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*Ph*AP protease from *Pseudoalteromonas haloplanktis* TAC125: Gene cloning, recombinant production in *E. coli* and enzyme characterization

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Abstract

Cold-adapted proteases have been found to be the dominant activity throughout the cold marine environment, indicating their importance in bacterial acquisition of nitrogen-rich complex organic compounds. However, few extracellular proteases from marine organisms have been characterized so far, and the mechanisms that enable their activity in situ are still largely unknown. Aside from their ecological importance and use as model enzyme for structure/function investigations, cold-active proteolytic enzymes offer great potential for biotechnological applications.

Our studies on cold adapted proteases were performed on exo-enzyme produced by the Antarctic marine bacterium *Pseudoalteromonas haloplanktis* TAC125. By applying a proteomic approach, we identified several proteolytic activities from its culture supernatant. *Ph*AP protease was selected for further investigations. The encoding gene was cloned and the protein was recombinantly produced in *E. coli* cells. The homogeneous product was biochemically characterised and it turned out that the enzyme is a Zn-dependent aminopeptidase, with an activity dependence from assay temperature typical of psychrophilic enzymes.

Keywords: Psychrophilic micro-organisms; Cold-active enzymes; Aminopeptidase

1. Introduction

Three quarters of the Earth's biosphere is cold and marine and most of the ocean's waters is yearly at temperatures below 15 °C. Although some marine bacteria have been studied, we have only limited knowledge about life at low temperatures in the seawater (Bölter et al., 2004). The huge number of cold-adapted microorganisms which successfully inhabits these regions, play fundamental roles in global elemental cycles and in the mineralization of organic matter. To perform this action, they have to possess enzymes with sufficient activity to catalyze chemical reactions at low *in situ* temperatures.

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Because the organic matter in marine environments is mainly composed of high-molecular-weight compounds the hydrolytic activity of extra-cellular enzymes plays a central role in bacterial acquisition of dissolved organic matter (Bölter et al., 2004) that could be not available for direct uptake by heterotrophic organisms. Proteases have been found to be the prevailing activity in the marine environment, demonstrating their fundamental role in the conversion and bacterial acquisition of nitrogen-rich organic compounds. Nevertheless, few secreted proteases from marine cold adapted bacteria have been characterized in order to improve our understanding on molecular mechanisms that allow their activity *in situ*.

Similarly to what observed in other extracellular psychrophilic enzymes, cold-active proteases are generally characterized by high-catalytic efficiencies at lower temperature and lower thermal stability. Several studies have indeed shown that cold-active proteases are often characterized by higher turnover numbers (*k*cat) and catalytic efficiencies (*k*cat/*K*m) at low temperatures compared to their mesophilic counterparts (Turkiewicz et al., 1999). These catalytic properties are useful in many different industrial applications, such as food industry, leather processing, and detergents (Georlette et al., 2004).

Our studies on cold adapted proteases were performed on exo-enzymes produced by the Antarctic marine bacterium *Pseudoalteromonas haloplanktis* TAC125 (*Ph*TAC125) which is able to secrete several proteases (Parrilli et al., 2008a, 2009). The molecular mechanisms of protein secretion in *Ph*TAC125 were investigated (Parrilli et al., 2008a,b). Recently, we reported the identification of the first component of a novel secretion system which is responsible of extracellular addressing of a psychrophilic alpha-amylase (Parrilli et al., 2009). The *in silico* analysis of *Ph*TAC125 genome (Medigue et al., 2005) demonstrated that the bacterium possesses also a type II secretion machinery (or T2SS) (Cianciotto, 2005) homologous to T2SS already described in many other Gram-negative bacteria (Parrilli et al., 2008a, 2009). A *Ph*TAC125 genomic mutant strain was constructed in which GSP-dependent secretion was completely abolished (Parrilli et al., 2009). This mutant was obtained by insertional mutagenesis of *gspE* gene that encodes a specialized ATPase whose inactivation in other Gram-negative bacteria resulted in the total loss of T2SS functionality (Parrilli et al., 2008b; Sandkvist et al., 1995). The *Ph*TAC125 *gspE* mutant strain resulted to secrete an extremely reduced number of proteases with respect to the wild type strain. Although the T2SS is responsible for the extracellular targeting of majority of secreted proteases, the analysis of the extra-cellular protein of *Ph*TAC125 *gspE* mutant revealed the presence of proteases that are translocated by secretion pathways different from T2SS.

In the present paper we report the identification of several proteolytic activities found in the culture supernatant of *Ph*TAC125 *gspE* mutant. One of them was selected for further investigations. The protein was recombinantly produced in *E. coli* cells, purified and its biochemical characterization was carried out.

2. Materials and methods

2.1. Partial purification of proteins from PhTAC125 gspE mutant supernatant

*Ph*TAC125 gspE mutant was grown at 4 °C in Typ medium (16 g/L Bacto tryptone, 16 g/L yeast extract, 10 g/L NaCl, pH 7.5) containing 100 μg/mL ampicillin till the medium exponential phase (about 72 h).

All procedures were performed at 4 °C. Culture supernatant (500 ml) was added with solid ammonium sulphate under stirring to bring the solution to 80% saturation. The suspension was incubated at 4 °C under gentle agitation for 4 h and then centrifuged at 20,000×*g* at 4 °C for 30 min. The pellet was dissolved in a minimal volume (50 ml) of 30 mM MES pH 5.0 (Buffer A). The resuspended pellet solution was dialysed against the same buffer and loaded onto a Resource Q 1 ml (GE Healthcare Life Science) column equilibrated with same buffer, and connected to an AKTA FPLC system (GE Healthcare Life Science). After extensive washing, a linear 0–1 M NaCl gradient in Buffer A was applied to separate the proteins. Active fractions for protease activity were eluted at approx. 250 mM NaCl, pooled, concentrated in a Savant centrifuge and dialysed against Buffer A containing 100 mM $CaCl_2$, 100 mM $MnCl_2$, 100 mM $MgCl_2$ and 50 mM $ZnSO_4$ at 4 °C.

2.2. In situ digestion

Protein bands were excised from the gel and destained by repetitive washes with 0.1 M NH_4HCO_3 pH 7.5 and acetonitrile. Samples were reduced by incubation with 50 μl of 10 mM DTT in 0.1 M NH_4HCO_3 buffer pH 7.5 and carboxyamidomethylated with 50 μl of 55 mM iodoacetamide in the same buffer. Enzymatic digestion was carried out with trypsin (12.5 ng/μl) in 10 mM ammonium bicarbonate pH 7.8. Gel pieces were incubated at 4 °C for 2 h. Trypsin solution was then removed and a new aliquot of the

digestion solution was added; samples were incubated for 18 h at 37 °C. A minimum reaction volume was used as to obtain the complete rehydration of the gel. Peptides were then extracted by washing the gel particles with 10 mM ammonium bicarbonate and 1% formic acid in 50% acetonitrile at room temperature. The resulting peptide mixtures were desalted using ZipTip pipettes from Millipore, following the recommended purification procedure.

2.3. NanoHPLC-chip MS/MS analysis

LCMSMS analyses were performed on a LC/MSD Trap XCT Ultra (Agilent Technologies, Palo Alto, CA) equipped with a 1100 HPLC system and a chip cube (Agilent Technologies). After loading, the peptide mixture was first concentrated and washed in 40 nL enrichment column (Agilent Technologies chip), with 0.2% formic acid in 2% acetonitrile as the eluent. The sample was then fractionated on a C_{18} reverse-phase capillary column (Agilent Technologies chip) at flow rate of 300 nl/min, with a linear gradient of eluent B (0.2% formic acid in 95% acetonitrile) in A (0.2% formic acid in 2% acetonitrile) from 7 to 60% in 50 min. Peptide analysis was performed using data-dependent acquisition of one MS scan (mass range from 300 to 1800 *m/z*) followed by MS/MS scans of the three most abundant ions in each MS scan. MS/MS spectra were measured automatically when the MS signal surpassed the threshold of 50,000 counts. Double charged ions were preferably isolated and fragmented over single charged ions.

2.4. Protein identification

The acquired MS/MS spectra were transformed in Mascot generic file format and used for peptides identification with a licensed version of MASCOT, in a local database.

Database searching of the MS/MS spectra was performed against the NCBInr database using an in house version of the software MASCOT (Matrix Science, London, UK) selecting *P. haloplanktis* TAC125 genome, previously inserted into the database. Parent ion tolerance was set at 600 ppm while fragment ion mass tolerance was set at 0.6 Da. Trypsin was chosen as enzyme.

The following protein modifications were considered as variable: oxidation of methionine to its sulfoxide counterpart, pyroglutamate formation (N-terminal Gln). Carbamidomethylation of cysteines was considered as a fixed modification.

2.5. Construction of the recombinant vector pET-22b-PhAP

DNA manipulation and isolation, amplification by PCR, and DNA sequencing were carried out by standard methods (Sambrook and Russell, 2001). Restriction enzymes, T4 DNA ligase, alkaline phosphatase, *Phusion* DNA polymerase were supplied by Boehringer-Roche, Promega, Fermentas or Finnzyme. DNA fragment purification was carried out by the QUIAEX II kit from Qiagen GmbH. *Ph*AP gene was amplified from *Ph*TAC125 genome in two separate PCR reactions. Primers used to amplify the first fragment of 1003 bp were designed to introduce a 5′ *Nde*I restriction site (*Ph*AP*NdeI*fw: 5′GTA*CATATG*AAAATCACACCTCT TTTTTTAAGC3′) and a 3′ *Spe*I restriction site (*Ph*AP*SpeI*rv: 5′GTA*ACTAGT*AAAACCTTCGTTT-AACCATAAATC3′) by silent mutagenesis of the *Ph*AP coding region. Primers used to amplify the second fragment of 840 bp were designed to introduce a 5′ *Spe*I restriction site (*Ph*AP*SpeI*fw: 5′TTTT*ACTAGT*TACG-TTGAAAACCGCATTATG3′) by silent mutagenesis of the *Ph*AP coding region restriction site and a 3′ *Not*I restriction site (*Ph*AP*NotI*rv: 5′ATAT*GCGGCCGC*-TTCAAATAATGCGTCTATAG3′). Each amplified fragment was cloned into pGEMT*easy* (Promega) vector and its nucleotide sequences were checked by sequencing to rule out the occurrence of mutations during synthesis. The two *Ph*AP fragments were then digested with *Nde*I/*Spe*I and *Spe*I/*Not*I, respectively, and ligated into pET22b expression vector (Novagen) previously digested with *Nde*I/*Not*I.

2.6. The PhAP recombinant production in E. coli cells

E. coli BL21(DE3) carrying pET-22b-*Ph*AP vector was grown in shake flask containing LB medium supplemented with 100 μg/mL ampicillin for 16 h at 37 °C. The obtained culture was diluted to a cell density of about 0.08 OD_{600} in 3 L of Terrific broth (12 g/L Bacto tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 12.54 g/L K_2HPO_4, 2.31 g/L KH_2PO_4, pH 7.2) containing 200 μg/mL ampicillin using a 7.5 L TechforsS fermentor (Infors HT Switzerland). The temperature of the medium was controlled at 28 ± 1 °C and the pH at 7.2 ± 0.5. Throughout the fermentation, the dissolved oxygen tension was maintained at approximately 30% of saturation by acting on air supply at constant agitation (250 rpm). Expression of enzyme was induced when culture density reached 3–4 at OD_{600} by addition of filter-sterilized IPTG to a final concentration of 1 mM. The culture was grown

for approximately 16 h post-induction. Cells were harvested by centrifugation at 32,000×*g* at 4 °C for 20 min, divided into 0.50 g aliquots and frozen at −80 °C.

2.7. Isolation and solubilization of PhAP inclusion bodies

The bacterial pellet (0.50 g) was frozen and thawed twice, resuspended in 10 mL of Buffer TN: 50 mM Tris–HCl, pH 7.5, containing 150 mM NaCl and subjected to sonication. Lysozyme (200 μg), RNase (200 μg), DNase (50 μg) and 30 mM $MgCl_2$ were added to the suspension and incubated for 30 min at 37 °C. The suspension was then diluted in 100 ml of 100 mM Tris–HCl, pH 8.0, 150 mM NaCl and gently shaken for 1 h at 4 °C. After 30 min centrifugation at 7000×*g*, the pellet containing the inclusion bodies was resuspended in 100 ml of 100 mM Tris–HCl, pH 8.0, and 50 mM β-mercaptoethanol. The suspension was gently shaken for 1 h at 4 °C and then centrifuged at 7000×*g* for 30 min. The pellet was solubilized in 10 ml of 6 M urea, 100 mM Tris–HCl, pH 8.0, 1 mM glycine, 1 mM EDTA, 50 mM β-mercaptoethanol at 4 °C with a gently shaking for 12 h.

2.8. PhAP refolding and purification

The solubilized material was centrifuged at 44,380×*g* for 40 min using a 60Ti rotor at 10 °C. The supernatant was then centrifuged at 49,000×*g* for 2 h using the same rotor at 16 °C. Renaturation of the supernatant containing the protease *Ph*AP was achieved by a 200-fold dilution of the denaturant in 100 mM Tris–HCl buffer, pH 8.0, and the resultant solution was concentrated again to about 50 ml in an Amicon ultrafiltration cell (Millipore) using a 10 kDa cut-off membrane. Starting from 0.50 g of cell pellet, about 5 mg of pure protein was obtained by this procedure and *Ph*APwas frozen at −20 °C for future use.

2.9. Electrophoretic analysis

Electrophoretic runs were performed with a Bio-Rad Mini-Protean II cell unit, at room temperature. Fractions from the fast protein liquid chromatography purification step were subjected to the SDS-PAGE analysis by using denaturing 12% polyacrylamide gel. The method used was performed essentially as described by Laemmli. "Page Ruler Prestained Protein Ladde" (Fermentas) was used as molecular weight standard.

2.10. N-terminal amino acid sequencing

The recombinant purified *Ph*AP enzyme was loaded on SDS-12% PAGE gel and then electroblotted onto polyvinylidene difluoride membrane. The band corresponding to the protein was cut and subjected to automatic Edman degradation to obtain the first 5N-terminal residues.

2.11. Enzyme assays

General proteolytic activity was measured by digestion of the macromolecular substrate azocasein; 200 μL of 0.7 mg/mL azocasein was incubated with 10 μg of enzyme solution for 30 min at 15 °C. After the incubation, the reaction was stopped by adding of 400 μL of 20% trichloroacetic acid (TCA) and shaking. Mixtures were then centrifuged at 1000×*g* for 15 min and finally, 325 μL of supernatant were added to 350 μL of 10 M NaOH. Absorbance of this solution was determined at 440 nm. *Ph*AP activity was also measured by using 200 μM L-alanine–alanine–valine–alanine-*p*-nitroanilide, L-leucine-*p*-nitroanilide, L-arginine-*p*-nitroanilide and L-alanine-*p*-nitroanilide as substrates. The reaction was followed by monitoring the linear release of *p*-nitroaniline (*p*-NAn) during 1 min at 410 nm in 1-cm path-length cells with a Cary 100 spectrophotometer (Varian, Australia) with an extinction coefficient of 8480 $M^{-1}\,cm^{-1}$. Initial rates were calculated by linear least-squares analysis of time courses comprising less than 10% of the total substrate turnover. Assays were performed at 25 °C in a mixture of artificial seawater (ASW) buffer (0.4 M NaCl, 9 mM KCl, 26 mM $MgCl_2$, 28 mM $MgSO_4$, 5 mM TAPSO {*N*-[tris(hydroxymethyl)methyl]-3-amino-2-hydroxypropanesulfonic acid} pH 7.2) with 5 mM $ZnCl_2$. Stock solutions at concentration of 100 mM of *p*-NA substrates were prepared by dissolving in water and stored at −20 °C.

2.12. Determination of pH and temperature optima

The dependence of the initial velocity on pH was monitored at 410 nm with a molar absorption coefficient of 8480 $M^{-1}\,cm^{-1}$. The buffers used were 5 mM MES over the range 5.0–7.0, 5 mM Tris–HCl over the range 7.0–9.0. The assays were carried out in duplicate or triplicate and the results were the means of independent experiments. The measurements of protease activity by using L-Leucine-*p*-nitroanilide at a final concentration of 200 μM in a reaction mixture 0.4 M NaCl, 9 mM KCl, 26 mM $MgCl_2$, 28 mM $MgSO_4$, 5 mM $ZnCl_2$ over the range 5.0–9.0. The dependence of protease activity on

temperature was studied over the range 5–35 °C; L-Leucine-*p*-NA at concentration of 200 μM was used as substrate in 5 mM Tris–HCl pH 8.0 containing 0.4 M NaCl, 9 mM KCl, 26 mM $MgCl_2$, 28 mM $MgSO_4$, 5 mM $ZnCl_2$.

2.13. Thermostability

The thermal stability of *Ph*AP was studied over the range 1–40 °C. Pure enzymes (0.2 mg/ml), in 0.1 mM Tris–HCl pH 8.0, were incubated in sealed glass tubes at different temperatures. Aliquots were withdrawn at 20, 40, 60, 80,100 and 120, min and assayed at 15 °C under standard condition described above.

2.14. Inhibition by several compounds of protease activity

Several compounds as divalent cations, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT) and EDTA were added to 10 μg of pure protein at final concentration of 0.1 and 5 mM and incubated at 4 °C for 1 h. The residual enzymatic activity was measured under standard condition as described above.

3. Results

3.1. Purification of extra-cellular proteases produced by PhTAC125 gspE mutant

*Ph*TAC125 *gspE* mutant cells were grown in TYP medium at 4 °C till the medium exponential phase and the culture medium was recovered. The extra-cellular proteases were purified from the culture supernatant by following the procedure described in Section 2. A single chromatography step allowed us to purify a pool of proteins with proteolytic activity. The SDS-PAGE analysis of purified sample revealed that it contains several proteins with a molecular weight ranging between 55 and 72 kDa (Fig. 1).

3.2. Identification of purified proteins

*Ph*TAC125 *gspE* purified proteins were excised from the gel shown in Fig. 1 into three gel slices (named 1, 2 and 3), reduced, alkylated and *in situ* digested with trypsin. The same procedure was applied to the corresponding (as regarding to the position on the gel) gel slices from a control void lane (Fig. 1, lane 2), in order to verify the absence of the identified proteins in the control lane.

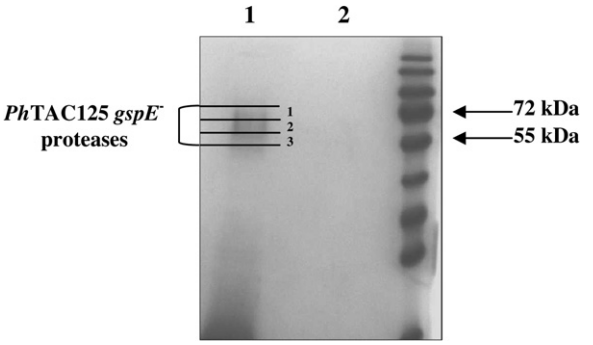

Fig. 1. SDS-PAGE (12% acrylamide) of the culture supernatant partially purified, utilised for the in situ digestion analysis. (*Lane 1*: proteases partially purified and *Lane 2*: control line).

The peptide mixtures were then directly analysed by nanoHPLC-chip MS/MS. The samples were resolved by nano liquid chromatography, and peptides were ionized on-line by nano-electrospray sources. The mass spectrometer acquires a mass spectrum, serially isolates the ions of choice, fragments them by collisions with an inert gas in collision cell and finally acquires a mass spectrum of the resulting ions (MS_2 spectrum) by three-dimensional ion trap system. MS_2 spectra consist mainly of N- and C-terminal fragments, produced by the breakage of the peptide amide bonds, named respectively y ions and b ions. MS/MS spectra were used to search for a non-redundant sequence database using the in house MASCOT software, taking advantage of the specificity of trypsin and the taxonomic category of the samples. The set of experimental mass values was compared to the theoretically predicted peptides from the proteins in the explored database leading to the reconstruction of the sequence and the protein identification.

As expected, more than one protein was identified in the same band, due the lack of resolution in the mono-dimensional SDS-PAGE. All the identified proteins are listed in the Table 1. Interestingly, only the products encoded by *PSHAa2184* and *PSHAa2915* are characterised by the presence of a clear signal peptide for the extra-cytoplasmic addressing of the encoded product. This feature makes the two proteins likely candidates to be substrates of the newly identified *P. haloplanktis* PSSA secretion system (Parrilli et al., 2009). *PSHAa2915* gene product was chosen for further investigations mainly due to the biotechnological potential related to aminopeptidase activity.

3.3. PhAP recombinant production and purification

pET-22b-*Ph*AP expression vector was constructed as described in Section 2. IPTG induction of recombinant

Table 1
Proteases identified in *Ph*TAC125 *gspE* mutant culture supernatant.

Accession number	Gene annotation	MW (Da)	SP	Protein
Q3IKD4	*PSHAa1089*	104.900	No	Putative peptidase
Q3IEB9	*PSHAa0935*	95.740	No	ATP-dependent protease
Q3IFX9	*PSHAa2492*	95.369	No	Putative Aminopeptidase
Q3IEU4	*PSHAa2184*	79.373	Yes	Peptidyl-dipeptidase dcp
Q3IJU7	*PSHAa2915*	68.997	Yes	Putative cold-active aminopeptidase;
Q3IGY7	*PSHAa1673*	51.309	No	Hypothetical protein
Q3ILI4	*PSHAa0562*	48.843	No	Proline aminopeptidase PII

E. coli BL21(DE3)(pET-22b-*Ph*AP) cells resulted in the accumulation of recombinant *Ph*AP as inclusion bodies (IB). IB were purified, solubilized with 6 M urea and the refolding was achieved by rapid dilution in 0.1 M Tris–HCl pH 8.0. Five milligrams of pure enzyme were obtained starting from 0.5 g of *E. coli* cell pellet. Purity of protein preparation was evaluated by SDS-PAGE analysis. The protein was recovered in quite homogeneous form (about 90–95% pure) as shown in Fig. 2. A unique band was observed with an apparent molecular mass of about 70 kDa, in agreement with a molecular mass of 68,997 Da predicted from the sequence. Recombinant *Ph*AP identification was carried out by N-terminal sequencing of the electroblotted protein band. The obtained N-terminal sequence was NAIDQ, thus confirming the signal peptide processing by the signal peptidase I and the periplasmic localization of *Ph*AP IB.

3.4. Determination of PhAP pH optimum, thermophilicity and thermostability

The dependence of *Ph*AP activity as a function of pH was examined at 25 °C towards the substrate L-leucine-*p*-NA. Kinetics were followed spectrophotometrically at 410 nm using two different buffers over the pH range 5.0–9.0 (Fig. 3). The highest relative activity was recorded at pH 8.0 in 5 mM Tris–HCl. The relationship between *Ph*AP activity and temperature in the range 5–35 °C was investigated using L-leucine-*p*-NA as substrate (Fig. 4). The apparent maximal activity was recorded at 15 °C. At 20 °C the recombinant enzyme exhibited about 60% of activity while at 35 °C the remaining activity drops down until 10%.

The *Ph*AP thermal stability was evaluated in the range 5–40 °C (Fig. 5). Enzyme samples were incubated at any given temperature for different lengths of time (up to 2 h) and residual activity was measured. After 2 h incubation at 4 °C, no change in activity was observed, while after 120 min of incubation at 40 °C the residual enzymatic activity resulted lower than 10%.

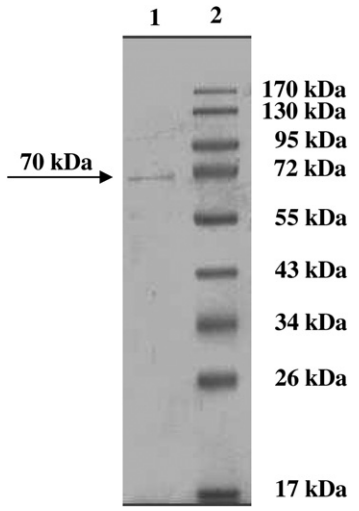

Fig. 2. SDS-PAGE (12% acrylamide) of recombinant *Ph*AP at the end of denaturation-refolding procedure (*Lane 1*: purified at homogeneity *Ph*AP and *Lane 2*: molecular weight markers).

3.5. PhAP substrate preference

Recombinant *Ph*AP displayed the highest activity toward *p*-nitroanilide substrate containing the Leucine residue (L-Leu-*p*-NA). Lower activity was detected using alanine in the substrates L-Ala-*p*-NA and in the tetra-peptide L-Ala–Ala–Val–Ala-*p*-NA (Table 2). About 70% of activity was detected using arginine (L-Arg-*p*NA).

The recombinant *Ph*AP activity was also assayed on a macromolecular substrate, such as Azocasein, to assess its proteolytic activity. This molecule resulted to

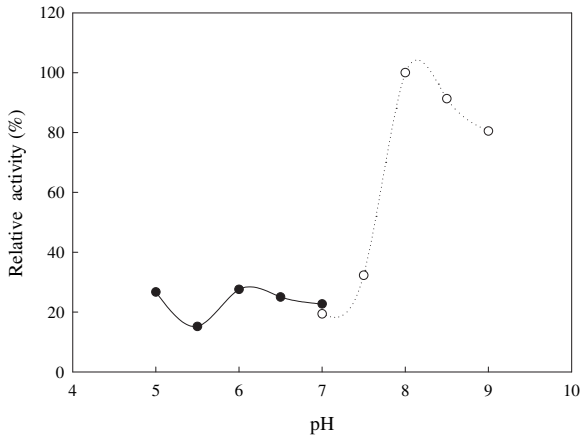

Fig. 3. Effect of pH on the activity of recombinant *Ph*AP. Measurements were performed in 5 mM MES (black symbols) and in 5 mM Tris–HCl (empty symbols).

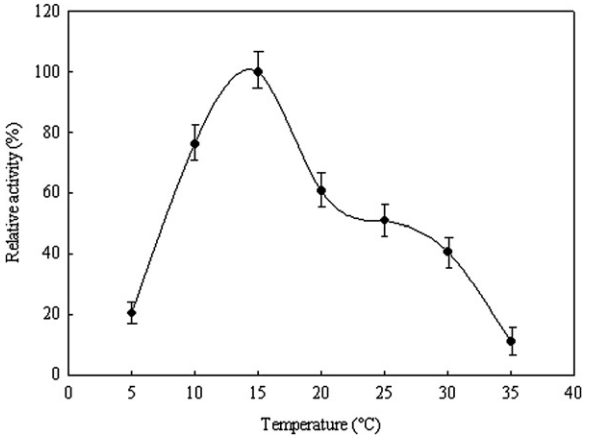

Fig. 4. Activity of recombinant *Ph*AP at various temperatures with 200 μM L-Leucine-*p*-nitroanilide as the substrate.

be a poor substrate for the psychrophilic enzyme (data not shown).

3.6. Effects of inhibitors on PhAP activity

Purified recombinant *Ph*AP was incubated 1 h in the presence of various denaturants or metal ions and the residual activity was measured using L-Leu-*p*-NA as substrate at 15 °C. Table 3 reports the observed residual activities. PMSF, a serine protease inhibitor, was found to completely inhibit *Ph*AP activity at concentration of 5 mM; the same effect was observed when the psychrophilic enzyme was subjected to DTT (a reducing agent) or EDTA (a metal-chelating agent) treatment. As concerns the effect of divalent cations addition, Li^{+}, Hg^{2+} and Ca^{2+} strongly inhibited *Ph*AP activity when added at a final concentration of 5 mM, while the adding of Ca^{2+} at lower concentration (1 mM) did not affect enzyme activity significantly. Mg^{2+} addition at 1 mM final concentration resulted to inhibit the enzyme activity of almost 60%.

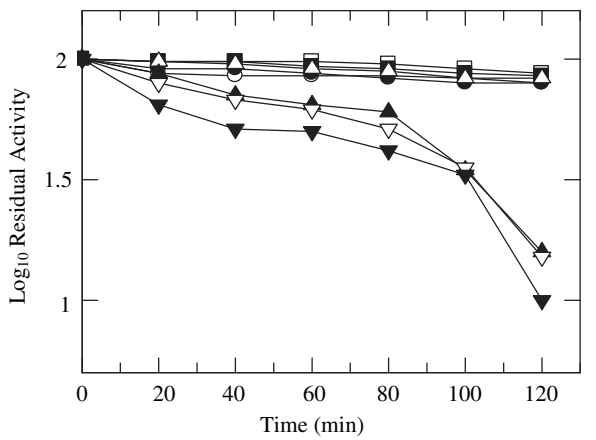

Fig. 5. Thermostability of *Ph*AP at various temperatures, such as 4 °C (○), 10 °C (●), 15 °C (□), 20 °C (■), 25 °C (△), 30 °C (▲), 35 °C (▽), 40 °C (▼). The enzyme was incubated in 0.1 M Tris–HCl pH 8.0 at the indicated temperatures for the times indicated. The residual activity was measured at 15 °C.

Table 2
Relative activities of recombinant *Ph*AP with various substrates at pH 8.0 and 15 °C.

Substrate	Hydrolysis rate (μmol min-1 mg-1)	Relative activity (%)
L-leucine-*p*-NA	0.150 ± 0.001	100
L-alanine-*p*-NA	0.135 ± 0.003	89.6
L-arginine-*p*-NA	0.112 ± 0.001	74.5
L-Ala–Ala–Val–Ala-*p*-NA	0.081 ± 0.001	54.0

3.7. PhAP sequence analysis

*Ph*AP is a 613 aa protein, characterised by the presence of a typical Type 1 Sec-dependent signal peptide of 21 aa devoted to protein export through the bacterial inner membrane.

The amino acid sequence of *Ph*AP was examined to determine its similarity to sequences available in the Swiss Prot database (http://www.expasy.ch/tools/blast). *Ph*AP exhibited the highest overall level of amino acid identity (from 55 to 65%) with putative M1 aminopeptidases from psychrophilic and mesophilic members of the same group of bacteria (γ-proteobacteria). The results of a multiple-sequence alignment (data not shown) indicated that there was perfect conservation of the putative substrate binding site (GGMEN) from Gly 282 to Asn 286 (the numbering refers to *Ph*AP sequence without the signal peptide), the zinc binding motif (HEXXH-X18-E), and catalytic residues involved in aminopeptidase activity (Glu 301 and Tyr 385 which are thought to act as a general base and a proton donor, respectively). Biocomputational sequence analysis revealed amino acid identities distributed throughout the entire *Ph*AP sequence that corresponded to secondary structural elements. Table 4 summarises some structural parameters derived from multiple alignment sequences of psychrophilic and mesophilic M1 aminopeptidases, in which we focused on structural features believed to increase the structural flexibility usually associated with low-temperature enzyme activity. In *Ph*AP and its psychrophilic homologs, the number of proline residues, which affect the backbone flexibility and thus the local mobility of the chain, is less than the numbers of proline residues in the mesophilic enzymes. The number of

Table 3
Purified *Ph*AP was incubated with various compounds for 1 h on ice in Tris–HCl 0.1 M pH 8.0 containing 0.4 M NaCl, 9 mM KCl, 26 mM $MgCl_2$, 28 mM $MgSO_4$, 5 mM $ZnSO_4$; residual activity was measured at 15 °C.

Compound	Concentartion (mM)	*Ph*AP activity (%)
Control buffer		100.0 ± 0.005
PMSF	0.1	77.7 ± 0.12
PMSF	5	0
DTT	5	0
EDTA	5	0
LiCl	5	0
$CaCl_2$	1	100.6 ± 0.017
$CaCl_2$	5	0
$MnCl_2$	5	0
$HgCl_2$	5	0
$MgCl_2$	1	39.5 ± 0.09

arginine residues, which have the potential to form multiple ion pairs and H bonds, is less than the number of lysine residues, as shown by the decrease in the Arg/(Arg + Lys) ratio. With respect to the other enzymes, *Ph*AP is also characterized by having a lower content of hydrophobic residues (A, F, G, I, L, M, P, V, and W) and a higher content of polar residues (C, N, Q, Y, S, and T) that, if exposed on the protein surface, could be involved in interactions with solvent molecules. Parameters resulting from biocomputation of the complete amino acid sequences of *Ph*AP and its mesophilic homologs with the Protparam tool (http://www.expasy.ch/tools/protparam.html) indicated that the grand average of hydropathicity (GRAVY index) of *Ph*AP and psychrophilic enzymes are relatively low, suggesting that the hydrosolubility of the psychrophilic proteases is higher with respect to the mesophilic counterparts and that these enzymes are predicted to have a better interaction with the solvent (Bauvois et al., 2008).

4. Discussion

The aim of this work was the identification, recombinant production, purification and biochemical characterization of *Ph*AP, an aminopeptidase isolated from the extracellular medium of a *P. haloplanktis* gspE mutant culture (Parrilli et al., 2008a,b). Being secreted by a T2SS mutant organisms, *Ph*AP could also represent a putative substrate of a psychrophilic secretion pathway, for which the first structural component (PssA) was recently identified (Parrilli et al., 2009).

To study *Ph*AP, we cloned the *PSHAa2915* gene into pET22b expression vector and we over-expressed the psychrophilic enzyme in *E. coli* BL21(DE3) strain. The amount of the recombinant enzyme in the whole-cell extract was very high, but the protein was poorly represented in the soluble fraction, because *Ph*AP largely accumulates as insoluble aggregates. Despite several attempts have been carried out to enhance the protein concentration in the soluble fraction (by changing production process parameters such as temperature, IPTG concentration, and specific *E. coli* strain), it always aggregates. Therefore, homogeneous *Ph*AP was obtained by inclusion bodies purification followed by *in vitro* refolding procedure. Applying this procedure, we obtained an average yield of 0.5 mg/L of protein, which was recovered in quite homogeneous form (about

Table 4
Summary of structural parameters of *Ph*AP, in comparison with psychrophilic and mesophilic homologous proteins, potentially involved in the cold-adaptation.

% of total residues										
Enzymes	Pro content	Arg/(Arg + Lys) ratio		Non Polar residues[a]		Polar residues[b]		Charged residues[c]		GRAVY[d] index
Psychrophilic enzymes										
*Ph*AP	30	5.1%	0.29	288	48.6%	162	27.3%	142	24.0%	−0.355
Colwellia AP	25	4.1%	0.36	294	48.4%	156	25.6%	155	25.6%	−0.343
P. atlantica T6c	39	6.4%	0.38	308	50.2%	159	25.9%	146	23.8%	−0.319
Shewanella baltica	31	5.0%	0.43	310	50.6%	171	27.4%	142	22.9%	−0.268
Alteromonas macleodii	37	5.9%	0.42	300	48.3%	163	26.1%	162	25.9%	−0.362
Mesophilic enzymes										
Xanthomonas oryzae	51	7.8%	0.43	357	54.9%	143	22.5%	149	22.9%	−0.249
Xanthomonas campestris	44	7.2%	0.39	331	53.9%	133	21.6%	151	24.6%	−0.291
Xylella fastidiosa M12	34	5.5%	0.42	335	54.2%	119	22.0%	151	24.3%	−0.145

[a] Non Polar 27 residues: Ala, Gly, Phe, Ile, Leu, Met, Pro, Val, Trp.
[b] Polar 28 residues: Cys, Asn, Gln, Tyr, Ser, Thr.
[c] Charged 29 residues: Asp, Glu, His, Lys, Arg.
[d] GRAVY index: Grand average of hydropathicity.

90–95% purity, as shown in Fig. 2). No additional purification steps were needed.

The refolded enzyme resulted to be active, being able to catalyse the efficient conversion of several substrates into products. Several criteria were evaluated to assess the quality of the purified recombinant *Ph*AP. The enzyme was analysed by CD spectroscopy and high percentages of the α and β structures were estimated (data not shown). These values are in agreement with data referring to properly folded forms of aminopeptidase belonging to the same family, such as the aminopeptidase from *Colwellia psychrerythraea* 34H (Huston et al., 2004).

The *Ph*AP apparent maximal activity was recorded at 15 °C (Fig. 4), while the enzyme retained only 10% of initial activity when incubated at 35 °C for 2 h (Fig. 5), values similar to those recorded for other enzymes from psychrophilic bacteria, such as the cold active protease from *C. psychrerythraea* 34H (Huston et al., 2004, 2008). Furthermore, in agreement with the *in silico* observation that *Ph*AP sequence contains the typical zinc-binding motif, the enzyme activity resulted to be dependent from the presence of zinc ions.

As for *Ph*AP substrate preferences are concerned, data reported in this paper suggest that this enzyme is an aminopeptidase, with a clear preference for substrates containing L-leucine residue (Table 2). In fact, azocasein, a macromolecular substrate, resulted to be a poor *Ph*AP substrate.

Taken together, our experimental results are strongly suggesting that *Ph*AP exhibits the classical traits of psychrophilic enzymes. Therefore, its amino acid sequence was subjected to several compositional analyses in comparison with other psychrophilic and mesophilic M1 aminopeptidases, with the aim of understanding which are the more relevant structural features related to cold-adaptation. In general, when cold-adapted proteins are compared to mesophilic proteins, they have fewer structural features that promote stability and more that increase protein flexibility. Structural features of cold-adapted proteins include fewer ion pairs, fewer arginine residues, fewer polar and H-bond-forming residues, fewer proline residues in protein loops, and fewer aromatic interactions than those in mesophilic proteins (Marx et al., 2007).

Amongst the several parameters analysed and reported in Table 4, its worth observing that *Ph*AP, and the other psychrophilic enzymes of the same family, is characterised by a higher percentage of polar residues with respect to the mesophilic counterparts. This remark, combined with the observation that the GRAVY index is lower in the cold adapted enzymes, is highly suggestive that *Ph*AP polar residues are likely exposed to the interaction with solvent molecules, a well known strategy to enhance the global protein flexibility (Aghajari et al., 2003; Arnórsdóttir et al., 2005; Georlette et al., 2004). If the flexibility in structure could justify the observed low-temperature activity of *Ph*AP, and in general of cold-adapted enzymes, however, it may be also responsible of the reduced thermal stability of the psychrophilic enzyme (Fig. 5).

Cold active proteases have proven to be promising enzymes to replace the conventional processes in biotechnological industries (Tutino et al., 2009). Moreover, these enzymes represent an extremely versatile group of catalysts that are capable of performing a variety of reactions thereby presenting a fascinating field for future research. The present study represents an obligatory step toward the solving of the issues related to the industrial implementation of these enzymes.

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