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Purification, Properties of a Cold-adapted Protease PA-1 Isolated from Marine *Pseudomonas* sp.

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A cold-adapted protease named PA-1 was produced by *Pseudomonas sp*. isolated from the yellow sea sediments. The protease was purified orderly by ammonium sulfate precipitation, DEAE-Sepharose ion exchange chromatography and Superdex 200 molecular sieve. SDS-PAGE displayed that the protein molecular weight was 47kDa. PA-1 was stable at temperature range of 20°C to 35°C, and the maximum activity was achieved at 30°C. The cold-adapted protease was stable in the pH range of 6.0–8.0, and the optimum pH was 7.0, indicating it belongs to the neutral protease. The influence of metal ions was evaluated. The addition of Mg^{2+}, K^{+} enhanced the proteolytic activity of PA-1, while Li^{+}, Na^{+} slightly inhibited the protease activity, Cu^{2+}, Zn^{2+}, Ag^{+} made the protease completely inactivated. Metalloprotease inhibitor EDTA and EGTA had no effect on the protease activity. The serine protease inhibitor PMSF and DFP significantly inhibited the protease activity which indicated that the cold-adapted protease belongs to the serine protease. Casein was chosed as substrate for determination of Michaelis-Menten kinetics. The K_m value and the V_{max} value were 1.95 g/L and 26.23mmol/min, respectively. These properties demonstrated PA-1 was an ideal choice in low temperature food processing.

Key words: *Pseudomonas* cold-adapted, Protease isolation, Purification serine.

Due to the long-term living in the environment of low temperature of seawater, most enzymes produced by marine microorganisms were provided with the characteristics of low temperature catalytic activity. The optimum temperature of cold-adapted enzymes is about 4°C to 30°C [1]. Under low temperature, cold-adapted enzymes produced by psychrophilic bacteria possess high catalytic efficiency, structure flexibility which give marine microbial enzyme special biochemical mechanisms and application prospects compared with terrestrial microbial enzymes[2].

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At present, it has become an important way to develop the marine biological resources by selecting low-temperature enzymes in the ocean. In many developed countries, especially the United States and Japan have invested enormous human and material resources to strengthen the research of marine low-temperature enzymes[3].

Protease is one of the three major industrial enzymes, the yield reached 60% of the world production of enzyme preparation[4]. The protease can effectively remove and decompose protein, and it has been widely used in food, beer, cosmetics, detergent and the pharmaceutical industry[5]. Cold-adapted proteases in many fields have better application prospects than mesophilic and thermophilic proteases[6]. For instance, in the meat industry, the cold-adapted proteases can contribute to retain the meat natural flavor because of low temperature catalytic activity[7]. But for the

late start, the study about cold-adapted proteases is not sufficient and extensive. Currently, most cold-adapted proteases studied were produced by bacteria such as *bacillus*, *pseudomonas*, *flavobacterium* etc[8,9,10]. Great interests have been devoted to the enzyme properties, cold-adapted mechanisms and applications in industry. In this report, we describe the purification and properties of a neutral cold-adapted protease PA-1 produced by *Pseudomonas sp.* isolated from the yellow sea sediments. All these results indicate that the protease PA-1 has potential application in low temperature food processing.

MATERIALS AND METHODS

Bacterial strain and culture conditions

Marine *Pseudomonas* sp. was isolated from deep sea sediment of the Yellow Sea. The strain was grown in GB medium (peptone 1.2%, east extract 2.4%, glycero 0.4%, NaCl 2.5%) at 18 °C.

The seed culture was inoculated in GB medium and cultured for 48h at 18 °C. The culture was centrifuged at 10000rpm for 30 minutes to obtain the supernatant as the protease source.

Purification of the enzyme

First, the supernatant was precipitated by adding filtered ammonium sulfate slowly up to 30% saturation with gentle stirring and left for 2 h at 4 °C. After centrifuged at 10000rpm for 30 minutes, the precipitate was discarded. Second, filtered ammonium sulfate was continually added to the supernatant up to 70% final concentration. Left for 2h at 4 °C, the precipitate was collected by centrifugation, air-dried, and re-dissolved in Tris buffer (10 mM Tris, 100 mM NaCl, pH 7.0) to obtain crude enzyme. Then the crude enzyme was dialyzed against the same Tris buffer for 24 h with 6–8 changes. Third, the crude enzyme of PA-1 was loaded onto a DEAE Sepharose fast flow column (2.5×30 cm) preequilibrated with above Tris buffer and eluted with a linear gradient of 0-0.5 M NaCl at a flow rate of 3 mL/min. The active fractions were pooled and concentrated by ultrafiltration. Last, the sample was reloaded onto a Sephadex G-200 column (1.6×90 cm) equilibrated with the same Tris buffer.The peak with protease activity was collected and used for SDS-PAGE analysis.

Enzyme assay

The protease activity was assayed using casein as substrate at pH=7.0 and 30 °C . After incubation for 10 min, the reaction was stopped by adding 20% (w/v) trichloroacetic acid. Left for 10 min in the room temperature, the reactive mixture was centrifuged at 10000rpm for 5 min. Then the supernatant was measured at 280nm by spectrophotometer[11]. One unit of enzyme activity is defined as the amount of enzyme that liberates 1μg tyrosine from the substrate per minute under the assay conditions.

Estimation of protein contents

The total soluble protein content was estimated by Lowry's method[12] using BSA as standard.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (SDS-PAGE) was carried out at 12% (w/v) isolation gel and 5% (w/v) concentration gel according to the method of Laemmli[13]. The molecular weight of PA-1 was calculated using the low protein molecular weight marker from Takara Biotechnology. Protein bands were visualised after staining and destaining.

Properties of protease PA-1

Determination of temperature optimum and thermal stability

In order to determine the optimum temperature, protease activity was measured using casein as substrate in 50 mM pH 7.0 Tris-HCl buffer solutions at 10, 20, 25, 30, 35, 40, 45, 50 °C, respectively. The thermal stability was studied by incubating the enzyme at above temperature for 1h.Then the residual enzyme activity was measured under standard assay conditions.

Determination of pH optimum and pH stability

Under the optimum temperature condition, the protease activity was measured at various pH buffers ranging from 3 to 12. The pH stability was studied by incubating the enzyme in different pH buffer (3-10) at 30°C for 1h. The residual activity was determined under standard assay conditions.

Determination of kinetic parameters

Purified enzyme was incubated in 50 mM pH 7.0 Tris-HCl buffer solutions at 30°C for 10 min. K_m and V_{max} values of the protease PA-1 were determined by measuring enzyme activity with various concentrations of casein as substrate. Kinetic constants were calculated from a Lineweaver-Burk reciprocal plot.

Effect of metal ions and inhibitors on protease activity

The effects of metal ions on the protease activity were studied by adding various metal ions to the assay buffer at the final concentration of 5mM under standard assay conditions and the assay buffer without metal ions was used as a control. The residual enzyme activity was measured as described above. The effects of inhibitors were also studied using EDTA and EGTA (metall oprotease inhibitor), PMSF and DFP (serine protease inhibitor) at final concentrations of 5 mM. The residual activity was measured as described above after incubation at 30°C for 10 min.

RESULTS AND DISCUSSION

Purification of PA-1

The purification results were summarized in Table 1. As methods described above, the PA-1 was precipitated between 30% and 70% ammonium sulfate saturation. By centrifugation, the precipitate was collected and redissolved in a minimum amount of Tris buffer and then dialyzed overnight against the same buffer. After ammonium sulfate precipitation, the purity of the enzyme was estimated to be about 2.78-fold greater than that of the crude extract. The crude enzyme of PA-1 was loaded onto a DEAE Sepharose fast flow column and eluted with a linear gradient of NaCl buffer. The results was shown in Fig.1 by making elution curve. In the process of elution, two protein peaks appeared and the first peak displayed protease activity. The sample with protease activity was collected, concentrated and loaded onto the Superdex 200 molecular sieve column. The elution result was shown in Fig.2. There were two protein peaks included in the elution curve and the second protein peak displayed protease activity. Through 3 steps operation, a 40.34-fold purification of protease PA-1 was achieved with a specific activity of 1843.56 U/mg. Electrophoresis under denaturing conditions (12% SDS-PAGE) revealed a single band at a molecular mass estimated to be 47 kDa (Fig.3) and this result was confirmed by gel

Table 1. Purification of cold-adapted protease PA-1

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold
Crude enzyem	52085	1151.56	45.23	1.00
Ammonium sulfate precipatation	45038	422.22	106.67	2.78
DEAE-Sepharose	31823	44.67	712.38	18.92
Superdex 200	9389	5.09	1843.56	40.34

Table 2. Effects of metal ions and inhibitors on enzyme activity

Ions and inhibitors	Concentration (mm)	Residual activity (%)
Non	-	100±0.2
Na+	5	98.6±2.1
K+	5	104.5±0.8
Li+	5	97.4±1.7
Mg2+	5	108.4±3.5
Ca2+	5	78.2±3.6
Zn2+	5	10.5±2.7
Cu2+	5	12.6±0.9
Mn2+	5	56.±3.4
Ba2+	5	59.2±2.8
Ag+	5	9.5±1.5
EDTA	5	90.3±4.1
EGTA	5	91.2±3.1
PMSF	5	5.9±0.5
DFP	5	8.2±1.5

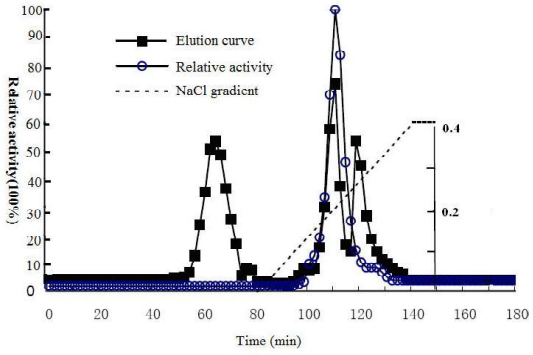

Fig. 1. DEAE-Sepharose ion exchange chromatography of cold-adapted protease PA-1. The sample from peak 2 was collected

filtration analysis, suggesting that PA-1 is monomeric.

Properties of protease PA-1

Effect of temperature on enzyme activity

As shown in Fig. 4A and 4B, the purified PA-1 exhibited thermal stability at temperatures ranging from 10 to 40°C and had an optimum at 30°C , while activity decreased rapidly above 40°C. The relative activity at 50°Cwas 20%. The results indicated that PA-1 was a cold-adapted protease and could be as a preferred candidate in industrial purpose, which required enzyme activity and stability in wide temperature range[14].Cold-adapted enzymes are characterized by high flexibility and specific activity at low temperatures[15], whereas mesophilic and thermophilic homologs have low activity and are structurally rigid at low temperatures[16]. Proteases are well known to be used as additives in detergents[17]. To reduce energy consumption and damage of clothing, cold-adapted proteases have obvious advantages in cold washing[18]. In the food industry, proteases

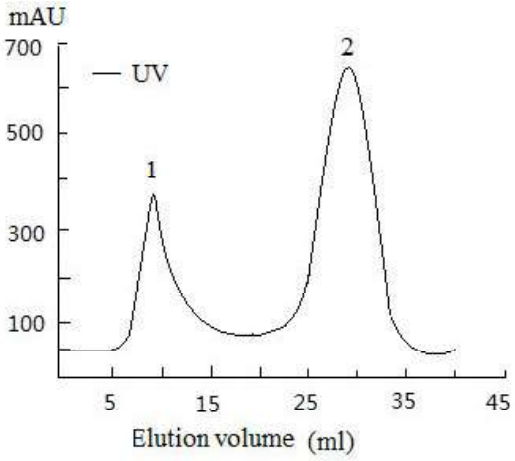

Fig. 2. Superdex 200 chromatography of the cold-adapted protease PA-1. The sample from peak 1 was collected

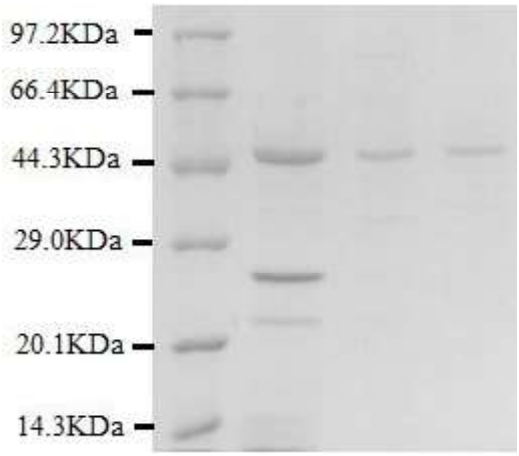

Fig.3. SDS-PAGE analysis of purified PA-1 from *Pseudomonas sp*. strain. Molecular mass (MW) standards (inkilodaltons) are shown in the left-hand lane; the purified PA-1from ammonium sulfate precipitation, DEAE-Sepharose ion exchange chromatography and Superdex 200 molecular sieveare shown in the right-hand lane, respectively

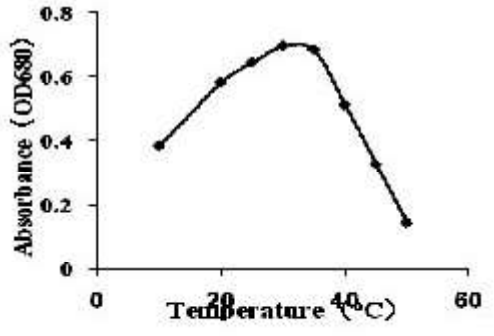

Fig. 4(a). The optimum temperature of purified PA-1. Protease activity was measured using casein as substrate in 50 mM pH 7.0 Tris-HCl buffer solutions at 10, 20,25,30,35 ,40,45,50°C,respectively.

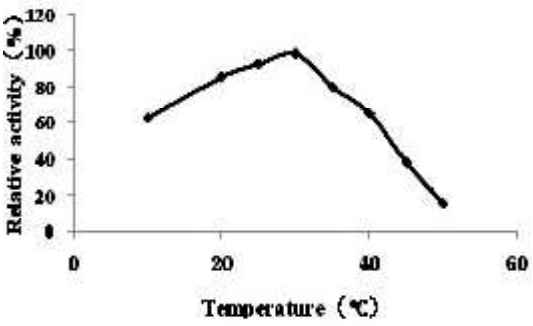

Fig. 4(b). The thermal stability of purified PA-1. The enzyme was incubated at 10,20,25,30,35, 40,45,50°C for 1h.Then the residual enzyme activity was measured under standard assay conditions

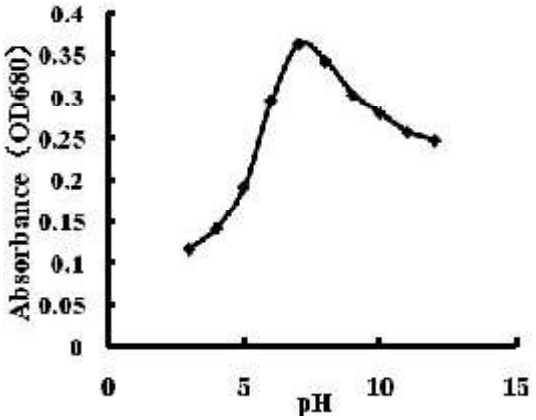

Fig. 5(a). The optimum pH of purified PA-1.Under the optimum temperature condition, the protease activity was measured at various pH buffers ranging from 3 to 12

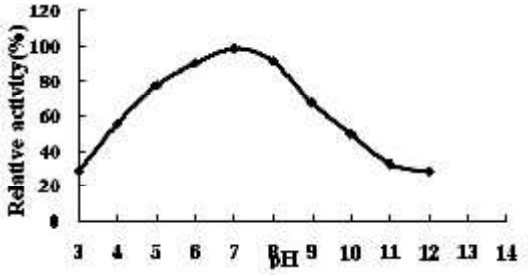

Fig. 5(b). The pH stability of purified PA-1. The enzyme was incubated in different pH buffer (3-10) at 30°C for 1h. The residual activity was determined under standard assay conditions

can be used to tenderize the meat, reduce the dough fermentation time and improve the properties of the dough and the crumb[19]. In addition, cold-adapted proteases have the advantages of delicious taste in brewing and wine industries.

Influence of pH on activity and stability of protease PA-1

Diluted protease with pH3-12 solution buffer, the enzyme activities were determined and the result was shown in Fig.5. PA-1 exhibited a optimally broad pH range of 6.0-8.0 for activity, and the maximal activity was observed at neutral pH7.0. The decrease in enzyme activity was obvious at pH above 9.0 and under 5.0 in which the relative protease activity retained 67.5% and 77.7% respectively. Compared with other neutral proteases, PA-1 was more efficient at neutral pH range. For example, cold-adapted protease ColAP retained only 10% and 5% of the maximal activity at pH 6.0 and 8.5 respectively, while no activity was detected at pH5.0 or 9.0 [20]. The protease from *Enterococcus faecalis* RQ15 was optimally active in pH7.5 and retained 47% and 45% of the maximal activity at pH 5.0 and 9.0, respectively[21].

Determination of kinetic parameters

Based on Lineweaver-Burk plotting method, the kinetic parameters of the purified protease were determined with various concentrations of casein as substrate. The K_m and V_{max} values of PA-1 were 1.95mg/ml and 26.23mmol/min, respectively, while the K_m values from haloalkaliphilic *Bacillus* sp. and *Pseudomonas aeruginosa* PseA were 2 mg/ml and 2.69 mg/ml[22]. Research reported that some cold-adapted enzymes from marine organisms had a lower K_m than thermostable homologs[23]. A chitobiase from an Antarctic marine bacterium, Km is 25-fold lower than a homolog from a mesophile[24]. The reason possessing a low K_m of cold-adapted enzymes may relate to the need to scavenge substrates that are at low concentrations in the environment[25, 26, 27]

Effects of metal ions and inhibitors on enzyme activity

The purified enzyme was mixed with different concentrations of metal ions and inhibitors and the residual enzyme activity was determined. As shown in Table2, the addition of Mg^{2+}, K^+ enhanced the proteolytic activity of PA-1, while Li^+, Na^+ slightly inhibited the protease activity, Cu^{2+}, Zn^{2+}, Ag^+ made the protease completely inactivated. Metalloprotease inhibitor EDTA and EGTA had no effect on the protease activity. The serine protease inhibitor PMSF and DFP significantly inhibited the protease activity which indicated that the cold-adapted protease belongs to the serine protease.

CONCLUSION

It is noticed that there is an increasing number of reports focusing on psychrophilic enzymes as models in basic studies and as potential

candidates for industrial and biotechnological applications[28, 29]. In this study, a novel cold-adapted protease PA-1 from marine bacterium *Pseudomonas sp*. was purified and the properties of which was studied. Unlike most neutral proteases belong to metalloprotease[30], PA-1 is a type of serine protease. Cold-adapted neutral protease has bright future in food industry. It can be used to tenderize and improve the taste of refrigerated meat products by removing undesirable tissues[31]. In addition, due to high catalytic activity at low temperature, cold-adapted proteases are particularly attractive for the processing of foods that minimize spoilage and save energy. Therefore, cold-adapted protease PA-1 maybe have a promising prospect.

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