rRNA gene analysis.

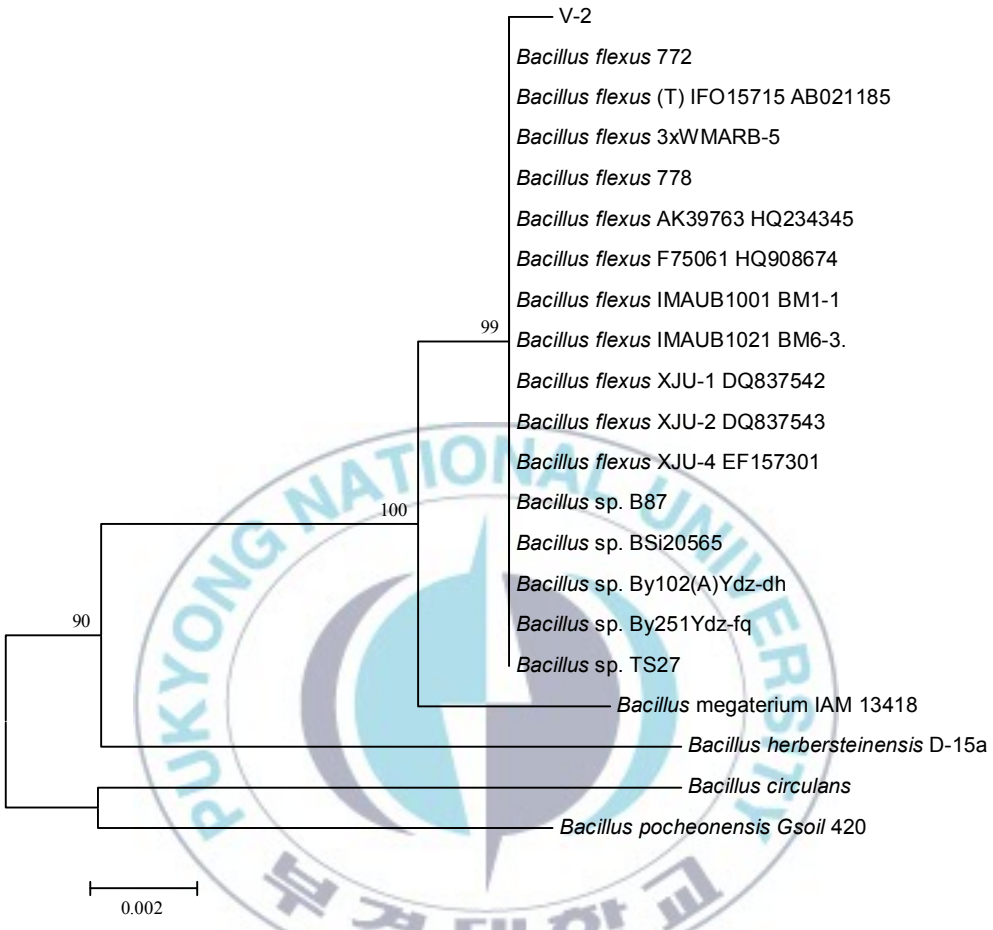


Fig. 4. Phylogenetic tree of strain V-2 on 16S rRNA gene sequences.

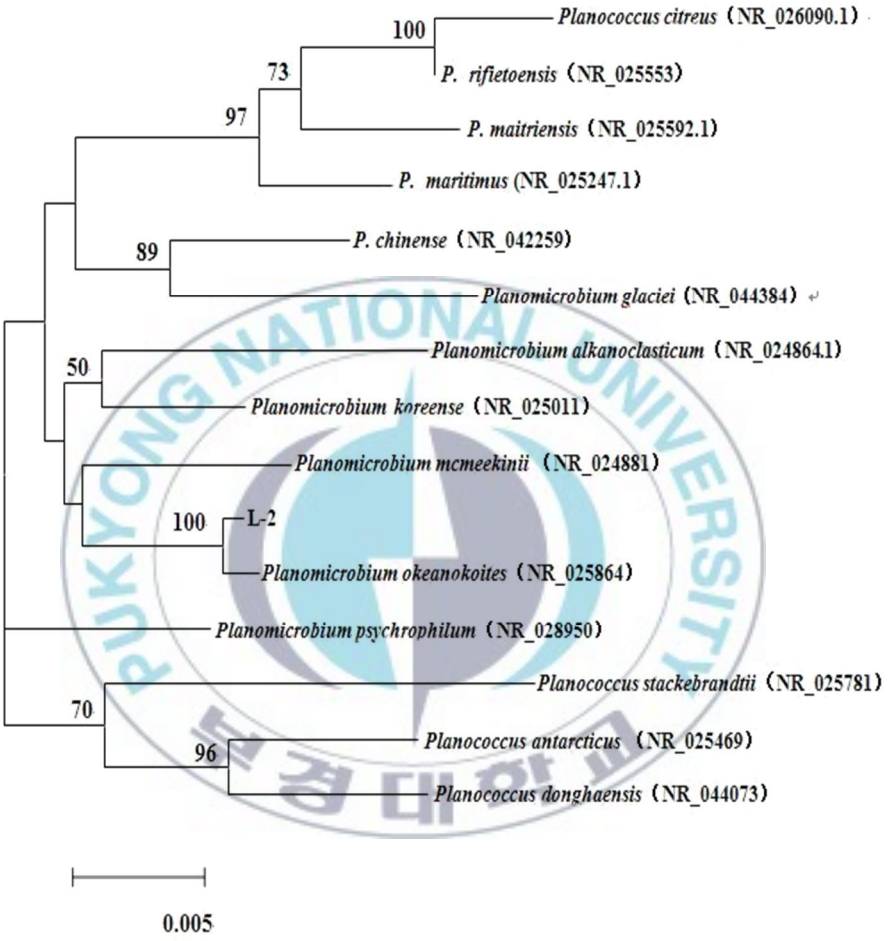


Fig. 5. Phylogenetic tree of strain L-2 on 16S rRNA gene sequences

et al. (2006) isolated bacilli from the alimentary tracts of Chinese grass carp and tilapia and identified them as *B. Megaterium* (CI3) and *B. circulans* (TM1), respectively. More recently, attempts have been made to identify enzyme-producing gut bacteria by 16S rRNA gene sequencing and subsequent comparison with data available in NCBI GenBank or RDP databases. In this study, 16S rRNA sequences of strain V-2 and L-2 are analyzed and their results shown in Fig.6 and Fig.7, respectively. Ray et al. (2010) identified the 10 most promising enzyme-producing strains isolated from the three Indian major carps by 16S rRNA gene sequence analysis of which five belonged to the genus *Bacillus*. Mondal et al. (2010) identified *B. licheniformis* (BF2) and *B. subtilis* (BH4) from the gut of bata on the basis of phenotypic characteristics as well as 16S rRNA sequence analysis. So far, the two protease-producing strains were the first time of bacteria isolation from the gut of octopus in the whole of China.

2.4.3. Optimum fermentation conditions

2.4.3.1. Growth curve

Enzyme synthesis is related to cell growth and therefore there is a co-relation between incubation period and enzyme production (Kaur et al., 1998). The growth curves of V-2 and L-2 strains were shown in Fig. 8 and 9, respectively. The V-2 strain grew slowly from 0 to 12 hrs during which the strain adopted environment tardily. Growing period was from 12 to 56 hrs when the strain grew exponentially using the nutrients provided by the medium. The period from 56 to 120 hrs was stable growth phase, while after 120 hrs the strain entered decline phase. In case of L-2 strain, the growth cycle was shorter than V-2 strain. The lag phase of L-2 strain was from 0 to 4 hrs. From 4 to 24 hrs the strain grew rapidly to achieve stable growth phase which ranged from 24 to 48 hrs. The decline phase was started from 48 hrs when the medium was out of nutrient.

1 AGAGTTTGAT CCTGGCTCAG GATGAACGCT GGCGGCGTGC CTAATACATG
51 CAAGTCGAGC GAACTGATTA GAAGCTTGCT TCTATGACGT TAGCGGCGGA
101 CGGGTGAGTA ACACGTGGGC AACCTGCCTG TAAGACTGGG ATAACTCCGG
151 CTAATACCGG ATAACATTTT CTCTTGCATA AGAGAAAATT GAAAGATGGT
201 GAAACCGGAG TTCGGCTATC ACTTACAGAT GGGCCCGCGG TGCATTAGCT
251 AGTTGGTGAG GTAACGGCTC ACCAAGGCAA CGATGCATAG CCGACCTGAG
301 AGGGTGATCG GCCACACTGG GACTGAGACA CGGCCCAGAC TCCTACGGGA
351 GGCAGCAGTA GGGAATCTTC CGCAATGGAC GAAAGTCTGA CGGAGCAACG
401 CCGCGTGAGT GATGAAGGCT TTCGGGTCGT AAAACTCTGT TGTTAGGGAA
451 GAACAAGTAC AAGAGTAACT GCTTGTACCT TGACGGTACC TAACCAGAAA
501 GCCACGGCTA ACTACGTGCC AGCAGCCGCG GTAATACGTA GGTGGCAAGC
501 GTTATCCGGA ATTATTGGGC GTAAAGCGCG CGCAGGCGGT TTCTTAAGTC
601 TGATGTGAAA GCCCACGGCT CAACCGTGGA GGGTCATTGG AAACTGGGGA
651 ACTTGAGTGC AGAAGAGAAA AGCGGAATTC CACGTGTAGC GGTGAAATGC
701 GTAGAGATGT GGAGGAACAC CAGTGGCGAA GGCGGCTTTT TGGTCTGTAA
751 CTGACGCTGA GGCGCGAAAG CGTGGGGAGC AAACAGGATT AGATACCCTG
801 GTAGTCCACG CCGTAAACGA TGAGTGCTAA GTGTTAGAGG GTTTCCGCCC
851 TTTAGTGCTG CAGCTAACGC ATTAAGCACT CCGCCTGGGG AGTACGGTCG
901 CAAGACTGAA ACTCAAAGGA ATTGGCGGGG GCCCGCACAA GCGGTGGAGC
951 ATGTGGTTTA ATTCGAAGCA ACGCGAAGAA CCTTACCAGG TCTTGACATC
1001 CTCTGACAAC TCTAGAGATA GAGCGTTCCC CTTCGGGGGA CGGAGTGACA
1051 GGTGGTGCAT GGTTGTCGTC AGCTCGTGTC GTGAGATGTT GGGTTAAGTC
1101 CCGCAACGAG CGCAACCCTT GATCTTAGTT GCCAGCATTT AGTTGGGCAC
1151 TCTAAGGTGA CTGCCGGTGA CAAACCGGAG GAAGGTGGGG ATGACGTCAA
1201 ATCATCATGC CCCTTATGAC CTGGGCTACA CACGTGCTAC AATGGATGGT
1261 ACAAAGGGCT GCAAGACCGC GAGGTCAAGC CAATCCCATA AAACCATTCT
1301 CAGTTCGGAT TGTAGGCTGC AACTCGCCTA CATGAAGCTG GAATCGCTAG
1351 TAATCGCGGA TCAGCATGCC GCGGTGAATA CGTTCCCGGG CCTTGTACAC
1401 ACCGCCCGTC ACACCACGAG AGTTTGTAAC ACCCGAAGTC GGTGGGGTAA
1451 CCTTTATGGA GCCAGCCGCC TAAGGTGGGA CAGATGATTG GGGTGAAGTC
1501 GTAACAAGGT AGCCGTA

Fig. 6. 16S rRNA sequence of Strain V-2

1	AGAGTTTGAT	CCTGGCTCAG	GACGAACGCT	GGCGGCGTGC	CTAATACATG
51	CAAGTCGAGC	GGAACCTTTG	GAGCTTGCTC	CATTGGTTTA	GCGGCGGACG
101	GGTGAGTAAC	ACGTGGGCAA	CCTGCCCTGC	AGATCGGGAT	AACTCCGGGA
151	AACCGGTGCT	AATACCGAAT	AGTTTTTTGC	CCCTCCTGGG	GCGAAACGGA
201	AAGACGGTTT	CGGCTGTCAC	TGCAGGATGG	GCCCGCGGCG	CATTAGCTAG
251	TTGGTGGGGT	AATGGCCCAC	CAAGGCGACG	ATGCGTAGCC	GACCTGAGAG
301	GGTGATCGGC	CACACTGGGA	CTGAGACACG	GCCCAGACTC	CTACGGGAGG
351	CAGCAGTAGG	GAATCTTCCG	CAATGGACGC	AAGTCTGACG	GAGCAACGCC
401	GCGTGAGTGA	CGAAGGTTTT	CGGATCGTAA	AACTCTGTTG	TGAGGGAAGA
451	ACAAGTACCA	AGTAACTACT	GGTACCTTGA	CGGTACCTCA	CCAGAAAGCC
501	ACGGCTAACT	ACGTGCCAGC	AGCCGCGGTA	ATACGTAGGT	GGCAAGCGTT
501	GTCCGGAATT	ATTGGGCGTA	AAGCGCGCGC	AGGCGGTCCC	TTAAGTCTGA
601	TGTGAAAGCC	CACGGCTCAA	CCGTGGAGGG	TCATTGGAAA	CTGGGGGACT
651	TGAGTGCAGA	AGAGGAAAGT	GGAATTCCAC	GTGTAGCGGT	GAAATGCGTA
701	GAGATGTGGA	GGAACACCAG	TGGCGAAGGC	GACTTTCTGG	TCTGTAACTG
751	ACGCTGAGGC	GCGAAAGCGT	GGGGAGCAAA	CAGGATTAGA	TACCCTGGTA
801	GTCCACGCCG	TAAACGATGA	GTGCTAAGTG	TTAGGGGGTT	TCCGCCCCTT
851	AGTGCTGCAG	CTAACGCATT	AAGCACTCCG	CCTGGGGAGT	ACGGCCGCAA
901	GGCTGAAACT	CAAAGGAATT	CGCACAAGCG	GTGGAGCATG	TGGTTTAATT
951	CGAAGCAACG	CGAAAAACCT	TACCAGGTCT	TGACATCCCG	CTGACCGCCT
1001	TAGAGATAAG	GCTTTCCCTT	CGGGGACAGC	GGTGACAGGT	GGTGCATGGT
1051	TGTCGTCAGC	TCGTGTCGTG	AGATGTTGGG	TTAAGTCCCG	CAACGAGCGC
1101	AACCCTTGAT	CTTAGTTGCC	AGCATTCAGT	TGGGCACTCT	AAGGTGACTG
1151	CCGGTGACA	AACCGGAGGA	AGGTGGGGAT	GACGTCAAAT	CATCATGCCC
1201	CTTATGACCT	GGGCTACACA	CGTGCTACA	ATGGACGGTA	CAAAGGGCAG
1261	CCAACCCGCG	AGGGGGAGCC	AATCCCAGAA	AACCGTTCTC	AGTTCGGATT
1301	GCAGGCTGCA	ACTCGCCTGC	ATGAAGCCGG	AATCGCTAGT	AATCGTGGAT
1351	CAGCATGCCA	CGGTGAATAC	GTTCCCGGGC	CTTGTACACA	CCGCCCGTCA
1401	CACCACGAGA	GTTTGTAACA	CCCGAAGTCG	GTGAGGTAAC	CCTTGTGGAG
1451	CCAGCCGCCG	AAGGTGGGAC	AGATGATTGG	GGTGAAGTCG	TAACAAGGTA
1501	TCAGCATGCC	CGTA			

Fig. 7. 16S rRNA sequence of Strain L-2

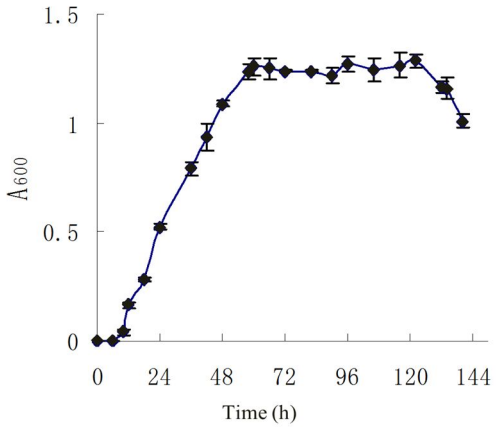


Fig. 8. Growth curve of strain V-2 which was inoculated into flask shaking medium (optimal condition), and were incubated under 30℃ at a speed of 180r/min.

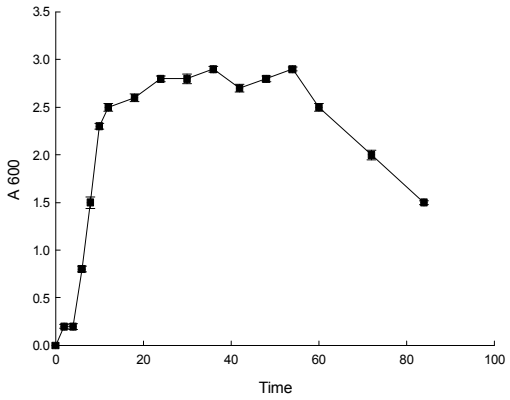


Fig. 9. Growth curve of strain L-2 which was inoculated into flask shaking medium (optimal condition), and were incubated under 19℃ at a speed of 180r/min

2.4.3.2. Effect of different carbon sources

As shown in Fig. 10, certain carbohydrates were introduced as carbon sources into the production medium of protease biosynthesis by strain V-2 and L-2. Our results indicated that the fructose was the best carbon source that induced the production of protease by strain V-2. In case of stain L-2, as the soluble starch was used as carbon source, the activity of protease produced by strain L-2 was the highest among those produced by the rest of the selected carbon sources. Variety enzymes can be produced to digest food by the alimentary canal colonies. Yang, et al., (1999) studied the effect of carbon sources on the production of protease by *Bacillus subtilis* growing in shrimp and crab shell powder medium containing one of the additional carbon sources; glucose, lactose, carboxymethyl cellulose, D(-) arabinose, D(+)xylose, and rice bran. They found that protease production was greatly enhanced by the addition of lactose or arabinose into the medium and that 1% (w/v) arabinose was the most effective substrate and concentration for protease production. On the other hand, Phadatare et al., (1993) evaluated various sugars such as glucose, ractose, lactose, maltose, sucrose, xylose, and sugar alcohols, glycerol, mannitol, and sorbitol for their effect on protease production. The results obtained revealed that sucrose gave maximum protease activity. Moreover, Andrade et al. (2002) found that the protease production reached to the maximum when added D-glucose to the medium especially when used at low concentrations (40 g/L). The results indicated that various carbon sources incorporated separately into production medium in absence of any other carbon sources except fructose and soluble starch succeeded to promote proteases productivity. For commercial production, sugars like fructose, lactose, mannitol, sucrose, and soluble starch will be prohibitive due to their cost. However, in this study fructose and soluble starch were selected for carbon sources used in the incubation of strain V-2 and L-2.

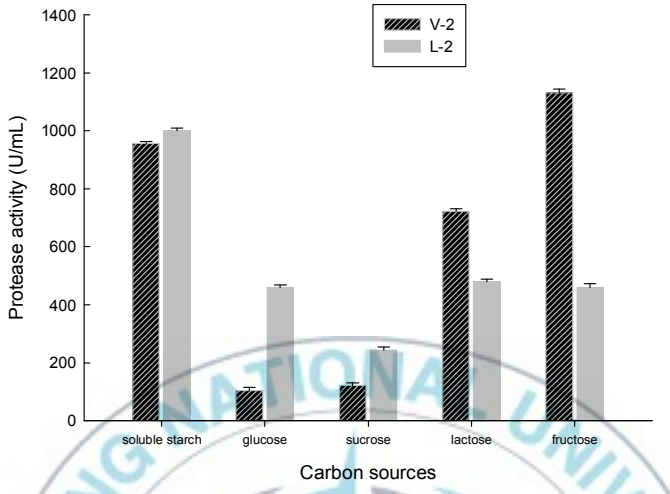


Fig. 10. Effects of various carbon sources on protease production for both strains

2.4.3.3. Effect of different nitrogen sources

The effects of different nitrogen sources applied on the two strains were shown in Fig. 11. For both strain of V-2 and L-2, peptone was detected to have the most obvious promoting effect on protease production. These findings were agreement with findings of Wang and Hsu (2005) who found out that casein and peptone were better nitrogen sources for protease production by *Prevotella ruminicolo*; Kalaiarasi and Sunitha (2009) reported that peptone was good nitrogen sources for alkaline protease production from *Pseudomonas fluorescens*; A.V. N Gupta et al. (2010) reported that peptone was good nitrogen sources for alkaline protease production from *Bacillus* sp. In Fig. 10, the activity value from beef extract shows a slight lower than that from peptone. Through SPSS software analysis, there was no significant difference on the acceleration effects of enzyme production from both peptone and beef extract ($p>0.05$). Hence, peptone was selected as nitrogen source for this work. Also, from the result, as ammonium sulfate and sodium nitrate were used, the protease production was decreased. This illustrates that these two nitrogen sources may have some inhibition on the enzyme production. Generally, both strains could use well the nutrient-rich organic nitrogen sources, and the utilization rate on inorganic nitrogen was not high because they can be metabolized rapidly into smaller compound which may be obstacles on enzyme production

2.4.3.4. Effect of different initial pH values

Different initial pH values of culture medium were tested for two strains. The results were given in Fig. 12. The protease production of stain V-2 could take place in a wide pH range from pH 5 to 9. Both neutral and alkaline conditions can

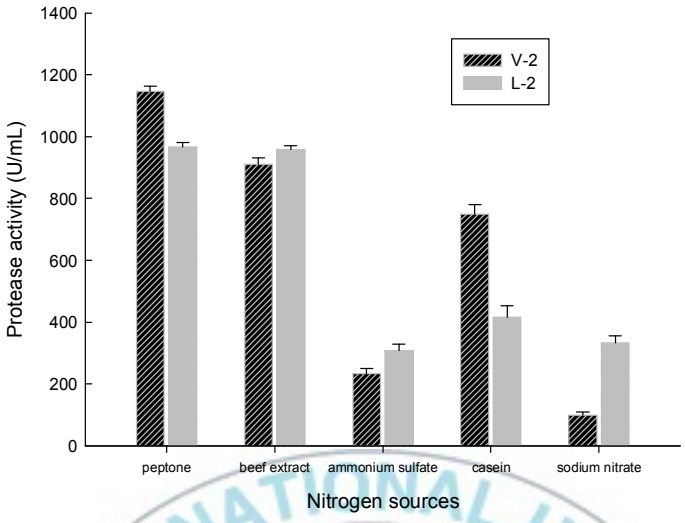


Fig. 11. Effects of various nitrogen sources on protease production for both strains

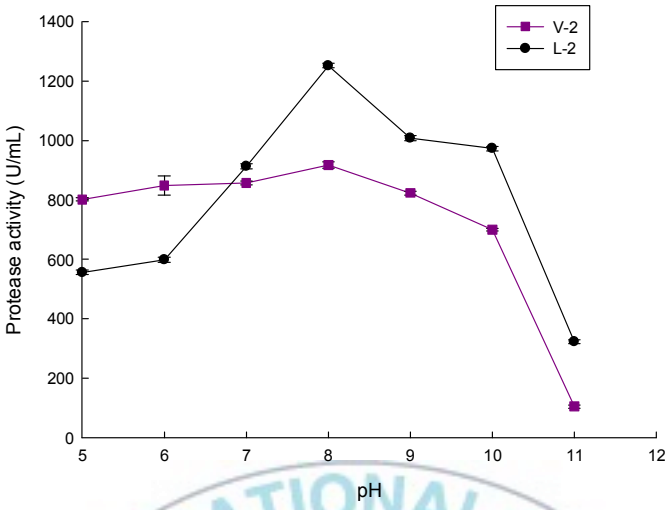


Fig. 12. Effects of different initial pH of medium on protease production for both strains

provide protease with high enzyme activity. Among those pH tested on V-2 strain, pH 8 shows the best effect on activity value. To stain L-2, obvious impact on enzyme production from initial medium pH could be found. Between pH 5 to 11, the protease activities were ranged from 339-1248 U/mL. The highest activity was at pH 8. The SPSS software analysis was carried out to show there was significant difference ($p<0.05$) on protease production obtained from pH 8 and other initial pH conditions ($p<0.05$). Therefore, pH 8 was chosen as the optimal pH 8 initial medium for this experiment. In view of the data of the other investigators, similarly, the optimal pH of protease activity produced by *E.coli* isolated from soil was 8.0 (Sonia Sethi et al., 2012). Johnvesly et al. (2002) reported that a high level of extra cellular thermostable protease activity produced by Thermoalkaliphilic *Bacillus* sp. JB-99 was observed at pH 11. The optimal pH of protease activity produced by *Clostridium bifermentans* NCTC 2914 was 7.0 (Macfarlane and Macfarlane, 1992). According to other studies, majority of microorganisms producing alkaline proteases show growth and enzyme production under alkaline condition (Tsujibo et al., 1990; Dunaevsky et al., 1996; D. J. Mukesh kumar et al., 2012).

2.4.3.5. Effect of incubation time

Fermentation time has a significant effect on enzyme production for both strains (Fig. 13). At the beginning, the two strains had different grow speed, when the fermentation time reached upto 3.5 days, the highest level of protease production was measured for both strains. However, Abdul et al. (1990) reported that both *Bacillus anthracis*, S-44 and *Bacillus cereus* var. *mycoides*, S-98 exhibited their maximum ability to biosynthesize proteases within 24 h incubation period since the productivity reached up to 126.09 U/mL^{-1} for *Bacillus anthracis*, S-44 corresponding to 240.45 U/mL^{-1} for *Bacillus cereus* var. *mycoides*, S-98 respectively. Myhara and Skura, (1990) investigated centroid search optimization of cultural conditions affecting the production of extracellular proteinase by

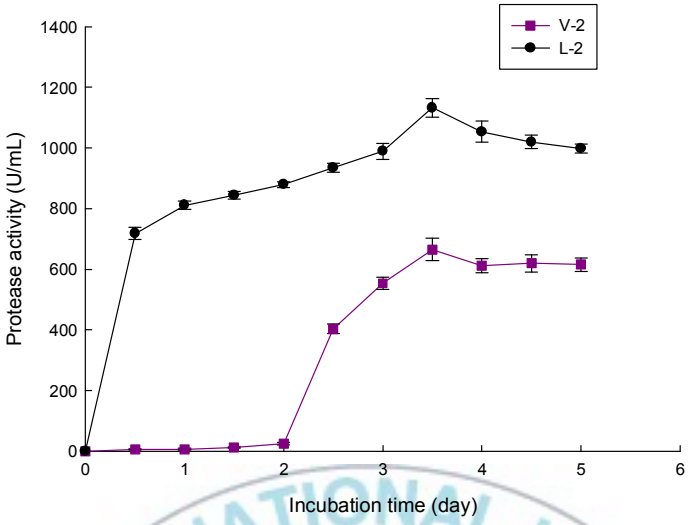


Fig. 13. Effect of different incubation time on protease production for both strains

Pseudomonas fragi ATCC 4973 and reported that the optimum incubation period for protease production by *Ps. Fragi* was 38 hr. On the other hand, Kohlmann et al, (1991) found that the detection of extracellular protease was made at 7 days of incubation at 7 ºC by *Pseudomonas fragi* and *P. fluorescens.* As the fermentation time increased, the amount of protease increased first and then decreased. This is probably due to the reduction of nutrients in the medium, the increasing accumulation of toxic metabolites, as well as the changes on pH, which inhibit the normal physiological activities of bacteria, and in turn result in the decline of the biosynthesis of the strain and cause the enzyme reduction. Since the protease-producing bacteria were descried from type to type, the difference of the individual incubation time should be accepted. Therefore, 3.5 days was used as the fermentation time for V-2 and L-2 strains.

2.4.3.6. Effect of incubation temperature

The optimum temperature has associated with the dietary habit and surroundings which the octopus has. The results of influences from different temperatures were presented in Fig. 13. Different temperature ranges were selected for two strains according to the actual result. In the case of V-2 strain, it could grow between 25 to 40℃ as shown in Fig. 14, so that 30℃ was defined as the optimal temperature and used for the further experiment. This result is in complete accordance with finding of many investigators (Vaskivyuk, 1981; Gomaa et al., 1987). Secades, et al. (2001) observe the same results that the optimum temperature for an extracellular protease produced by *Flavobacterium psychrophilum* was at temperatures between 25 and 40℃. Jobin and Grenier (2003) investigated the production of proteases by *Streptococcus suis* serotype 2 and recoded that the optimum temperature for protease production ranged from 25 to 42℃. Smaller range of temperature from 13 to 30℃ was applied to strain L-2, in which the protease activity came to the highest when 19℃ was used. After

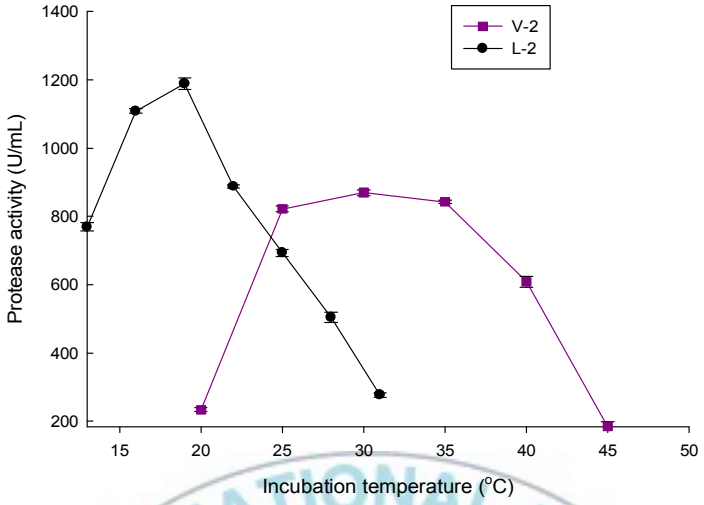


Fig. 14. Effects of different temperature on protease production for both strains

19℃, the activity value was decreased gradually with the increasing temperature. For most of protease-producing strains, the culture temperature of 19℃ was relative lower than that of others. However, the optimum temperature could depend on many aspects such as the living environment and feeding habit. In a study isolating bacteria from intestinal contents of Arabesque greenling (*Pleurogrammusazonus*), one of the isolates showed strong proteolytic activity (Hoshino et al. 1997). The isolate was identified to genus *Pseudomonas* and displayed highest protease production at 10℃. In addition to that, the production of extracellular protease by the optimum temperature for proteinase production by *Pseudomonas fragi* ATCC 4973 was 12.5℃ (Myhara and Skura, 1990). Moreover, many investigators study the relation of temperatures and protease production the temperature ranging from 2-70℃ or more all depends on the type of organism, the medium conditions and the type of enzyme (Wery, et al., 2003; Garcia de Fernando et al., 1991; Michalik et al. 1997). Finally, 30℃ and 19℃were accepted as the best culture temperature for strain V-2 and L-2, respectively.

2.4.3.7. Effect of metal ions and surfactants on protease production

As the results shown in Table 5, Mn^{2+} and Ba^{2+} could promote the protease production of V-2 strain. The appropriate amount of Mn^{2+} can increase the bacteria growth and enzyme formation. At the same time, Zn^{2+} and Cu^{2+} showed significant inhibition effect on activity value of strain V-2. There was no significant effect on protease production with the addition of surfactants. The results are similar with the properties of protease-producing strains screened from wild Dingwei gut obtained from Irtysh (Feng, 2008). To strain L-2, Ca^{2+} and Ba^{2+} were measured to have slight promotion effect on protease activity. Similar effect with control was found in other metal ions used in the experiment. Nascimento and Martins (2004) reported that protease produced by *Bacillus* spp. was

Table 5. Effects of metal ions and surfactants on protease production.

Metal ion & surfactant	Concentration	Relative protease activity (%)	
		V-2 strain	L-2 strain
Control	-	100	100
Ca^{2+}	2mM	93.3	104
Mg^{2+}	2mM	94.1	97
Mn^{2+}	2mM	102	94
Ba^{2+}	2mM	106	102
Zn^{2+}	2mM	3.8	100
Cu^{2+}	2mM	2.7	46
Polyethylene glycol	0.04g/mL	100	69.3
Tween-80	0.04g/mL	98.5	40

enhanced at different levels by Mn^{2+}, Ca^{2+} and Ba^{2+}. These metal ions protected the enzyme from thermal denaturation and maintained its active conformation at the high temperature. In addition, protease required a divalent cation like Mn^{2+}, Ca^{2+} and Ba^{2+} or combination of these cations for its maximum activity (Qasim and Rani, 2003; Kumar et al., 1999). In the case of L-2 strain, Cu^{2+} ion was detected to have significant inhibition for enzyme production. Similarly, the inhibitory effects of heavy metals Cu^{2+}, Zn^{2+}, and Hg^{2+} on alkaline protease production were widely reported, and the inhibition caused by these metal ions might suggests the relevance of sulfhydryl residues for the catalytic action of the protease (Vallee and Ulmer, 1972; Johnvesly et al., 2002; Mei and Jiang, 2005; Venugopal and Saramma, 2006). The results also present that polyethylene glycol and Tween-80 could affect the activity negatively. In the case of strain L-2, the addition of the two surfactants prior to the inoculation affected the growth of organism to different extents. The inhibition of Polyethylene glycol might caused by the destruction of the hydration shell of some proteins, while Tween-80 may have negative effect on the molecular structure of certain enzyme.

2.5. Conclusions

Two protease high-yield strains were successfully isolated from octopus gut, and named as V-2 and L-2. Morphological, physiological, and biochemical analysis of these two strains were taken place, as well as the 16S rRNA sequences identification. As the results, strain V-2 was Gram-positive bacteria, which was identified to belong to *bacteria, firmicutes, proteobacteria, bacillus mesh, bacillus brance,* and *bacillus.* There was 99.2% homology with *Bacillus flexus* 3xWMARB-5. The strain V-2 was named as Bacillus sp. V-2. L-2 strain was identified as *Pseudoalteromonas okeanokoites,* which belongs to *bacteria, firmicutes, bacilli, bacillales, planococcaceae,* and *planomicrobium*.

The fermentation conditions were also studied for both two strains. The results show that strain V-2 with fructose and carbon source, peptone as nitrogen source, medium initial pH 8.0, under temperature 30℃, for 3.5 days incubation, could produce the highest activity level of protease. In addition, soluble starch was used as a carbon source, peptone was used as nitrogen source, pH 8.0 was selected to be the initial medium pH, 19℃ was applied as culture temperature, and 3.5 days was defined to be the best suitable culture time. Under the optimal culture condition described above, the greatest activity level could be obtained from strain L-2.

Several metal ions were checked in this experiment. Mn^{2+} and Ba^{2+} were found to promote the protease production of V-2 strain, while Zn^{2+} and Cu^{2+} showed significantly inhibition effect. In case of strain L-2, Cu^{2+} ion was detected to have significant inhibition for enzyme production. Ca^{2+} and Ba^{2+} were measured to have slight promotion effect. The polyethylene glycol and Tween-80 could have inhibition effect on protease activity of strain L-2. However, there was no significant influence on strain V-2.

Chapter 3

Purification of protease obtained from two strains and its enzymatic properties

3.1. Introduction

Protease widely exists in the digestive tract of animals and humans. It plays a very important role in the protein digestion and absorption (Ding et al., 2008). Currently protease is an integral part of important industrial enzymes in modern manufacture, source of 60% of total in the world. Protease has wide range of applications in food and drug processing, chemicals and leather goods, fur industry and many other fields (Gupta et al., 2002). Currently, commercialized protease has been divided into animal protease, plant protease, and microbial protease. With the development of fish farming and marine product industry, fish gut proteases have been attracted with a growing number of attentions. Compared to protease produced from other animals intestinal, aquatic animal gut protease has its unique features such as high catalytic properties at low temperatures, low heat stability and high acid-resisting and alkali-resisting characteristics (Yu et al., 2012). Early in the last century, scientists have conducted on fish gut protease purification and characterization. The main source of industrial enzymes is gastrointestinal proteases extracted from whales, sharks and other marine organisms (Wang & Feng, 2008; Xu, 2008; Feng et al., 2008; Yan, 2010; Wang et al., 2011).

There are many domestic and foreign studies on microbial protease. The foreign scholar Kanekar (2002) has isolated 2 protease-producing strains from lake. The optimum pH of protease produced from strain *A. ramosus* was pH 11.0, and that from strain *B. alcalophilus* was pH10.0. BanerjeeU (1999) reported that the best temperature for an alkaline protease obtained from strain *bacillus brevis* was 37℃ and optimal pH was pH 10.5. Strain bacillus mojavensis A 21 was another protease producing bacteria isolated by Anissa (2009). The protease has been applied to acetone precipitation, gel chromatography, and ion exchange purification. An electrophoresis grade protease was obtained with optimal

temperature at 60℃, optimal pH 8.0, and the molecular weight was 20kDa that was measured through SDS-PAGE.

Although a certain scale has been formed recently in protease production in China, the market demand can still not meet, so every year a large amount of proteases have to be imported from abroad. In this chapter, the extracellular proteases produced by V-2 and L-2 strains were isolated and purified. The studied enzyme properties were optimum temperature, optimum pH, thermal stability, molecular weight, as well as the effects of metal ions and protease inhibitors.

3.2. Materials and apparatus

3.2.1. Material

Strain V-2 and L-2 were prepared.

3.2.2. Reagents and apparatus

Azocasein, casein, acrylamide, coomassie brilliant blue, sodium dodecyl sulfate (SDS), ammonium persulfate (AMPS), tetramethylethylenediamine (TEMED), methylene diacrylamide, trihydroxymethyl aminomethane (Tris), protein standards, and DEAE-Sephadex A50 used in this chapter were purchased from Sigma, USA. Sephadex G-100 was from Suolaibao Bio-tech. Co., Ltd, China. Cellulose CM-52 was from GE Healthcare, Sweden. DU-800 Ultraviolet spectrophotometer was from Beckman, USA. TGL-16M Benchtop high speed refrigerated centrifuge was from Xiangyi Lab instr. Dvpt. Co., Ltd., China. DHP-9032 Electro-heating standing-temperature cultivator was from Senko apparatus Co., Ltd, China. Criterion Cell was from BIO-RAD, USA. DYY-6D Electrophoresis meter was from WoDeLife Sci. Instr. Co., Ltd, China. pH 211 desktop acidity meter was purchased from HANNA, Italia.

3.3. Methods

3.3.1. Preparation of crude enzyme solution

The liquid medium for each strain was prepared referring to the optimal conditions for two strains obtained in Chapter 2. Strain V-2 was incubated for 3.5 days at initial pH 8.0 medium and 30℃ culture temperature. Strain L-2 was cultured in temperature oscillation incubator at 19℃ for 3.5 days with initial medium pH 8.0. The fermentation broth was centrifuged at a speed of 10,000 rpm for 10 min to remove cells and other insoluble materials. The supernatant was referred to crude enzyme solution, which has to be placed in 4℃ before using.

3.3.2. Ammonium sulfate precipitation

Crude enzyme solution was prepared and placed in an ice bath. An ammonium sulfate solution with saturation of 80% was gradually added into the enzyme solution with slowly stirring until it was dissolved completely.

3.3.3. Dialysis and concentration

The mixture after ammonium sulfate precipitation was centrifuged at a speed of 10,000 rpm, under 4℃, for 10 min. The supernatant was discarded while the precipitate was added into 8 − 14 kDa MWCO dialysis bag. The dialysis bag was placed into a beaker with distilled water at 4℃ refrigerator. The water was stirred with a magnetic stirrer. The water was changed every 3 hrs at the first 12 hrs, after that the interval was 12 hrs. Three days after dialysis, the dialysis bag was taken out and applied with glycol 6000 to concentrate. After concentration the sample was stored for chromatographic injection.

3.3.4. Cellulose CM-52 cation exchange chromatography

The concentrated sample was loaded into the cellulose CM-52 cation exchange column (1.2 cm x 40 cm). Firstly the enzyme was washed with a 0.02 M pH 7.0

phosphate buffer, and then it was eluted with sodium chloride solution at a gradient range from 0 to 2 M. The flow rate was 60 mL/h. A fraction collector was used to collect the purified protease, and the enzyme activity from each receiving tube was measured under a wavelength of 280 nm. The proteases of high activity were combined together.

3.3.5. DEAE-Sephadex A50 anion exchange chromatography

For further purification, the activated constituents received from Cellulose CM-52 cation exchange chromatography were isolated by DEAE-Sephadex A50 anion exchange column (1.6 cm x 40 cm). After sample loading, the compound was eluted at a flow rate of 60 mL/h firstly with a 0.02 M pH 8.5 Tris-HCl buffer. Then, a sodium chloride solution with a gradient from 0 to 2 M was applied. Protease of each receiving tube was detected at a wavelength of 280 nm, and the activated constituents were combined for further experiment.

3.3.6. Sephadex G-100 gel chromatography

In the case of strain L-2, the active fractions collected from DEAE-Sephadex A50 anion exchange chromatography were depurated with Sephadex G-100 gel chromatography column (2.0 cm x 40 cm). A pH 7.0, 0.02 M phosphate buffer was selected as elution buffer with a flow rate at 30 mL/h. Enzyme of each receiving tube was detected at a wavelength of 280 nm. The activated compounds were mixed.

3.3.7. SDS-PAGE electrophoresis

A 3.75% stacking gel and a 12% separation gel (containing 0.05% of casein substrate) with a thickness of 0.75 mm were prepared. The active ingredients collected from DEAE-Sephadex A50 anion exchange chromatography were analyzed by electrophoresis. After completion of the electrophoresis, the gel with protein standard bands was cut down for conventional staining and destaining.

The gel where the sample bands existed was placed into refolding buffer (2.5% TritonX-100). The gel was soaked for 30 min at 4℃ to recover protease activity, and then placed into a reaction buffer (0.1 M Tris-HCl, pH 8.0) at 37℃ for 3 hrs. The color level of protease on the gel was observed. There were bright white bands around the protease reaction site and the rest is dark blue.

3.3.8. Determination of protease activity

Refer to 2.3.2. Measurement of protease activity

3.3.9. Measurement of protein content

Method of Bradford was used to measure the protein content. For standard curve, 0, 25, 50, 75, 100, 125, 150, 175 μg/mL of BSA standard 100 μL were prepared. One milliliter coomassie bright blue working solution was added into each standard solution. The mixture was kept at room temperature for 2 min, and the absorbance was measured at 595 nm.

3.3.10. Protease stability

3.3.10.1. Effects of temperature on proteases

The proteases were placed at different temperatures of 20, 30, 40, 50, and 60℃ for 1 hr. The protease was then mixed with substrate azocasein and the reaction was carried out at 37℃. Protease activities were measured from different conditions to observe the effect of temperature. The highest point of enzyme activity referred to 100 % with which the relative activity was calculated.

3.3.10.2. Effects of pH on proteases

Different pH values of buffer were set at 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0, and the buffers were mixed with the purified protease at a ratio of 1:1. The mixture was stored at 4℃ for 1 hr and reacted with individual azocasein which has corresponding pH value at 37℃. The Azocasein method was utilized as the

measurement for enzyme activity value. The protease that did not go through the processing was defined as control. The highest point was denoted as 100% and the protease activity was calculated.

3.3.10.3. The thermal stability of proteases

Different methods were applied on this aspect. The two proteases were incubated under 40, 50, 60, and 70℃ for 0, 10, 20, 30, 40, 50, and 60 min, respectively. The Azocasein method was used to observe the relative activity. The fermentation broth without high temperature treatment was considered as control and the activity value was denoted as 100% which was used to calculate the activity.

3.3.10.4. Effects of metal ions and surfactants on proteases

Different metal ions solutions, including Ca^{2+}, Mg^{2+}, Ba^{2+}, Mn^{2+}, Cu^{2+}, and Zn^{2+}, with the same concentration of 2 mM/L were mixed with protease solution, respectively. The solution of 5 mL /L PMSF and 2.5 mM/L EDTA were also tested by adding into purified protease solutions, respectively. The mixtures were kept at 37℃ for 1 hr, and the relative activities of enzyme were analyzed by the method of Azocasein. Distilled water was used as a control to calculate the results.

3.4. Results and discussions

3.4.1. Cellulose CM-52 cation exchange chromatography

Protease from the two strains was purified by the three-step procedure described above. The result of fractions purified through Cellulose CM-52 cation exchange chromatography was shown in Fig. 15. Two active peaks were obtained, and labeled as Fa and Fb. The protease activities of two active constituents were 213.0 U/mL and 225.1 U/mL, respectively. Fb showed a higher specific activity than Fa. However, it contained lower amount of protein and there was no polypeptide band under SDS-PAGE measurement. The active component (Fa)

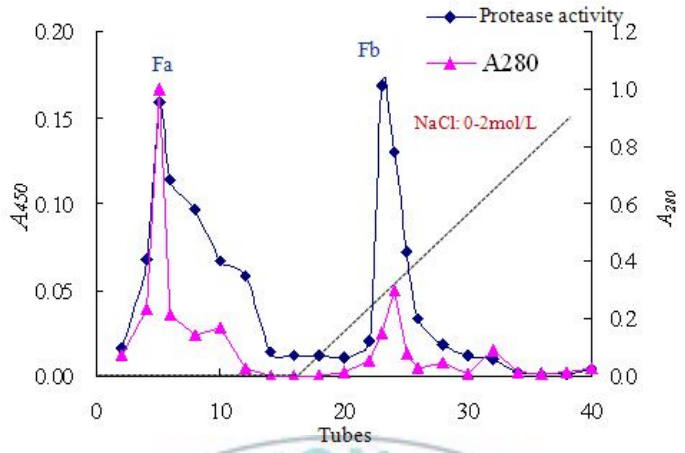


Fig. 15. Elution profile of proteases from strain V-2 on Cellulose CM-52 cation exchange column

was collected and stored at -20℃ after freeze drying.

3.4.2. DEAE-Sephadex A50 anion exchange chromatography

The Fa fraction isolated from Cellulose CM-52 cation exchange chromatography was further purified by DEAE-Sephadex A50 anion exchange column, and the elution profiles of protease activity and proteins from DEAE-Sephadex A50 was shown in Fig. 16. Fractions containing protease activity were pooled and loaded on the column pre-equilibrated with 0.02 M pH 8.5 Tris-HCl buffer, and bingding proteins were eluted with a liner gradient of 0-2 M NaCl in the same buffer. This procedure yielded four activity peaks isolated from the original compound.

The four ingredients were named as Fa-1, Fa-2, Fa-3, and Fa-4, whose protease activities were 6.8, 22.5, 16.7, and 22.9 U/mL, respectively. Ion exchange chromatography was used to remove contaminating protein and to separate different protease isoforms. Two proteases from the digestive tract of anchovy (*Engraulis encrasicholus*) were purified by using an ion exchange column, DEAE –sephadex at the final step of purification (Martinez et al., 1988). Kishimura, Hayashi, Miyashita, and Nonami (2005) also isolated two protease isozymes from visera of Japanese anchoy (*Engraulis japonica*) using DEAE-cellulose in the final step. The proteases obtained from strain L-2 could not be filtered with Cellulose CM-52 chromatography. Therefore, after ammonium sulphate precipitation, the supernatant was subjected to DEAE-Sephadex A50 anion exchange column.

The elution profiles of protease activity and proteins from DEAE-Sephadex A50 were presented in Fig. 17. The fractions with protease activity were loaded on the exchange column with a 0.02 M pH 7.0 phosphate buffer, and binding proteins were eluted with a linear gradient of 0 – 1 M NaCl at a flow rate of 1 mL/min. Samples were passed through DEAE-Sephadex A50 anion exchange column, and resulted in one peak of proteolytic activity which was named as F1.

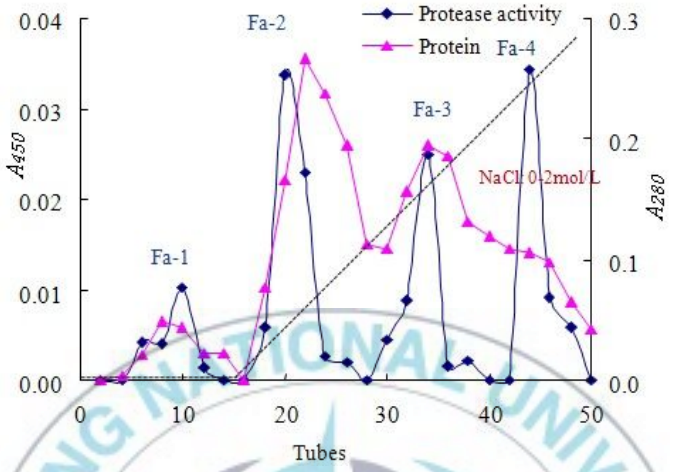


Fig. 16. Elution profile of Fa protease on DEAE-Sephadex A 50 of protease

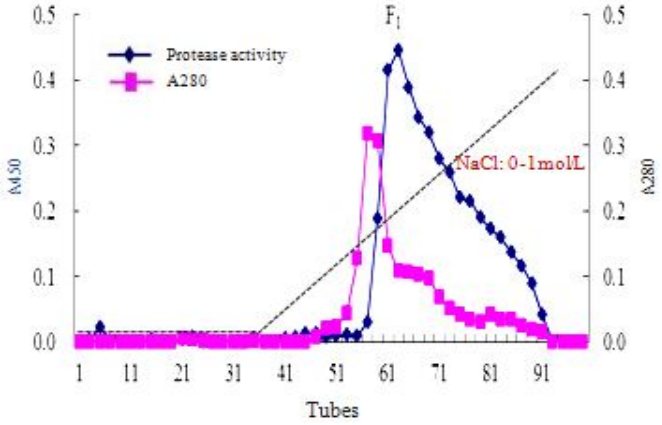


Fig. 17. Elution profile of proteases from strain L-2 on DEAE-Sephadex A50 ion-exchange column

The enzyme activity of F1 was detected as 594.3 U/mL. Active fractions were combined, concentrated and subjected to a second Sephadex G-100 gel filtration.

3.4.3. Sephadex G-100 gel chromatography

Sample F1 collected from DEAE-Sephadex A50 anion exchange column was further purified by Sephadex G-100 (Fig. 18) equilibrated and eluted with a 0.02 M phosphate buffer (pH 7.0). This procedure yielded a single active peak, which was detected to have high protease activity as 347.3 U/mL. It was donated as F1-1. After Sephadex G-100 gel chromatography, a large amount of contaminated proteins was removed, resulting in an increase in purification fold. Sephadex gel columns are well established gel filtration medium to purify protein by desalting and buffer exchange of peptides. One trypsin from the viscera of Sardinelle(*Sardinella aurita*) was purified by using Sephadex G-75 at the final step of purification (Hayet et al., 2008). Ali et al. (2007) also isolated one protease from visera of Sardine (*Sardinella pilchardus*) using sephadex G-100 in the final step.

The purification of extracellular protease from the culture supernatant is summarized in Table 6. Extracellular proteases were purified through, a three-step purification protocol of centrifugation, ammonium salt precipitation, and filtration chromatography. All the purification steps were performed at 4℃. The crude enzymes were precipitated with 80% ammonium sulphate with recoveries of 79.4% (strain V-2) and 26.2% (strain L-2) of activity that amounted to nearly 1.1 (strain V-2) and 1.3 (strain L-2) fold purification. The precipitated enzymes were loaded onto different filtration columns (Cellulose CM-52 cation exchange column, DEAE-Sephadex A50 ion-exchange column, and Sephadex G-100 gel column). The chromatogram showed several elution peaks with a single activity peak at the final purification step, which gave 12.5% recovery of the enzyme activity with nearly 2.5 fold purification to strain V-2, and 7.5% recovery of

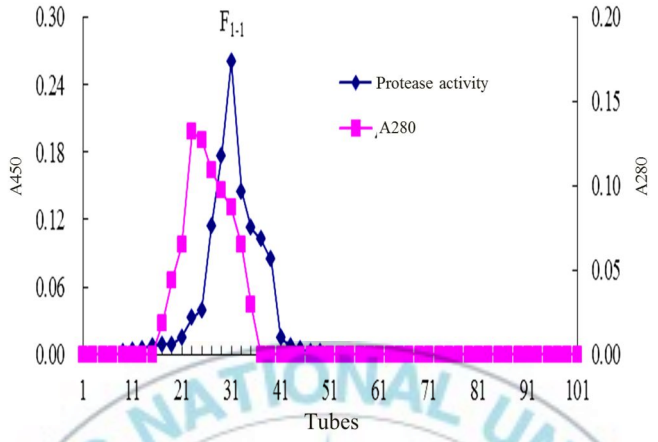


Fig. 18. Elution profile of F1 protease on Sephadex G-100 chromatography

Table 6. Summary of purification of protease

Precedure	Enzyme activity (U)		Total Protein (mg)		Specific activity (U/mg)		Purification fold		Recovery (%)	
	V-2	L-2	V-2	L-2	V-2	L-2	V-2	L-2	V-2	L-2
Crude protease	246107.8	661316.8	68.0	91.7	3619.2	7211.7	1.0	1.0	100.0	100.0
Amm. Sul. Precipitation	195485.4	173250.6	47.8	18.7	4089.6	9264.7	1.1	1.3	79.4	26.2
Cellulose CM-52	89765.4	135270.2	10.8	13.7	8311.6	9873.7	2.3	1.4	36.5	20.5
DEAE- Sephadex A50	30675.3	-	3.3	-	9075.5	-	2.5	-	12.5	-
Sephadex G-100	-	49577.9	-	4.0	-	12394.5	-	1.7	-	7.5

activity with 1.7 fold purification to strain L-2. Boonyaras et al. (2000) purified two thermostable proteases from *Bacillus stearothermophilus* TLS33 through a 3-step purification procedure (centrifugation, ammonium salt precipitation, and filtration chromatography). The purification was 2.57- and 2.74-fold over the culture supernatant, and the specific activities were 25.2 and 26.9 U/mg of protein, with recoveries of 6.62 and 26.6% of the total proteolytic activity in the culture supernatant, respectively.

3.4.4. SDS-PAGE electrophoresis

To check the purification and molecular weight determination, fractions from all purification steps were run on SDS-PAGE. The SDS-PAGE results of the fractions obtained during the purification of protease from strain V-2 were shown in Fig. 19. Three proteases were detected from Fa which was isolated from Cellulose CM-52 cation exchange chromatography. The molecular weights of three proteases were 96.6 kDa, 75.8 kDa, and 61.6 kDa, respectively. Fa-2 was purified from Fa through DEAE-Sephadex A50 anion exchange column, and an electrophoresis grade-pure protease was obtained, whose molecular weight was estimated to be 61.6 kDa.

The SDS-PAGE results of the fractions obtained during the purification of protease from strain L-2 were also shown in Fig. 19. After purified by DEAE-Sephadex A50 anion exchange column, two bright bands were found on the gel for sample F1 (Fig. 19), whose molecular weights were measured as 61.4 kDa and 53.2 kDa, respectively. An electrophoresis grade-pure protease F1-1 was obtained with the molecular weight of 61.4 kDa after Sephadex G-100 gel chromatography. Earlier low molecular-weight proteases had been isolated from *Bacillus* sp. APR-4 (16.9 kDa) by Kumar and Bhalla (2004) and *Micrococcus* sp. INIA 528 (19.4 kDa) by Fernandez et al. (1996). However, two proteases isolated from *Bacillus* sp. TLS33 had molecular masses of 53 and 71 kDa (Boonyaras et al., 2000). In general, the molecular masses of previously found proteases are rarely more

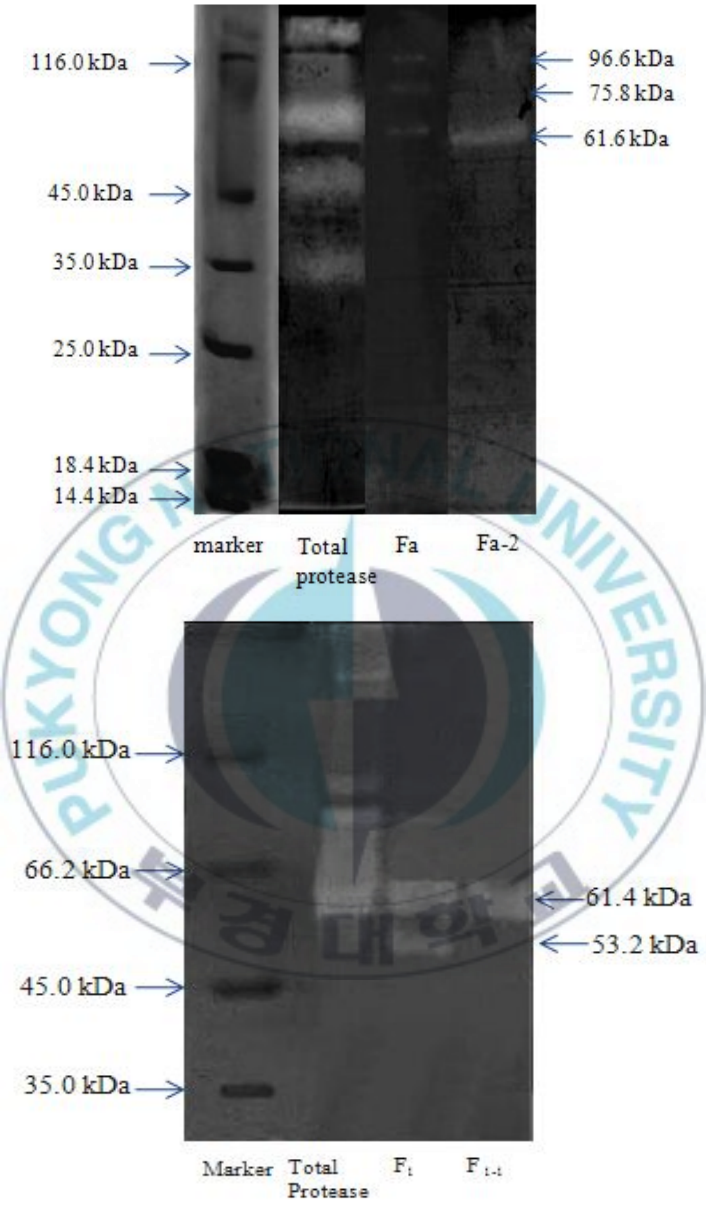


Fig. 19. SDS-PAGE of proteases obtained from V-2 strain and L-2 strain.

than 50 kDa (Watanabe et al., 1993; Dozie et al., 1994; Klingeberg et al., 1995). However, molecular masses of proteases Fa-2 and F1-1 are about 61.6 and 61.4 kDa, respectively, indicating that both proteases are novel proteases.

3.4.5. Detection of protein content

For determining the protein concentration, the method of Bradford was used. The standard curve was shown in Fig. 20. The curve equation was Y=0.0019x + 0.0509 with a standard deviation of 0.99. According to the regression equation, the amount of protein could be calculated through the corresponding absorbance. The enzyme broth of 100 μL was added into 1 mL Coomassie blue. The mixture was placed at room temperature for 2 min, and then the absorbance was measured at 595 nm.

3.4.6. Protease stability

3.4.6.1. Effect of temperature on proteases

The effects of temperature on Fa-2 and F1-1 were shown in Fig. 21. The optimum temperature for both proteases to have the highest activity was 40℃, which indicated that the proteases belong to mesophilic enzyme. Generally, microbial proteases have a broader optimal temperature range from 30 to 75℃, depending on different genera of bacteria. Uttam et al (1999) isolated protease-producing *Bacillus subtilis* from water and the optimal temperature of the protease was 37℃. Another researcher of Jung (1996) also has reported a *Pseudomonas* sp KFCC 10818 strain which could secrete protease of which optimum temperature was 70℃.

3.4.6.2. Effects of pH on proteases

Significant influence of pH on protease Fa-2 has been detected (Fig. 22). The most suitable pH for protease Fa-2 ranged from pH 9.0 to pH 9.5, belonging to alkaline protease. Currently, Optimum pH values range for most of the alkaline

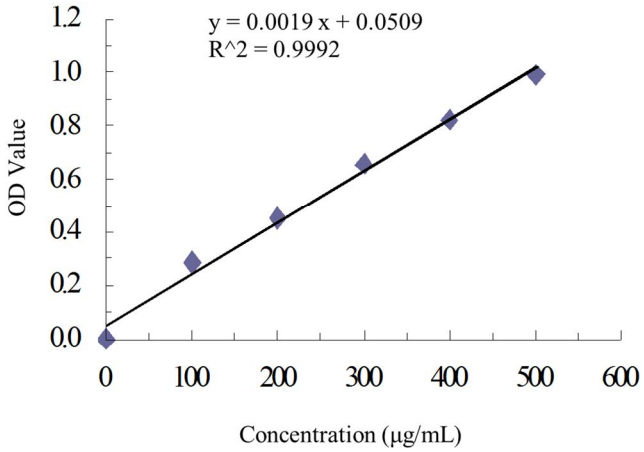


Fig. 20. Standard curve of different concentrations of bovine serum albumin

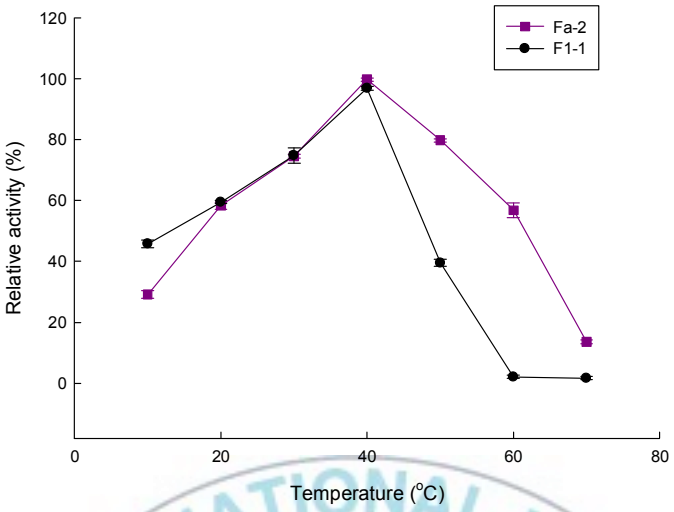


Fig. 21. Effects of different temperature on protease Fa-2 and F1-1

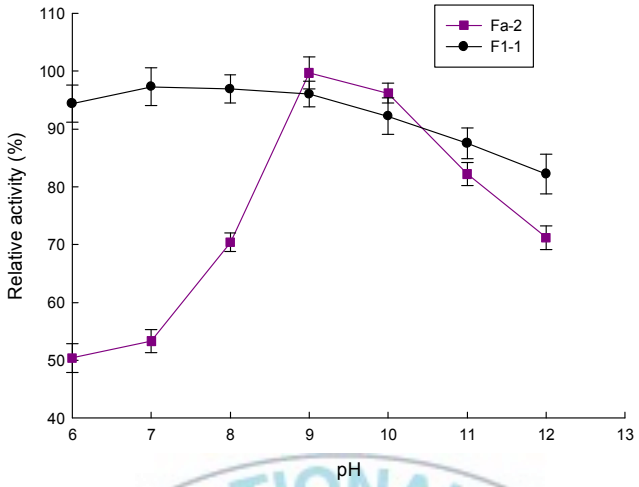


Fig. 22. Effects of different pH values on protease Fa-2 and F1-1

proteases were from 8.5 to 10.0. Only a few is more than 11.0 and even up to 12.0.

Shiha et al. (1999) have reported that a strain of *bacillus pantotheneticus* could produce protease with an optimum pH at 8.5. Kumar et al. (1999) have isolated bacillus sp. NCDC180 from soil which could secrete two kinds of proteases named as AP1 and AP2. The optimum pH values for both proteases were 11.0 and 12.0, respectively. However, to protease F1-1, in the pH range of pH 6.0 to pH 12.0, pH value no large fluctuation has been observed. The optimum pH of the experimental enzyme is comparable with protease isolated from *Azospirillum* sp., which was also active in a broad pH range, but the enzyme showed maximum activity to pH 8.5 (Oh et al., 1999). The result may verify that pH effect on F1-1 protease activity was not significant.

3.4.6.3. The thermal stability of proteases

Protease stability was tested at a broad range of temperatures from 40℃ to 70℃. The results of thermal stability of two proteases were shown in Fig. 23 and 24. The two proteases showed similar thermal stability trend. The purified proteases remain almost fully activity even after 60 min of incubation at 40℃. At the 50℃ for 30 min, there was a significant reduction in activity for both proteases. At 70℃ the enzymes retained none of their initial activity after 20 min(Fa-2) and 10 min (F1-1) incubation. The optimal activity temperature of experimental enzyme was found to be higher than that of earlier reported proteases from *Azospirillum* sp. (Oh et al., 1999) and *Shewanella* strain Ac10 (Kulakova et al., 1999). However, the optimal temperature of protease from *Bacillus* sp. APR-4 is reported to be 60℃ (Kumar and Bhalla, 2004). The results verified that the protease has good thermal stability. The stability of the enzyme at higher temperatures suggests its usefulness in industrial applications (Ramesh et al., 2007).

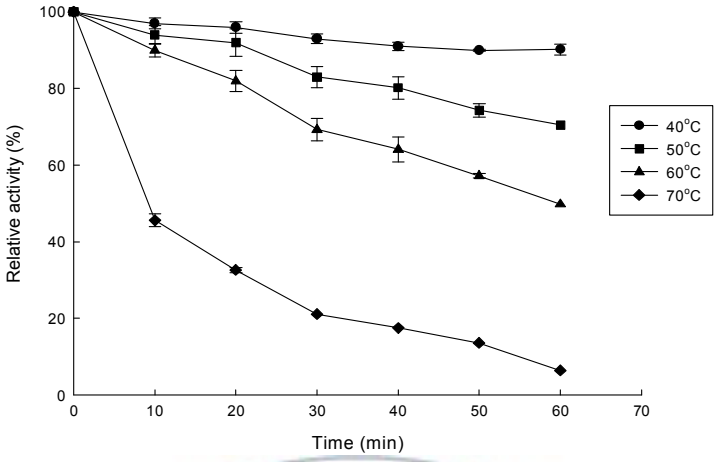


Fig. 23. The thermal stability of Fa-2 at different temperatures

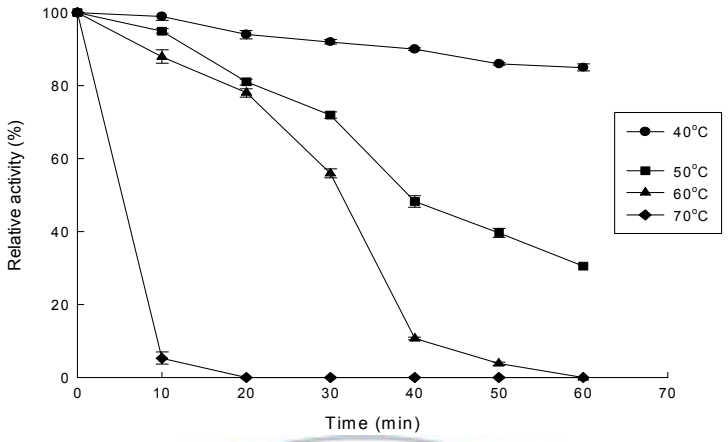


Fig. 24. The thermal stability of F1-1 proteases at different temperatures

3.4.6.4. Effects of metal ions and surfactants on proteases

Proteases are classified as serine protease, cysteine protease, aspartic protease, and metalloprotease according to the involvement of different amino acids and metal ions in the catalysis (Ramesh et al., 2007). Different metal ions and surfactants have been analyzed on Fa-2 and F1-1. The results were shown in Table 7. For both proteases, the Serine protease inhibitor (PMSF) has completely inhibited the activities of Fa-2 and F1-1. This indicates that the active center of the two proteases contain a serine which belongs to the serine proteases. According to the research report, most of the alkaline proteases produced by microorganism were found to be this kind of protease (Anissa et al., 2009). EDTA was also has been detected to slightly inhibit the activity of protease Fa-2, as well as Ba^{2+}, Zn^{2+}, and Cu^{2+}. However, in presence of Mg^{2+} and Mn^{2+} ions, the Fa-2 protease showed a maximum increase in the activity, and the activities have reached to 115% and 189%, respectively. Similarly *Bacillus* sp. protease showed a strong promotion by Mg^{2+} and Mn^{2+} (Manachini et al., 1988; Takami et al., 1990; Ramesh et al., 2007). Mn^{2+}, Zn^{2+}, and Cu^{2+} ions have been observed to have inhibition on protease F1-1. Ba^{2+} and Ca^{2+} showed a slight promotion effect. There was no significant inhibition of EDTA analyzed on F1-1. Other results were also reported in proteases from the pyloric caeca of mandarin fish (*Siniperca chuatsi*) by Lu et al. in 2008. Enzymatic activity of the two proteases was also strongly inhibited by Mn^{2+} and partially inhibited by Cu^{2+}, Zn^{2+}, and Co^{2+} at 1 mM.

3.5. Conclusions

In this chapter, the proteases obtained by strain V-2 and L-2 were purified through ammonium sulfate precipitation, CM-52 cellulose cation exchange chromatography, DEAE-Sephadex A50 anion exchange chromatography, and Sephadex G-100 gel chromatography. The purified proteases were named as Fa-2

Table 7. Effects of inhibitions and metal ions on Fa-2 and F1-1

Reagents	Concentration (mM)	Relative activity (%)	
		Fa-2	F1-1
Ca^{2+}	5	97	103
Mg^{2+}	5	115	89
Mn^{2+}	5	189	56
Ba^{2+}	5	55	106
Zn^{2+}	5	70	87
Cu^{2+}	5	68	15
EDTA	5	85	96
PMSF	2.5	0	10

and F1-1.

After the three-step purification, the purification folds of Fa-2 and F1-1 were 2.5 and 1.7, respectively, and their activity recoveries were 12.5% and 7.5%, respectively. The enzymatic properties were also analyzed. To Fa-2, its molecular weight was 61.6 kDa. Its optimal temperature was 40℃, which meant that it belongs to mesophilic enzyme. The optimum pH of Fa-2 was between pH 9.0 and pH 9.5 which indicated it is an alkaline protease with a good thermal stability at 50℃.

In the case of protease F1-1, the results of enzyme properties showed that the molecular weight was 61.4 kDa with an optimum temperature at 40℃, which is also a mesophilic enzyme. The effect of pH was not obvious, and it has a great thermal stability at 60℃.

Serine protease inhibitors could completely inhibit Fa-2 activity which indicates that the enzyme may be serine protease family. Similar results were obtained of the effect of surfactants on F1-1. Ba^{2+}, Zn^{2+}, and Cu^{2+} ions showed inhibition on Fa-2 activity, while Mg^{2+} and Mn^{2+} ions have been detected to activate the protease. Mn^{2+}, Zn^{2+}, and Cu^{2+} ions have been measured to have inhibition on protease F1-1, and Ba^{2+} and Ca^{2+} showed a slight promotion effect. The effects of protease activities on the metal ions also explain that they may associate with the diet habits of octopus which mainly includes shrimps and crabs. This study will provide basic information for industrial application of octopus intestinal proteases.

Chapter 4

Application of Stain V-2 and L-2

4.1. Introduction

A small amount of oxygen-free radicals would be produced in human body under normal physiological processes. These radicals present in the body could be eliminated through natural antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT), or antioxidants such as vitamin C, vitamin E and other components of the antioxidant system. Therefore, under normal conditions, the level of free radicals can be maintained on a certain level in a dynamic equilibrium. However, the activity of antioxidative enzymes would be decreased with age or some certain pathological conditions, which could result in pathobolism and followed with large amount of reactive oxygen species production, and cell death and tissue damage.

Since free radical theory has been put forward, people have started to know that the oxidative free radicals generated with human aging and many diseases. Therefore, more and more attentions have been paid on antioxidants research. Antioxidative peptides were one of the modern antioxidants, which have anti-aging effect and could reduce the incidence of a variety of age-related diseases and other physiological functions. They could prevent oxidative deterioration of food, improve food stability and extend food shelf life (Wu & He, 2008). Therefore, they can be used as food additives and extend food hygiene factors applied in food, health products, and cosmetic IDM (Adi & Mohammed, 2000).

There were a large number of by-products in the process of octopus products which are usually very rich in protein. The by-products would not only cause the squander of protein resources, but also would result in environment pollution. However, those proteins could provide nutrients during the process of microbial growth, and the substrate of proteins would be decomposed into polypeptide (Yee et al. 1980). The preparation of peptides used as bio-fermentation method has been reported as early as 1980 (Nabil et al., 2008). Liu et al. (2007) have studied the antioxidant activity of soybean peptides produced with strains of *Bacillus*

subtilis. Li (2003) and Qin (2010) used *Mucor* sp. and *Aspergillus niger* to ferment soybean and they obtained soybean peptides, respectively.

In this study, strain V-2 and L-2 have been used to ferment octopus scraps. The antioxidative peptides were prepared through ultrafiltration and fed the subacute-aging mode mice injected with D-galactose. Their antioxidative activities were also measured after the isolation. The content of malondialdehyde (MDA) was detected in the serum and liver tissue of mice. The OH• free radical-scavenging activity and the activity of SOD, glutathione peroxidase (GSH-Px) and CAD were also measured as evaluation factors in the study of *in vivo* antioxidant activity of enzymatic hydrolysate of octopus scraps.

4.2. Materials and apparatus

4.2.1. Materials

Octopus scraps were provided by Shandong Ronxin Aquatic Food Co. LTD. The scraps were stored at negative 20℃. Kunming male mice (SPF) were purchased from Qingdao Experimental Animal Center.

4.2.2. Reagents and apparatus

Trihydroxymethyl aminomethane (Tris) and 2,2-Diphenyl-1-picrylhydrazyl free radical (DPPH•) were purchased from Sigma, USA. Dithiothreitol (DTT) was purchased from Suolaibao Bio-Tech. Co., Ltd., China. DU-800 Ultraviolet spectrophotometer was from Beckman, USA. D-galactose was purchased from Sinopharm Chemical Reagent Co., Ltd., China MDA kit, T-SOD kit, GSH-Px kit and CAT kit were purchased from Nanjing Jiancheng Bioengineering Institute, China. FD-1D-80 freeze dryer was purchased from Beijing Bo Kang Experimental Medical Instrument Co., Ltd., China. TGL-16M high-speed desktop refrigerated centrifuge was purchased from Hunan Xiangyi Laboratory Instrument Co., Ltd., China. PPM-18S/T membrane separator was purchased from Sandamo (Xiamen) Co., Ltd., China.

4.3. Methods

4.3.1. Preparation of fermentation broth

The strains were inoculated into liquid fermentation mediums with individual optimal parameters for one day as the seed broth. The octopus scraps were grinded, and 5 g of the sample was weighted and added into the seed broth. The pH value was adjusted, and the mixture was incubated at 25℃ for 24 hrs. The fermentation broth was centrifuged at 10,000 rpm for 15 min, and the supernatant liquid was subjected for further experiments.

4.3.2. Ultrafiltration

The fermentation broth was passed through 3 kDa membrane filter to remove unhydrolyzed protein molecules, and then, was filtered with 250 Da membrane to remove salts. The filtrate was collected and concentrated with freeze-dryer to obtain polypeptide powder of octopus scraps.

4.3.3. Antioxidant activity of octopus scraps peptides

4.3.3.1. Determination of DPPH• free radical-scavenging activity

DPPH• has great stability in organic solvents, and its lone pair electrons have strong absorption at 517 nm. However, the absorption would be disappeared or diminished, when the scavengers present with which the lone pair electrons could be paired. The DPPH radical-scavenging activity of the hydrolysates was determined as described by Bersuder et al. (1998).

The sample of 0.5 mL was mixed with 1.2 mL of 0.2 mM DPPH• solution (in ethanol), and deionized water was added to make total 4 mL. The mixture was placed at dark for 30 min and its absorbance was measured at 517 nm. The equation of DPPH• removal rate (%) was as follows: DPPH• removal rate (%) = $(1-\frac{A_1-A_2}{A_0})\times 100\,\%$

Where, A_1 is the absorbance of the sample after the reaction with DPPH•. A_2 is the absorbance of the sample itself. A_0 is the absorbance of the unreacted DPPH•.

4.3.3.2. Determination of OH• free radical-scavenging activity

The solution of 3.25 mL of 0.15 M phosphate buffer (pH 7.4) was mixed with 0.25 mL of 7.5 mM phenanthroline. The 0.25 mL of 7.5 mM ferrous sulfate was added into the mixture, followed by 0.25 mL of 0.1% hydrogen peroxide. The mixture were mixed well and denatured at 37℃ water bath for 90 min, and the absorbance was measured at 536 nm. The equation of OH· removal rate (%) was as follows: $\text{OH· removal rate（%）} = \frac{A_1 - A_0 - A_2}{A_3 - A_2} \times 100\,\%$

Where A_1 is the absorbance of sample after the reaction with phenanthroline, ferrous sulfate and hydrogen peroxide; A_2 is the absorbance of phenanthroline, ferrous sulfate and hydrogen peroxide after reaction; A_3 is the absorbance of phenanthroline and ferrous sulfate reaction; A_0 is the absorbance of the sample

4.3.3.3. Determination of Superoxide radicals (O_2^-•) scavenging activity

The 50 μL sample was mixed with 5 mL of 0.1 M Tris-HCl solution with pH 8.2 (preheated to 25℃). The 40 μL of 25 mM pyrogallol was added into the mixture. The reaction was allowed to carry out for accurate 3 min under 25℃. The reaction was terminated by quickly dropping 50 μL of 50 mg/mL DTT. The mixture was allowed to stay at room temperature for 10 min, and the absorbance was measured at 316 nm. O_2^- removal rate $\text{（%）} = \frac{A_0 - A_1 + A_2}{A_0} \times 100\,\%$

Where, A_1 is the absorbance of sample after the reaction with pyrogallol; A_2 is the absorbance of the sample; A_0 is the absorbance of pyrogallol autoxidation.

4.3.4. Determination of peptide concentration

Bicinchoninic acid assay (BCA) method was used to determine the concentration of peptide. Protein Quantitative Reagent Kit-BCA was applied to the BCA method. The peptide purified by DEAE-Sephadex A50 colomn was adjusted to an appropriate content. BCA working solution A and B were mixed with 50 : 1 ratio. The sample of 20 μL was added into 200 μL mixture of BCA working solution and vortexed well. The solution was incubated at 37℃ water bath for 1 hr. The absorbance was measured at 562 nm. The standard curve was prepared with bovine serum albumin (BSA).

4.3.5. Preparation of experimental rats

The antioxidant activity *in vitro* experiments confirmed the high antioxidant activity of the octopus scrapes hydrolysates, where P1 has more antioxidant activity. To further validate the antioxidant activity of hydrolysates *in vivo*, P1 was applied for animal experiments.

The 36 Kunming mice of 8-week-old male were selected with body weight of 20±2 g. They were randomly divided into three groups. There were 12 mice in each group. The groups were labeled with blank group, D-galactose-induced model group and octopus scraps hydrolysate group. The rats were raised under temperature of 18-22℃ with natural light and self-feeding. After 1 week adaptation period, the animals were given one of the following daily subcutaneous injections for 6 weeks: (1) 0.1 mL / (10 g d) of saline for blank group; (2) D-galactose at 200 mg/(kg bw d) for D-galactose-induced model group; (3) D-galactose at 200 mg/(kg bw d) plus Octopus scraps hydrolysate P1 at 300 mg/(kg bw d) by gavage for hydrolysate group. The signs of mice were observed and the body weights were recorded once a week. Mice were sacrificed at the end of treatment and serum was collected and placed for 3 hrs. The serum was centrifugated under 3,000 rpm for 15 min, and the supernatant was collected for

further experiment. The mice were dissected and liver was separated from body. The 0.2 g of liver tissue was sampled and homogenized completely with 9 times saline to make 10% liver homogenate.

4.3.6. Determination of protein content of the liver tissue of mice.

Bradford method kit was used for measuring protein content of liver tissue in mice. The liver homogenate was diluted to 0.4% with saline. The 50 μg of 0.4% liver homogenate was mixed with 3 mL diluted coomassis brilliant blue chromogenic reagent. The mixture was placed for 10 min, and the absorbance value was detected under 595 nm.

4.3.7. Determination of antioxidant activity of hyderolysate on rats' plasma and liver tissue.

4.3.7.1. Determination of MDA concentration in rat plasma and liver homogenate

MDA is a degradation product of lipid peroxidation, which could be condensed with thiobarbituric acid. It yields a red product which has a special absorption peak at 532 nm. Original rat plasma and 10% liver tissue were used with MDA test kit to determine MDA concentration.

4.3.7.2. Determination of OH• free radical scavenging activity using rat plasma and liver homogenates

OH• free radical could react with almost all of the organic matter *in vivo*. Therefore, to determinate the OH• free radical scavenging activity of rat plasma and liver homogenates are very important. The rat serum was 20-fold diluted and 0.05% liver homogenate were applied to OH• free radical-scavenging test kit.

4.3.7.3. Determination of T-SOD activity in rat plasma and liver homogenates

Superoxide dismutase (SOD) plays a vital role in equilibrium of oxidation and antioxidation. SOD is the natural enemy of oxygen free radicals, and also SOD concentration is a key indicator to evaluate the effectiveness of any antioxidants.

The SOD activity was defined as: a SOD activity unit (U) in plasma (or liver homogenate) means the amount of SOD corresponding to the 50% inhibition rate in every milliliter of reaction solution. The original rat plasma and 0.25% liver homogenate were prepared for the detection of total superoxide dismutase (T-SOD) activity using a SOD test kits.

4.3.7.4. Determination of GSH-Px activity in rat plasma and liver homogenates

The reaction between H_2O_2 and reduced GSH could be catalyzed by GSH-Px. It yields H_2O and oxidized glutathione (GSSH). GSH can be used to remove peroxide metabolites, block lipid peroxidation chain reaction, and to protect cell membrane structure. The GSH-Px activity could be expressed by its enzymatic reaction rate. The activity of GSH-Px was obtained according to the consumption rate of GSH. The GSH-Px activity was defined as: a unit of enzyme activity (U) in plasma (or liver homogenate) means the every 1 μM deduction of GSH per minute at 37℃ in every 0.1 milliliter of reaction solution for 5 min (or in every milligram of protein tissue for 1 min. The non-enzymatic reaction should be neglected. In this experiment, 5-fold diluted rat plasma and 0.3% liver tissue were carried out for the detection of GSH-Px activity using glutathione peroxidase test kit.

4.3.7.5. Determination of CAT activity in rat plasma and liver homogenates

OH• free radical could be decomposed by catalase (CAT), thereby protecting the stability of the intracellular environment. CAT activity level is very important for the evaluation of the antioxidant capacity of antioxidants. Every decomposition of 1 μmol H_2O_2 in 1 milliliter of plasma (or 1 milligram of protein tissue) for 1 second was considered as one CAT activity unit (U). The original rat plasma and 10% of liver homogenate were applied to this method using catalase test kit to obtain the CAT activity.

4.3.8. Data processing

SPSS 17.0 statistical software was used to compare the signification single factor variance analysis and the difference significance test between the two groups.

4.4. Results and discussions

4.4.1. Standard curve of peptide concentration

Bovine serum albumin (BSA) was used as a substrate. The standard curve was shown in Fig. 25, with linear equation and the correlation coefficient of 0.9995.

4.4.2. Determination of free radical-scavenging activity

4.4.2.1. Determination of DPPH• free radical-scavenging activity

These peptides are present in the raw materials or are generated during food processing and protein hydrolysis by digestive enzymes. Many antioxidative peptides were extracted by enzymatic hydrolysis using various enzymes. One of the approaches for the effective release of bioactive peptides from protein sources is enzymatic hydrolysis, which is widely applied to improve and upgrade the functional and nutritional properties of protein (Kim, Je, and Kim, 2007). In the present study, we also utilised the two strains (V-2 and L-2) to ferment the octopus scraps and extract the antioxidative peptides, and we evaluated their antioxidative activity using free radical scavenging capacity, as described previously.

There were two peptides obtained from the two strains, labeled as P1 and P2. DPPH is a stable free radical that shows maximum absorbance at 517 nm. When DPPH radicals encounter a proton-donating substrate such as an antioxidant, the radicals would be scavenged and the absorbance is reduced (Shimada et al., 1992). The DPPH• scavenging-capacities of the two peptides at different concentrations were shown in Fig. 26. With the increased peptide concentration, DPPH• radical-

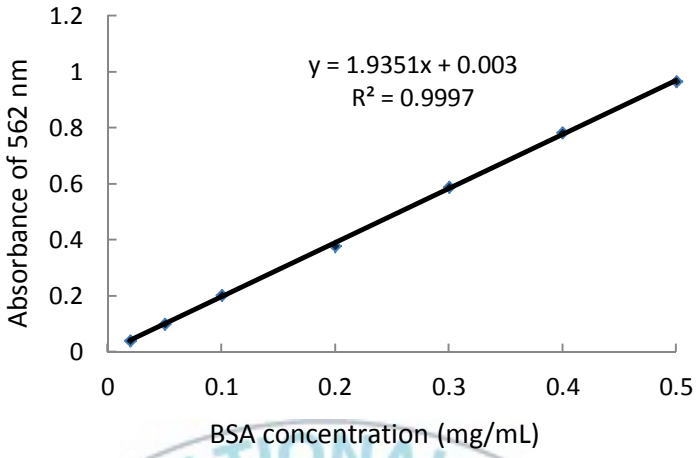


Fig. 25. Standard curve of peptide concentration

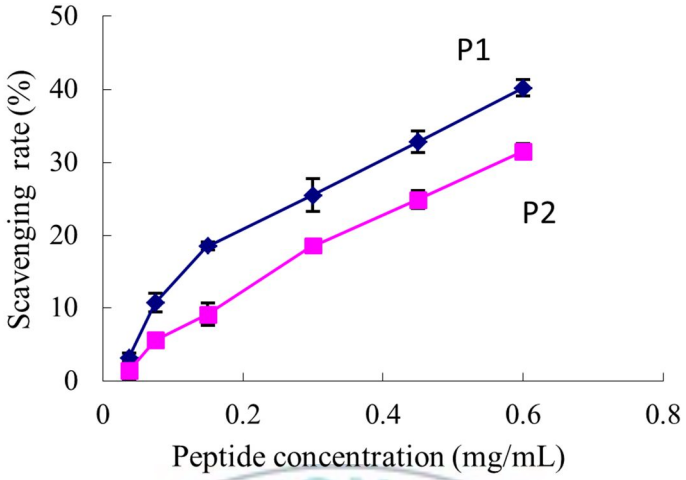


Fig. 26. DPPH· scavenging effects of polypeptide P1 and P2

scavenging rates of P1 and P2 were increased. At the peptide content of 0.6 mg/mL, the removal rates of P1 and P2 were 40% and 31%, respectively. The IC_{50} values were determined. The lower IC_{50} indicates higher free radical-scavenging ability. Hydrolysate P1 obtained by treatment with Fa-2 protease was found to be active radical-scavenger (IC_{50}= 0.73 mg/mL) followed by F1-1 protease hydrolysate P2 (IC_{50}= 0.93 mg/mL). Protein hydrolysates have been prepared and characterized from smooth hound (*Mustelus mustelus*) muscle showed similar DPPH• scavenging activities. Hyrolysate obtained by treatment with LMW protease showed the highest scavenging activity of 76.7% at 3 mg/mL, and the highest IC_{50} was 0.6 mg/mL among all the hydrolysates obtained from LMW protease (Ali et al., 2009).

4.4.2.2. Determination of OH• free radical-scavenging activity

The hydroxyl radical is an extremely reactive oxygen species that can react with everything in living organisms, especially with proteins, DNA and lipids. Hydroxyl radicals are capable of rapid initiation of the lipid peroxidation process by abstracting hydrogen atoms from unsaturated fatty acids (Aruoma, 1998). When the hydroxyl radical, generated by the Fenton reaction (containing $FeCl_3$, EDTA, H_2O_2 and ascorbic acid), attacks deoxyribose, it generates fragments that on heating with TBA form a pink chromogen, which can be quantified spectrophotometrically at 536 nm (Halliwell and Guttridge, 1989). Therefore, removal of hydroxyl radicals can protect humans against some diseases (Je, Park, and Kim, 2005).

The OH• free radical-scavenging capacities of different peptide concentrations of P1 and P2 were shown to have great scavenging rates. As the peptide concentrations increased, hydroxyl radical-scavenging rates of P1 and P2 increased. The scavenging activity of P1 is higher than that of P2 (Fig. 27). When the peptide concentration was 0.6 mg/mL, the clearance rates of P1 and P2 were 60.1% and 42.3%, respectively. Naourez et al. (2012) reported that the ZPHs

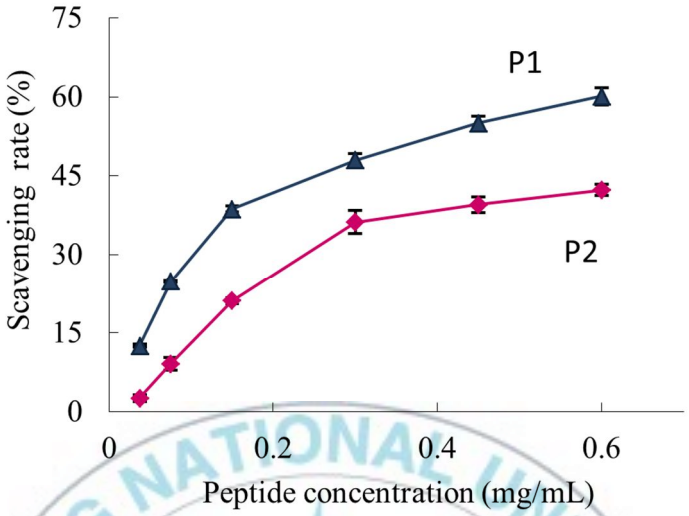


Fig. 27. OH· scavenging effects of polypeptide P1 and P2

hydrolysates extracted from muscle of zebra blenny (*Salaria bacilisca*) showed relatively good hydroxyl radical-scavenging abilities ranged from 51% to 56% at a concentration of 6 mg/mL. In this study, the hydroxyl radical-scavenging abilities of P1 and P2 were higher than those of the report. The IC_{50} values of hydroxyl radical-scavenging ability were 0.4 mg/mL and 0.62 mg/mL for P1 and P2 hydrolysates, respectively. You, Zhao, Regenstein, and Ren (2011) reported that loach peptide showed hydroxyl radical scavenging ability with IC_{50} of 17.0 mg/mL. However, The IC_{50} values of the both experimental hydrolysates were lower than those of PYFNK (0.24 mg/mL) from ethanol-soluble protein hydrolysate of *S. lewini* (Wang et al., 2012), but were similar with NGLEGLK (0.313 mg/mL) and NADFGLNGLEGLA (0.612 mg/mL) from giant squid protein hydrolysate (Rajapakse, Mendis, Byun, and Kim, 2005). So, peptides P1 and P2 had good antioxidant activities against hydroxyl radicals, which indicated that they could be used as the scavenging agents for protecting hydroxyl radical-induced damage in living body.

4.4.2.3. Determination of O_2^{-}• free radical-scavenging activity

Superoxide radical is known to be very harmful to cellular components as a precursor of more reactive oxidative species, such as single oxygen and hydroxyl radicals (Aurand et al., 1977). Furthermore, superoxide radical is considered to play an important role in the peroxidation of lipids (Dahl and Richardson, 1978). Therefore, studying the scavenging effects of peptides P1 and P2 on superoxide radicals is one of the most important ways of clarifying the mechanism of antioxidant activity.

The O_2^{-}• free radical-scavenging capacities of different peptide concentrations of P1 and P2 were shown in Fig. 28. With the increases of peptide concentrations of P1 and P2, the scavenging rates of superoxide anion radicals also were increased by following a flat upward trend. When the polypeptide concentration reached up to 0.3 mg/mL, the upward trend started to level off. The clearance rate

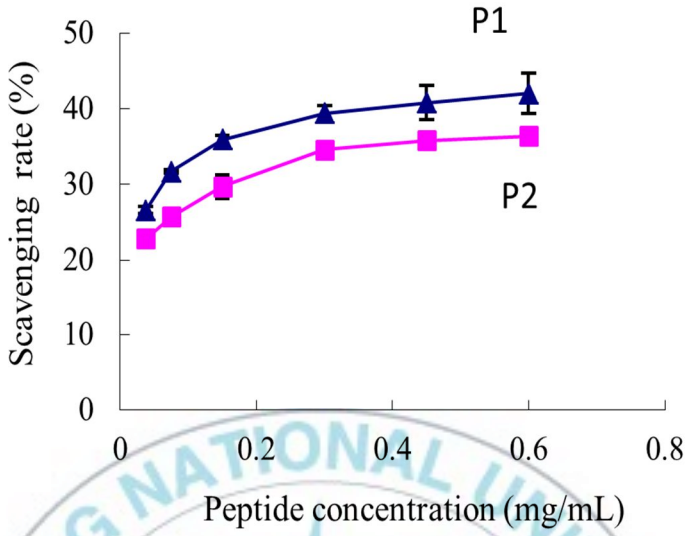


Fig. 28. O_2^{-}•scavenging effects of polypeptide P1 and P2

of P1 was stronger than that of P2. The scavenging rates of P1 and P2 were 43% and 36%, respectively, while the concentration of peptide went up to 0.6 mg/mL. The IC_{50} values were 0.83 mg/mL and 1.08 mg/mL for each peptide. It was reported that peptides from protein hydrolysates could strong eliminate superoxide radicals, such as WGPH (0.4 mg/mL) obtained from wheat germ (Kexue et al., 2006), NADFGLNGLEGLA (IC_{50} 0.864 mg/mL) and NGLEGLK (IC_{50} 0.419 mg/mL) from giant squid muscle protein hydrolysate (Rajapakse, Mendis, Byun, et al., 2005). Compared with those reported peptides, P1 and P2 showed strong superoxide radical scavenging activity.

4.4.3. Effect of octopus scraps enzymatic hydeolysate on body weight of rats.

Aging is the result of metabolism disorder. The disorder of glycometabolism will lead to the metabolic abnormality of heart, liver, kidney, brain and other vital organs, finally aging appears (Cai, Shen, Qu, He, and Wang, 2008). Change in body weight is one of important physiological indicators of aging. Experimental mice were bred at temperature of 20 ± 2℃, with natural light and self-feed. Daily subcutaneous injections and gavage were carried out for 6 weeks as described before. The body weights of rats were recorded once a week, and the change of body weight in rats was shown in Fig. 29. From the results, the body weight of each rat group shows a growth trend. However, the growth rate of model group lagged behind the hydrolysates group and the control group since the second week. It was found that the body weight of mice was much lower in all treatment groups compared to the hydrolysate group ($P<0.01$), the decrease ratios were 33.49%. It may probably be that the effect of D-galactose-induced aging has trickled in gradually, which the octopus scarps enzymatic hydeolysate could really slow the aging caused by D-galactose from the results. Chen et al. (2007) reported that the polypeptide extracted from peanut could also display a capability to maintain the grow speed of body weight of oxidative-injured rats. These results indicate that

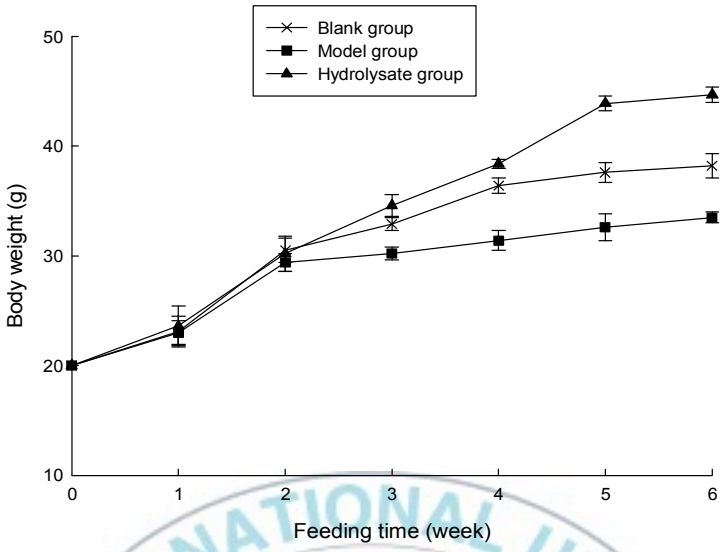


Fig. 29. Effect of octopus scraps hydrolysate on body weight of rats.

octopus scarps hydrolysate P1 displays significant anti-aging activity. Recently, many research papers also indicate that the aging is relative to body weight and the free radicals in the metabolism (Balz et al. 1988; Beckman et al. 1998; Grune 2000; Stadtman 1992), and our results are in agreement with reports of other workers which suggest that feeding a protease hydrolysate-enriched diet to experimental animals with the treatment of D-galactose can help to maintain body weight and keep healthy. (Anila and Vijayalakshmi, 2003).

4.4.4. Effect of antioxidant activities of hydeolysate on serum and liver tissue.

4.4.4.1. Effect of hydrolysate on MDA concentration of serum and liver tissue.

MDA is a hallmark of lipid peroxidation and displays cytotoxic activity. The amount of MDA reflects the formation of lipid peroxides, which can damage cell membranes and hepatic tissue (Chen et al., 2011b). The concentration of MDA is an important indicator of the evaluation of the antioxidant effects. The mice were sacrificed after the last time gavage and subcutaneous injection. The concentration of MDA in plasma and liver homogencates of mice were measured and the results were shown in Table 8. As the results, MDA contents of serum and liver homogenate in the aging model had a significant higher level than that in other groups ($P<0.01$). That result indicates that the subacute aging model was successfully established by D-galactose. The MDA content of hydrolysate group decreased, compared with that of model group with a significant difference ($P<0.01$), showing 31.91% decrease in plasma and 35.65% in liver tissue, respectively. The MDA concentration is almost same with blank group, which verifies that octopus scarps peptide could significantly inhibit the MDA level in plasma and liver tissue of the D-galactose-induced rats, and maintain the MDA level within the normal range. Hayet et al. (2012) reported that protein hydrolysates WSP and SPHs from sardinelle (*Sardinella aurita*) showed similar antioxidant activities. Administration of WSP and SPHs at 5% (SPHA1, SPHA21

Table 8. Effect of hydrolysate on MDA concentration of serum and liver tissue.

Groups	MDA content in plasma (nmol/mL)	MDA content in liver tissue (nmol/mgprot)
Blank	10.48 ± 0.32	0.68 ± 0.23
Model	15.98 ± 0.83**	1.15 ± 0.37**
Hydrolysate	10.88 ± 0.94$^{\Delta\Delta}$	0.74 ± 0.09$^{\Delta\Delta}$

*: There was significant difference compared to blank group (P<0.05)
**: There was markedly significant difference compared to blank group (P<0.01)
$^{\Delta}$: There was significant difference compared to model group (P<0.05)
$^{\Delta\Delta}$: There was markedly significant difference compared to model group (P<0.01)

and SPHEE) to rats fed D-galactose decreased significantly (ranged from 38 to 56%) the level of MDA compared with the model group. Wang and Ren (2010) observed that peptide (BSP, 800 mg/kg bw d) from black soybean also exhibited strong capability to decrease the level of MDA in serum (24.29%) and liver tissue (21.19%) of D-galactose-induced aging mice with a significant difference ($P < 0.01$). According to the results, we could draw a conclusion that the P1 hydrolysate from octopus scraps could evidently decrease MDA level in the blood serum, liver.

4.4.4.2. Effect of hydrolysate on OH• free radical-scavenging activity of serum and liver tissue.

D-galactose, which has the ability to induce oxidative stress *in vivo*, can be used to mimic natural aging in order to screen antioxidants (Luo et al., 2010; Qiao et al., 2009). It has been demonstrated that aging, as a result of diminished antioxidant protection, is associated with a reduction in antioxidants and incremental increases in lipid peroxidation (Katrin et al., 2006). The major antioxidant enzymes, acting as the first line of defense against antioxidants, can prevent the formation of toxic compounds so as to protect against oxidative stress and tissue damage (Chen et al., 2011).

Compared to the model group, the OH• free radical-scavenging activities in serum and liver tissue of blank group and hydrolysate group were distinctly increased with a significant difference at the level of $P<0.05$ (Table 9). The increase rates were 36.37% and 44.69% for plasma and liver tissue in hydrolysate group, respectively. It has been observed that the scavenging activity of hydrolysate group was slightly higher than that of blank group, which could be explained that the active polypeptide of octopus scarps hydrolysates was directly absorbed in mice, and entered the blood and tissues to provide anti-oxidant for the body. The reason why the increase rate of scavenging activity of liver homogencates was the highest is probably the active peptides of octopus scraps

Table 9. Effect of hydrolysate on OH• free radical-scavenging activity of serum and liver tissue

Groups	OH•scavenging activity in plasma (U/mL)	OH•scavenging activity in liver tissue (U/mgprot)
Blank	1684.89 ± 34.85	632.88 ± 32.89
Model	1238.68 ± 54.90*	523.42 ± 34.67*
Hydrolysate	1689.03 ± 26.95$^{\Delta}$	757.33 ± 44.85$^{\Delta\Delta}$

*: There was significant difference compared to blank group (P<0.05)
**: There was markedly significant difference compared to blank group (P<0.01)
$^{\Delta}$: There was significant difference compared to model group (P<0.05)
$^{\Delta\Delta}$: There was markedly significant difference compared to model group (P<0.01)

hydrolysate could be accumulated in rat liver tissue. Wang et al. (2013) demonstrated that administration of fucoidan hydrolysate (200 mg/kg bw d) from *Laminaria japonica* was able to prove the OH• free radical-scavenging activities in the serum and liver level in D-galactose-induced aging rat with markedly significant difference ($P<0.01$). The results indicated that the hydrolysate P1 from octopus scarps displayed potential antioxidant properties.

4.4.4.3. Effect of hydrolysate on T-SOD activity of rats plasma and liver tissue.

Enzymatic antioxidant systems are important in scavenging free radicals and their metabolic products, as well as in maintaining normal cellular physiology, promotion of immunity, and prevention of various diseases (Sözmen et al., 2001). SOD is one of the main enzymes used to scavenge oxygen free radicals *in vivo*.

After daily subcutaneous injections and gavage for 6 weeks as described before, the mice were sacrificed and the blood serum and liver tissue were applied to the measurement of T-SOD activity. The results were shown in Table 10. The T-SOD activities in plasma and liver tissue of blank group and hydrolysate group were obviously higher than those of model group with a significant difference ($P<0.05$). Compared with model group, the activity increase rate was 35.56% in plasma and 27.34% in liver tissue of hydrolysate group. Hayet et al. (2012) also studied the correlation between hydrolysates from sardinelle (*Sardinella aurita*) and SOD activity in D-galactose-induced aging rats. The treatment of model group rats with hydrolysates WSP and SPH from muscle of sardinelle increased the SOD activity significantly (upto 96%). Ding et al. (2011) studied the anti-fatigue and anti-oxidation of jellyfish collagen hydrolysate (JCH) *in vivo*, the results showed that mice in the Aging Model was showed a decreased level in SOD activity of serum compared with the hydrolysate group ($P<0.05$) at a dose of 10 mg/kg bw d. The decrease rate was 15.32%. The antioxidant activity of proteins is due to complex interactions between their ability to inactivate reactive oxygen species, scavenge free radicals, chelate prooxidative transition metals (Stadtman and Levine, 2003).

Table 10. Effect of hydrolysate on T-SOD activity of serum and liver tissue

Groups	T-SOD activity in plasma (U/mL)	T-SOD activity in liver tissue (U/mgprot)
Blank	204.33 ± 14.55	214.23 ± 9.45
Model	146.38 ± 11.53*	159.67 ± 12.26*
Hydrolysate	198.43 ± 7.34$^{\Delta}$	203.33 ± 5.85$^{\Delta}$

*: There was significant difference compared to blank group (P<0.05)
**: There was markedly significant difference compared to blank group (P<0.01)
$^{\Delta}$: There was significant difference compared to model group (P<0.05)
$^{\Delta\Delta}$: There was markedly significant difference compared to model group (P<0.01)

Therefore, different antioxidant activities obtained from various administrations treated with different enzymatic hydrolysates would be changed from type to type. In the present study, the results estimate that the hydrolysate P1 from octopus scraps could significantly ($P<0.05$) improve the T-SOD activity in serum and liver tissue of D-galactose-induced aging model rats, which verified that the hydrolysate P1 plays an important role in protecting biological system.

4.4.4.4. Effect of hydrolysate on GSH-Px activity of rats plasma and liver tissue.

The effect of administration of hydrolysate P1 to rats fed cholesterol-enriched diet on antioxidant enzyme activities of GPx was also determined. D-galactose was used to induce oxidative stress *in vivo* by diminishing the antioxidant protection, which is associated with a reduction in antioxidants and incremental increases in lipid peroxidation (Katrin et al., 2006). GSH-Px could catalyze the reaction of H_2O_2 and reduced GSH. H_2O and oxidized glutathione (GSSH) could be produced, which can be used to remove and protect cell membrane structure. As the results shown in Table 11, the GSH-Px activities in plasma and liver tissue of blank group and hydrolysate group were much higher than those of model group ($P<0.01$). The increase rates of hydrolysate group were 22.83% in plasma and 16.97% in liver homogenate, showing higher values than those of model group. The anti-oxidative role of protein hydrolysates from sardinelle (*Sardinella aurita*) have been intensively studied in D-gal-induced aging (Hayet et al., 2012). In the investigation antioxidative enzyme of GSH-Px significantly decreased in D-galactose treated group as compared to hydrolysate-treated group ($P<0.01$) at a concentration of 5%. The GSH-Px activities of protein hydrolysates (SPHA1, SPHA21 and SPHEE) increased by 60%, 81.4% and 55%, respectively. Fan et al. (2009) investigated the protective effect of troxerutin against D-galactose-induced mice, and the results showed that D-gal-treated mice markedly decreased antioxidant ability of SOD, CAT and GSH-Px activities ($P<0.01$), compared to the vehicle control at a dose of 150 mg/kg bw d. Even different compounds have

Table 11. Effect of hydrolysate on GSH-Px activity of rats plasma and liver tissue.

Groups	GSH-Px activity in plasma (U/mL)	GSH-Px activity in liver tissue (U/mgprot)
Blank	825.44 ± 19.43	342.56 ± 6.67
Model	664.32 ± 11.28**	288.76 ± 15.47**
Hydrolysate	815.98 ± 25.46$^{\Delta\Delta}$	337.75 ± 19.12$^{\Delta\Delta}$

*: There was significant difference compared to blank group (P<0.05)
**: There was markedly significant difference compared to blank group (P<0.01)
$^{\Delta}$: There was significant difference compared to model group (P<0.05)
$^{\Delta\Delta}$: There was markedly significant difference compared to model group (P<0.01)

been compared, troxerutin (a rutoside derivative) has been identified to have effectively antioxidative and anti-inflammatory activities in many studies (Yang, Wang, and Hu, 2006; Maurya et al., 2005; Li et al., 2005), could serve as a comparison object for the assessment of antioxidant ability of octopus by-produce hydrolysate P1 *in vivo* experiment, and these suggested that the protective effect of octopus by-produce hydrolysate P1 may be related to the increased ability of scavenging H_2O_2. Hence, the results released that the octopus scarps hydrolysates could strongly promote the GSH-Px activity in plasma and liver tissue of D-galactose-induced aging mice.

4.4.4.5. Effect of hydrolysate on CAT activity of rats plasma and liver tissue.

CAT catalyzes the breakdown of H_2O_2 to form water and O_2. The antioxidant defense mechanisms become weaker during chronic aging and other disease conditions (Powers & Lennon, 1999). So the CAT activity was also determined as a common indicator for antioxidative effect *in vivo* experiment. After daily subcutaneous injections and gavage for 6 weeks, the mice were sacrificed and the blood serum and liver tissue were applied to the measurement of CAT activity. As the results shown in Table 12, the CAT activity in plasma of hydrolysate group was 33.85% showing higher value than that of model group ($P<0.01$). In liver tissue, the CAT activity of hydrolysate group was 40.34% showing higher value than that of model group. Our results are in agreement with reports of other workers which suggest that injecting a D-galactose to experimental animals depresses their antioxidant system due to increased lipid peroxidation and formation of free radicals (Ho, Liu, and Wu, 2003). Hayet et al. (2012) reported that the protein hydrolysates from sardinelle (*Sardinella aurita*) showed greater antioxidant activity in D-gal-induced aging. The results demonstrated that the level of CAT activity in the liver tissue of D-galactose-treated mouse was significantly lower ($P<0.01$) than that in the hydrolysate-treated mouse at a dose of 5%. The D-galactose-induced group supplemented with protein hydrolysates of

Table 12. Effect of hydrolysate on CAT activity of rats plasma and liver tissue.

Groups	CAT activity in plasma (U/mL)	CAT activity in liver tissue (U/mgprot)
Blank	5.34 ± 0.35	13.65 ± 1.34
Model	3.87 ± 1.21**	9.42 ± 0.67*
Hydrolysate	5.18 ± 0.54$^{\Delta}$	13.22 ± 1.66$^{\Delta\Delta}$

*: There was significant difference compared to blank group (P<0.05)
**: There was markedly significant difference compared to blank group (P<0.01)
$^{\Delta}$: There was significant difference compared to model group (P<0.05)
$^{\Delta\Delta}$: There was markedly significant difference compared to model group (P<0.01)

SPHA1, SPHA21 and SPHEE has improved CAT activities in liver at the rates ranged from 58 to 72.3%. The present results are also in agreement with that of You, Zhao, Regenstein and Ren (2011) which showed that peptides from loach (*Misgurnus anguillicaudatus*) prepared by papain digestion reduce oxidative stress *in vivo* and they increased the SOD, CAT and GSH-Px activities. The increase may have been due to the activation of enzymes by hydrolysates, thereby resulting in a lower superoxide anion level. The result proves that the decrease of CAT activity in serum and liver tissue could be induced effectively by the octopus scraps hydrolysates.

SOD dismutates superoxide radicals to form hydrogen peroxide. CAT and GSH-Px prevent the formation of hydroxyl radicals by decomposing hydrogen peroxide into water and oxygen. Therefore, these enzymes act cooperatively against free radicals in the defense of active oxygen compounds (Li et al., 2007; Pan and Mei, 2010). Meanwhile, MDA is a cytotoxic product of lipid peroxidation. The formation of lipid peroxides can damage hepatic tissue by causing incomplete cell membranes (Chen et al., 2011; Huang et al., 2011). Our results showed that the antioxidant enzyme activities in mice serums and livers were markedly decreased in the D-galactose-induced aging mice, while the MDA levels both in serums and livers significantly increased.

Many protein antioxidant mechanisms are dependent on amino acids composition, and all 20 amino acids found in proteins have the potential to interact with free radicals (Elias et al., 2008). The aromatic amino acid residues are prime to targets for oxidation, such as phenylalanine residues, tyrosine residues. Methionine has been proposed to be an important free radical scavenger in proteins in biological systems (Stadtman & Levine, 2003). This is because methionine residues are very labile to oxidation and can scavenge radicals before they are able to attack other amino acid residues. The role of methionine as an

important free radical scavenger in biological systems can also be argued based on the ubiquity of methionine sulfoxide reductases (Elias et al., 2008).

In this study, the main composition was the mixture of peptides and amino acids. The antioxidant and anti-fatigue activity of octopus scraps hydrolysates were related to amino acids and unique properties of peptides contributed by their chemical composition and physical properties.

4.5. Conclusions

The strains obtained from octopus tracts were fermented with octopus scraps to get a crude peptide, which was purified by ultrafiltration. P1 and P2 peptides were obtained. The DPPH• radicals and hydroxyl radicals-scavenging activities were studied at different peptide concentrations of P1 and P2. The results showed that P1 and P2 have scavenging capability on all of the radicals. The scavenging activities of 0.6 mg/mL P1 and P2 were 40% and 31% in DPPH• radical, and 60.1% and 42.3% in OH• radical, and 43% and 36% in O_2^-• radical, respectively.

The antioxidant activity *in vitro* experiments indicated that the octopus scrapes hydrolysates had high antioxidant activity. P1 was applied for animal experiments to validate the antioxidant activity of hydrolysates *in vivo*.

The 200 mg/(kg bw d) of D-galactose was injected into the model group rats. Compared with the blank group, the MDA concentration was increased obviously in plasma and liver tissue, while the OH• radical scavenging activity, T-SOD activity, GSH-Px activity and CAT activity were observed to decrease significantly. The results showed that, with 200 mg/(kg bw d) of D-galactose daily injection, it is feasible to establish the D-galactose-induced subacute aging model.

In hydrolysate group, 200 mg/(kg bw d) of D-galactose and 300 mg/(kg bw d) of octopus scraps hydrolysate P1 were used for hydrolysate group daily treatment. After 6 weeks, the MDA levels in rats plasma and liver homogenate of hydrolysate group were decreased significantly, compared with those of model

group. In addition, the OH• radical-scavenging activity, T-SOD activity, GSH-Px activity, and CAT activity of hydrolysate group were observably increased. The results demonstrated that the antioxidative capability of octopus scraps hydrolysate was strong *in vivo*, which could significantly inhibit the symptom of D-galactose-reduced aging. It probably because the active peptide component of octopus scraps hydrolysate could be degraded and absorbed directly after ingestion. Therefore, the excess free radicals in rats could be removed, the lipid peroxidation reaction could be inhibited, and the endogenous antioxidant enzymes in the course of their metabolism could be active to maintain a balance against *in vivo* oxidation and antioxidation.

Summary

According to all the studies described above, we conclude that:

1. Two protease high-yield strains were successfully isolated from octopus gut named as V-2 and L-2. Strain V-2 has 99.2% homology with *Bacillus flexus* 3xWMARB-5. L-2 strain was identified as *Pseudoalteromonas okeanokoites*.

2. Two proteases, named as Fa-2 and F1-1, were obtained by strain V-2 and L-2 were purified, and two electrophoresis grade-pure proteases were obtained.

3. The strains obtained from octopus tracts were fermented with octopus scraps to get a crude peptide which was purified by ultrafiltration, and P1 and P2 were obtained.

4. The DPPH• radicals, hydroxyl radicals and superoxide radicals scavenging activities were studied at different peptide concentration of P1 and P2. Good antioxidant activity *in vitro* experiments verified that the octopus scrapes hydrolysates had high antioxidant activity.

5. The P1 was applied for animal experiments to validate the antioxidant activity of hydrolysates *in vivo*. The D-galactose-induced subacute aging model was established by injecting D-galactose into the model group rats.

6. After 6 weeks, the MDA levels in rats plasma and liver homogenate of hydrolysate group were decreased significantly, compared with those of model group. In addition, the OH• radical-scavenging activity, T-SOD activity, GSH-Px activity, and CAT activity of hydrolysate group were observably increased.

7. The results demonstrated that the antioxidative capability of octopus scraps hydrolysate was strong *in vivo*, which could significantly inhibit the symptom of D-galactose-reduced aging.

With an increasing number of wastes during the processing of marine food product in China, the two strains could be applied to these scraps and which could

be made into high value-added products through some suitable methods, so that the problem of environmental pollution can be solved. In the meantime, greater economic benefits shall be created for the company.

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Octopus vulgaris 의 장관으로부터 분리한 단백질분해효소 생산 균주와 생성된 효소의 특성

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초록

프로티아제는 자연에서 널리 분포되어있으며 미생물은 이런 효소들의 우선적 원천이다. 미생물에서 추출된 효소들은 광범위한 생화학적 다양성, 대량 배양의 실현가능성과 유전적 조작의 용이성 때문에 널리 연구되어 왔다. 이 연구에서, *Octopus vulgaris* 의 장관으로부터 프로테아제 생성 박테리아를 분리시키고, 확인하였다. 분리된 균주에서 프로테아제 최적 생산 조건을 조사하였다. 분리된 균주에서 생산된 프로테아제는 황산암모늄 침전 반응을 이용하여 정제하였고, 이온 교환 크로마토그래피를 사용하였다. 한편, 정제된 프로테아제의 특성 역시 연구되었다.

분리된 균주의 잠재적 산업 적용 가치를 연구하기 위해서, 균주는 문어 가공 부산물을 이용하여 배양되었다. 그리고 가수분해물의 항산화 활성은 *in vivo* 와 *in vitro* 조건에서 측정되었고, 그 결과로서 V-2 와 L-2 의 프로테아제 수율이 높은 두 균주가 성공적으로 분리되었다. V-2 는 *Bacillus flexus* 3xWMARB 와 99.2%의 상동함을 보였고, L-2 균주는 *Psedoalteromonas okeanokoites* 로 확인되었다. V-2 균주는 탄소원으로 fructose 와 질소원으로서 펩톤을 이용하여 초기 pH 8.0, 배양온도 30℃ 조건에서

3.5 일에서 배양하였을 때, 높은 활성의 프로테아제 생산하였다. L-2 균주는 수용성 전분을 탄소원으로, 펩톤을 질소원으로 사용하였고, 초기 pH 8.0 에서 배양온도는 19℃에서 최적 조건이었다.

두 가지의 균주 V-2 와 L-2 에서 얻은 정제된 프로테아제를 Fa-2 와 F1-1 으로 명명하였다. 정제배수는 각각 2.5 와 1.7 이었으며, 활성 회복도는 각각 12.5%와 7.5%이었다. Fa-2 의 경우에는 분자량 61.6kDa 이고 최적 온도와 pH 는 40℃와 pH 9.0 이며, 열안전성을 가지고 있었다. F1-1 은 61.4kDa 의 분자량을 가지고 있고, 최적온도는 40℃이며, 뛰어난 열안전성을 나타내었다. 두 가지의 균주와 문어의 부산물을 이용하여 얻은 가수분해물을 P1 과 P2 라고 명명하고 DPPH· radical, hydroxyl radical, superoxide radical 소거능을 조사하였다. P1 이 P2 보다 높은 radical 소거능을 나타내었고, 결과에 따라 P1 가수 분해물을 이용하여 *in vivo* 상태에서의 항산화 능력을 측정하였다. 쥐의 D-galactose 유도 노화 모델을 설정하고, P1 가수분해물이 혈청과 간에서의 항산화 효과는 노화증상을 약화시키는 잠재적 능력을 가지고 있다고 결정지었다. 이 결과 두 균주가 우선 문어의 장관에서 성공적으로 분리되었고, *in vitro, in vivo* 모두 높은 항산화능을 가지고 있으며, 상업적 부분에 있어서도 적용할 수 있는 뛰어난 잠재력을 가지고 있다. 이 결과는 앞으로의 연구에 기초로 제공될 것이다.

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