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Purification and biochemical characterization of a protease secreted by the *Salinivibrio* sp. strain AF-2004 and its behavior in organic solvents

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Abstract A metalloprotease secreted by the moderately halophilic bacterium *Salinivibrio* sp. strain AF-2004 when the culture reached the stationary growth phase. This enzyme was purified to homogeneity by acetone precipitation and subsequent Q-Sepharose anion exchange and Sephacryl S-200 gel filtration chromatography. The apparent molecular mass of the protease was 31 kDa by SDS-PAGE, whereas it was estimated as approximately 29 kDa by Sephacryl S-200 gel filtration. The purified protease had a specific activity of 116.8 μmol of tyrosine/min per mg protein on casein. The optimum temperature and salinity of the enzyme were at 55°C and 0–0.5 M NaCl, although at salinities up to 4 M NaCl activity still remained. The protease was stable and had a broad pH profile (5.0–10.0) with an optimum of 8.5 for casein hydrolysis. The enzyme was strongly inhibited by phenylmethyl sulfonylfluoride (PMSF), Pefabloc SC, chymostatin and also EDTA, indicating that it belongs to the class of serine metalloproteases. The protease in solutions containing water-soluble organic solvents or alcohols was more stable than that in the absence of organic solvents. These characteristics make it an ideal choice for applications in industrial processes containing organic solvents and/or salts.

Keywords Halophiles · *Salinivibrio* · Serine metalloprotease · Organic-solvent tolerant protease

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Introduction

Proteases represent a class of enzymes with important roles in physiological process. Besides this, they have commercial applications, being one of the three largest groups of industrial enzymes, accounting for about 60% of the total world-wide sale of enzymes (Anwar and Saleemuddin 1998). The application of proteases in detergent, leather, silk, bakery, soy processing, meat tendering and brewery industries is well documented (Rao et al. 1998; Gupta et al. 2002). On the other, the operation of proteases in organic media is an interesting developing area of biotechnology and biochemistry (Ogino and Ishikawa 2001). Proteases can catalyze synthetic reactions which are the reverse reactions of hydrolyses, and also transestrification reactions in the presence of organic solvents. However, the use of proteases in organic media for the synthesis of peptides, esters and other biologically active organic molecules has been rather limited by the specificity and the instability of these enzymes in the presence of organic solvents. Several methods for stabilizing proteases in organic solvent media such as chemical modification (Takahashi et al. 1984), immobilization (Clark and Bailey 1984; Kise et al. 1990), site-directed mutagenesis (Martinez et al. 1992) and directed evolution (Chen and Arnold 1991) have been used to obtain organic solvent-stable proteases. However, if enzymes are naturally stable and exhibit high activities in the presence of organic solvents, such stabilization methods and/or isolation is not necessary. So far, only a few natural enzymes which are organic solvent stable have been reported. Organic solvent-tolerant proteases from *Pseudomonas aeruginosa* PST-01, Pse A and *Bacillus cereus* BG1 were isolated and characterized (Ogino et al. 1999; Gupta et al. 2005; Ghorbel et al. 2003). Also, an amylase with organic solvent-tolerant property from the extremely halophilic archaea, *Haloarcula* sp. strain S-1, has been reported (Fukushima et al. 2005). However, behavior of enzymes from moderately halophilic species in organic

media has not been reported so far. Usually, moderately halophilic bacteria that grow optimally in media containing 3–15% NaCl produce halo-tolerant enzymes which are able to deal with high ionic strength in their environments (up to 4 M NaCl) and also in contrast to extremely halophilic counterparts can be active in the absence of salts (Ventosa et al. 1998). Therefore, investigation on the behavior of enzymes from moderately halophilic bacteria in the presence of organic solvents may be lead to obtain new enzymes with organic solvent-tolerant potential.

In our research for proteolytic moderately halophilic bacteria, a strain of *Salinivibrio* sp. was isolated from the Bakhtegan salt lake in the south of Iran. The strain AF-2004 secreted a considerable amount of extracellular proteases in culture media. Therefore, extensive studies were made on purification and characterization of its proteolytic activities. In our earlier studies we purified and characterized a zinc-metalloprotease with haloalkaline properties from the strain AF-2004 (Karbalaei-Heidari et al. 2006). In this paper, we report the purification processes and some biochemical properties of another extracellular protease of this strain, including the organic solvent-tolerant characteristics of the protease.

Materials and methods

Bacterial strain and culture conditions for protease production

Salinivibrio sp. strain AF-2004, a moderately halophilic bacterium that produced extracellular proteases, was isolated from the hypersaline lake as described previously (Amoozegar et al. 2006). For maximum protease production, subculture was prepared by inoculating a loopful of stock culture of strain AF-2004 in the medium (pH 8.0) containing (g/l): Tryptic Soy Broth, 30; Na_2HPO_4, 14.19; $CH_3COONa{\cdot}3H_2O$ 13.61; and NaCl, 50; followed by incubation at 32°C and 220 rpm. This overnight grown culture (1 ml) was used to inoculate 100 ml of fermentation medium (with the same conditions) in a 500 ml Erlenmeyer flask. The incubation was done at 32°C in an orbital shaker at 220 rpm. After 40 h of growth, the cells were harvested by centrifugation at 8,000 *g* for 20 min at 4°C (Ultracentrifuge, L5–50, USA), and the supernatant was collected and used for further studies.

Protease purification

All the purification steps were performed at 4°C. At the first step, pre-chilled acetone was gradually added to the supernatant up to 50% concentration and after discarding the precipitate, further acetone up to 80% saturation was added to the solution with gentle stirring and left for 1 h. The precipitate formed between 50 and 80% saturation of acetone was collected by centrifugation at 12,000 *g* for 20 min, dissolved in a minimum amount of 20 mM Tris-HCl, pH 8.0 containing 50 mM NaCl and 0.5 mM $CaCl_2$ (buffer A) and dialysed against the same buffer for 24 h. The dialysed enzyme preparation was applied to a Q-Sepharose HP column (1.6 × 20 cm) equilibrated with buffer A. The column was washed with the same buffer until no absorbance at 280 nm was detectable and bound proteins were eluted by applying a linear gradient of 0.05–0.75 M NaCl in buffer A at a flow rate of 1 ml/min. Active fractions were pooled and concentrated by freeze-drying. Samples were dissolved in a minimal volume of 20 mM Tris-HCl, pH 8.0 containing 0.3 M NaCl and 5 mM $CaCl_2$ (buffer B), and dialysed against the same buffer for 24 h with two buffer changes. The dialysed sample was finally loaded on a Sephacryl S-200 gel filtration column (1.6 × 65 cm; Pharmacia Biotech.), equilibrated with buffer B. Fractions of 3 ml were collected at a flow rate of 0.33 ml/min. The active fractions were pooled and concentrated by ultrafiltration (Centricon, Amicon, USA) and used as the purified enzyme for further analysis. The molecular mass of the purified enzyme was determined using the same column calibrated previously with a range of reference proteins: bovine serum albumin (BSA, 67,000 Da), bovine carbonic anhydrase (29,000 Da) and Cytochrome C (12,400 Da). Blue Dextran was used to determine the void volume of the column.

Protease assay

Protease activity was routinely determined by the casein hydrolysis method, as described previously (Karbalaei-Heidari et al. 2006). In brief, a reaction mixture of 0.48 ml of buffer A containing 1% (w/v) Hammarsten casein (E. Merck Darmstadt, Germany) and 0.02 ml of the enzyme solution with suitable concentration was incubated at 50°C for 5 min. The reaction was stopped by the addition of 0.5 ml of 10% (w/v) trichloroacetic acid (TCA), kept at room temperature for 15 min and then centrifuged at 14,000 *g* (Eppendorf 5415 C, Germany) for 8 min. The absorbance was measured against a blank at 280 nm. In some cases (effect of inhibitors), to 0.5 ml of the TCA precipitated supernatant, 1.5 ml of Na_2Co_3 solution and 0.5 ml of 1 N Folin-Ciocalteu reagent were added and mixed thoroughly. The color developed after 20 min of incubation at 37°C was measured at 660 nm. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μmol of tyrosine in 1 min at 50°C. The specific activity is expressed in the units of enzyme activity per milligram of protein.

Protein determination

Protein concentration was measured by the method of Bradford (1976) using BSA as the standard. During

chromatographic purification steps, protein concentration was estimated by observing the absorbance at 280 nm.

Polyacrylamide gel electrophoresis

Native PAGE was performed by the method of Davis (1964) in a 10% (w/v) polyacrylamide gel with Tris/glycine buffer, pH 8.3. SDS-PAGE was carried out according to the method of Laemmli (1970) after heating the samples at 80°C for 4 min, for the determination of molecular mass of the protease in 12% polyacrylamide gel containing 0.1% sodium dodecyl sulfate at 4°C and 10 mA/gel. A ready-to-use molecular marker (Fermentase; SM#0661) was used as a standard. Following native and SDS-PAGE, the proteins were stained with Commassie Brilliant Blue (0.2%). The demonstration of a protease activity band was done after electrophoresis in native PAGE gel which was placed in 1% BSA in buffer A for 1.5 h at 50°C and then stained with amido black 10B. A clear band of BSA hydrolysis was observed after destaining of the gel in water–methanol–acetic acid (60:30:10).

Influence of temperature, pH and NaCl

The optimum temperature for enzyme activity was determined by conducting the assay at various temperatures from 25 to 75°C. The effect of pH on protease activity was examined by incubating the reaction mixture at pH values ranging from 5.0 to 12.0, in the following buffer systems: 0.1 M sodium acetate (pH 4.0–5.5); 0.1 M sodium phosphate (pH 6.0–7.5); 0.1 M Tris-HCl (pH 8.0–9.0); 0.1 M glycine–NaOH (pH 9.5–12.0). Also, the effect of NaCl on protease activity was measured in the presence of 0–4 M NaCl in enzyme reaction mixture.

Effect of inhibitors and metal ions on proteolytic activity

The effect of inhibitors on the protease activity was examined after the protease had been pre-incubated with inhibitor for 30 min at 37°C, and the residual activity was determined by the standard assay method. The concentration of inhibitors (in the pre-incubation mixture) is listed in Table 2. For determining the influence of metal ions, the same procedure was used. The level of inhibition was expressed as a percentage of the activity remaining (with inhibitor) of the control activity (with out inhibitor).

Organic solvent stability of the protease

The stability tests in the different organic solvents were performed in buffer A containing 3 U/ml of the protease at 30°C. One volume of organic solvent was added to 4.0 volumes of the protease solution with constant shaking at 150 rpm for 7 days. The effect of higher concentration of solvents on the protease stability (40 and 80% v/v) was also studied at the same manner. The samples were incubated for appropriate periods of time, aliquots were then withdrawn and the residual activities of the enzyme were determined by using the assay procedure described above.

Results

Purification and properties of the protease

By applying a linear gradient of 0.05–0.75 M NaCl on Q-Sepharose anion exchange chromatography column, a chromatogram with two protease activity peaks was achieved. Further purification for the first proteolytic activity peak was then performed by loading on a Sephacryl S-200 gel filtration column. Based on the purification steps results, the protease was purified about 12.1-fold from the initial culture broth with a specific activity of 116.8 U/mg on casein and an overall yield of 6%. The results of the purification procedure are summarized in Table 1. The purified enzyme migrated as a single band in SDS-PAGE (Fig. 1a) and apparent molecular mass of the protease was determined to be 31 kDa from the semi-logarithmic plot of molecular mass versus mobilities for the standard proteins, and it was estimated as approximately 29 kDa by Sephacryl S-200 gel filtration. A zymogram, carried out under native conditions, showed a band with high-level activity of the purified protease similar to the control enzyme solution (50–80% acetone precipitation). A clear band concerning to another protease of this strain is also seen in the control enzyme solution vel (Fig. 1b).

The substrate specificity of the protease indicated that it was active on a variety of natural proteins (BSA, gelatin, hemoglobin and casein), but exhibited the

Table 1 Purification of the serine metalloprotease from *Salinivibrio* sp. strain AF-2004

Purification steps	Total activity (units)	Total protein (mg)	Sp. activity (units/mg)	Purification (fold)	Yield (%)
Culture supernatant	992	103.33	9.6	1.0	100
50–80% Acetone	861	15.95	54.0	5.6	87
Q-Sepharose	192	2.94	92.3	9.6	19
Sephacryl S-200	58	0.54	116.8	12.1	6

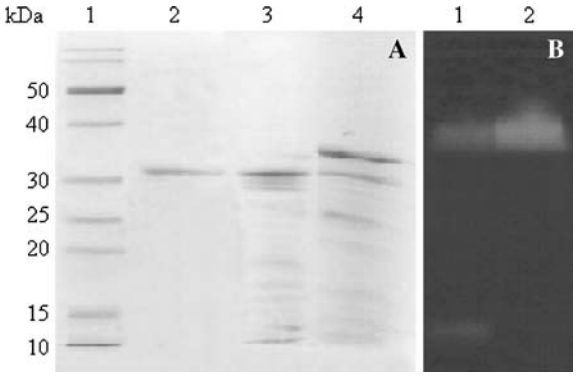

Fig. 1 **a** SDS-PAGE analysis of various purification steps pf protease produced by strain AF-2004. *Lane 1* Molecular markers, *lane 2* Sephacryl S-200 purified protease, *lane 3* Q-Sepharose column, *lane 4* acetone precipitated (50–80%); **b** zymogram of the purified protease in native PAGE as described in Materials and methods; *lane 1* acetone precipitated (50–80%), *lane 2* purified protease after Sephacryl S-200 column

highest activity toward casein. K_m and V_{max} values of the pure enzyme were determined by the measurement of enzyme activity in the presence of various concentrations of the casein substrate. Kinetic constants were calculated to be 264 U/mg and a K_m value of 1.4 mg/ml.

Effect of temperature, pH and NaCl concentration on the activity of the protease

The characterization of the purified protease revealed that the enzyme exhibited optimum temperature for maximum protease activity at 55°C (Fig. 2a) at pH 8.5. The protease undergoes thermal activation above 40°C with a maximum activity between 50 and 60°C, followed by thermal inactivation above 65°C. The protease from strain AF-2004 showed activity over a wide pH range for casein hydrolysis with an optimum pH of 8.5, and retained 85% of initial activity at pH 10 (Fig. 2b). Also maximal protease activity was measured at a concentration of 0–0.5 M NaCl (at 50°C and pH 8.5); although at higher salinities up to 4 M, activity still remained (Fig. 2c).

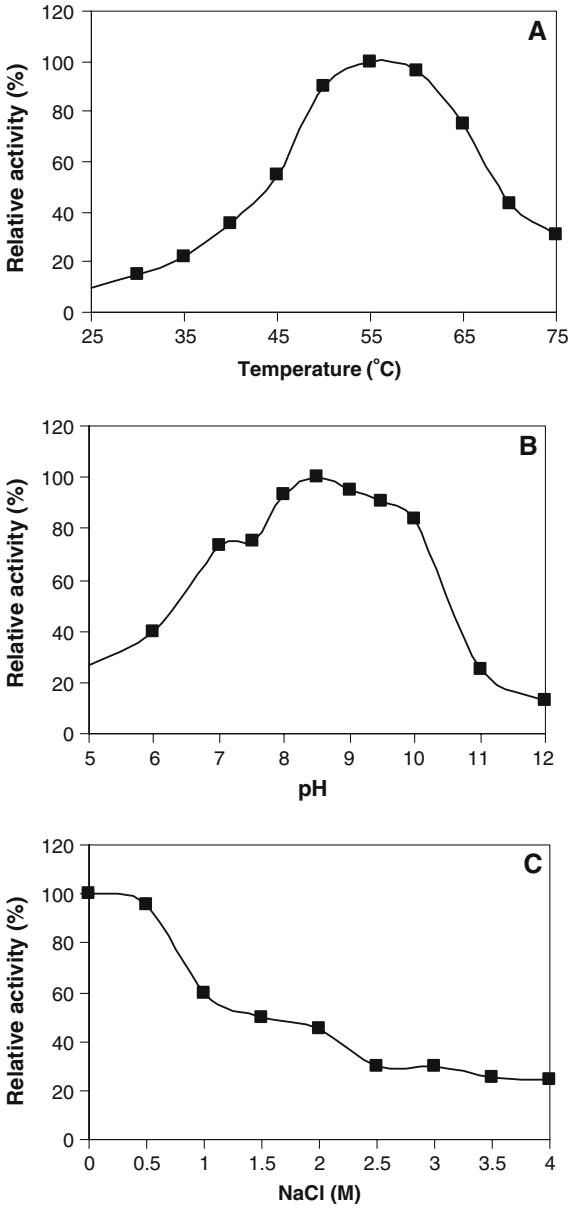

Fig. 2 Effect of temperature (pH 8.5) (**a**), pH (at 50°C) (**b**) and NaCl concentration (**c**) on the caseinolytic activity of the serine metalloprotease of strain AF-2004. The relative activity was defined as the percentage of activity detected with respect to the maximum protease activity. See Materials and methods for further details

Effect of different inhibitors and metal ions on proteolytic activity

The effect of chelating agents and group-specific reagents on purified protease activity is summarized in Table 2. The complete inhibition of caseinolytic activity by serine protease inhibitors such as Pefabloc SC and PMSF at a concentration of 4 and 3.3 mM, respectively, indicating the involvement of serine residue at its active site. Similarly, the protease was inactivated by the addition of 100 μM chymostatin, a chymotrypsin-like protease inhibitor. Enzyme activity was inhibited to 70% by 1 mM EDTA, while 1,10-phenanthroline (a zinc-specific chelator) had no significant effect. Antipain (a papain-like protease inhibitor) at 74 μM inhibited the protease activity by 50%. Also phosphoramidon, the competitive peptide inhibitor for thermolysin-like metalloprotease, had slight inhibitory effect on protease activity. Other specific protease inhibitors such as bestatin, E-64, leupeptin, pepstatin and aprotinin (BPTI) had no effect. Among metal ions, Zn^{2+}, Ca^{2+} and Ni^{2+} marginally stimulated the protease activity up to 34% at 1 and 5 mM (Table 3); whereas Mg^{2+} ions had slight inhibitory effect.

Table 2 Effect of different inhibitors on purified protease activity

Inhibitors	Final concentration	Residual activity (%)
Antipain	74 μM	50
Bestatin	130 μM	85
Chymostatin	100 μM	3
E-64	28 μM	94
Leupeptin	10 μM	80
Pepstatin	1 μM	101
	16.6 μM	97
Pefabloc SC	4 mM	5
PMSF	3.3 mM	0
Aprotinin (BPTI)	0.3 μM	93
	5 μM	100
Phosphoramidon	0.6 mM	81
EDTA-Na2	1 mM	30
	10 mM	15
1,10-phenanthroline	1 mM	105
	10 mM	94

The enzyme was incubated with different inhibitors at 37°C for 30 min. The residual protease activity was measured as described in Materials and methods. The values shown are the percentages of the activity without inhibitors

Table 4 Half-life of the serine metalloprotease of strain AF-2004 in the presence of various organic solvents

Organic solvent	Log K_{ow}	Half-life		
		20%	40%	80%
None	–	2 h	2 h	2 h
Ethanol	−0.235	> 50 days	6 days	4.4 days
1,4-Dioxane	−0.27	17.6 days	2 days	1.1 days
Benzene	2.13	1 h	5 h	8 h
Cyclohexane	3.3	12 h	1 days	14 h
Toluene	2.73	1.5 h	10 h	1 day
Cyclohexanol	1.23	16 days	18 days	10.3 days
1-Butanol	0.88	3.3 days	3 days	2.1 days
1-Hexanol	2.03	> 30 days	> 30 days	22 days
1-Octanol	2.97	> 50 days	15 days	24 days
1-Decanol	4.10	13.4 days	10 days	11.5 days

The enzyme was incubated in buffer A at 30°C with shaking at 150 rpm in the presence or absence of different concentrations of organic solvents for 7 days. The residual activity was measured after 0, 0.04, 0.75, 1, 2, 4, and 7 days. The half-life was calculated from the exponential regression curve

Effect of organic solvents on protease stability

The effect of different organic solvents at 20, 40 and 80% (v/v) concentrations on the stability of the protease in buffer A at 30°C was examined. Mixtures of the protease (3 U/ml) and organic solvents were incubated at 30°C with shaking and the remaining activity was measured at appropriate time intervals. As indicated in Table 4, the half-life of the enzyme in the absence of an organic solvent was about 2 h at 30°C. In the presence of 20% water-soluble organic solvents or alcohols tested, the half-lives were longer than 3 days. In particular, in the case of 20% ethanol and 1-octanol, the half-life of the enzyme was more than 50 days. However, the half-life of the enzyme in the presence of 20% water-insoluble organic solvents such as benzene and toluene was shorter than that in the absence of organic solvent. Also, in the presence of 20% cyclohexane, the half-life of the enzyme was 12 h. At higher concentration of solvents (40% and 80%), a reverse pattern of the stability in the presence of water-soluble or alcohols and water-insoluble organic solvents was seen. The half-life in water-soluble organic solvent or alcohol solutions decreased, but in the case of water-insoluble organic solvents especially benzene and toluene a little increased. Moreover, in the further experiments, the stability of the protease at 50°C was investigated in the presence of 20% ethanol and 1-octanol and was compared with that in the absence of organic solvent or alcohol. At 50°C, the enzyme activity in

Table 3 Effect of metal ions on the purified protease from S*alinivibrio* sp. strain AF-2004

Ions	Concentration (mM)	Residual activity (%)
None	–	100
$ZnCl_2$	0.5	131
	1	134
	5	127
$CaCl_2$	1	95
	5	115
$MgCl_2$	1	91
	5	92
$MnCl_2$	1	110
	5	98
$CoCl_2$	1	104
	5	94
$NiCl_2$	1	107
	5	120

The purified protease was dialyzed against Tris-HCl 20 mM buffer (pH 8.5). Samples were pre-incubated with various metal ions at different final concentrations at 37°C for 30 min. The protease activity was tested as described in Materials and methods

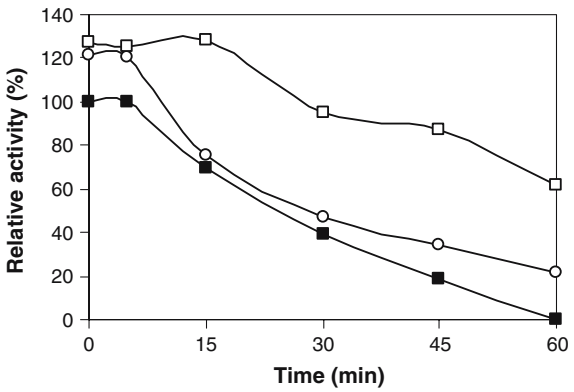

Fig. 3 Effect of organic solvents on stability of the serine metalloprotease. Purified protease was incubated at 50°C with constant shaking in the absence (*filled square*) or presence of 20% (v/v) 1-octanol (*open square*) and ethanol (*open circle*) for 60 min. The protease activity of the non-solvent containing control has been taken as 100%

the absence of organic solvents was virtually lost after 1 h of incubation. However, in 20% 1-octanol, the enzyme retained more than 60% of its activity in the 1 h incubation period (Fig. 3).

Discussion

A classical method to isolate enzymes with new industrial capabilities is to screen microorganisms which are normal inhabitants of extreme environments. A group of these extremophiles are halophilic microorganisms and among them, moderately halophilic bacteria exist which have been studied very rare. This paper describes the purification and characterization of the protease secreted by the newly isolated moderately halophilic bacterium, *Salinivibrio* sp. strain AF-2004. Like many other bacteria, the isolate showed proteolytic activity at the post-exponential phase of growth and reached to a plateau during the stationary phase (data not shown). The purification results revealed two kinds of proteases; a zinc-metalloprotease that was reported earlier (Karbalaei-Heidari et al. 2006) and a serine metalloprotease that will discuss here. Acetone precipitation, anion exchange and gel filtration chromatography were used to obtain the protease in its pure form. The molecular weight of the protease was about 31 kDa on SDS-PAGE. This value is smaller than that reported for certain other moderately halophilic proteases (Sánchez-Porro et al. 2003; Lama et al. 2005). However, similar molecular masses were shown by other serine alkaline proteases from *bacillus* species (Khalil Beg and Gupta 2003; Prakash et al. 2005). Biochemical properties of the enzyme such as temperature and pH profile revealed a moderate thermoactive and alkalophilic character (optimal activity at 55°C and pH 8.5). These characteristics are very similar to the protease CP1 from *Pseudoalteromonas* sp. strain CP76 (Sánchez-Porro et al. 2003). However, in comparison with another protease from strain AF-2004, this protease has lower temperature optimum and similar optimal pH. Also, the protease was active even without NaCl and over a wide range of NaCl concentrations (up to 4 M). This property is an important point from the view of biotechnology and helps to use the protease in both situations in industrial processes. For determining type of the protease, inhibition tests were done. The protease was strongly inhibited by PMSF, Pefabloc SC and chymostatin (see Table 2), indicating that it is a serine protease. This behavior is similar to semi-alkaline proteases (Luisetti et al. 1991). On the other hand, inhibition of the enzyme by EDTA suggested that this protease is a metalloprotease and no effect of O-Phe revealed that Zn^{2+} ions do not have an important role in its catalytic function. Also, the reactivation of the protease inhibited by 1 mM EDTA was done by the addition of 5 mM concentration of various metal ions (Ca^{2+}, Zn^{2+}, Mg^{2+}, Ba^{2+}); but none of them were effective for the reactivation of the enzyme (data not shown). Perhaps, some irreversible processes during denaturation step of the protease are responsible of this behavior. Taken together, these data suggest that probably this protease place in the class of serine metalloproteases. Similar behavior was shown by a serine metalloprotease from *Burkholderia pseudomallei* (Lee and Liu 2000).

When the polarity of the medium surrounding the enzyme molecules is reduced by introducing an organic solvent, enzymes are usually inactivated in response to the increase of hydrophobic environment. According to our knowledge, most of proteases have been introduced as an organic solvent-stable protease are metalloprotease (Ogino et al. 1999; Ghorbel et al. 2003; Gupta et al. 2005). For this reason, the behavior of this metalloprotease from strain AF-2004 in the presence of some organic solvents was tested. This is the first protease from a moderately halophilic bacterium that has been tested in organic media. As shown in Table 4, the enzyme revealed a good stability in the presence of 20% water-soluble organic solvents or alcohols such as ethanol, 1,4-dioxane, 1-hexanol, cyclohexanol, 1-butanol, 1-octanol and 1-decanol; since the half-lives were longer than that in the absence of these chemicals. This property of the protease which in the presence of water-soluble organic solvent or alcohol solutions is more stable than without it is unique. In recently reported for a stable protease in organic solvents from solvent-tolerant strain of *P. aeruginosa*, the stability of the protease without organic solvents is more than that in the presence of it (Gupta and Khare 2006). However, in the case of water-insoluble organic solvents, the same behavior to organic solvent-stable PST-01 protease was observed (Ogino et al. 1999). Similar to previous observations by Ogino et al. and Gupta et al., no relationship between the stability against organic solvent and the polarity (log K_{ow} value) of the added organic solvent was found. In order to see whether stability was retained if higher concentration of solvents was used, the effect of 40 and 80% (v/v) of organic solvents was also studied (Table 4). It was found that in the presence of water-soluble organic solvents and alcohols up to concentration as high as 80%, the half-life of the protease is longer than at least 24 h. Also, the results of the thermal stability at 50°C (see Fig. 3) confirmed the positive effect of alcohols on the stability of the protease. Other alcohols such as 1-hexanol, 1-decanol and even cyclohexane as a water-insoluble organic solvent had positive effect on its stability at 50°C (data not shown).

Thus, in brief, it can be summarized that the strain AF-2004 serine metalloprotease is a potential candidate for its use as a biocatalyst for peptide syntheses in the presence of hydrophilic organic solvents. Considering its stability in the presence of high concentration of salt and even without salt may also find potential application in the treatment of proteinaceous waste solutions with or without salt contents.

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