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ORIGINAL PAPER

Molecular and biochemical characterization of an extracellular serine-protease from *Vibrio metschnikovii* J1

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Abstract A protease-producing bacterium was isolated from an alkaline wastewater of the soap industry and identified as *Vibrio metschnikovii* J1 on the basis of the 16S rRNA gene sequencing and biochemical properties. The strain was found to over-produce proteases when it was grown at 30°C in media containing casein as carbon source (14,000 U ml^{-1}). J1 enzyme, the major protease produced by *V. metschnikovii* J1, was purified by a three-step procedure, with a 2.1-fold increase in specific activity and 33.3% recovery. The molecular weight of the purified protease was estimated to be 30 kDa by SDS-PAGE and gel filtration. The N-terminal amino acid sequence of the first 20 amino acids of the purified J1 protease was AQQTPYGIRMVQADQLSDVY. The enzyme was highly active over a wide range of pH from 9.0 to 12.0, with an optimum at pH 11.0. The optimum temperature for the purified enzyme was 60°C. The activity of the enzyme was totally lost in the presence of PMSF, suggesting that the purified enzyme is a serine protease. The kinetic constants K_m and K_{cat} of the purified enzyme using *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide were 0.158 mM and 1.14×10^5 min^{-1}, respectively. The catalytic efficiency (K_{cat}/K_m) was 7.23×10^8 min^{-1} M^{-1}. The enzyme showed extreme stability toward non-ionic surfactants and oxidizing agents. In addition, it showed high stability and compatibility with some commercial liquid and solid detergents. The *apr*J1 gene, which encodes the alkaline protease from *V. metschnikovii* J1, was isolated, and its DNA sequence was determined. The deduced amino acid sequence of the preproenzyme differs from that of *V. metschnikovii* RH530 detergent-stable protease by 12 amino acids, 7 located in the propeptide and 5 in the mature enzyme.

Keywords *Vibrio metschnikovii* · Alkaline serine protease · Purification · Characterization

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Introduction

Protease constitutes one of the most important groups of industrial enzymes, accounting for more than 60% of the total industrial enzyme market [13]. Proteases are used in many areas of applications, such as the detergent, food, agrochemical, and pharmaceutical industries [17].

The industrial demand for highly active preparations of proteolytic enzymes with appropriate specificity and stability of pH, temperature, metal ions, surfactants, and organic solvents continues to stimulate the search for new enzymes sources. Bacteria, molds, and yeasts are some of the microorganisms that produce proteases. Microbial proteases, especially from *Bacillus* sp., are the most widely exploited industrial enzymes [8, 12]. Among them, alkaline proteases are of particular interest due to their potential applications in the detergent industry as a cleaning additive [1, 11].

Some of the gram-negative *Vibrio* species have been known to secrete proteases into the extracellular medium. Kwon et al. [19] reported the secretion of two SDS-resistant alkaline serine proteases (VapT and VapK) from *V. metschnikovii* RH530. *V. metschnikovii* DL33-51 was found to produce a 29.5-kDa alkaline protease useful for detergent formulations [25].

Isolation and screening of microorganisms from naturally occurring alkaline habitats or from alkaline wastewaters is

expected to provide new strains producing enzymes active and stable in high alkaline conditions. During a screening program on protease-producing strains, *V. metschnikovii* J1 producing an alkaline protease was isolated.

This paper reports the purification and characterization of the extracellular alkaline protease (AprJ1) from the *V. metschnikovii* J1 strain. The nucleotide sequencing of the corresponding gene (*apr*J1) was also performed.

Materials and methods

Reagents

N-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (SAAP NA), casein sodium salt from bovine milk, ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), dithio-bis-nitrobenzoic acid (DTNB), trichloroacetic acid, glycine, ammonium sulphate, bovine serum albumin, and markers for molecular weights 14,200–66,000 Da were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium dodecyl sulphate (SDS), acrylamide, ammonium persulphate, *N*,*N*,*N*′,*N*′-tetramethyl ethylenediamine (TEMED), and Coomassie Brilliant Blue R-250 were from Bio-Rad Laboratories (Mexico). Sephadex G-75 and diethylaminoethyl (DEAE)-cellulose were from Pharmacia Biotech (Uppsala, Sweden). Tris (hydroxymethyl) aminomethane was procured from Panreac Quimica SA (Spain). All other reagents were of analytical grade.

Bacterial strain

V. metschnikovii J1 was isolated from an alkaline wastewater of soap industry in Sfax, Tunisia. The collected sample was plated onto skim-milk agar plates containing (g l^{-1}): peptone 5, yeast extract 3, bacteriological agar 12, and skim milk 250 ml, pH 10.0. The plates were incubated 24–48 h at 37°C. A clear zone of skim-milk hydrolysis gave an indication of protease-producing strains. Individual colonies were purified through repeated streaking on fresh agar plates. Among 20 strains, showing different proteolytic activities, isolate J1 was selected. The strain was identified as *V. metschnikovii* according to the methods described in Bergey's Manual of Determinative Bacteriology and on the basis of the 16S rDNA sequence analysis. The nucleotide sequence of the 16S rRNA gene has been submitted to the GenBank database and assigned accession no. FJ752498.

Culture and growth conditions

V. metschnikovii J1 was cultivated in M1 medium consisting of (g l^{-1}): casein 10.0, yeast extract 5.0, and NaCl 5.0, pH 8.0. Inocula were routinely grown in Luria–Bertani (LB) broth medium [26] composed of (g l^{-1}): casein peptone 10, yeast extract 5, and NaCl 5, pH 8.0. Media were autoclaved at 121°C for 20 min. For LB agar plates, media were solidified with 1.8% (w/v) agar [28].

Cultivations were performed on a rotatory shaker (150 rpm) for 18 h at 30°C, in 250-ml Erlenmeyer flasks with a working volume of 25 ml. The culture medium was centrifuged at 12,000×*g* for 15 min at 4°C, and the cell-free supernatant was used for estimation of proteolytic activity. The growth of the microorganism in M1 medium was determined by measuring absorbance at 600 nm.

Protease assay

Protease activity was measured by the method of Kembhavi et al. [15] using casein as a substrate. A 0.5-ml aliquot of the culture supernatant or the purified enzyme, suitably diluted, was mixed with 0.5 ml of 100 mM glycine–NaOH (pH 11.0) containing 1% (w/v) casein and incubated for 10 min at 60°C. The reaction was stopped by addition of 0.5 ml trichloroacetic acid (20%, w/v). The mixture was allowed to stand at room temperature for 15 min and then centrifuged at 10,000×*g* for 15 min to remove the precipitate. The acid-soluble material was estimated spectrophotometrically (T70, UV/VIS spectrophotometer, PG Instruments Ltd., Wiftaft, UK) at 280 nm. A standard curve was generated using solutions of 0–50 mg l^{-1} tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine per minute under the experimental conditions used. Protease activities represent the mean of at least two determinations carried out in duplicate.

Purification of the alkaline protease

The culture supernatant containing the extracellular enzyme was first concentrated by ultrafiltration with a stirred ultrafiltration cell (Millipore 8400) using a 10-kDa MW cut-off membrane (PBGC membrane, Millipore Co., Bedford, MA). The concentrated enzyme was then subjected to gel filtration on a Sephadex G-75 column (2.5 × 80 cm) equilibrated with 25 mM Tris–HCl buffer, pH 8.0, containing 0.05% (v/v) Triton X-100. Fractions of 5 ml each were collected at a flow rate of 30 ml h^{-1} with the same buffer and analyzed for protease activity and protein concentration. Fractions exhibiting protease activities were pooled and applied to a DEAE-cellulose column (3 × 25 cm) that had been equilibrated with 25 mM glycine–NaOH buffer (pH 9.0). After washing the column with equilibration buffer until the absorbance at 280 nm reached baseline, bound proteins were eluted with a linear gradient of sodium chloride in the range of 0–0.5 M in the equilibration buffer.

Fractions of 5 ml each were collected at a flow rate of 72 ml h^{-1} and analyzed for protease activity and protein concentration. All the purification steps were conducted at temperatures not exceeding 4°C.

Polyacrylamide gel electrophoresis

SDS-PAGE was carried out to determine the purity and molecular weight of the enzyme as described by Laemmli [20] using a 5% (w/v) stacking and a 15% (w/v) separating gel. The molecular weight of the enzyme was estimated using a low-molecular weight calibration kit as markers consisting of bovine serum albumin (66 kDa), glyceraldehyde-3-deshydrogenase (36 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa), and bovine α-lactoalbumin (14.2 kDa). Protein bands were visualized after staining with Coomassie Brilliant blue R-250.

Detection of proteolytic activity on polyacrylamide gels (protease zymogram)

Zymography was performed in conjunction with SDS-PAGE according to the method described by Garcia-Carreno et al. [10] with slight modification. The sample was not heated before electrophoresis. After electrophoresis, the gel was submerged in 100 mM glycine–NaOH buffer (pH 11.0) containing 2.5% Triton X-100 for 30 min at 4°C, with constant agitation to remove SDS. Triton X-100 was then removed by washing the gel three times with 100 mM glycine–NaOH buffer (pH 11.0). The gel was then incubated with 1% (w/v) casein in 100 mM glycine–NaOH buffer (pH 11.0) for 20 min at 50°C. Finally, the gel was stained with Coomassie Brilliant Blue R-250. The development of clear zones on the blue background of the gel indicated the presence of protease activity.

Protein concentration

Protein concentration was determined by the method of Bradford [4] using bovine serum albumin as standard and during the course of enzyme purification by measuring the absorbance at 280 nm.

N-terminal amino acid sequence of the purified protease

The purified enzyme, from DEAE-cellulose anion exchange chromatography, was subjected to SDS-PAGE and electrophoretically transferred to a polyvinylidiene difluoride (PVDF) membrane. After brief staining with Coomassie Brilliant Blue R-250, the PVDF band corresponding to the protease was excised, and the N-terminal amino acid sequence was determined by the Edman degradation method on an ABI Procise 494 protein sequencer (Applied Biosystems, Roissy, France).

Effect of pH on protease activity and stability

The optimum pH of the purified enzyme was studied over a pH range of 6.0–12.0 at 60°C. For the measurement of pH stability, the enzyme was incubated for 1 h at 25°C in buffers of various pH values, and the residual proteolytic activity was determined under standard assay conditions. The following buffer systems were used: 100 mM phosphate buffer, pH 6.0–7.5; 100 mM Tris–HCl buffer, pH 8.0–8.5; 100 mM glycine–NaOH buffer, pH 9.0–13.0.

Effect of temperature on protease activity and stability

The effects of temperature on J1 protease activity were studied at temperatures ranging from 40 to 75°C in 100 mM glycine–NaOH buffer (pH 11.0), using casein as a substrate. Thermal stability was examined by incubating the enzyme for 1 h at 55 and 60°C in the absence and presence of 5 mM $CaCl_2$. Aliquots were withdrawn at desired time intervals to test the remaining activity at pH 11.0 and 60°C. The non-heated enzyme was considered as control (100%).

Effect of metal ions and enzyme inhibitors

The effects of various metal ions (5 mM) on enzyme activity were investigated using $CaCl_2$, $MnSO_4$, $ZnSO_4$, $CuSO_4$, $BaCl_2$, $HgSO_4$, and $MgSO_4$. The activity of the enzyme incubated in the absence of metal ions was considered as 100%.

The effects of enzyme inhibitors on protease activity were studied using PMSF, DTNB, β-mercaptoethanol, and EDTA. The purified enzyme was pre-incubated with inhibitors for 30 min at 25°C, and then the remaining enzyme activity was estimated using casein as a substrate. The activity of the enzyme assayed in the absence of inhibitors was taken as 100%.

Kinetic studies

The activity of the purified protease was evaluated at 25°C with different final concentrations of *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide, ranging from 0.02 to 0.3 mM. The determinations were repeated twice, and the respective kinetic parameters, including K_m and V_{max}, were calculated from Lineweaver–Burk plots [22]. The value of the turnover number (K_{cat}) was calculated from the following equation: $K_{cat} = V_{max}/[E]$, where [E] is the active enzyme concentration and V_{max} is the maximal velocity.

Effect of surfactants and oxidizing agents on protease activity

The effect of some surfactants (Triton X-100, Tween 20, Tween 80, and SDS) and oxidizing agents (sodium perborate) on enzyme stability was studied by pre-incubating the purified protease for 1 h at 40°C. The residual activity was measured at pH 11.0 and 60°C. The activity of the enzyme without any additive was taken as 100%.

Detergent compatibility

The compatibility of the purified alkaline protease with commercial solid and liquid laundry detergents was studied. The solid detergents used were Dixan (Henkel, Spain), Nadhif (Henkel-Alki, Tunisia), Ariel (Procter and Gamble, Suisse), New Det (Sodet, Tunisia), and Axion (Colgate-Palmolive, France). Commercial detergents were diluted in tap water to give a final concentration of 7 mg ml^{-1} to simulate washing conditions. The liquid detergents used were Dixan (Henkel-Spain), Nadhif (Henkel-Alki-Tunisia), and Lav^{+} (Tunisia), and they were diluted 100-fold in tap water to simulate washing conditions. The endogenous proteases contained in these detergents were inactivated by heating the diluted detergents for 1 h at 65°C prior to the addition of the enzyme preparation. The purified enzyme was incubated with various detergent solutions for 1 h at 30 and 40°C, and then the remaining activities were determined under the standard assay conditions. The enzyme activity of a control, without detergent, incubated under the similar conditions, was taken as 100%.

DNA amplification

Genomic DNA from *V. metschnikovii* J1 was purified using Wizard® Genomic DNA purification kit (Promega, France). Three oligonucleotides, designed on the basis of the published sequence of the alkaline protease gene of *V. metschnikovii* RH530 [6], were synthesized and used for isolation and determination of the protease gene sequence. The complete *apr*J1 gene, which encodes the alkaline protease of *V. metschnikovii* J1, and its flanking regions were amplified using the upstream primer F1J1 (5′-TAACCAATAGGT AACAATTCA-3′) and the downstream primer R1J1 (5′-G CTGGAACCTGTCAAATTCC-3′) to generate a fragment of 1.4 kb. The internal primer F2J1 (5′-TGAGGATCCCG CACAACAGACCCCATAC-3′) was used with R1J1 to amplify and sequence the mature region of the *apr*J1 gene (1.0 kb).

DNA amplification was carried out using the TechGene® TC-312 Thermal Cycler (TECHNE, Cambridge, England). The PCR program was as follows: 1 cycle of 94°C for 2 min, 50°C for 45 s, and 72°C for 1 min 30 s; 30 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 1 min 30 s. Amplified PCR products were analyzed on an agarose gel and purified by the PureLink™ PCR Purification Kit (Invitrogen).

Nucleotide sequence determination

DNA sequencing was determined by the dideoxynucleotide chain termination method [29] using the ABI PRISM 3100 Avant (Applied Biosystem, France) sequencer according to the instructions of the manufacturer. In order to confirm the fidelity of the sequence, two independent PCR products were sequenced in both directions. Sequence comparison with the databases was performed using BLAST through the NCBI server. Alignment of protein sequences was done with the CLUSTALW program at the European Bioinformatics Institute server (http://www.ebi.ac.uk/clustalw) [30].

Nucleotide sequence accession number

The nucleotide sequence of the gene encoding AprJ1 has been submitted to the GenBank database and assigned accession no. EU443196.

Results and discussion

Purification of *V. metschnikovii* J1 alkaline protease

Alkaline protease (AprJ1), the major protease produced by *V. metschnikovii* J1, was purified by the three-step procedure described in "Materials and methods." The results of the purification procedure are summarized in Table 1. In the first step, the cell-free supernatant was concentrated by ultrafiltration. The specific activity at this step was 74,400 U mg^{-1}. Zymographic analysis of the concentrated enzyme revealed three clear bands of casein hydrolysis on the gel, indicating the presence of at least three proteases (data not shown). The concentrated enzyme preparation was then successively subjected to Sephadex G-75 gel filtration and DEAE-cellulose anion exchange chromatography. After the final purification step, the enzyme was purified 2.1-fold with a recovery of 33.3% and a specific activity of 124,040 U mg^{-1}.

The purified AprJ1 was homogenous on SDS-PAGE, and its molecular weight was estimated to be 30 kDa (Fig. 1), corresponding with that determined by gel filtration. Purity of the enzyme was also evaluated by zymography. As shown in Fig. 1, a unique clear band of casein hydrolysis was observed in the gel, indicating the homogeneity of the purified enzyme. Some proteases of other *Vibrio* species have been also reported. Two surfactant-stable

Table 1 Summary of the purification of J1 alkaline protease

Purification steps	Total activity (10^3 U)	Total protein (mg)	Specific activity (10^3 U mg^{-1})	Yield (%)	Purification factor
Culture supernatant	1129.84	19.08	59.2	100	1
Ultrafiltration	929.85	12.49	74.4	82.3	1.25
Sephadex G-75	680.16	6.99	97.25	60.2	1.64
DEAE-cellulose	376.23	3.03	124.04	33.3	2.1

All operations were carried out at 4°C

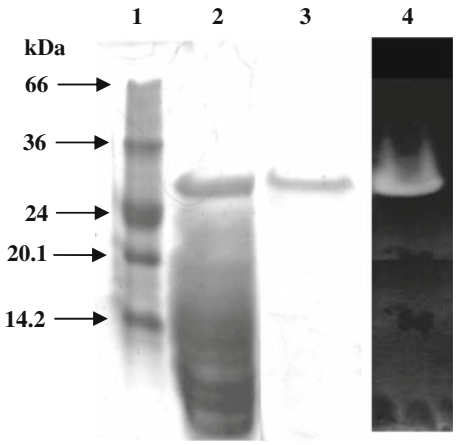

Fig. 1 SDS-PAGE of the purified alkaline protease from *V. metschnikovii* J1. *Lane 1* molecular weight markers, *lane 2* J1 crude extracellular proteases, *lane 3* purified J1 protease (25 µg), *lane 4* zymography of the purified J1 protease

alkaline metalloproteases 31 and 49 kDa were purified from *V. fluvialis* TKU005 [32]. Mei and Jiang [25] reported the purification of a 29.5-kDa serine-protease from *V. metschnikovii* DL33-51.

The N-terminal sequence of the first 20 amino acid residues of the purified enzyme from *V. metschnikovii* J1 was determined to be AQQTPYGIRMVQADQLSDVY. The 20 N-terminal amino acid sequence showed uniformity, indicating that it was isolated in a pure form.

Effect of pH on protease activity and stability

The pH activity profile of the purified AprJ1 was determined using different buffers of varying pH values. The purified enzyme was active in the pH range of 8.0–12.0, with an optimum at pH 11.0 (Fig. 2a). The relative activities at pH 10.0 and 12.0 were about 87 and 56%, respectively, of that at pH 11.0. The optimum pH for activity was slightly higher than that of the detergent protease of *V. metschnikovii* RH530 (pH 10.5) [19]. The high activity of the AprJ1 at high pH solutions is a very important characteristic for its eventual use as a laundry detergent additive, because the pH of laundry detergents is generally in the range of 9.0–12.0 [14]. The pH stability profile showed that the purified protease is highly stable in the pH range of

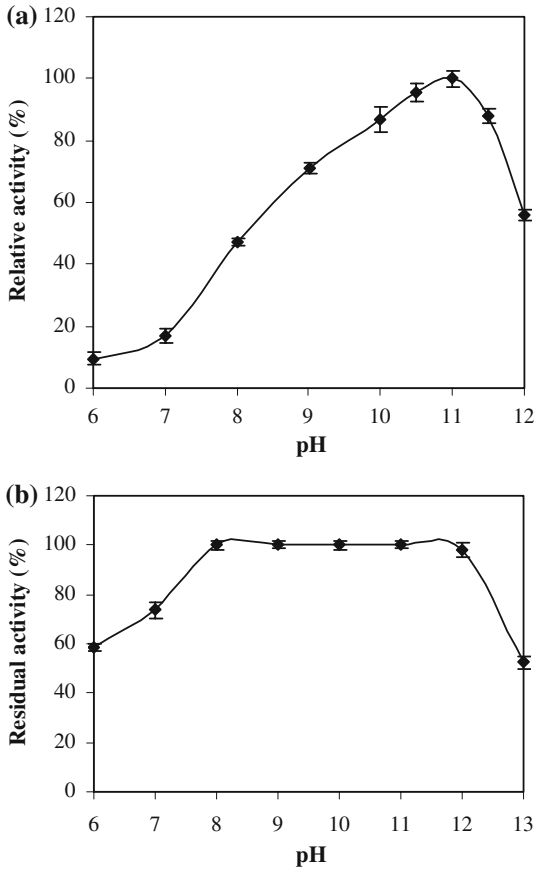

Fig. 2 Effect of pH on the activity (**a**) and stability (**b**) of the purified J1 protease. pH optimum was measured by incubating the enzyme with the substrate at different pH values at 60°C. The maximum activity obtained at pH 11.0 was considered as 100% activity. The pH stability was determined by incubating the enzyme in different buffers for 1 h at 25°C, and the residual activity was measured at pH 11.0 and 60°C. The activity of the enzyme before incubation was taken as 100%. Buffer solutions used for pH activity and stability are presented in "Materials and methods"

8.0–12.0, maintaining 100% of its initial activity after 1 h incubation at 25°C (Fig. 2b).

Effect of temperature on enzyme activity and stability

The effect of temperature on the activity of the purified AprJ1 was examined at various temperatures at pH 11.0. The AprJ1 was active between 50 and 75°C, with an

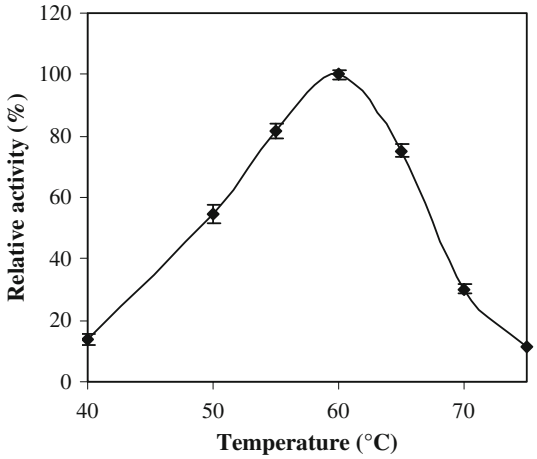

Fig. 3 Effect of temperature on the activity of the purified J1 protease. The temperature profile was determined by assaying protease activity at temperatures between 40 and 75°C. The activity of the enzyme at 60°C was taken as 100%

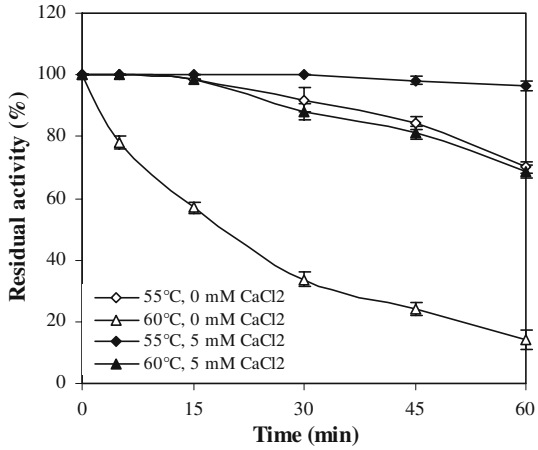

Fig. 4 Effect of temperature on thermal stability of J1 protease. The enzyme was preincubated at 55 and 60°C in the presence and absence of 5 mM $CaCl_2$, and residual enzyme activities were determined from 0 to 60 min at regular intervals under standard conditions. The non-heated enzyme was considered as control (100%)

optimum around 60°C (Fig. 3). The relative activities at 55 and 65°C were about 81.6 and 75.1%, respectively, of that at 60°C. The optimum temperature of AprJ1 was similar to the values reported earlier for proteases from other *Vibrio* strains [19, 31, 32].

In order to investigate the role of Ca^{2+} in the enhancement of the thermal stability during the thermal inactivation, the time courses on the inactivation of the enzyme was examined at 55 and 60°C in the absence and presence of 5 mM $CaCl_2$. The thermal stability profiles showed that Ca^{2+} was required for enzyme stability (Fig. 4). In the presence of $CaCl_2$, the enzyme retained 96.4 and 68.5% of its initial activity after 60 min incubation at 55 and 60°C, respectively. However, in the absence of $CaCl_2$ the enzyme retained 70 and 14.2% of its activity at 55 and 60°C, respectively. At 60°C, the addition of $CaCl_2$ extended the half life of the enzyme from 20 to 100 min. The improvement in protease thermostability against thermal inactivation in the presence of $CaCl_2$ may be explained by the strengthening of interactions inside protein molecules and probably by the binding of Ca^{2+} to the autolysis site [2, 21]. These results are in accordance with several works that reported the major role of the calcium ion in the stabilization of enzymes at high temperatures [7, 24].

Effect of enzyme inhibitors on protease activity

The effects of a variety of enzyme inhibitors, such as chelating agent, and a group-specific reagents on the AprJ1 activity are summarized in Table 2. Thiol reagent (DTNB, 2 mM) was without influence on the activity of the purified enzyme. However, the enzyme was completely inhibited by the serine protease inhibitor (PMSF, 2 mM), indicating that the purified enzyme is a serine protease. It is also interesting to note that the chelating agent (EDTA, 5 mM) inhibited the activity by 31%. Partial inhibition of most serine enzymes by EDTA, indicating the importance of cations as stabilizing agents, has been reported [3, 25].

Table 2 Effect of some enzyme inhibitors on the activity of J1 protease

Inhibitors	Concentration (mM)	Residual activity (%)
None	–	100
PMSF	2	0
EDTA	5	69 ± 1.2
DTNB	2	100 ± 0.9

Purified enzyme was pre-incubated with various inhibitors in 100 mM glycine–NaOH buffer (pH 11.0) for 30 min at 25°C, and then the residual activity was determined at pH 11.0 and 60°C. Activity of the enzyme pre-incubated in the absence of any inhibitor was taken as 100%. Protease activities represent the mean of at least two determinations carried out in duplicate

Effect of metal ions on enzyme activity

The effects of some metal ions, at a concentration of 5 mM, on the activity of the purified AprJ1 were studied at pH 11.0 and 60°C by the addition of the respective cations to the reaction mixture (Table 3). The addition of $CaCl_2$ increased the enzyme activity by 20% of the control. $BaCl_2$ and $MgCl_2$ showed no influence on the enzyme activity. The addition of Mn^{2+}, Zn^{2+} and Cu^{2+} had little effect on enzyme activity, whereas Hg^{2+} greatly affected the enzyme activity with more than 74.5% inhibition. These results are similar to those reported for other alkaline proteases from

Table 3 Effect of various metal ions (5 mM) on purified J1 protease activity

Metal ions	None	Ca^{2+}	Ba^{2+}	Zn^{2+}	Cu^{2+}	Mg^{2+}	Mn^{2+}	Hg^{2+}
Relative activity (%)	100	120 ± 1.75	100 ± 1.42	83 ± 1.88	90 ± 1.52	102 ± 2.1	87.7 ± 1	25.5 ± 0.88

The activity of the protease was determined by incubating the enzyme in the presence of various metal ions for 10 min at 60°C and pH 11.0. The activity is expressed as a percentage level in the absence of metal ions. Protease activities represent the mean of at least two determinations carried out in duplicate

Vibrio species [25, 31]. The effect of $CaCl_2$ concentrations on the enzyme activity was also studied. Maximum activity was observed with 2 mM $CaCl_2$ (128%) compared to that realized without metallic ions (data not shown).

Kinetic properties

Kinetic constants K_m and K_{cat} of the AprJ1 were determined using Lineweaver–Burk plots. The K_m and K_{cat} of the enzyme for *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide were 0.158 mM and 1.14×10^5 min^{-1}, respectively. In comparison with AprP from *Pseudomonas* sp. KFCC 10818, AprJ1 has a two-fold increased affinity to the used substrate [16]. Although the K_{cat} value of AprJ1 was similar to that of *Pseudomonas* enzyme (1.46×10^5 min^{-1}), the catalytic efficiency of AprJ1 (7.23×10^8 min^{-1} M^{-1}) for the synthetic substrate was 1.6 times higher that that of AprP (4.45×10^8 M^{-1} min^{-1}). From this result, it is evident that AprJ1 has a significantly higher substrate specificity toward the synthetic substrate than does AprP, which shares high sequence homology with the AprJ1 [16].

Effect of oxidizing agents and surfactants on protease stability

The high activity of the AprJ1 at high pH solutions is a very important characteristic for its eventual use as a laundry detergent additive. Further, in order to be effective during washing, a good detergent protease must be compatible and stable with all commonly used detergent compounds, such as surfactants, oxidizing agents, and other additives, that might be present in the formulation [12, 17].

The purified enzyme was incubated 60 min at 40°C in the presence of additives, and then the residual activity was assayed at pH 11.0 and 60°C. As shown in Table 4, the purified enzyme was highly stable in the presence of the non-ionic surfactants, retaining 100% of its activity. However, AprJ1 was completely inactivated by the strong anionic surfactant SDS (1%). On the contrary, the VapK protease from *V. metschnikovii* RH530, which shows high sequence homology with AprJ1, retained 100% of its initial activity after incubation with 1% SDS under the same conditions (30 min at 25°C) [6, 19].

We also investigated the influence of propylene glycol (PG) as a stabilizer toward inactivation by SDS. The enzyme was incubated for 1 h at 40°C in the presence of SDS (1%) supplemented with PG, and then the residual enzyme activity was determined. Addition of PG improved enzyme stability toward the inactivation by SDS. Further, the protective effect increased with increasing PG concentration. When AprJ1 was incubated with 1% SDS in the presence of 15% PG, 100% of activity was maintained.

Table 4 Stability of J1 protease in the presence of various surfactants and bleaches

Surfactants/oxidizing agents	Concentration (%)	Residual activity (%)
None	–	100
Triton X-100	5 (v/v)	103.5 ± 1.4
Tween 20	5 (v/v)	100 ± 1.4
Tween 80	5 (v/v)	103.7 ± 1
Sodium perborate	1 (w/v)	110.5 ± 2
	2	106.7 ± 1.7
SDS	1 (w/v)	0
SDS + propylene glycol	1 (w/v), 10 (v/v)	44 ± 2.4
	1 (w/v), 15 (v/v)	102 ± 1.4

The enzyme was incubated with different additives for 1 h at 40°C, and the remaining activity was measured under standard conditions. The activity is expressed as a percentage of the activity level in the absence of additives. Protease activities represent the mean of at least two determinations carried out in duplicate

Interestingly, the activity of AprJ1 was enhanced when preincubated in the presence of the oxidizing agents. The activities after 1 h incubation at 40°C were about 110 and 106% in the presence of 1 and 2% (w/v) sodium perborate, respectively. The stability of the enzyme in the presence of oxidizing agents is a very important characteristic for its eventual use in detergent formulations.

Stability of AprJ1 with commercial detergents

The high activity and stability of the purified alkaline protease in the pH range from 8.0 to 11.0, as well as its relative stability toward surfactants and oxidizing agents, are very useful for its eventual application as detergent additive. To check the compatibility and stability of the alkaline protease toward detergents, the enzyme was preincubated in the presence of various commercial laundry detergents of different compositions for 1 h at 30 and 40°C. The data

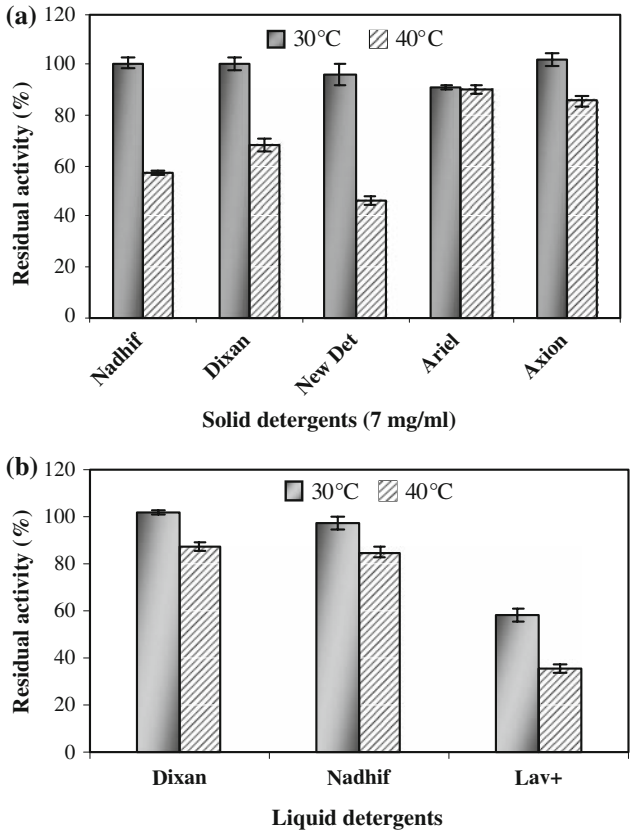

Fig. 5 Stability of the alkaline protease from *V. metschnikovii* J1 in the presence of various commercial solid (**a**) and liquid (**b**) detergents. The alkaline protease, at 500 U/ml, was added to solid detergent solutions at 7 mg/ml in tap water (pH 10.0) and to liquid detergent solutions diluted 100-fold in tap water (pH 9.0), and incubated 60 min at 30 and 40°C. The residual enzyme activity was determined at 60°C and pH 11.0. Enzyme activity of the control sample without any detergent, incubated under the similar conditions, was taken as 100%

presented in Fig. 5a show that the enzyme is highly stable in the presence of all solid detergents tested at 30°C. The AprJ1 retained 100% of its initial activity in the presence of Axion, Dixan, and Nadhif, and more than 92% with New Det and Ariel. At 40°C, the enzyme exhibited higher stability in Axion (90%) than Ariel (85%).

The data presented in Fig. 5b also show that the enzyme is relatively stable in the presence of liquid detergents, retaining more than 85% of its activity in the presence of Dixan and Nadhif, even after 1 h incubation at 30 and 40°C. However, the enzyme was found to be affected by Lav+, retaining only 58.3 and 35.7% of its initial activity at 30 and 40°C, respectively.

The obtained results clearly indicated that the performance of enzymes in detergents depends on number of factors, including the detergent's compounds, since the proteolytic stability of J1 protease varied with each laundry detergent tested.

Sequencing of *apr*J1 gene and deduced amino acid sequence

Analysis of the nucleotide sequence of the *apr*J1 gene (accession no. EU443196) revealed the presence of an open reading frame of 1,269 bp, starting with an ATG codon at nucleotide position 1 and terminating with a TAA stop codon at position 1,269. The G + C content of *apr*J1 gene is 47%.

The deduced amino acid sequence shows that AprJ1 is synthesized as a preproenzyme consisting of 422 amino acids with a predicted molecular weight and isoelectric point of 44,883.16 Da and 5.73, respectively. The protease was expressed as a precursor form, which contains pre- and pro-sequences. This result is similar to that of VapK RH530 [6]. The active mature protease consists of 283 amino acids with a calculated molecular weight of 29.524 kDa and a predicted isoelectric point of 7.26. The molecular weight of the purified enzyme (30 kDa), determined by SDS-PAGE and gel filtration, is in good agreement with the predicted value. In addition, the 20 residues of the N-terminal amino acid sequence of the purified AprJ1 perfectly matched with the deduced amino acids sequence which was found at position 140, confirming that the isolated gene encoded for the purified enzyme.

Comparison of J1 alkaline protease with other proteases

The deduced amino acid sequence of the mature enzyme was compared with those of other known alkaline proteases (Fig. 6). The deduced amino acid sequence showed 98, 97, and 68% identity with the sequences of alkaline serine proteases from *Pseudomonas* sp. [16], *V. metschnikovii* RH530 [6], and subtilisin-like serine protease from *Alkalimonas collagenimarina*, respectively [18].

Compared with VapK RH530, detailed sequence analysis revealed areas of sequence differences that led to 12 different amino acids, 7 located in the propeptide and 5 in the mature enzyme. For example, in VapK RH530 Lys_{139} and Arg_{273}, two basic amino acids are replaced by Asn_{139} and Ala_{273}, two neutral amino acids, respectively. In addition, the phylogenetic tree showed high homology between alkaline proteases from *V. metschnikovii* J1 and RH530 as they have the same root (Fig. 7). In comparison with the AprP from *Pseudomonas* sp. reported by Ko et al. [16], three amino acids (Thr_{98}, Val_{173}, and Arg_{272}) in the mature AprJ1 differ from the corresponding residues in the AprP (Asn_{98}, Ile_{173}, and Ser_{272}). In addition, the Ser_{272} residue, a neutral amino acid, in AprP is replaced by Arg_{272}, a basic amino acid. It is interesting to note that the modification in position 98 is present only in AprJ1 in comparison with all the protein sequences of the related *Vibrio* species. The residues involved in the active site (Asp_{30}, His_{63}, and Ser_{219}) are strictly conserved.

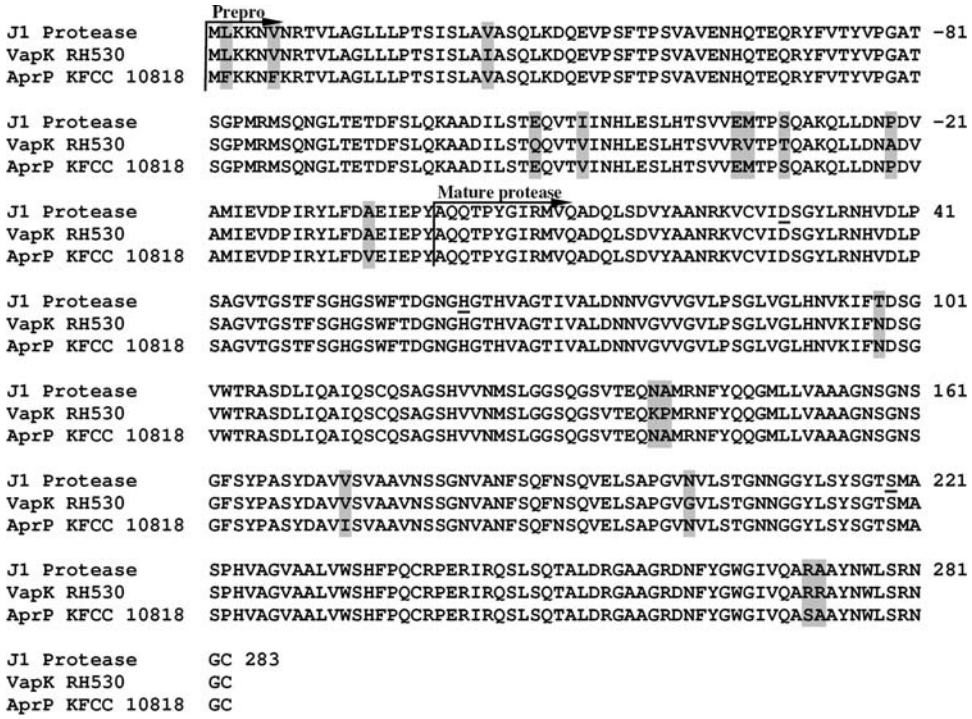

Fig. 6 Amino acid sequence alignment of J1 protease with those of VapK RH530 of *V. metschnikovii* RH530 [6] and AprP KFCC 10818 of *Pseudomonas* sp. [16]. The first amino acid of the mature protease, Ala, is counted as +1. X shows amino acid changes in J1 protease with other enzymes. The active site residues Glu_{30}, His_{63}, and Ser_{219} are *underlined*

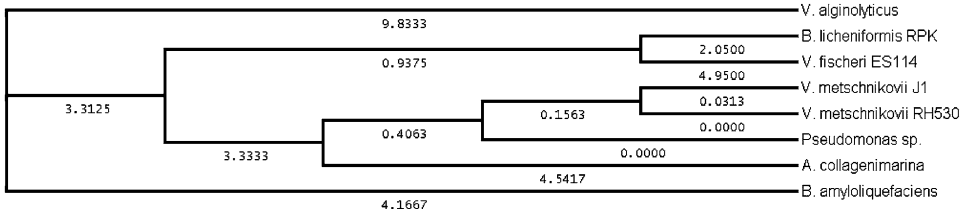

Fig. 7 Phylogenetic tree of protease from *V. metschnikovii* J1 (ACA49722) in relation to alkaline proteases from *V. metschnikovii* RH530 (AAF23173) [6], *Pseudomonas* sp. (AAA84886) [16], *A. collagenimarina* (BAF80056) [18], *B. licheniformis* RPK (EU502844) [9], *V. alginolyticus* (ABA28307) [5], *V. fischeri* ES114 (YP_204732) [23], and *B. amyloliquefaciens* (AAZ66858) [27]. The phylogenetic tree was generated using the neighbor-joining method in the MEGA program. The distances for branch lengths are presented along each branch

Conclusion

In this study, a novel alkaline protease (AprJ1) from *V. metschnikovii* J1 was purified and characterized, and the gene encoding this enzyme was completely sequenced. The purification to homogeneity of the enzyme was achieved by ultrafiltration, gel filtration through Sephadex G-75, and ion-exchange chromatography on DEAE-cellulose. After the final purification step, the enzyme was purified 2.1-fold with a specific activity of 124,040 U mg^{-1} and 33.3% recovery. The purified enzyme was homogenous on SDS-PAGE, and its molecular weight was estimated to be 30 kDa. The optimum temperature for proteolytic activity was 60°C. Its thermostability was enhanced in the presence of Ca^{2+}. Interestingly, the enzyme was highly active and stable over a wide range of pH from 8.0 to 12.0. Additionally, J1 alkaline protease was extremely stable toward non-ionic surfactants and oxidizing agents, and showed high stability and compatibility at 30°C with a wide range of commercial liquid and solid detergents. Considering the high activity and stability in high alkaline pH and in the presence of various commercial laundry detergents, AprJ1 may find potential application in laundry detergents. Further works are needed to improve the stability of the alkaline protease.

The *apr*J1 gene, which encodes the alkaline protease, was isolated and sequenced. The gene consisted of 1,269 bp encoding a protein of 422 aa, composed of a signal peptide and propeptide (139 aa) and a mature enzyme (283 aa). The amino acid sequence deduced from the nucleotide sequence revealed that the mature protease differs from that of VapK from RH530 by five amino acids.

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