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A new cold-adapted serine peptidase from Antarctic *Lysobacter* sp. A03: Insights about enzyme activity at low temperatures

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ABSTRACT

Currently, there is a great interest for customized biocatalysts that can supply the ongoing demand of industrial processes, but also deal with the growing concern about the environment. In this scenario, cold-adapted enzymes have features that make them very attractive for industrial and biotechnological purposes. Here, we describe A03Pep1, a new cold-adapted serine peptidase isolated from *Lysobacter* sp. A03 by screening a genomic library. The enzyme is synthesized as a large inactive prepropeptidase that, after intramolecular processing, gives rise to the active form, of 35 kDa. The heterologous expression of A03Pep1 was carried out in *E. coli* cells harboring the vector pGEX-4T-2-*a0301*. Its activity was optimal at pH 9.0 and 40 °C, in the presence of 25 mM Ca^{2+}, which may contribute to the thermal stability of the enzyme. The 3D structure modelling predicted a less deep and more open binding pocket in A03Pep1 than that observed in the crystal structure of its mesophilic homologous AprV2, presumably as a way to enhance the probability of substrate binding at low temperatures. These results provide possible approaches in developing new biotechnologically relevant peptidases active at low to moderate temperatures.

1. Introduction

The current demand for sustainable alternatives to environmentally damaging chemical processes has led to an increasing interest in the use of enzymatic biocatalysts both in industrial as well as in consumer applications [1,2]. Therefore, diverse environmental niches have been investigated to search for enzymes that can be able to withstand very specific conditions of temperature, pH, salinity and pressure found in many industrial processes [3,4].

In recent years, it has increased the number of studies showing the advantages of using enzymes derived from psychrophilic microorganisms, i.e. those organisms able to live in permanently cold environments as the Polar Regions, the depths of the oceans and the high altitudes [5]. Cold active enzymes showed to have features that make them very attractive for industrial and biotechnological purposes. They have a high activity at lower concentrations, reducing the amount of enzyme needed in a reaction. In addition, cold active enzymes are heat labile, being easily and selectively inactivated under moderate heat input, and remain efficient at ambient temperature, offering a way to deal with the ongoing need to reduce energy consumption [5–7]. It was demonstrated that such characteristics are due to their flexible structure, which compensates the low kinetic energy at cold environments. This structural flexibility is achieved by some features such as decreased core hydrophobicity, while there is an increased surface hydrophobicity, weaker inter-domains and inter-subunits interactions, decreased secondary structure content and a bias toward some amino acid residues rather than others [8–10]. Nevertheless, there are still many questions about structure-function relationships in cold adapted enzymes to be answered that could be valuable to understand microbial life at low temperatures and to design customized biocatalysts through enzyme engineering.

Lysobacter is a genus of Gram-negative gliding bacteria that belongs to the Gammaproteobacteria Class, generally isolated from soil and freshwater habitats. *Lysobacter* species are known by the production of many biotechnologically relevant compounds, such as biocontrol agents with activity against plant pathogens [11], antibiotics like lysobactin [12] and enzymes, giving up attention to those produced by strains of *Lysobacter enzymogenes* [13]. Recently,

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our research group described a psychrophilic strain of *Lysobacter*, named as *Lysobacter* sp. A03, isolated from penguin feathers in Antarctica [14]. This strain produces a large amount of proteolytic enzymes preferentially when cultivated in keratinous substrates at temperatures below 20 °C, being a promising source of industrially important enzymes.

In this study, we describe a new cold-adapted peptidase isolated from *Lysobacter* sp. A03, named A03Pep1, which was successfully cloned and expressed in *E. coli*, being highly active at low to moderate temperatures. The biochemical parameters together with *in silico* analysis indicated that the enzyme belongs to the S8 family of serine peptidases and is composed of five domains. Through homology modelling, it was predicted that the substrate binding site of the mature A03Pep1 peptidase may be more accessible than that of homologous mesophilic enzymes, a relevant characteristic to improve enzymatic catalysis at low temperatures.

2. Materials and methods

2.1. Bacterial strains and plasmids

The genomic library samples were prepared using *Escherichia coli* EPI300 (Epicentre, Madison, WI, USA) and *E. coli* JM109 (Promega, Fitchburg, WI, USA) as hosts for Fosmid pCC1FOS (Epicentre, Madison, WI, USA) and plasmid pGEM-T easy (Promega, Fitchburg, WI, USA), respectively. The *a0301* gene cloning was made using *E. coli* DH5α (New England Biolabs, MA, USA) as host for pGEM-T easy vector. The enzyme expression was made using *E. coli* ArcticExpress (Agilent Technologies) as host for pGEX-4T-2 vector.

2.2. DNA extraction and fosmid library construction

The strain *Lysobacter* sp. A03 was isolated from decomposing penguin feathers and cultivated in 10 g L^{-1} Feather Meal Agar (FMA) as sole carbon and nitrogen source (Pereira et al., 2014). The genomic DNA was extracted using the Wizard Genomic DNA Purification kit (Promega) according the manufacturer's protocol and was quantified by using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc.). Approximately 30 μg of DNA was randomly sheared by pipetting to generate fragments of about 30 Kb, which were gel-purified, their ends were repaired and then linked to fosmid pCC1FOS (Epicentre). After phage-packaging, fosmids were propagated in *E. coli* EPI300, which was then plated on Luria Bertani (LB) agar containing 12.5 μg mL^{-1} chloramphenicol. A negative control was made transforming pCC1FOS in *E. coli* EPI300 without DNA insert. The resulting clones were suspended in 2 mL of LB medium and stored at −70 °C in 20% (v/v) glycerol until use.

2.3. Library screening

The clones from *Lysobacter* sp. A03 library were replicated in LB plates amended with 12.5 μg mL^{-1} chloramphenicol and 10% skimmed milk and incubated during 24- to 48-h at 28 °C. The proteolytic activity was verified by the presence of a clear halo around the colonies, as an indicative of the milk casein hydrolysis. The positive clones were isolated and cultivated in LB broth containing 12.5 μg mL^{-1} chloramphenicol plus 0.1% arabinose for fosmid extraction and partial sequencing to select unique clones.

2.4. Sub-cloning of the protease-encoding gene

The unique fosmids, positive for proteolytic activity, were cleaved with different combinations of *Eco*RI, *Nsi*I, *Pst*I and *Sph*I (Promega), enzymes, then fragments around 3–5 kb were ligated into pGEM-T easy vector (Promega) cleaved with the same pair of enzymes. The resulting sub-cloned vectors were used to transform *E. coli* JM109 cells that were screened for proteolytic activity in LB plates containing 100 μg mL^{-1} ampicillin, 0.5 mM IPTG, 80 μg mL^{-1} X-Gal and 10% skimmed milk. The positive clone, chosen also based on its stable and higher protease production was isolated and cultivated in LB broth containing 100 μg mL^{-1} ampicillin for plasmid extraction and insert sequencing using M13 Forward and Reverse primers (Promega), as well as internal specific primers designed to obtain the full sequence of the protease-containing fragment.

2.5. Sequence analysis of gene coding for a0301 peptidase

To define the Open Reading Frame of the *Lysobacter* sp. A03 peptidase gene, named *a0301*, and its amino acid sequence, the ORF Finder tool was used and the search for homologous sequences was made using the nucleotide-nucleotide Basic Local Alignment Search Tool (BlastN) and the protein-protein basic local alignment search tool (BlastP), from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). To predict the presence and cleavage site of an N-terminal signal peptide, the software SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) was used with a cutoff of 0.8. The prediction of conserved domains was performed by the BLAST analysis of conserved domains, also implemented at the NCBI website and through the Pfam database of protein families [15]. The catalytic domain was further verified by comparing the sequence from A03Pep1 with its homologues using the T-Coffee tool [16]. The classification of the A03Pep1 protease was performed by comparing the sequence to the MEROPS peptidase database (http://merops.sanger.ac.uk) [17]. The prediction of the A03Pep1 polypeptide secondary structure was performed through the PSIPRED Protein Sequence Analysis Workbench (http://bioinf.cs.ucl.ac.uk/psipred/) and the phyre2 protein folding recognition server [18].

2.6. Heterologous expression of the gene coding for a0301 peptidase

Based on the ORF sequence defined by the Orf Finder tool, the gene encoding for *a0301* peptidase was entirely amplified using two primers containing *Eco*RI and *Xho*I (Promega) restriction sites. The amplified product was gel-purified using the Wizard SV Gel and PCR Clean-Up System (Promega) and inserted into a pGEM-T easy vector used to transform *E. coli* JM109 cells. To facilitate the selection of the peptidase-bearing plasmids, the proteolytic colonies were screened in LB plates amended with 100 μg mL^{-1} ampicillin, 0.5 mM IPTG, 80 μg mL^{-1} X-Gal and 10% skimmed milk. After the confirmation of the insert sequence, the plasmid containing the peptidase gene was digested with *Eco*RI and *Xho*I and then ligated into the pGEX-4T-2 expression vector, cleaved with the same restriction enzymes, and then used to transform *E. coli* ArcticExpress (Agilent Technologies) cells. The selection was made in LB plates containing 100 μg mL^{-1} ampicillin and 20 μg mL^{-1} gentamycin, and the resistant clones were isolated and cultivated in LB broth containing the same antibiotics at 37 °C until the OD_{600} value reaches 0.6. Then the temperature was reduced to 15 °C and the expression of the recombinant protein was induced by the addition of 0.5 mM IPTG following cultivation for additional 24 h. The construction pGEX-4T-2-*a0301* vector was expressed in *E. coli* ArcticExpress. As the attempts to purify the peptidase from the cell lysate were unsuccessfully and a large amount of the target protein was released to the supernatant, an unusual two-steps purification protocol was adopted with an Amicon Ultra-15 10,000 MWCO filter (Millipore) followed by another step of ultrafiltration with Amicon Ultra-15 30,000 MWCO filter. To verify the purification, samples were analyzed by 12% SDS-PAGE and zymography [19].