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Purification and Characterization of a Protease Produced by a *Planomicrobium* sp. L-2 from Gut of *Octopus vulgaris*

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ABSTRACT: Protease widely exists in the digestive tract of animals and humans, playing a very important role in protein digestion and absorption. In this study, a high protease-producing strain *Planomicrobium* sp. L-2 was isolated and identified from the digestive tract of *Octopus variabilis*. The strain was identified by physiological and biochemical experiments and 16S rDNA sequences analysis. A protease was obtained from the strain *Planomicrobium* sp. L-2 through ammonium sulfate precipitation, dialysis and enrichment, DEAE-Sephadex A50 anion-exchange chromatography, and Sephadex G-100 gel chromatography. The molecular weight and properties of the protease were characterized, including optimum temperature and pH, thermal stability, protease inhibitions and metal ions. According to our results, the protease from *Planomicrobium* sp. L-2 strain designated as F1-1 was obtained by three-step separation and purification from crude enzyme. The molecular weight of the protease was 61.4 kDa and its optimum temperature was 40°C. The protease F1-1 showed a broad pH profile for casein hydrolysis between 5.0~11.0. No residual activity was observed after incubation for 40 min at 60°C and 60 min at 50°C. F1-1 protease was inhibited by Mn^{2+}, Hg^{2+}, Pb^{2+}, Zn^{2+}, and Cu^{2+} ions, as well as PMSF, indicating that the protease F1-1 was a serine protease. Additionally, research basis provided by this study could be considered for industrial application of octopus intestinal proteases.

Keywords: enzymatic properties, identification, intestinal bacteria, octopus, purification

INTRODUCTION

Protease is mainly produced from animals, plants, and microorganisms, where microbial production of protease accounts for about 60% of total output throughout the world (1). Protease is widely used in detergent, food processing, pharmaceutical industry, silk industry, and waste utilization (2,3). Many enzyme-producing bacteria have been found in marine organisms (4-8), which could be explained by many marine organisms taking in bacteria-contaminated food and water through their gills and orals. The physiological and biochemical activities and even the metabolites of the bacteria were changed dramatically by the environment. Eventually, ecological balance was achieved between the marine organisms and the intestinal flora.

However, any protease producing intestinal bacteria obtained from octopus is rarely reported. In this study, protease producing strains were isolated, screened, and identified from octopus gut. Meanwhile, the protease obtained by the intestinal bacteria was purified through ammonium sulfate precipitation, dialysis and enrichment, DEAE-Sephadex A50 anion-exchange chromatography, Sephadex G-100 gel chromatography, and gel chromatography and its properties were characterized, including molecular weight of the protease, optimum temperature and pH, thermal stability, metal ions and surfactants. This work provides theoretical foundation for the future industrial application of protease.

MATERIALS AND METHODS

Materials

Octopus vulgaris purchased from a seafood market in Qingdao (China) was bred for 3 days in the laboratory, at the culture temperature under 10°C and water changed twice a day, before experiments were performed.

Methods

Sample preparation: The sterilized mortar, sea water, cultural medium, and *Octopus vulgaris* were prepared. The

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octopus was dissected and the gastrointestinal tracts were separated from the octopus and rinsed five times with sterile sea water to remove intestinal debris. The tracts were placed into the mortar and shredded and grinded with 3 mL sterilized sea water.

Screening: The bacterial suspension was diluted and spread on VNSS and 2216E mediums (Hope Biochemistry Technology Co. Ltd., Qingdao, China) (9). The mediums were sealed and incubated at 25°C for three days. The well-growing colonies were inoculated into casein mediums with an inoculating loop ring. The casein mediums (Hope Biochemistry Technology Co. Ltd.) were sealed and placed in a 25°C constant temperature incubator for 24 h. According to the observation of proteolytic transparent circle around the strains, the protease producing bacteria could be selected.

Measurement of protease activity: Azocasein method (10) was used for protease activity measurement. 1% Azocasein (Sigma, St. Louis, MO, USA) was mixed in 0.02 M phophate buffered saline (PBS, pH 7.0) to make the substrate. Crude enzyme (50 μL) (Sigma) was added into the azocasein buffer and the mixture was incubated in a water bath oscillator (CTI Co., Addison, TX, USA) at 140 rpm and 37°C for 1 h. The reaction was terminated by adding 300 μL 10% (w/v) trichloroacetic acid (TCA) (Sigma) to the mixture. After 15 min at room temperature, the mixture was then centrifuged at 10,000 rpm for 5 min, and 100 μL of supernatant was added into 100 μL of 1 M NaOH. The mixture was mixed well with 3 min vortexing, and the absorbance (A) was analyzed under 450 nm wavelength to measure enzyme activity.

Identification of strains

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 by using a Genome Extraction Kit (Bioteke, Beijing, China). The primers for the PCR reaction were universal bacteria primer 27F (5'AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The amplification was conducted by subjecting the samples to an initial denaturation step of 5 min at 98°C and then 35 cycles of 35 seconds of denaturation at 95°C, annealing at 55°C for 35 seconds, and 1.5 min at 72°C for extension. The final step consisted of 8 min at 72°C and storage at 4°C. The amplified 16S rRNA was cloned into strain *E. coli* 110 (Sangon Biotech Co. Ltd., Shanghai, China) and sequenced by Sangon Biotech Company.

Preparation of crude enzyme solution: To prepare the cultural media, optimal culture conditions were tested previously: 25 g peptone, 25 g soluble starch, 5 g yeast extract, 0.5 g phosphate, and 5 L sea water were mixed at room temperature and pH was adjusted to 8.0. Every 100 mL of the medium was divided into 500 mL flasks. The flasks were sterilized at 121°C for 20 min and cooled until room temperature. The isolated strains were inoculated into the medium and incubated at 19°C for 3.5 days. The fermentation broth was centrifuged at a speed of 10,000 rpm for 10 min, and the supernatant was collected and stored at 4°C for further experiments.

Purification of protease

An ammonium sulfate solution with saturation of 80% was gradually added into the enzyme solution while slowly stirring until completely dissolved. The whole precipitation took place in an ice bath. The precipitated protein was dissolved in 0.05 M Tris-HCl buffer and dialyzed against the same buffer.

The concentrated protease was isolated by a DEAE-Sephadex A50 anion exchange column (1.6 cm×40 cm) (Solarbio Science & Technology Co. Ltd., Beijing, China). Firstly, the protease was eluted with a 0.02 M Tris-HCl buffer (pH 8.5) at a flow rate of 1 mL/min, and then the same buffer with a linear gradient of 0～2 M sodium chloride was applied. The fractionated proteases were collected with receiving tubes and each portion was detected at a wavelength of 280 nm.

The enzyme sample collected from DEAE-Sephadex A50 anion exchange chromatography was loaded onto a Sephadex G-100 gel chromatography column (Solarbio Science & Technology Co. Ltd.). A 0.02 M phosphate buffer (pH 7.0) was selected as elution buffer with a flow rate at 0.5 mL/min. Enzyme of each receiving tube was detected as mentioned above. The protease-active fractions were pooled together for further use.

Determination of the molecular weight of proteases

The molecular weights of purified proteases were detected using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). 5% (w/v) stacking and 12% (w/v) separating gels were prepared. Samples were prepared by mixing purified enzymes in the ratio of 1:5 (v/v) with glass distilled water containing 10 mM Tris-HCl (pH 8.0), 2.5% SDS, 5% β-mercaptoethanol and 0.002% bromophenol blue. All chemicals used were purchased from Sigma.

Effects of pH and temperature on proteases and thermal stability

The pH effect of the protease was studied from pH 5.0 to pH 11.0, and the buffers with different pH were mixed with the purified protease at a ratio of 1:1. All pH values were adjusted at room temperature. The enzyme activity was determined by the assay method described previously.

The reaction mixture was incubated at different temperatures ranging from 20 to 60°C for 1 h after which the protease was added into substrate azocasein and the reaction was carried out at 37°C. The activity values of proteases received from different temperatures were measured. To study the thermal stability at different temperatures, the protease was pre-incubated at 50 and 60°C for 60 min and rapidly cooled, and residual activities were measured as the assay procedure mentioned above. Residual activity is a ratio of the enzyme activity after heating and the initial activity of the enzyme extract.

Effects of metal ions and surfactants on proteases

50 μL protease solution was mixed with different metal ions solutions, including Hg^{2+}, Ca^{2+}, Mg^{2+}, Ba^{2+}, Mn^{2+}, Cu^{2+}, Fe^{3+}, and Zn^{2+}, with the same concentration of 2 mM. 50 μL of 5 mM phenyl methyl sulfonyl fluoride (PMSF) (Sigma) and 2.5 mM ethylene diamine tetraacetic acid (EDTA) (Sigma) were also tested by adding into 50 μL of purified protease solutions. The mixtures were kept at 37°C for 1 h, and the relative activity values were measured. Distilled water was used as control to calculate the results.

RESULTS

Screening of protease producing bacteria from *Octopus vulgaris* gut and identification of strain L-2

In this experiment, a total of 4 strains were detected to produce transparent circles on casein medium, which were named L-1, L-2, L-3, and L-4. According to the results shown in Table 1, the largest diameter of transparent circle of strain L-2 was obtained with the highest protease activity of 1,211.4 U/mL. Therefore, strain L-2 was subjected for further studies.

The extracted genomic DNA of strain L-2 was tested with 1% and 1.5% agarose gel electrophoresis. The products were around 1,500 bp with clear bands, which were used for the determination of the strain 16S rRNA sequence (data not shown). The alignment of the 16S rRNA sequence indicated that strain L-2 was most closely related to the genera *Planococcus* and *Planomicrobium*, which both belong to *Planococcaceae* with more than 99% similarity. Phylogenetic analysis based on the 16S rRNA sequence indicated that strain L-2 showed the highest homology with *Planomicrobium okeanokoites* (Fig. 1). The bacteria located in the neighboring clades on the phylogenetic tree all belonged to the genus *Planomicrobium*. Although the 16S rRNA sequences of the *Planococcus* strains showed high similarity to that of strain L-2, they

Table 1. Screening results of strains from tract of *Octopus vulgaris*

Strains	L-1	L-2	L-3	L-4
Transparence circles (cm)	0.80	1.58	0.10	0.79
Protease activity (U/mL)	146.40	1,211.40	1,024.60	746.90

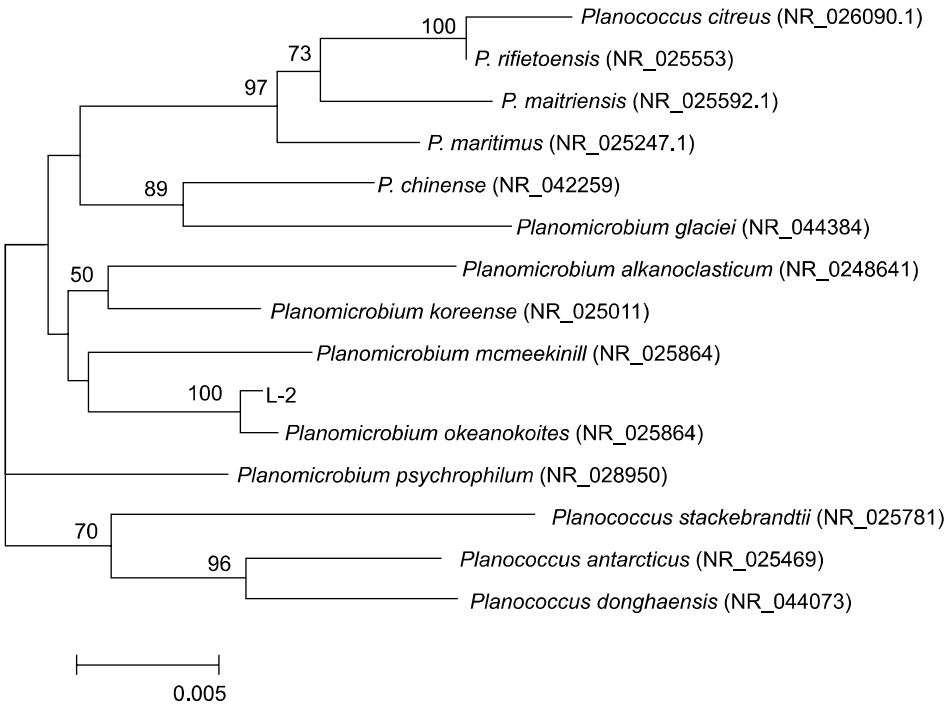

Fig. 1. Phylogenetic tree of strain L-2 on 16S rRNA gene sequences.

were located in different clades on the phylogenetic tree. Therefore, we placed strain L-2 in the genus *Planomicrobium*, as *Planomicrobium* sp. L-2.

Purification of protease and molecular weight determination

The extracellular protease was purified with a recovery of 26.2% of activity that amounted to nearly a purification fold of 1.3. The precipitated enzyme was loaded onto a DEAE-Sephadex A50 anion exchange column. The chromatogram showed a single activity peak named F1 (Fig. 2), which gave 20.5% recovery of the enzyme activity with nearly 1.4 fold purification. Also, F-1 active fractions were then concentrated and analyzed (Fig. 3). A single active protease F1-1 was attained with a purification fold of 1.7. The final recovery of 7.4% was obtained with a specific activity was found to be 12,394.5 U/mg (Table 2).

The molecular weights of the purified proteases F1 and F1-1 by SDS-PAGE were 61.4 kDa and 53.2 kDa, respectively. However, reasons not yet clear. The crude enzyme did not show a 53.2 kDa band before the DEAE-Sephadex A50 anion exchange chromatography analysis. The protease F1 was further purified through the Sephadex G-100 gel chromatography column, and F1-1 was obtained as the only active fraction with a single clear band in SDS-PAGE. The molecular weight of F1-1 was determined to be 61.4 kDa.

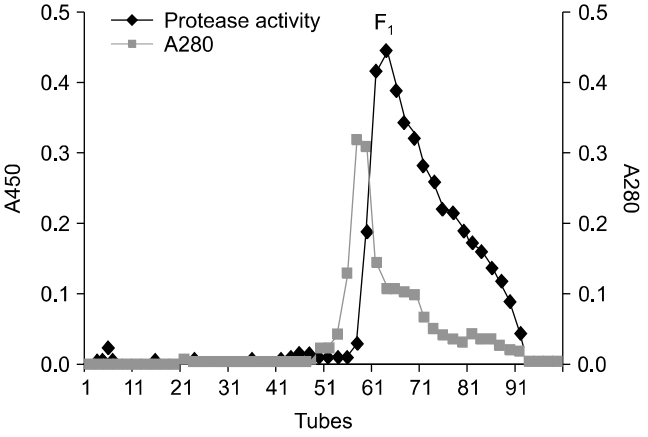

Fig. 2. DEAE-Sephadex A50 ion-exchange chromatography.

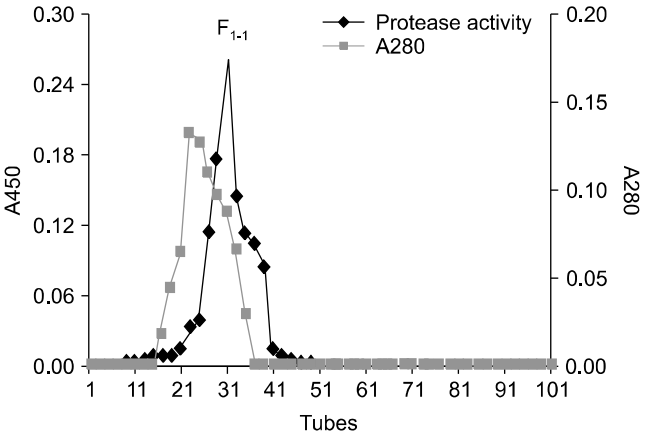

Fig. 3. Sepahdex G-100 chromatography.

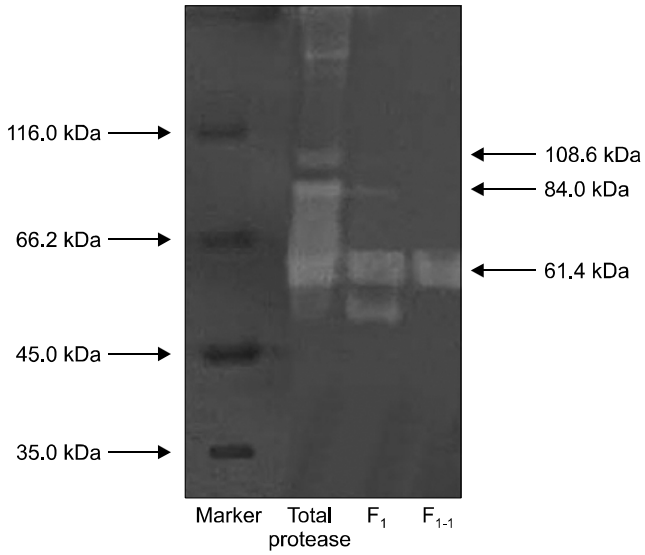

Fig. 4. SDS-PAGE analysis of F1-1 proteases.

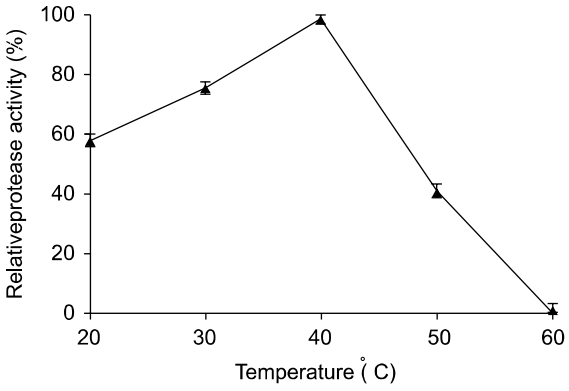

Fig. 5. Effects of reaction temperature on the activity of F1-1 proteases.

Table 2. Summary of purification of protease

Purification step	Enzyme activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude protease	661,316.8	91.7	7,211.7	1.0	100.0
$(NH_4)_2SO_4$ precipitation	173,250.6	18.7	9,264.7	1.3	26.2
DEAE-sephadex A50	135,270.2	13.7	9,873.7	1.4	20.5
Sephadex G-100	49,577.9	4.0	12,394.5	1.7	7.5

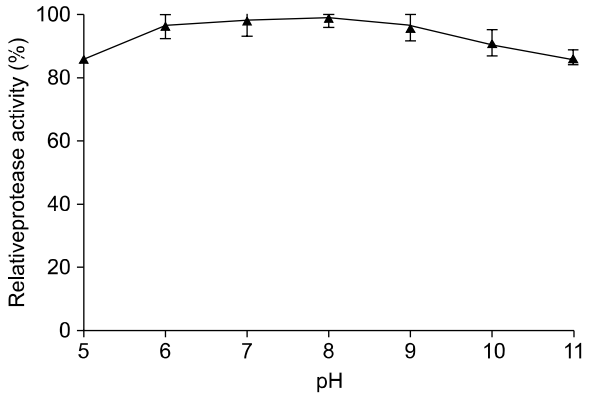

Fig. 6. Effects of pH on the activity of F1-1 proteases.

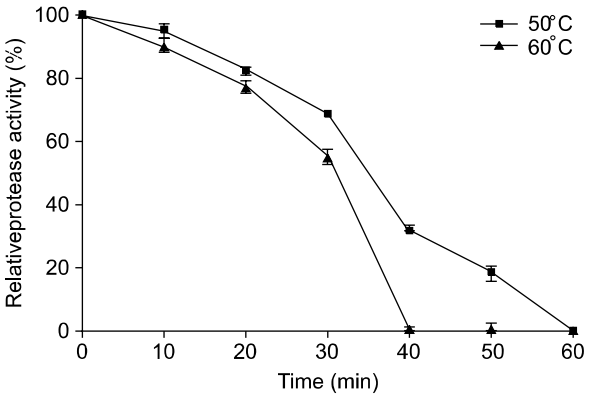

Fig. 7. The thermal stability of F1-1 proteases.

Effect of temperature and pH on proteases

The effect of temperature on F1-1 was shown in Fig. 5. The optimum temperature for F1-1 protease to have the highest activity was 40°C, which was lower than that of mesophilic protease. The enzyme showed approximately 60% of its residual activity at 20°C. After 40°C the activity decreased rapidly with increasing temperature, and there was almost no activity at 60°C. Generally, microbial proteases have a broader optimal temperature range from 30 to 75°C depending on the genera of bacteria.

The strain *Planomicrobium* sp. L-2 protease showed a broad pH profile for casein hydrolysis with an optimal pH of 7.0 (Fig. 6). However, the enzyme retained more than 80% of its activity between pH 5~11; no significant fluctuation of pH had been observed, which verified that the effect of pH on F1-1 protease activity was not significant.

The thermal stability of proteases

Regarding the percentages of residual activity after incubation for 20 min at 50 and 60°C, strain *Planomicrobium* sp. L-2 protease retained approximately 80% of the activity (Fig. 7). No residual activity was observed after incubation for 40 min at 60°C and 60 min at 50°C. Some isolated strains (*Pseudoalteromonas* spp. IE3-1 and IE7-4) produced low levels of protease activity under the culture conditions mentioned previously, whereas the thermal stability of their extracellular proteases was not analyzed (11).

Table 3. Inhibitory effects of metal ions on F1-1

Reagents	Concentration (mM)	Relative activity (%)
Control	–	100
Cu^{2+}	2	15
Pb^{2+}	2	85
Mg^{2+}	2	89
Ba^{2+}	2	106
Zn^{2+}	2	87
Mn^{2+}	2	56
Ca^{2+}	2	103
Fe^{3+}	2	107
Hg^{2+}	2	29
PMSF	5	10
EDTA	2.5	96

Effects of metal ions and surfactants on proteases

Among the metal ions tested, Fe^{3+}, Ba^{2+}, and Ca^{2+} activated the enzyme, while Mn^{2+}, Hg^{2+}, Zn^{2+}, Pb^{2+}, and Cu^{2+} ions inhibited the protease F1-1. As shown in Table 3, 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 F1-1 of *Planomicrobium* sp. L-2 was a serine protease.

DISCUSSION

Proteases act as processing enzymes that participate in regulatory or catabolic mechanisms in the cell or as extracellular enzymes that play an important role in the degradation of proteinaceous substrates that serve as carbon or energy source (12). As the largest superfamily of proteases, the subtilisin-like proteases, which are also known as subtilases, have been widely studied (13-15). Many reports describe subtilases as proteases from alkaliphilic *Bacillus* sp. No. 221 (16), Savinase™ from *Bacillus lentus* (17), and an oxidatively stable alkaline serine protease from *Bacillus* KP-43 (18), *Bacillus alcalophilus* PB92 (17). However, only a few studies on *Planomicrobium* sp. have been reported.

Four protease producing strains were successfully isolated from octopus gut named L-1, L-2, L-3, and L-4. According to the screening results, strain L-2 was selected as the start strain. In general, two bacteria can be identified as members of the same genus if they share more than 99% homology in their 16S rRNA gene sequences (19,20). In this study, 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. Some *Planococcus* strains have been re-identified as *Planomicrobium* (21-23), indicating that these genera have a high degree of similarity. Based on the alignment and phylogenetic analysis, strain L-2 was placed in genus *Planomicrobium* as *Planomicrobium* sp. L-2.

The proteases obtained by strain L-2 were purified through ammonium sulfate precipitation, DEAE-Sephadex A50 anion exchange chromatography, and Sephadex G-100 gel chromatography. After the three-step purification process, the purified protease named F1-1 was successfully obtained from crude enzyme. Two bright bands with molecular weights of 61.4 kDa and 53.2 kDa were obtained from F1 protease, according to Fig. 4. However, the 53.2 kDa band of the crude protease before the DEAE-Sephadex A50 anion exchange chromatography analysis was not observed. The unknown band was speculated to be contamination from environment, although further experiments are needed. In general, the molecular weights of previously found proteases are rarely more than 60 kDa (24-26). However, molecular mass of protease F1-1 is about 61.4 kDa, indicating that the protease is novel protease.

The optimal temperature of F1-1 protease was 40°C, which was lower than many mesophilic proteases. Specifically, F1-1 retained more than 80% of its activity between pH 5.0 and 11.0. Moreover, the protease thermostabilities analyzed in this study shows that F1-1 protease retained 80% of its activity at 50 and 60°C for 20 min. As the incubation time increases the activity of F1-1 protease decreases. No residual activity was observed after incubation for 40 min at 60°C and 60 min at 50°C.

Serine protease inhibitors could completely inhibit F1-1 activity which indicates that the enzyme may belong to the serine protease family. Mn^{2+}, Hg^{2+}, Pb^{2+}, Zn^{2+}, and Cu^{2+} ions have been measured to inhibit protease F1-1, while Fe^{3+}, Ba^{2+} and Ca^{2+} showed a slight promotion effect. The metal ion effects on F1-1 may be associated by the diet habits of octopus, which mainly include shrimps and crabs. Therefore, this study provides a research basis for industrial application of octopus intestinal proteases.

The outcome of this study indicated that the strain *Planomicrobium* sp. L-2 isolated from the gut of *Octopus vulgaris* was capable of producing proteolytic enzymes in varying quantities and can be beneficially applied for nutrient enrichment of different feed or food ingredients. One scope is the refinement of fermentation process by using the strain for nutrient enrichment of different food or feed ingredients. The potentiality of these enriched ingredients to replace others is a subject of further study.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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