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ORIGINAL ARTICLE

Cloning, expression and decoding of the cold adaptation of a new widely represented thermolabile subtilisin-like protease

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Abstract

Aims: Cloning, expression and characterization of a new cold-adapted protease with potential biotechnological application, isolated from Antarctic bacteria.

Method and Results: A subtilisin-like gene was isolated from several Antarctic bacterial genus using CODPEHOP-designed primers and a genome walking method. This gene encodes a precursor protein, which undergoes an autocatalytic cleavage resulting in a 34·6 kDa active cold-adapted protease with a maximum activity at 25–35°C and optimum pH of 8·0–9·0. It showed a higher catalytic efficiency at lower temperatures compared to its mesophilic counterpart. Heat-induced inactivation resulted in a very low melting point. Local packing analysis using the homology model indicated Ala284 as an important cold-adaptation determinant, which was corroborated by the site-directed mutagenesis.

Conclusions: A new thermolabile subtilisin-like protease has been successfully cloned and analysed, and an important hot spot in the evolution of the cold adaptation and substrate specificity of this enzyme was identified and tested.

Significance and Impact of the Study: This work reports a new cold-adapted protease with a vast representation amongst Antarctic genus, suggesting therefore its evolutionary success in this cold environment. Likewise, important sites for genetic potentiation have been identified, which are extrapolated to other enzymes of the same kind.

Introduction

Temperature is one of the most important environmental factors affecting the efficiency of biochemical reactions. Low-temperature environments are the most widespread extreme condition present on the planet, and they have high diversity of cold-adapted micro-organisms. Cold-adapted micro-organisms known as psychrophiles survive over a wide range of temperatures, being the upper limit close to 35°C (Feller *et al.* 1996; Margesin 2009). These cold-adapted micro-organisms are known to produce cold-active enzymes, which have high levels of biocatalytic activity at low temperatures, but rapid inactivation at moderate temperatures (Feller *et al.* 1996).

The molecular basis of the adaptation at low temperatures still remains unclear; however, authors agree that the decreasing number and strength of intramolecular interactions such as noncovalent interactions, salt bridges, hydrogen bonds, hydrophobic interactions and aromatic–aromatic interactions are responsible for the high flexibility of the protein structure. These enzymes present high levels of activity at low temperatures and reduced thermostability (Russell 2000; Smalas *et al.* 2000; Lonhienne *et al.* 2001). These characteristics make cold-active enzymes

very interesting candidates for modelling in studies of thermal stability and molecular adaptation of proteins.

When compared with their mesophilic counterparts, cold-adapted enzymes have higher specific activity (k_{cat}) and catalytic efficiency ($k_{cat}\,K_m^{-1}$) at temperatures between 0 and 30°C. These characteristics provide cold-active enzymes with interesting advantages for biotechnological applications and huge potential for some industrial processes. For instance, due to their high enzymatic activity at low temperature, less enzyme concentration is enough to obtain sufficient biochemical conversion in a particular reaction. Also, the use of cold-adapted enzymes can decrease the consumption of energy (Gomes and Steiner 2004; Hoyoux *et al.* 2004).

The super family of subtilisin-like serine proteases (subtilases) is one of the largest groups of serine proteases and very important for industrial applications given their biochemical characteristics (Siezen and Leunissen 1997).

The extended use of proteases in industry has increased the demand for the discovery of new proteases adapted to the specific condition, for example, high catalytic efficiency at low temperature for laundry detergents (Yang *et al.* 2000; Gupta *et al.* 2002).

In this work, a new subtilisin-like protease gene was isolated, cloned and recombinantly expressed. The recombinant protease, P6, was biochemically characterized and compared with a mesophilic counterpart. Molecular models of P6 and mesophilic subtilisin-like proteases phylogenetically related with P6 were compared to find some of the principal factors, which may explain the cold adaptation.

Materials and methods

Strain selection

Psychrophilic bacteria were isolated from sea water and krill (*Euphausia superba Dana*) collected at Base Frei Montalva (Lat 62°11′S Long 58°58′W), King George Island, Chilean Antarctic. The bacterioneuston (bacterial community within the neuston or sea surface microlayer) were isolated through direct streaking onto marine agar 2216 (Difco, Sparks, MD) plates and incubated at 4°C. Other strains were isolated from the thorax, abdomen and head of Antarctic krill through direct streaking onto marine agar 2216 plates and incubated at 4°C. Sixty-two different strains (differentiated by colony characterization) were isolated, grown in marine broth 2216 and stored in 10% glycerol at −80°C. Twenty strains were selected according to the proteolytic activity of the supernatant using Succinyl-Ala-Ala-Pro-Phe-p-Nitroanilide (Succ-AAPF-pNa) as substrate. The bacterial strain of P6 protease was designated as *Pseudoalteromonas* sp. strain 2–10 after analysis of its 16S rDNA sequence.

Nucleic acid manipulation

Individual strains for DNA manipulation were harvested by centrifugation at 4°C after 4 days of cultivation in marine medium 2216 (BD 279110) at 4°C. DNA manipulation was carried out as described in Sambrook *et al.* (1989). PCR products were purified from agarose gel after electrophoresis by QIAEXII supplied from Qiagen Inc. (CA, USA). PCR-purified products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced by Macrogen (Seoul, Korea). Primers and restriction enzymes were purchased from Invitrogen (Carlsbad, CA, USA) and New England Biolabs (Ipswich, MA, USA), respectively. Taq DNA polymerase and elongase were obtained from Promega and Invitrogen, respectively.

Amplification of subtilase-encoding genes by PCR using consensus-degenerated primer design with CODEHOP and whole gene cloning

Subtilisin-like (subtilase) sequences of different families (subtilisin, proteinase K, thermitase, kexin and pyrolisin) were randomly selected and obtained from NCBI database (http://www.ncbi.nih.gov). They were aligned using the CLUSTAL X program to identify areas of consensus; then, after alignment of the protein sequences, consensus-degenerate PCR primers were designed according to the CODEHOP algorithm (Rose *et al.* 1998, 2003) using the WWW access at http://blocks.fhcrc.org/blocks/codehop.html. Degenerated primers and amplification reaction have been previously described for the subtilisin-like enzymes in Acevedo *et al.* 2008 (forward primer: B2F 5′-GGCCACGGCACCCAYGTBGCSGG-3′ and reverse primer: B2R 5′-CGTGAGGGGTGGCCATRSWDGT-3′). The amplification reaction was performed using 20 ng of genomic DNA from a pool of 20 bacteria strains previously selected. The main amplification bands were excised, purified, TA cloned into pGEM-T Easy vector and finally sequenced.

A selected partially sequenced gene was completed by a previously described genome walking technique (Acevedo *et al.* 2008). One microgram of genomic DNA was digested with an appropriated restriction enzyme (*Eco*RI for 5′ end amplification and *Hin*dIII for 3′ end amplification); then, all steps described by the genome walking technique were made: elongation of digested DNA end, ligation of digested DNA to an oligo-cassette adaptor, single-specific-primer polymerase chain reaction with the first specific primer and a second PCR with the oligo-cassette primer and the second specific primer. The first

specific primer used for the 5′ end amplification of the gene was P6R1 (5′-TGATAGAACGCCTTCAGAGTCGTTTGC-3′) and the second was P6R2 (5′-CGGCCCACTGATGCTACGTTTACTACAC-3′). The first specific primer for the 3′ end amplification was P6F1 (5′-CGCAAAAGTAGTGCCTGTTCGTGTACTTG-3′) and the second was P6F2 (5′-GGCAGCGGTTCATGTAGCTCTACAAC-3′). We obtained a DNA fragment of approximately 4·0 kb for the 5′ end of the gene and a DNA fragment of approximately 3·1 kb for the 3′ end. All PCR fragments were cloned directly into the pGEM-T Easy vector (Promega) and sequenced. After overlapping the sequences obtained from the CODEHOP PCR and genome walking, the whole nucleotide sequence of the gene was obtained. The completed nucleotide sequence was checked by complete PCR amplification and sequencing. The nucleotide sequence has been submitted to the GenBank database (http://www.ncbi.nlm.nih.gov/) (Benson *et al.* 2005). The nucleotide sequence encoding for P6 subtilisin-like protein was submitted under the following accession number: EF670003 (Acevedo *et al.* 2008).

Analysis of 16S rDNA

Primers P16F (5′-AGCTAGTTGGTAGGGTAAAG-3′) and P16R (5′-CCGCGATTACTAGCGATTCC-3′) were used to partially amplify 16S ribosomal sequences from culturable bacterial DNA that has the new subtilase genes. The amplified DNA of 900 bp was cloned into the pGEM-T Easy vector (Promega) according to the manufacturer's instructions. Finally, the cloned DNA fragments were sequenced by Macrogen Inc. A BLAST (NCBI) search was performed using the amplified DNA sequence to determine the most closely related 16S rRNA species.

Recombinant expression in *Escherichia coli*

The *E. coli* expression vector pET22b(+) (Invitrogen) was used to express the recombinant subtilisin-like protease P6. Based on the upstream and downstream sequences obtained from the genome walking method, two primers were used to generate PCR products carrying a *Nco*I restriction site at its 5′ end and a *Xho*I site at its 3′ end. P6FC (5′-TCTGCCATGGCTCAATCAGTTTCAAGTTCAG-3′) was used to generate the 5′ end in all variants of P6 expression vectors. For the 3′ end generation, the following primers were used: P6RC (5′-CTTTCTCGAGCGGTTGGTATTGCCC-3′), which allows the expression of the recombinant P6 fusioned to a C-terminal hexahistidine tag, P6RCsH (5′-AAACCTCGAGTTACGGTTGGTATTGCCC-3′) for the expression without the C-terminal hexahistidine tag, P6RsC (5′-TTGTCTCGAGACTTGCTGCTGCAACAGC-3′) for the expression of P6 fused to C-terminal hexahistidine tag, but without the pre-peptidase C-terminal domains (PPC) and P6RsCsH (5′-GAGTCTCGAGTTAACTTGCTGCTGCAACAGC-3′) for the expression of P6 without a C-terminal hexahistidine tag and without the PPC domains. The complete ORF (open reading frame) was amplified directly from the genomic DNA of the selected strain. The PCR products of P6 gene were digested with *Nco*I and *Xho*I restriction enzymes and then cloned into the expression vector pET22b(+) forming the different recombinant plasmids.

Electrocompetent cells of *E. coli* BL21 were prepared according to Sambrook *et al.* (1989). Recombinant plasmid was mixed with electrocompetent cells and electroporated. Spread LB plates were incubated at 37°C to screen for transformants. *Escherichia coli* BL21 transformed with the expression plasmids was grown overnight with shaking (200 rev min^{-1}) at 30°C in 20 ml of Luria broth (Difco). Expression was performed according to the supplier's instructions (pET system; Invitrogen). Five hundred millilitre of culture was grown at 30°C until the OD600 reached 0·8. The cells were harvested by centrifugation at 5000 ***g*** for 5 min and then resuspended in 500 ml of terrific broth medium (TB). The induction was performed 15–20 h at 14°C, using an inducer (IPTG, isopropyl β-D-1-thiogalactopyranoside) concentration of 0·3 mmol l^{-1}. To generate the P6-ΔPPC Ala284Ser mutant, QuikChange® site-directed mutagenesis was performed following the supplier instructions (Stratagene, LaJolla, CA).

Protein purification and characterization

The cells were harvested by centrifugation at 5000 ***g*** for 10 min at 4°C. The supernatant was modified to a concentration of 50 mmol l^{-1} NaH_2PO_4, 300 mmol l^{-1} NaCl, 2.5 mmol l^{-1} imidazole and pH 8·0. Then, 5 ml of Ni-NTA superflow (Qiagen) was added to 500 ml of supernatant. The mixture was gently stirred for 3 h at 4°C. Resin Ni-NTA loaded with the P6 recombinant protease was recovered and packed in a chromatographic column thermoregulated at 4°C. Affinity chromatography was performed using an ÄKTA purifier 10 (GE Healthcare, Piscataway, NJ). Packed resin was washed with the buffer 50 mmol l^{-1} NaH_2PO_4, 300 mmol l^{-1} NaCl, 2·5 mmol l^{-1} imidazole, pH 8·0. Protease P6 recovery was performed using a linear gradient of mobile phase from wash buffer to elution buffer, the last containing 50 mmol l^{-1} NaH_2PO_4, 300 mmol l^{-1} de NaCl, 250 mmol l^{-1} imidazole, pH 8·0. Fractions with high proteolytic activity were used as aliquot and stored at −80°C.

To estimate apparent molecular mass, analytical SDS-PAGE was performed using 12% acrylamide gels, and to estimate the isoelectric point, an isoelectric focusing on PhastGel 4–6·5 was performed using the Phast-System

(Pharmacia, Uppsala, Sweden). Protein concentrations were determined according to the Bradford method using a BSA calibration curve (Bradford 1976), and for the purified enzymes, concentrations were also calculated measuring the absorbance of the protein solution at 280 nm and using the molar absorption coefficient, which was predicted through the procedure described by Pace *et al.* (1995). Subtilisin-like activity was determined by the Erlanger method (Erlanger *et al.* 1961). This method comprises the measurement of the degradation of Succinyl-Ala-Ala-Pro-Phe-p-Nitroanilide (Succ-AAPF-pNa) in buffer 50 mmol l^{-1} Tris HCl, 2 mmol l^{-1} $CaCl_2$ and pH 8·0 for 20 min at 25°C. Released p-nitroanilide groups can be detected by their preferable absorbance at 410 nm. Temperature dependence or maximum catalytic activity was determined after the measurement of the proteolytic activity for 5 min at different temperatures in the range of 5–40°C, previous 5-min incubation of the reaction mixture at the corresponding temperature without the enzyme aliquot. The pH dependence of the cold-adapted P6 was determined on proteolytic assays at 25°C using 40 mmol l^{-1} Britton–Robinson buffer (40 mmol l^{-1} boric acid, 40 mmol l^{-1} acetic acid and 40 mmol l^{-1} phosphoric acid) supplemented with 2 mmol l^{-1} $CaCl_2$ and adjusted to pHs between 4·5 and 12. Temperature and pH dependence were calculated using both proteolytic activity, the method described above and the following method.

To show the effect of the oxidant on the proteolytic activity, proteases were incubated in the presence of 0·1 mol l^{-1} H_2O_2 during different intervals of time at 4°C. Residual activities were measured using the succinylated casein method (Baragi *et al.* 2000). Assays with nonoxidized proteases were used as controls, and assays without proteolytic substrate were used to obtain the baseline. The concentration of succinylated casein in these proteolytic assays was 1·44 mg ml^{-1}.

Kinetic parameters at various temperatures (5–40°C) were determined using succinylated casein as substrate (concentration 1% w/v in 50 mmol l^{-1} sodium borate, pH 8·0) and at different intervals during 40 min. The release of amino acids or the appearance of new NH_2 groups was measured using 2,4,6-trinitrobenzene sulfonic acid (TNBSA). Interaction between TNBSA and NH_2 groups can be detected by measuring the absorbance at 340–450 nm (Baragi *et al.* 2000). To obtain the kinetic parameter values, observed initial rate reactions were modelled by the nonlinear function of Michaelis–Menten for enzyme kinetics $V_i = \frac{V_{max} \cdot S}{S + K_m}$. The iterative method of least squares was used to model numerical data (observed V_i) by adjusting the parameters V_{max} and K_m. Substrate concentrations or independent variable used for this regression analysis were between 0 and 6 mg ml^{-1}.

Molecular modelling of P6-ΔPPC protease and analysis

A molecular three-dimensional model of the mature protease P6 was built using the MODELLER software (Sali and Blundell 1993). This modelling was based on multiple templates to improve the quality of the final homology model (Larsson *et al.* 2009). Only the mature sequence of P6 was modelled, excluding the N-terminal prosequence and two pre-peptidase C-terminal domains (PPC domains). Four previously defined three-dimensional structures of subtilisin-like proteins were used as templates. The structures included were as follows: *Bacillus lentus* subtilisin (PDB code 1GCI), *Bacillus alcalophilus* serine protease (1AH2), *Bacillus* sp. thermostable serine protease (1DBI), *Tritirachium album* proteinase K (1IC6) and *Thermoactinomyces vulgaris* (1THM). The templates share at least 37% sequence identity with P6. The modelling process started with the generation of multiple structure alignment using the MODELLER command SALIGN. Then, using the same command SALIGN, but keeping the structural alignment, the amino acid sequence of the protease P6 was aligned with the templates. Then, this was improved using a second multisequence alignment, which besides the above-mentioned proteins included the following amino acid sequences: EDO69450.1, YP_438579.1, 1ST3, 1C9M, 1Q5P, P23314, P42779, P29143, P04072, Q45670, P29118, P6 and O-7. The command MALIGN was used with more restricted gap penalties (gap opening and extension penalties of −1000 and −500, respectively). These strategies ensure an appropriate alignment amongst the secondary structures of the templates and the P6-ΔPPC amino acid sequence. The first alignment of structures and protease P6, improved by the adjustment with the second multisequence alignment, was used to build the homology model of P6-ΔPPC. The three-dimensional modelling was carried out using the MODEL command of the MODELLER program. This gave 20 possible models that were evaluated through another routine of the MODELLER software, named DOPE. The models were also evaluated using the web server VERIFY3D (http://nihserver.mbi.ucla.edu/Verify_3D/) (Bowie *et al.* 1991; Lüthy *et al.* 1992). Additionally, the subtilisin-like protein of *Xanthomonas campestris pathovar campestris* (GenBank accession code: P23314), a mesophilic counterpart of P6, was homology modelled using the same strategy and adjusted alignment. The catalytic domain of Xanthomonas subtilisin had a 71% sequence identity with P6-ΔPPC.

Comparative analysis of local packing

Local packing analysis of every residue in the X-ray structure models and homology models was evaluated and compared. As a measure of local packing, we used

the previously described contact density concept (Halle 2002; Gunasekaran and Nussinov 2007), which corresponds to the number of inter-residue atomic contacts observed in every residue. We define atomic contacts as any pair of nonhydrogen atoms positioned closer than a cut-off distance value of 4·5 Å to each other. Similar cut-off distances have been used to depict subtle local differences in homologous structures (Robinson-Rechavi *et al.* 2006; Böde *et al.* 2007; Gunasekaran and Nussinov 2007). Comparisons were made on the basis of structurally equivalent residues, identified from structure-assisted multiple sequence alignments using the software Expresso (3DCoffee) (Poirot *et al.* 2004). To avoid trivial information, atomic contacts between contiguous residues in the primary structure were not considered. Every homology model was compared to a group of homologous mesophilic subtilisin-like structures (PDB codes: 1SVN, 2ID8, 1YU6, 2IXT and 1GNS). Differences were expressed as the ratio between the average packing score of the mesophilic group and the score of the evaluated homology model for equivalent residues. Only the positions showing statistically significant differences through a Student's *t*-test were considered. In this work, both homology models, P6-ΔPPC and the catalytic domain of Xanthomonas subtilisin, were used to compare their residual local packing to an average of the group of mesophilic subtilisin-like proteins. Both homology models were compared using their local packing ratio differences, allowing the identification of the most likely residues that could explain the cold-adaptation behaviour of P6-ΔPPC.

Results

Cloning of partial subtilisin-like sequences by PCR amplification using consensus-degenerate hybrid oligonucleotide primers (CODEHOP)

DNA extracted from 20 Antarctic marine bacterial strains was used as a template for the amplification of subtilisin-like gene fragments in a PCR. These strains were selected from a collection of culturable strains isolated from samples of Antarctic sea water and krill. This selection was made according to their supernatant proteolytic activity using Succ-AAPF-pNa as substrate. Partial gene amplifications and sequencing of subtilisin-like genes were performed according to the previous publication (Acevedo *et al.* 2008).

Bands of approximately 500 pb were TA cloned into pGEM-T Easy vector and sequenced; results showed that from 13 selected products, 10 were identified as subtilisin-like genes and named from P1 to P10 accordingly. Although there were phenotypical differences in the colonies amongst the strains, eight of these nucleotides bands had at least 98% calculated amino acid sequence identity (P1, P2, P3, P5, P6, P8, P9 and P10). P6 from strain 4 (ABS01328) was used as an example of these genes for complete cloning. The sequencing results also showed that P4 (GenBank accession number: EF670002 (p4)) from strain 10 and P7 (EF670004 (p7)) from strain 20 were also subtilisin-like genes, but distantly related to the others. An analysis of the 16S rDNA sequences was performed to see whether the selected strains were from different phylogenetic origin. This analysis indicated that strain 4 has the highest 16S identity with *Pseudoalteromonas* sp.; strain 10 presents the highest 16S identity to *Marinobacter* sp.; strain 13, which presented the amplification P9, has the highest 16S identity with *Psychrobacter* sp.; and strain 20, which had the amplification P10, showed the highest identity with *Polaribacter* sp. Although the nucleotide sequences of P6, P9 and P10 are very similar, they come from different bacterial species according to the 16S rDNA analysis. Finally, P6 was chosen to complete the adjacent unknown sequences through an improved genome walking technique developed by our group (Acevedo *et al.* 2008).

Analysis of the calculated amino acid sequence of the subtilisin-like P6

The amino acid sequence of the new subtilase gene was analysed using the predictor programs Psortb, ver. 2.0.4 (Gardy *et al.* 2003, 2005); SignalP 3.0 (Bendtsen *et al.* 2004); and LipoP, ver. 1.0 (Juncker *et al.* 2003). The results indicated that P6 has a potential signal peptide sequence of 27 amino acids at its N-terminal. Blast search analysis revealed that the gene encoding P6 has a putative prosequence adjacent to the signal peptide and also two other pre-peptidase C-terminal domains (PPC domains) (see Fig. S1). P6 was predicted as a secreted subtilisin-like protein, and its amino acid sequence showed high similarity to peptidases from *Pseudoalteromonas* sp SM9913 (ABD92880, 86% identity), *Pseudoalteromonas haloplanktis* TAC125 (CAI87697, 84% identity) and *Pseudoalteromonas piscicida* O-7 (BAA18912, 71% identity) (Tsujibo *et al.* 1996). This type of subtilisin-like protein has also been found in genus such as *Idiomarina, Stigmatella, Xanthomonas* and *Stenotrophomonas*. Multisequence alignment including the new subtilase gene and other subtilisin-like proteases such as BPRV_DICMO, SEPR_THESR, THES_BACSJ, HLY_HAL17 and PROA_VIBAL helped to predict an N-terminal prosequence. All these subtilases were previously characterized as having an N-terminal sequence that is excised in the mature enzyme.

Protein P6 purification

Different variants of P6 were constructed in the expression vector pET-22b(+), some of them including a hexahistidine tag at the C-terminal. Some of the constructs included PPC domains, whereas others not. *Escherichia coli* BL21 (DE3) was transformed, and the recombinant P6 expression was induced adding IPTG to the culture. Samples of supernatant were assayed using the synthetic substrate Succ-AAPF-pNa. Proteolytic activities were measured at 25°C, and they showed an unusual behaviour. None of the P6 variants showed activity in their supernatant immediately after the assays started. The initial activity rate in the assay progressively increased during the first 80 min (Fig. 1). Proteases with or without 6XHis, as well as proteases having or not having the PPC domains, showed approximately the same activity value and activation behaviour.

P6 variants without PPC domains (P6-ΔPPC) and fused to a C-terminal hexahistidine tag were easily purified from the supernatant through a Ni-NTA His-Bind affinity chromatography (see Fig. S2). P6-ΔPPC showed a molecular mass of ≈32 kDa, an isoelectric point of pH 5·55 ± 0·05 estimated by SDS-PAGE analysis and Phast-Gel IEF 4–6·5, respectively. Further studies in this publication were performed using exclusively the P6-ΔPPC 6XHis-tag construct because it did not present apparent differences with the other constructions and was easily isolated.

Biochemical and enzymatic characterization of P6-ΔPPC

Proteolytic assays of P6-ΔPPC using succinylated casein were reduced more than 90% after incubation with 1 mmol l^{-1} PMSF (phenylmethylsulphonyl fluoride). The same result was observed for Carlsberg subtilisin, a mesophilic counterpart. The presence of 1% SDS was highly deleterious for the P6 proteolytic activity, reaching 90% inactivation; meanwhile, for Carlsberg subtilisin, the inactivation was only about 20%. High PMSF sensitivity suggests the presence of a reactive serine residue, which is the main catalytic amino acid in the active site of subtilisin-like proteins. On the other hand, incubation of P6-ΔPPC with 5% β-mercaptoethanol did not have significant effects on the proteolytic performance.

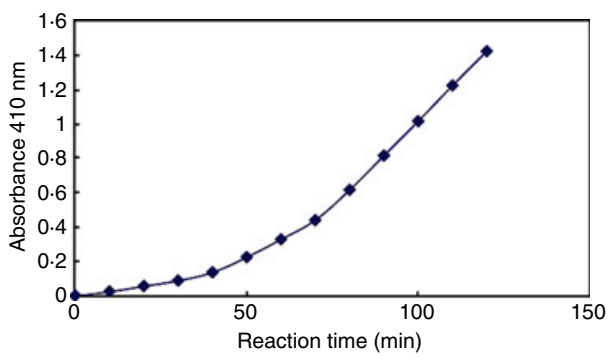

Figure 1 Proteolytic activity of P6 protease without the PPC domain (P6-ΔPPC). Measurements were performed in the supernatant of an IPTG-induced culture, using Succ-AAPF-pNa as substrate.

Pre-incubation with the strong oxidant H_2O_2 produced a high and rapid inactivation effect on the proteolytic activities of P6-ΔPPC and Carlsberg subtilisin. P6-ΔPPC appears to be more susceptible to the oxidant than Carlsberg subtilisin, showing only 15% residual activity after 5 min of incubation (Carlsberg subtilisin 30%). After 30 min, the inactivation did not increase for P6-ΔPPC, but the residual activity of Carlsberg subtilisin was reduced to 20%. P6-ΔPPC rapidly achieved a maximal inactivation compared to Carlsberg subtilisin, which took more time to reach lower states of activity.

The optimum pH for the catalytic reaction of P6-ΔPPC was between 8·0 and 9·0 (data not shown), and the temperature with maximum catalytic activity measured after 5-min reaction was 25–35°C. P6 suffers rapid denaturation, even at temperatures below 25°C (see Fig. 2a). Owing to the heat sensitivity, it is very difficult to measure initial rate activity, especially at higher temperatures, and it is necessary to follow the reaction every 5 s to avoid the effect of heat inactivation after minutes of reaction. This problem for P6-ΔPPC is even more critical than other cold-adapted enzymes, because it denatures at lower temperatures. During this study, we did not check autolysis of the enzyme, which is a common phenomenon amongst subtilisin-like proteins. We controlled this problem and the heat inactivation by expressing the enzyme at low temperatures (14°C) and performing the protein purification at 4°C. This would ensure the integrity of the enzyme before characterization. After Ni-NTA His-Bind affinity chromatography, SDS-PAGE analysis showed mainly a unique band of 32 kDa. However, autolysis and heat inactivation probably exert their effect during enzymatic reactions at different temperatures, but they are difficult to isolate in this context. During short reaction times in the order of second or few minutes, most likely autolysis is not significant.

P6-ΔPPC seems to be considerably more thermolabile than its counterpart Carlsberg subtilisin (see Fig. 3). It seems to be less thermostable than S41 Antarctic subtilisin or the subtilisin-like protease from the Vibrio sp strain PA-44 (Davail *et al.* 1994; Kristjansson *et al.* 1999), which showed values of maximum activity at temperatures 10°C above the maximum for P6-ΔPPC.

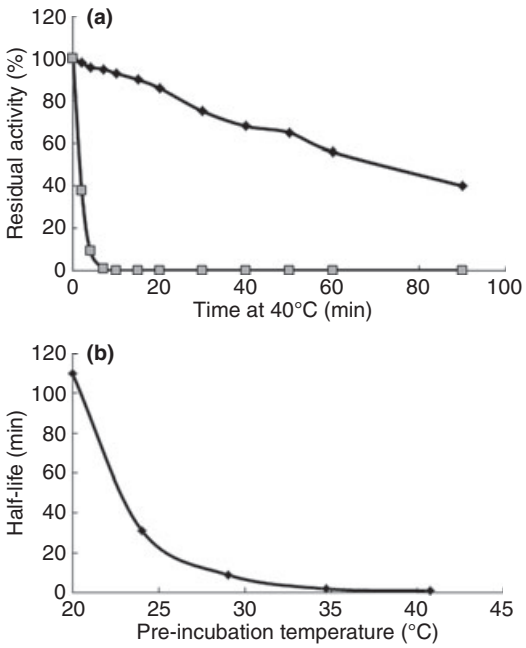

Figure 2 Thermostability analysis of P6-ΔPPC. (a) Residual activity of P6 and Carlsberg subtilisin after incubation at 40°C. (b) The half-life of P6-ΔPPC protease at different temperatures. P6-ΔPPC was incubated at different temperatures, and the time taken for the activity to reduce to half of the original activity was measured. (♦) P6-PΔPPC and (■) S. Carlsberg.

Due to technical difficulties in measuring initial rates at critical temperatures for cold-adapted enzymes, kinetic parameters were calculated up to 25°C (Fig. 3). Both k_{cat} and catalytic efficiency were higher for P6-ΔPPC than Carlsberg subtilisin at lower temperatures, even when K_m was higher for the P6 protease.

P6-ΔPPC substrate specificity was studied and compared to Carlsberg subtilisin using different synthetic substrates: Succ-AAPF-pNa, Succ-AAPL-pNa and Succ-AAVA-pNa. Succ-AAPF-pNa was the best synthetic substrate for P6-ΔPPC and Carlsberg subtilisin. Using Succ-AAPL-pNa, P6-ΔPPC showed 5% activity compared to Succ-AAPF-pNa; meanwhile, Carlsberg subtilisin showed 65%. P6-ΔPPC does not hydrolyse Succ-AAVA-pNa, and Carlsberg subtilisin showed only 5% of the activity previously exhibited towards Succ-AAPF-pNa. Carlsberg seems to have much more activity than P6, comparing their activities using Succ-AAPF-pNa as the proteolytic substrate, but when a general substrate such as succinylated casein was used, P6-ΔPPC showed four times higher proteolytic activity than Carlsberg.

Analysis of the P6-ΔPPC protease model

Based on a multiple structure alignment of different template structures of subtilisin-like proteins, a three-dimensional model of P6-ΔPPC was built. Also, a homology model of the catalytic domain of *Xanthomonas* subtilisin (P23314) a mesophilic counterpart of P6-ΔPPC was built using the same structural alignment used for the P6-ΔPPC model. Comparing both homology models, which share 71% amino acid sequence identity, few differences appeared. P6-ΔPPC showed an additional loop, which can be an important element of thermolability for

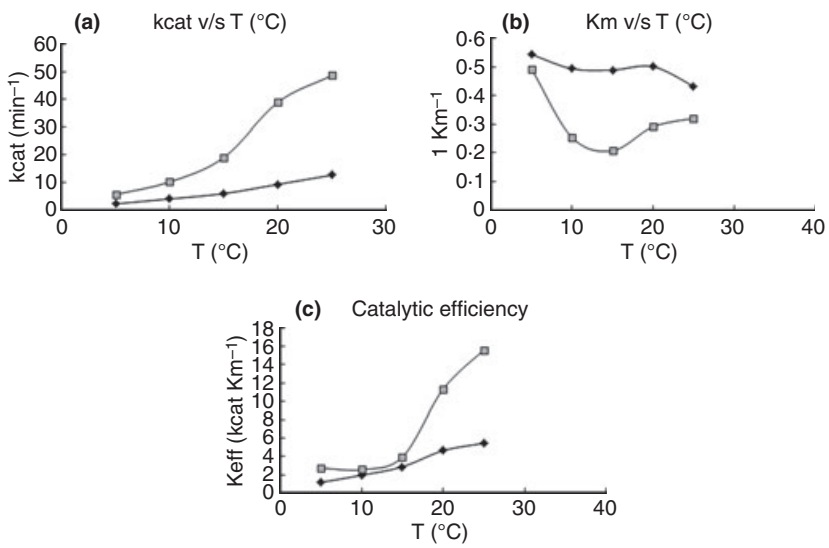

Figure 3 Kinetic parameter analysis of P6-ΔPPC using succinylated casein hydrolysis. (a) k_{cat} of P6-ΔPPC and Carlsberg subtilisin at different temperatures (b) K_m; (c) catalytic efficiency (k_{cat} K_m^{-1}). Proteolytic activities were measured at pH 8·0 under the standard conditions described. (♦) Carlsberg and (■) T7.

the entire structure. Both models showed no significant structural differences in terms of salt bridges, hydrogen bonds, ion pairs, aromatic interactions and charge–dipole interactions in α-helices. Also, they did not show differences in the amino acidic composition.

Through a comparative approach, we also analysed the local packing differences at the residue level between each homology model and a group of mesophilic homologous structures (PDB codes: 1SVN, 2ID8, 1YU6, 2IXT and 1GNS).

Afterwards, local packing differences found in the P6-ΔPPC model and in the model of the closely related mesophilic counterpart, *Xanthomonas* subtilisin, were analysed to compare their structural flexibility. Three residue positions showing significant lower local packing in the psychrophilic P6-ΔPPC were identified. These residues were Gly 217, Ala 284 and Ala 297, which correspond to the residues Asn 173, Thr 224 and Lys 237, respectively, in the experimentally determined structures of savinase (1SVN). In the mesophilic *Xanthomonas* subtilisin homology model, those residues correspond to Asp 354, Ser 412 and Val 425 based on the complete amino acidic sequence (Swiss-Prot accession number: P23314). In the mesophilic group, position 173 (based on the savinase sequence) is occupied by Asn or Ser, position 224 by Thr or Ser and position 237 by Lys, Leu or Glu. In the psychrophilic enzyme P6-ΔPPC, those positions are occupied by Gly, Ala and Ala, respectively. There is a clear tendency towards a reduction in the residue size and charge in the identified positions of the cold-adapted enzyme. The corresponding position 284 of P6-ΔPPC in the mesophilic subtilisin-like protease from *Bacillus sphaericus* (SSII) (Wintrode *et al.* 2000) was identify as a cold-adapting residue upon substitution from Thr to Ala. To unveil the possible role of Ala284 in the cold adaptation of P6 protease, the reverse mutation Ala284Ser was performed in P6-ΔPPC, and the possible increase in its thermostability quickly evaluated. Incubation at 25°C during 1 h showed almost complete heat-induced inactivation in the case of the enzyme P6-ΔPPC, whereas in the variant P6-ΔPPC Ala284Ser, the activity slightly decreased, inferring a higher thermostability in this case (see Fig. 4). Further experiments are necessary for a deeper and more conclusive analysis of the more thermostable mutant, but at this point these are beyond the scope of this work.

Discussion

In this work, several subtilisin-like proteins were identified from Antarctic bacterial strains. Most of these

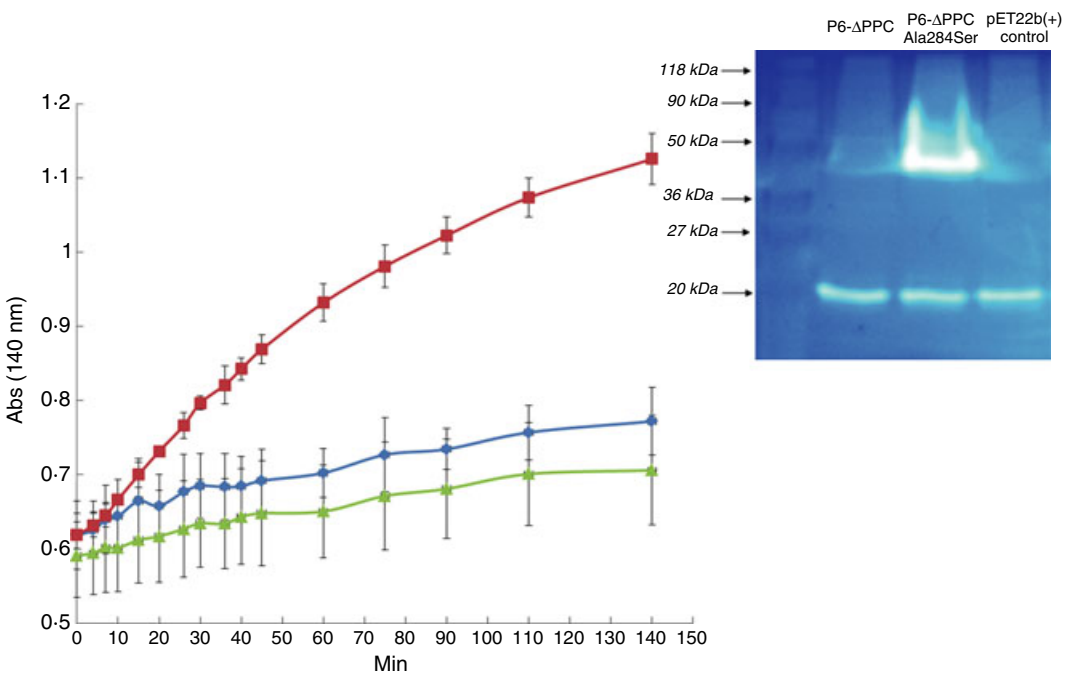

Figure 4 Proteolytic activity of P6-ΔPPC and mutant P6-ΔPPC Ala284Ser. Purified proteases were submitted to 1-h incubation at 25°C. Afterwards, the enzymatic activities were measured, showing complete heat-induced inactivation for P6-ΔPPC, whereas P6-ΔPPC Ala284Ser appears to be still highly active. pET22b(+) control corresponds to a transformed *Escherichia coli* with the expression vector excluding the protease gene. In all cases, the purification procedure was the same. The same purified enzymes were tested using Zimogram assays with gelatin as the protease substrate. (–◆–) P6-ΔPPC; (–■–) P6-ΔPPC Ala284Ser and (–▲–) pET22b(+) control.

subtilisin-like genes had at least 98% identity in their calculated amino acid sequences. This type of natural coincidence is not unusual, considering that these bacterial isolates share a common ecosystem, although many belong to different genus. Bacteria usually have high levels of horizontal gene transfer, especially with genes that involve evolutional advantages (Whittam 1992).

Few successful attempts of recombinant expression of protease genes have been published, probably due to the toxicity and lethality caused by the proteolytic activity on the host cells of the expression system. We believe that P6 protease was possible to produce as a recombinant protein due to its initial expression as an inactive protease followed by an unusually slow activation process taking place in the supernatant. P6-ΔPPC and its other recombinant versions showed important proteolytic activity at low temperature.

A slow activation process was observed both for P6 and P6-ΔPPC, suggesting that this activation could be due to an N-terminal excision rather than to a pre-peptidase C-terminal maturation processing. Previous reports have suggested that this characteristic N-terminal prosequence in other subtilisins could have important functions in the folding and activation process (Shinde *et al.* 1999).

P6-ΔPPC showed higher catalytic constants (k_{cat}) than Carlsberg protease at low and moderate temperatures (5–15 and 15–25°C, respectively), although K_m was also higher for the psychrophilic enzyme. It is well known that cold-adapted enzymes often have an increased k_{cat} and higher K_m (Xu *et al.* 2003). K_m usually represents a measure of the affinity of the enzyme for the substrate, and it is indirectly related to the number and strength of the stabilizing interactions between the substrate and the binding site. Weaker molecular interactions with the substrate in cold-adapted enzymes are accompanied by a less stable enzyme–substrate complex, which is consistent with a higher K_m. Reduction in the reaction temperature to 5°C allowed a substantial decrease in the K_m of the cold-adapted protease P6-ΔPPC. This can be explained by the decline of vibrational molecular movements produced by heat, which facilitates the structural stabilization of the enzyme–substrate complex. This hypothesis makes more sense if we considered as an important factor of thermolability in P6 the increased local flexibility in the active site, which was predicted by the previous comparative studies of residues' contact density. Alternatively, the reduction in K_m could indicate that this temperature (5°C) is probably close to the best conditions for the enzyme, where decrease in the hydrophobic and ionic interactions could facilitate the substrate binding.

According to the comparative substrate specificity study, there is an important difference in the substrate acceptance between P6-ΔPPC and Carslberg subtilisin. S-AAPF-pNa was an excellent and specific substrate for Carlsberg protease, whereas the activity registered for P6-ΔPPC using S-AAPF-pNa was six times lower than the activity using the general proteolytic substrate, succinyl casein. Comparing the enzymatic activity of P6-ΔPPC and Carlsberg protease using succinylated casein, P6-ΔPPC subtilisin had about threefold more activity, but when S-AAPF-pNa was used, Carlsberg protease activity was 20 times superior to the P6-ΔPPC activity. The synthetic substrates used in this work were far from being specific for P6-ΔPPC, in contrast to the observed results with Carlsberg subtilisin. These results suggest that the substrate specificity is quite different between P6-ΔPPC and the Carlsberg subtilisin; further studies are necessary to show the exact differences.

More flexible structures often allow better solvent accessibility. Therefore, a greater inactivation by H_2O_2, which can penetrate to deeper regions of the structure (Davies 2005), is not surprising. A more open conformation may result in better access to susceptible amino acids located in the inner regions of the enzymes. Methionine residues, known for their susceptibility to oxidation, are located in internal positions of the 3D structure in P6-ΔPPC as well as in Carlsberg subtilisin, including the methionine located close to the active site. Their possible conversion to more polar sulfoxide can produce important effects on enzyme conformations; oxidation of methionines closer to the active site can generate almost complete inactivation of the enzymatic activity. The rapid deleterious effects of H_2O_2 on the proteolytic activity of P6-ΔPPC suggest the occurrence of larger conformational changes around the active serine and methionine. This is again in agreement with our results of comparative contact density analysis, which define a less compact environment in the position Ala 284 of the cold-adapted P6-ΔPPC.

It is well known that the study of spatial variations in local packing density can predict protein flexibility with very good accuracy. This is also predicted by complex studies such as atomic mean-square displacements (AMSDs) or B factors profiles based on X-ray structures (Halle 2002).

Usually cold adaptation is a multifactorial phenomenon. In the case of P6-ΔPPC, Ala 284 could be one of the important residues to explain the cold adaptation. This is suggested by the low local packing density and structural location. Ala 284 is located in close contact with the catalytic serine at one turn down in the α-helix (third residue after the serine). In the *Xanthomonas* subtilisin and other mesophilic subtilisin-like proteins, the identity of this amino acid is serine or threonine, both contributing to the stability of the α-helix where the

catalytic serine is located. Ala in position 284 could permit a more flexible structure in the catalytic site of the cold-adapted P6-ΔPPC, explaining in part the observed incremented values of k_{cat} and K_m compared to its mesophilic counterpart. Wintrode *et al.* (2000) described a directed evolution strategy to convert a mesophilic subtilisin-like protease from *Bacillus sphaericus* (SSII) into a cold-adapted enzyme, which showed much higher increments of activity at low temperature than at moderate temperature and lower thermal stability compared to the wild type. The naturally psychrophilic subtilisin S41 shares 77·4% identity with SSII, so one possible route for the cold adaptation would be the acquisition of cold-activating mutations present in S41. However, the new cold-adapted variants of SSII did not show any of the possible mutations that appear in S41. Instead, a single amino acid substitution of Thr-253 to Ala conferred most of the increment of activity at low temperature, showing a k_{cat} 3·9 times higher than the wild type at 10°C and higher K_m values (Wintrode *et al.* 2000). Although this cold-adapted variant showed improved activity especially at low temperature, it presented only about 3-min decrease in half-life at 70°C. This indicates that the effects of the substitution of Thr-253 to Ala could be more related to a local effect in the active site, which is reflected mainly in the k_{cat} and K_m values, as mentioned before in the case of P6-ΔPPC. However, the results presented by Wintrode *et al.* (2000) were from the study that used a small synthetic substrate, and the increased activity at low temperature could eventually be only the result of a structural modification with more activity towards this substrate. This variant still shows a much better improvement in activity at low temperature than at moderate temperature, suggesting that the effect of this mutation on the activity for this specific substrate is more important at lower temperatures.

The substitution of Thr-253 to Ala corresponds structurally to the same Ala-284 in P6-ΔPPC. Thr-253, or Ser-276 in the case of *Xanthomonas* subtilisin, would form a hydrogen bond with a Thr adjacent to the catalytic serine (see Fig. 5). Disruption of this hydrogen bond by the replacement of threonine with a nonpolar residue such as alanine could lead to structural rearrangements or increased mobility in the active site. Such disruption could account for the large increase in activity at low temperature. In an attempt to demonstrate the importance of this alanine residue in the cold adaptation of P6-ΔPPC, the reverse mutation to serine was performed. Mutant P6-ΔPPC Ser284Ala presented no activity using the previously tested synthetic substrates such as AAPF. However, the proteolytic activity on succinylated casein remained high. Interestingly, long incubation at moderate temperature abolished proteolytic activity in the

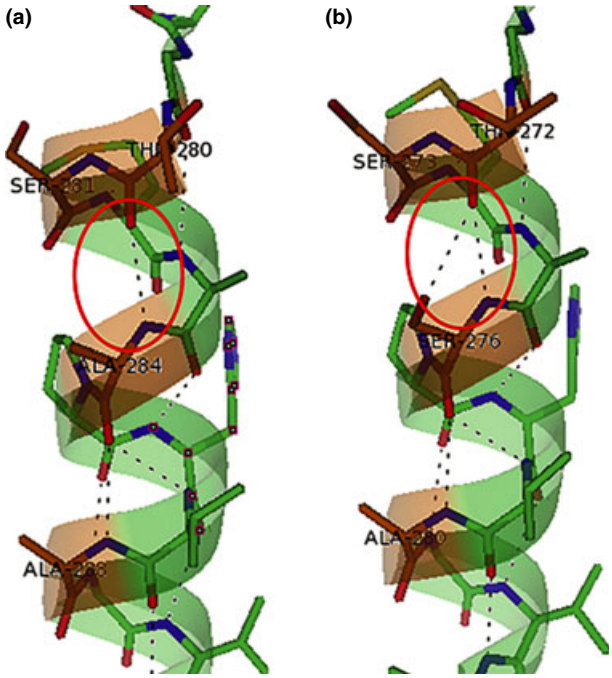

Figure 5 The catalytic α-helix of P6-ΔPPC (a) and *Xanthomonas* (b) homology models. The modelled H-bonds that could produce important effects on the stability of the whole α-helix are circled.

P6-ΔPPC protease, whereas mutant P6-ΔPPC Ser284Ala keeps its enzymatic function. These results prove to a certain extent our hypothesis; however, more detailed and elaborated experiments are needed to evaluate the increase in thermostability and decrease in activity at lower temperatures and to go deeper in molecular explanation of cold adaptation in the case of mutant P6-ΔPPC Ser284Ala. Cold adaptation is thought to be a multifactorial phenomenon, which is structurally achieved after complex evolutionary process. In this case, we suggest that one substitution or modification in an enzyme could explain part of its high activity at low temperature, but it needs to be coupled to other mutations allowing structural and functional protein integrity.

In this work, we have identified a new thermolabile subtilisin-like serine protease, which appear to be widely present amongst different genus of Antarctic bacteria. It is characterized for having higher proteolytic activity at low to moderate temperatures and higher heat-induced inactivation. In an attempt to identify important residues in the cold adaptation of this type of enzymes using local packing analysis and site-directed mutagenesis, Ala284 appears as a promising candidate and was also identified in the cold adaptation of a mesophilic protease using laboratory evolution. The position of this Ala residue could be an important hot spot in the evolution of subtilisin-like proteases, where modification at the level of this

residue would change important enzymatic parameters such as temperature adaptation and substrate specificity.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 P6 has a potential signal peptide sequence of 27 amino acids in the N-terminal.

Figure S2 P6 variants without PPC domains (P6-ΔPPC) and fused to a C-terminal hexahistidine tag were easily purified from the supernatant through a Ni-NTA His•Bind affinity chromatography.

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