

## Protease Properties of Protease-Producing Bacteria Isolated from the Digestive Tract of *Octopus vulgaris*

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A high protease-producing strain was isolated and identified from the digestive tract of *octopus vulgaris* by detecting a hydrolysis circle of protease and its activity. The strain was identified by morphology observation, biochemical experiments, and 16S rRNA sequence analysis. The protease obtained from the strain was purified by a three-step process involving ammonium sulfate precipitation, carboxy methyl-cellulose (CM-52) cation-exchange chromatography, and DEAE-Sephadex A50 anion-exchange chromatography. The properties of protease were characterized as well. The strain *Bacillus* sp. QDV-3, which produced the highest activity of protease, was isolated. On the basis of the phenotypic and biochemical characterization and 16S rRNA gene-sequencing studies, the isolate was identified as follows: domain: *Bacteria*; phylum: *Firmicutes*; class: *Bacilli*; order: *Bacillales*; family: *Bacillaceae*; and genus: *Bacillus*. The isolate was shown to have a 99.2% similarity with *Bacillus flexus*. A high active protease designated as QDV-E, with a molecular weight of 61.6 kDa, was obtained. The enzyme was found to be active in the pH range of 9.0-9.5 and its optimum temperature was 40°C. The protease activity retained more than 96% at the temperature of 50°C for 60 min. Phenylmethylsulfonyl fluoride (PMSF) inhibited the enzyme activity, thus confirming that this protease isolated from *Bacillus* sp. QDV-3 is an alkaline serine protease. Metal ions, Mn<sup>2+</sup> and Mg<sup>2+</sup>, were determined to enhance the protease activity, whereas Ba<sup>2+</sup>, Zn<sup>2+</sup>, and Cu<sup>2+</sup> were found to inactivate the enzyme.

**Key words** : Identification, intestinal bacteria, octopus vulgaris, protease, purification

### Introduction

Enzymes have attracted attention from researchers all over the world because of the wide range of physiological, analytical and industrial applications. Among these enzymes, proteases execute a large variety of functions and have important biotechnological applications. Proteases represent one of the three largest groups of industrial enzymes and find application in detergents, leather industry, food industry, pharmaceutical industry and bioremediation processes [10]. For instance, Fish protein hydrolyzate, Soy protein hydrolyzate, and zein have been produced with high nutritive value and therapeutic effects by the application of microbial protease in food [8, 17, 21].

Protease are widespread in nature, 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 [12]. *Bacillus* produces a wide variety of extra-cellular enzymes, including proteases. Several *Bacillus* species involved in protease production are e.g. *B. cereus*, *B. sterothermophilus*, *B. mojavensis*, *B. megaterium* and *B. Subtilis* [1, 2, 9, 20, 23, 24].

The *octopus vulgaris* (common octopus) is the most studied commercial octopus of all species. The major consumer countries of octopus are Japan, South Korea, followed by Argentina and China. During the process of octopus digestion, the endogenous digestive enzymes, which are secreted to the lumen of the alimentary canal, originate from the oesophageal, gastric, phloric caeca and intestinal mucosa and from the pancreas [20]. The presence of endogenous digestive enzymes in aquatic organisms has been reported in many studies [4, 5]. Although many protease-producing bacterial have been studied, the discovery of new ones is still significant for both commerce and research. This is especially true for protease-producing bacterial from extreme environments such as the deep sea and Antarctica, because of

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their novel characteristics.

In this study, intestinal protease producing strains were isolated from octopus itself. Morphological and molecular biological identifications were carried out. The protease produced by the intestinal bacteria was purified and different parameters were detected to obtain the protease properties.

## Materials and Methods

### 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°C, and water has to be changed twice a day.

### Methods

#### 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.

#### Screening

The bacterial suspension prepared above was diluted and applied on screening medium [3]. The mediums were sealed and put into the thermostat incubator under 25°C for 3 days. The well growing colony which has the largest proteolytic transparent circle on the casein medium was selected.

#### Measurement of protease activity

Method used for protease activity measurement was Azocasein method [25]. 1% Azocasein was dissolved in 0.02 mol/l pH 7.0 Phosphate Buffered Saline (PBS) as the substrate, 50 µl of crude enzyme was mixed with azocasein buffer thoroughly, the mixture was incubated at water bath oscillator at a speed of 140 rpm, under 37°C for 1 hour. The reaction was terminated by adding 300 µl 10% (w/v) trichloroacetic acid (TCA) 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 mixed with 100 µl of 1 mol/l NaOH. After vortexing, the absorbance (A) was analyzed under 450 nm wavelength to measure enzyme activity.

#### Identification of strains

##### 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".

##### Genomic DNA extraction and 16S rRNA analysis

The identification was conducted by 16S rRNA analysis. Genomic DNA from strain obtained from the tract of octopus was prepared using a Genome Extraction Kit (Bioteke, China). The primers for the PCR reaction were universal bacteria primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTACCTTGTTACGACTT-3'). The amplification was conducted by subjecting the samples to an initial denaturation step of 4 min at 98°C and then 30 cycles of 35 seconds of denaturation at 95°C, annealing at 55°C for 1.5 min, and 1.5 min at 72°C for extension. The final step consisted of 10 min at 72°C and storage at 4°C. The amplified 16S rRNA was cloned into strain *E.coli* 110 and sequenced by Sangon Biotech Company (China)

##### Preparation of crude enzyme solution

Strain QDV-3 was incubated for 3.5 days with initial medium pH 8.0 and culture temperature 30°C. The fermentation broth was centrifuged at a speed of 10,000 rpm for 10 min to remove cells and other insoluble materials, and the supernatant was referred to as crude enzyme solution, which has to be placed in 4°C before using.

##### Purification of protease

The organism was grown for 3.5 days as described previously. The cells were separated by centrifugation, and the supernatant was fractionated by precipitation with an ammonium sulfate solution of 80% of saturation. The ammonium sulfate was gradually added into the enzyme solution with slowly stirring until it dissolved completely. All the subsequent steps were carried out at 4°C. The protease was resuspended in 50 mM phosphate buffer, pH 7.0, and dialyzed against the buffer for three days.

The sample after ammonium sulfate precipitation was loaded into the cellulose CM-52 cation exchange column (1.2 cm × 40 cm). Firstly, the enzyme was eluted with a 0.02 mol/l phosphate buffer (pH 7.0), and then was fractionated with a linear gradient of 0 to 2 mol/l sodium chloride in the same buffer at a flow rate of 60 ml/h. A fraction collector was used to collect the purified protease, and the enzyme activity from each receiving tube was measured.

For further purification, the activated resultant protease received from Cellulose CM-52 cation exchange chromatog-

raphy was loaded onto a DEAE-Sephadex A50 anion exchange column (1.6 cm × 40 cm) which had been equilibrated with a 0.02 mol/l Tris-HCl buffer (pH 8.5), then the unadsorbed materials were washed from the column with the same buffer. The rest was eluted with a linear gradient of 0 to 2 mol/l sodium chloride in the same buffer at a flow rate of 60 ml/h. The protease fractions were collected and scanned for their  $A_{280}$ , and assayed for protease activity.

#### Determination of molecular weight

The molecular weight of purified protease was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with casein as standard protein. 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 purified protein samples collected from DEAE-Sephadex A50 anion exchange chromatography were run on a SDS-PAGE. After completion of the electrophoresis, the gel with protein standard bands was cut down for conventional staining and destaining.

#### Effects of temperature on proteases and thermal stability

The proteases were placed at different temperatures from 10 to 70°C for one hour, and the activity of proteases was measured. For thermal stability, the purified proteases were put under the condition in which the temperature ranged from 10 to 80°C respectively for 60 min. The reaction was stopped in ice water and the residual protease activity was measured under standard assay condition mentioned before.

#### Effects of pH on proteases

The pH effect of the protease was studied from 7.0 to 13.0, and the buffers with different pH were mixed with the purified protease at a ratio of 1:1. The mixture was stored at 4°C for one hour, and the method mentioned before was utilized as the measurement for enzyme activity value.

#### Effects of metal ions and surfactants on proteases

The protease was pre-incubated for 30 min with different metal ions including  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Zn}^{2+}$ , at an optimized concentration of 2.0 mM and the remaining activity was determined after the incubation period. Phenyl methyl sulfonyl fluoride (PMSF) and ethylene diamine tetra acetic acid (EDTA) were also tested against the enzyme under optimum reaction conditions. Aliquots of the protease were pre-incubated with the different protease inhibitors at 5.0 and 2.5 mM for 30 min at 37°C and the residual activity of the enzyme was assayed.

## Results and Discussion

Screen of protease producing bacteria from octopus vulgaris gut

The ability of enzyme production was demonstrated by the diameter of transparent circle in screening medium produced by protease producing bacteria. In this experiment, there were total 6 strains were detected to produce transparent circles on casein medium, and the protease activity of each strain was detected. As the results shown in Table 1, the D/d value of QDV-3 and QDE-5 were higher than that of the others. The activity produced by stain QDV-3 was observed to be the highest, followed by QDE-5 and QDE-7. Therefore, QDV-3 was selected for further experiments.

#### Identification of strains

The bacterial morphology was observed after 24 hours by microscope QDV-3 strain was rod-shaped, rounded at both ends, with spores. The Colony was white, round, convex, smooth and with neat edges (results not show). The QDV-3 strain was identified as Gram-positive bacteria.

Further characterization was confirmed with its 16S rRNA gene sequence, where the extracted genomic DNA of the strain QDV-3 was tested with 1% and 1.5% agarose gel

Table 1. 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	Protease activity (U/ml)
QDV-1	15.9±0.4	6.7±0.3	2.2±0.2	182.0
QDV-3	22.7±0.6	5.4±0.5	4.2±0.1	865.2
QDE-1	8.2±0.2	4.6±0.4	1.5±0.3	155.6
QDE-2	10.0±0.2	5.2±0.2	1.9±0.1	1298.8
QDE-5	28.8±0.5	6.3±0.4	4.4±0.1	655.1
QDE-7	19.5±0.9	5.9±0.2	3.3±0.1	539.2

electrophoresis. An approximately 1500 bp of the gene sequence showed 99.2% similarity with *Bacillus flexus* (DQ376024). The NJ-based phylogenetic tree showed that the isolated QDV-3 stood alone in unidentified strains of *Bacillus* sp. clade (Fig. 1). Hence, strain QDV-3 of the present study was identified as *Bacillus* sp. QDV-3 and the sequence was submitted to GenBank under the accession number JQ836666.

Purification of protease and molecular weight determination

An extracellular alkaline protease was purified from the 3.5 days culture filtrate of *Bacillus* sp. QDV-3. A three-step purification process was followed for the purification of the protease. The crude enzyme was precipitated with 80% ammonium sulphate with a recovery of 79.4% of activity that amounted to nearly a purification fold of 1.1. The precipitated enzyme was loaded onto a Cellulose CM-52 cation exchange column. The chromatogram showed two activity peaks named FI and FII were detected (Fig. 2), which gave 36.5% recovery of the enzyme activity with nearly 2.3 fold

purification. In the final step, the active fraction FI collected from the gel filtration were loaded to the DEAE-Sephadex A50 anion exchange column and eluted for the active fractions. Such active fractions were then concentrated and analyzed, and then four active fractions were obtained as shown in Fig. 3, which were named as FI-1, FI-2, FI-3, and FI-4. The enzyme was purified up to 2.5 fold with a final recovery of 12.5% whose specific activity was found to be 9075.5 U/mg proteins.

The purified protease FI was shown to have three clear bands by SDS-PAGE electrophoresis with apparent molecular masses which were estimated to be approximately 96.6 kDa, 75.8 kDa, and 61.6 kDa (Fig. 4). The protease FI was further purified through DEAE-Sephadex A50 anion exchange column, and FI-2 was obtained as one of the activity fractions with a single clear band in SDS-PAGE. The molecular weight of FI-2 was determined to be 61.6 kDa, and it was named as QDV-E. In general, the molecular weights of previously found protease are rarely more than 60 kDa [6, 13, 24]. However, molecular mass of protease QDV-E is

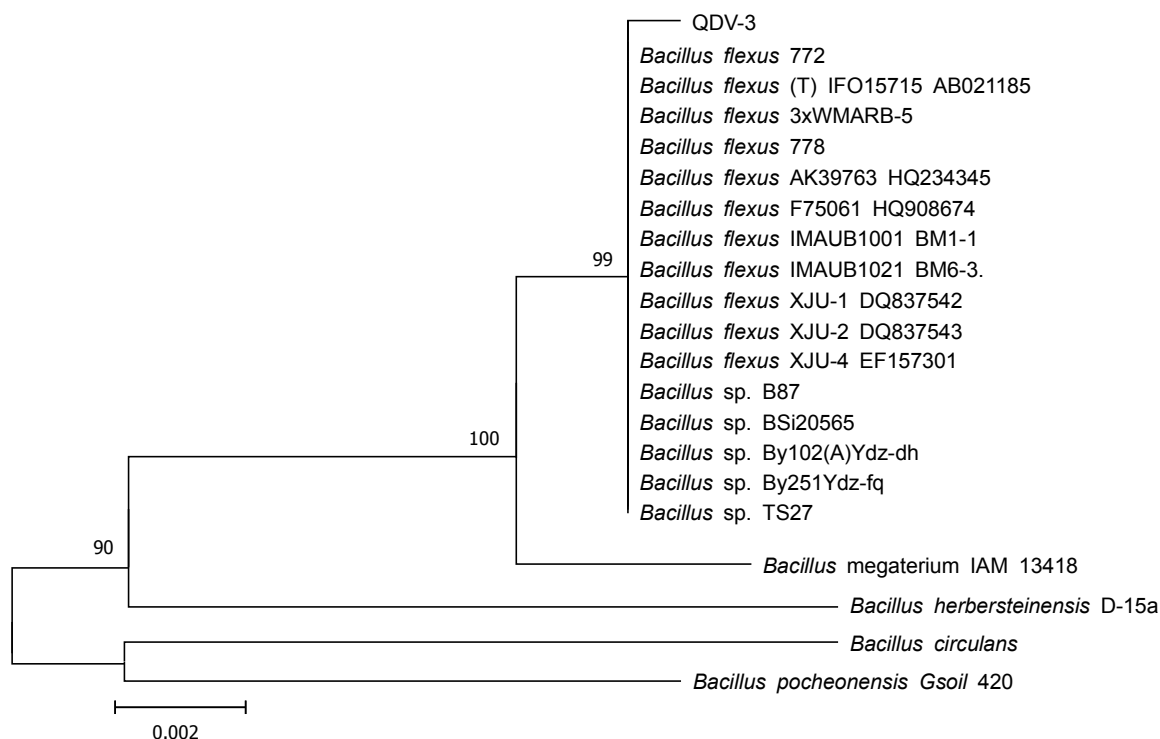


Fig. 1. Phylogenetic tree of strain QDV-3 on 16S rRNA gene sequences. Genomic DNA was prepared using a Genome Extraction Kit (Biotek, China). The primers for the PCR reaction were universal bacteria primer 27F and 1492R. The amplification was conducted by subjecting the samples to an initial denaturation step of 4 min at 98°C and then 30 cycles of 35 seconds of denaturation at 95°C, annealing at 55°C for 1.5 min, and 1.5 min at 72°C for extension. The final step consisted of 10 min at 72°C and storage at 4°C. The amplified 16S rRNA was cloned into strain *E. coli* 110 and sequenced by Sangon Biotech Company (China).

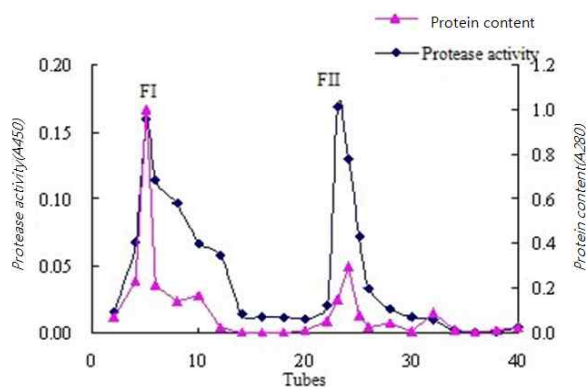


Fig. 2. Ion-exchange chromatogram on Cellulose CM-52 of protease. The sample was loaded into the cellulose CM-52 cation exchange column (1.2 cm × 40 cm). Firstly, the enzyme was eluted with a 0.02 mol/l phosphate buffer (pH 7.0), and was fractionated with a linear gradient of 0 to 2 mol/l NaCl in the same buffer at a flow rate of 60 ml/h. A fraction collector was used to collect the purified protease. Protease activity was measured by Azocasein method. 1% Azocasein was dissolved in 0.02 mol/l pH 7.0 PBS, 50  $\mu$ l of crude enzyme was mixed with azocasein buffer and was incubated at a speed of 140 rpm, under 37°C for 1 hour. After adding 300  $\mu$ l 10% (w/v) TCA, it stood at room temperature for 15 min, and centrifuged at 10,000 rpm for 5 min, 100  $\mu$ l supernatant was mixed with 100  $\mu$ l of 1 mol/l NaOH. The absorbance was analyzed under 450 nm wavelength to measure enzyme activity.

about 61.6 kDa, indicating that the protease is novel protease. The overall results of the purification procedures are summarized in Table 2.

#### Effect of pH on proteases

The QDV-E protease was active in the pH range of 7.0-13.0, with optimum activity at pH 9.0 (Fig. 5), suggesting that it belongs to alkaline protease. At the range of pH 9.0-9.5, the enzyme showed more than 96% activity, whereas at pH 7.0 and 13.0, the protease activities were decreased by nearly 53.3% and 64.2%, respectively. Currently, most of the alkaline proteases' optimum pH range from 8.5 to 10.0, only a few is more than 11.0, even up to 12.0. Shikha et

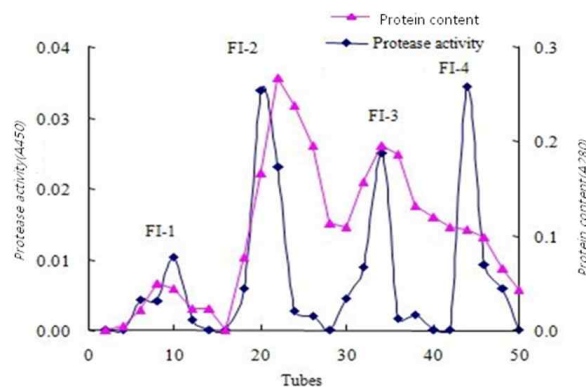


Fig. 3. Ion-exchange chromatogram on DEAE-Sephadex A 50 of protease SDS-PAGE electrophoresis strain QDV-3. The activated resultant protease received from Cellulose CM-52 cation exchange chromatography was loaded onto a DEAE-Sephadex A50 anion exchange column (1.6 cm × 40 cm) which had been equilibrated with a 0.02 mol/l Tris-HCl buffer (pH 8.5), then the unadsorbed materials were washed from the column with the same buffer. The rest was eluted with a linear gradient of 0 to 2 mol/l sodium chloride in the same buffer at a flow rate of 60 ml/h. The protease fractions were collected and scanned for their  $A_{280}$ , and assayed for protease activity.

al. have reported a strain of *Bacillus pantotheneticus* could produce protease with an optimum pH at 8.5 [22]. Kumar et al. 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 [15]. The protease of *B. licheniformis* NH1 has also been reported to have similar properties [26]. The alkaline proteases which have high commercial value are those that have pH optima in the range of 9.0-12.0.

#### Effect of temperature on proteases

The effect of temperature on QDV-E was shown in Fig. 6. The optimum temperature for protease QDV-E to have the highest activity was 40°C, which indicated the proteases belong to mesophilic enzyme. Generally, microbial proteases have a broader optimal temperature range from 30 to 75°C

Table 2. Summary of purification of protease

Purification step	Enzyme activity (U)	Total Protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude protease	246107.8	68.0	3619.2	1.0	100.0
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> Precipitation	195485.4	47.8	4089.6	1.1	79.4
Cellulose CM-52	89765.4	10.8	8311.6	2.3	36.5
DEAE- Sephadex A50	30675.3	3.3	9075.5	2.5	12.5

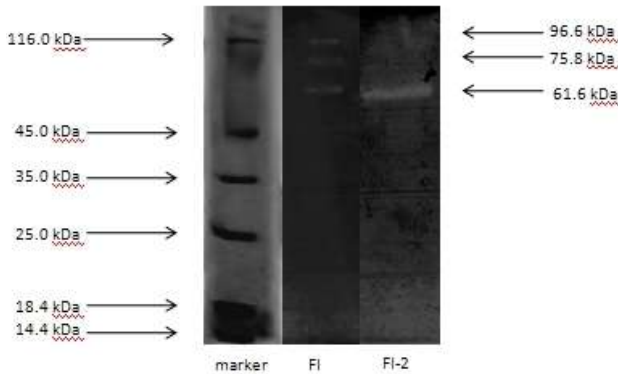


Fig. 4. SDS-PAGE of FI & FI-2 fraction by strain QDV-3. The molecular weight of purified protease was determined by SDS-PAGE with casein as standard protein. A 3.75% stacking gel and a 12% separation gel with a thickness of 0.75 mm were prepared. The purified protein samples collected from DEAE-Sephadex A50 anion exchange chromatography were run on a SDS-PAGE. After completion of the electrophoresis, the gel with protein standard bands was cut down for conventional staining and destaining.

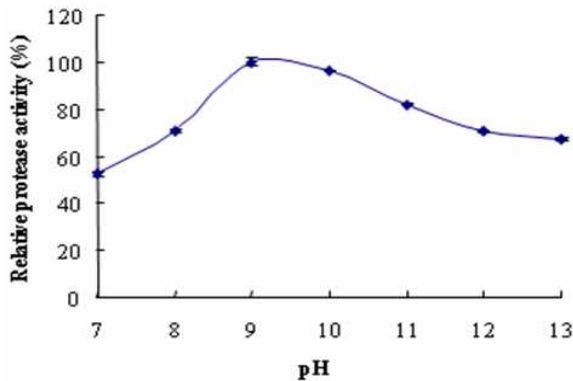


Fig. 5. Effects of different pH values on protease activity of QDV-E by strain QDV-3. The pH effect of the protease was performed from 7.0 to 13.0, and the buffers were mixed with the purified protease at a ratio of 1:1. The mixture was stored at 4°C for one hour, and azocasein method used for protease activity.

depend on different genera of bacteria. Uttam et al have isolated a protease producing short *Bacillus subtilis* from water sample, the optimal temperature of the protease was 37°C [26]. Another researcher Jung also has reported a *Pseudomonas* sp. KFCC 10818 strain which could secrete protease whose optimum temperature was 70°C [11].

The thermal stability of proteases

To examine the thermal stability of the QDV-E protease, the enzyme solution was allowed to stand for 60 min at vari-

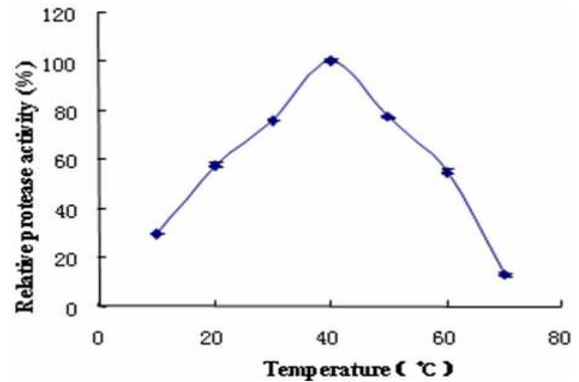


Fig. 6. Effects of different temperatures on protease activity of QDV-E by strain QDV-3. The proteases were placed at different temperatures from 10 to 70°C for one hour, and the activity of proteases was measured by azocasein method.

ous temperatures, and the residual activity was measured. The QDV-E protease showed good stability at 50°C (Fig. 7) with 82% retained activity. However, as the temperature was increased to 60°C, the retained activity was dropped to 40%, and was rapidly inactivated at higher temperature. The protease activity was completely inactivated at 70°C. The temperature of best thermal stability was same with the protease isolated from *Exiguobacterium* sp. SKPB5 [17]. However it was found to be higher than that of earlier reported protease from *Azospirillum* sp. [16] and *Shewanella* strain Ac10 [14].

Protease is desirable to be stable in the presence of commercial detergents. The suitability of any protease in detergent formulation is dependent on its stability and compatibility with the detergent components. Besides, the enzyme

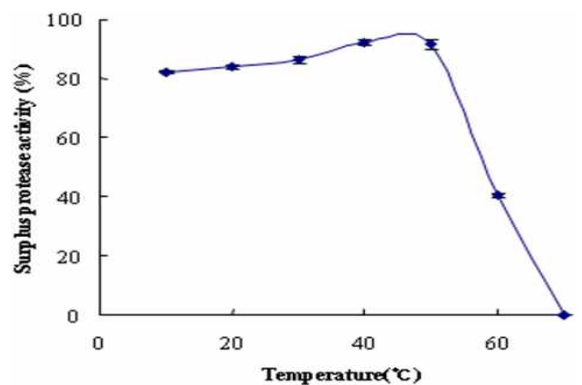


Fig. 7. The thermal stability on protease activity of QDV-E by strain QDV-3. The purified proteases were put under the condition in which the temperature ranged from 10 to 80°C respectively for 60 min. The reaction was stopped in ice water and the residual protease activity was measured by azocasein method

Table 3. Effects of inhibitions and metal ions on QDV-E

Reagents	Concentration (mM)	Relative activity (%)
Ca <sup>2+</sup>	2	97
Mg <sup>2+</sup>	2	115
Mn <sup>2+</sup>	2	189
Ba <sup>2+</sup>	2	55
Zn <sup>2+</sup>	2	70
Cu <sup>2+</sup>	2	68
EDTA	5	95
PMSF	2.5	0

should be alkaline and thermostable in nature. However, the stability and compatibility of the enzyme with the components alone should not be considered as the only pre-requisite for its inclusion in detergent formulation. Therefore, more experiments about active state of protease at room temperature are necessary to be taken place in the future.

#### Effects of metal ions and inhibitors on proteases

The effects of various metal ions, at a concentration of 2.0 mM, on the activity of QDV-E were analyzed (Table 4). It was observed that activity of the enzyme was enhanced by nearly 15% and 90% in the presence of Mg<sup>2+</sup> and Mn<sup>2+</sup> ions. On the other hand, the heavy metals like Ba<sup>2+</sup>, Zn<sup>2+</sup>, and Cu<sup>2+</sup> had inactivated the enzyme. Among the different protease inhibitors, EDTA did not completely inhibit the enzyme. In contrast, the protease was strongly inhibited by the PMSF, thus confirming the finding that the protease QDV-E of *Bacillus* sp. QDV-3 was that of serine protease.

The effect of metal ions and various compounds on protease isolated from *Azospirillum* sp. and *Bacillus* sp. APR-4 have been reported. *Azospirillum* sp. was stabilized by Mg<sup>2+</sup> [18], and that from *Bacillus* sp. APR-4 was stabilized by Ca<sup>2+</sup> [16]. In this study, a stimulatory effect on the activity was shown by Mg<sup>2+</sup> and Mn<sup>2+</sup> ions. Between two of them, Mn<sup>2+</sup> showed the maximum increase in activity. Because EDTA had no much effect on the activity, the enzyme could not be classified as a metalloprotease. This also suggests that metal ions were not the essential requirement for the activity, but at their addition, the enzyme showed a stimulatory effect. An 89% increase in activity was observed when the enzyme was incubated in presence of Mn<sup>2+</sup>.

Traditionally, microbial proteases have been applied in many areas in the food industry. Alkaline protease is used in the production of hydrolyzed protein, which has high nutritional values on the blood regulation and infant milk formula. After that, treatment food products, fortified juices,

and soft drinks have been developed with the alkaline proteases. 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 food.

Currently, the use of microorganisms or proteolytic enzymes for deproteinization of marine produce wastes is a modern trend in conversion of waste into useful biomass. It is a simple and inexpensive alternative to chemical methods employed in the preparation of various products such as: peptide and chitin. Selection of organism for the commercial production of protease relies not only on its ability to produce the enzyme with desired characteristics, but also on the cost-effective methods of production of the enzyme. The results in the present study indicated that the protease production pattern varied with the type of marine-residues. This could be attributed to the marine products, which play a dual role; that is, supply of nutrients to the microbial culture and anchorage for the growing cells.

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초록 : *Octopus vulgaris*의 장관으로부터 분리한 단백질 분해효소 생성 균주와 생성된 효소의 특성

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*Octopus vulgaris*의 장관으로부터 단백질 가수분해력과 활성을 측정함으로써 높은 단백질분해효소 생성능을 가진 균을 분리하여 동정하였다. 균이 생성한 단백질분해효소는 황산암모늄침전, cellulose CM-52 양이온 교환 크로마토그래피, DEAE-Sephadex A50 음이온 교환 크로마토그래피의 3단계를 통해 정제하였다. 장관으로부터 분리한 균중 가장 높은 단백질분해효소 생성능을 가진 균은 *Bacillus* sp. QDV-3로 나타났으며 이균을 분리한 후 표현형 분석, 생화학적 특성, 16S rRNA 유전자염기서열분석을 통해 *Bacteria*역, *Firmicutes*문, *Bacilli*강, *Bacillales*목, *Bacillaceae*과, *Bacillus*속으로 *Bacillus flexus*와 99.2%의 유사성을 보이는 것으로 확인하였다. 균이 생성한 단백질분해효소를 QDV-E로 지정하였으며 61.6 kDa의 분자량을 나타내었다. 이 효소는 pH 9.0~9.5에서 활성을 나타내었고 최적온도는 40°C였으며 50°C에서는 60분간 96% 이상의 활성을 보유하였다. Phenyl methyl sulfonyl fluoride (PMSF)에 의하여 활성이 억제 되었으므로 세린 알칼리성 단백 분해 효소인 것으로 결론지었다. 금속이온인  $Mn^{2+}$ 와  $Mg^{2+}$ 에 의하여 효소활성 상승효과를 보였으며  $Ba^{2+}$ ,  $Zn^{2+}$ , 그리고  $Cu^{2+}$ 에 의하여 활성이 억제되었다.