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The unique cold-adapted extracellular subtilase from psychrophilic yeast *Leucosporidium antarcticum*

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Abstract

The extracellular subtilase synthesized by a strain of *L. antarcticum* isolated from the Admiralty Bay waters (depth of 200 m) was purified to homogeneity by means of 3-step column chromatography and characterized. The enzyme appeared to be kinetically and thermodynamically adapted to cold (T_{opt} 25 °C, poor thermostability, high catalytic efficiency at 5–25 °C, low values of ΔG^*, ΔH^*, ΔS^* and E_a). The sequence of 35 N-terminal amino acid residues of the *L. antarcticum* proteinase shows 31 and 37% homology with that of proteinase K and *A. oligospora* subtilase, respectively, thus indicating that the *L. antarcticum* serine proteinase belongs to the subfamily C clan SB. It is the first psychrophilic yeast subtilase in this subfamily.

Keywords: Subtilase; Yeast; *Leucosporidium antarcticum*

1. Introduction

The perspectives of biocatalysis have been markedly widened, due to investigation into various areas, including studies on enzymatic apparatus of extremophilic microorganisms, the most numerous group of which are psychrophiles (>80% of biosphere is the psychrosphere, characterized by an average temperature below 5 °C). Though cold-adapted enzymatic proteins, synthesized by psychrophiles, act according to the same catalytic mechanisms as homologous enzymes produced by other organisms [1], they display some specific kinetic and structural features, which confer efficient function in energy-deficient environments [2].

Enzymes from Antarctic psychrophilic bacteria are the main objective of studies in the field of low-temperature biocatalysis (it is believed that true psychrozymes are synthesized by microorganisms, which permanently exist at low temperatures). On the other hand, only two enzymes from Antarctic yeast, such as an aspartyl proteinase from a psychrophilic strain of *Candida humicola* [3], and a xylanase from psychrophilic strain of *Cryptococcus adeliae* [4], have been purified and characterized to date. An extracellular serine proteinase from psychrophilic marine yeast *Leucosporidium antareticum*, presented in this communication, is the third reported enzyme of that type. It is a particularly interesting enzyme since it is produced by the Antarctic endemic species of yeast [5]. Furthermore, the only two extracellular yeast serine proteinases (neutral and alkaline), which have been described to date, were synthesized by mesophilic strains [6]. One of these enzymes (an alkaline serine proteinase of *Yarrowia lipolytica*) has been classified into the group of subtilases of the *Tritirachium album* proteinase K subfamily [7].

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2. Purification and general characterization of the proteinase

The enzyme was isolated from the culture broth of *L. antarcticum* 171, which was isolated from sub-glacial waters (depth of 200 m) in the Admiralty Bay (Antarctica) and classified by Donachie [8]. Submerged cultures of the strain (12 days, 6 °C) have been run in the medium containing 0.5% bactopeptone, 0.3% yeast extract, 1% saccharose and 3.5% marine salt. The homogeneous proteinase (Fig. 1) has been obtained by means of a multi-step procedure, including precipitation of proteins with acetone, gel filtration on Sephadex G-75, ion-exchange chromatography on DEAE-Sephacel (pH 5.69) and molecular sieving on Sephacryl S-100. The homogeneous enzyme is a serine proteinase since it is completely inhibited by 1 mM PMSF. It is also partially inhibited by 1 mM chymostatin (60% of inhibition), 5 mM TPCK (45% of inhibition) and SBTI (50% of inhibition corresponds to the concentration of 0.125 mg ml^{-1}). EDTA, iodoacetate and pepstatin do not affect the activity, similarly to Ca^{2+}, Zn^{2+} and Mg^{2+}. The most substantial properties of the proteinase are summarized in the Table 1. The enzyme displays a broad subtilisin-like specificity and splits peptide bonds on the carboxyl sides of Tyr (preferentially), Ala and Leu (Table 1). Like the true subtilisins, the *L. antarcticum* proteinase shows a weak activity against elastine (1 mg of the enzyme liquefies 0.1 mg of elastine

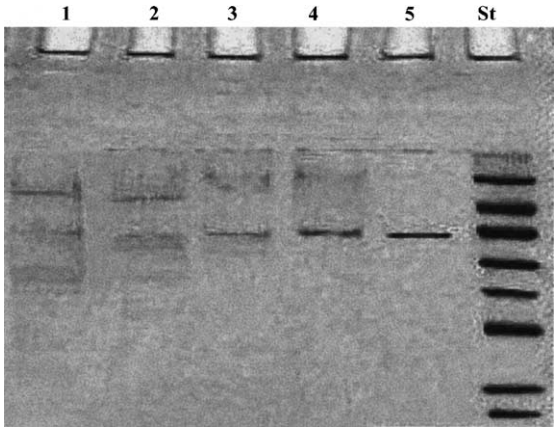

Fig. 1. SDS–PAGE protein patterns of culture broth (lane 1), solution of proteins precipitated with acetone (lane 2), fractions after: gel filtration on Sephadex G-75 (lane 3), ion-exchange chromatography on DEAE-Sephacel (lane 4), and molecular sieving on Sephacryl S-l00 (lane 5). Molecular mass standards: BSA 66,000, ovoalbumin 45,000, glyceraldehyde-3-phosphate dehydrogenase 36,000, carbonate anhydrase 29,000, trypsinogen 24,000, trypsin inhibitor 20,000, α-lactoglobulin 14,200, aprotinin 6500 (lane St).

Table 1
Properties of the proteinase from *L. antarcticum*

Molecular weight (kDa)	
SDS–PAGE	35.0
MALDI-TOF	34.4
Isoelectric point	5.62
Carbohydrate content (%)	25.0
Absorbancy index $A_{280}^{1\%}$	1.1–1.2
Specific activity (U mg^{-1} of protein[a])	
Urea denatured hemoglobin (pH 6.5)	84.70
Casein (pH 7.0)	28.20
BzTyrOEt (pH 8.0)	8.84
$Suc(Ala)_3pNA$ (pH 8.0)	0.30
$Z(Gly)_2Leu$ pNA (pH 8.0)	0.27
Optimal pH	
Hemoglobin hydrolysis	6.5–6.8
Casein hydrolysis	7.0–7.3
BzTyrOEt	8.0–8.5
Effect of temperature	
Maximum activity (°C)	25
Percentage of maximum activity	
At 0 °C (%)	25
At −20 °C (%)	18
Thermal stability ($\tau_{1/2}$, min)	
30 °C	147.6
35 °C	52.8
55 °C	0.6
pH-stability range	
5 °C	4–10
25 °C	6–9
35 °C	7–9[b]
Kinetic constants (BzTyrOEt, 5–25 °C)	
K_m (mM)	0.18–0.16
k_{cat} (s^{-1})	3.4–5.9
k_{cat}/K_m (mM^{-1} s^{-1})	19.1–35.6
Thermodynamic constants (BzTyrOEt, 5 °C)	
E_a (kJ mol^{-1})	22.5
ΔG^a (kJ mol^{-1} K^{-1})	52.4
ΔH^a (kJ mol^{-1} K^{-1})	23.1
ΔS^a (kJ mol^{-1} K^{-1})	−95.5

[a] Note: 1 unit of activity (U) corresponds to 1 μmol of the reaction product released from substrate for 1 min at 30 °C.
[b] But the activity is 30% lower than at 5 °C.

for 1 min at pH 7.0 and 30 °C) and Suc(Ala)$_3$ pNA (Table 1). An interesting feature of the proteinase is its halophilic character, since the enzyme is stable in 7.5% NaCl, and retains 50% of activity for 30 min in 15% solution of sodium chloride. NaCl is also essential for the enzyme biosynthesis. The highest yields of the proteinase have been observed for 2.5–3.5% salt solutions, corresponding to the natural *L. antarcticum* habitat. The enzyme requires no protectants and retains its total activity at −22 °C for 5–6 months.

3. Cold adaptation

The *L. antarcticum* serine proteinase is a true psychrophilic enzyme, both kinetically and thermodynamically adapted to cold, typical of the natural habitat of the yeast, as proved by the following properties:

- low optimal temperature (25 °C) when compared to not only the mesophilic subtilisin Carlsberg (60 °C) but also to the Antarctic subtlisin S41 (40 °C, [9]),
- a high relative activity at 0 °C and below (Table 1),
- a high degree of thermolability,
- a dependence of pH-stability on temperature (the lower the temperature the broader range of pH-stability, Table 1),
- high catalytic efficiency (k_{cat}/K_m) at physiological temperature of *L. antarcticum*,
- low values of free energy (ΔG^*), enthalpy (ΔH^*), entropy (ΔS^*) and Arrhenius energy of activation (Table 1).

Moreover, the yield of proteinase biosynthesis is also temperature-dependent and twice higher at 6 °C than at the optimal temperature of *L. antarcticum* growth (T_{opt} = 15 °C). At the higher cardinal temperature of the strain (T_{max} = 20 °C), the proteolytic activity has not been detected in the culture broth.

4. N-terminal sequence and initial classification of the *L. antarcticum* proteinase

The sequence of 35 N-terminal amino acids of the proteinase (the analysis was performed at the BioCenter of the Jagiellonian University, Cracow, Poland) using a gas-phase sequencer (Model 491,

Table 2
Comparison of N-terminal amino acid sequence of the *L. antarcticum* serine proteinase with that of two typical subtilases

L. antarcticum	DIQADA- - - - -TWGLQRITQQ- - - - - -EKLDNTDPFALTYEYPFVA
Tr. album	AAQTNA- - - - -PWGLARISST- - - - - - - - - - -SPGTSTYYYDES-
A. oligospora	AEQTDS- - - - -TWGLDRISHE- - - - - - - - - - -DYSAPYTYEYDETA

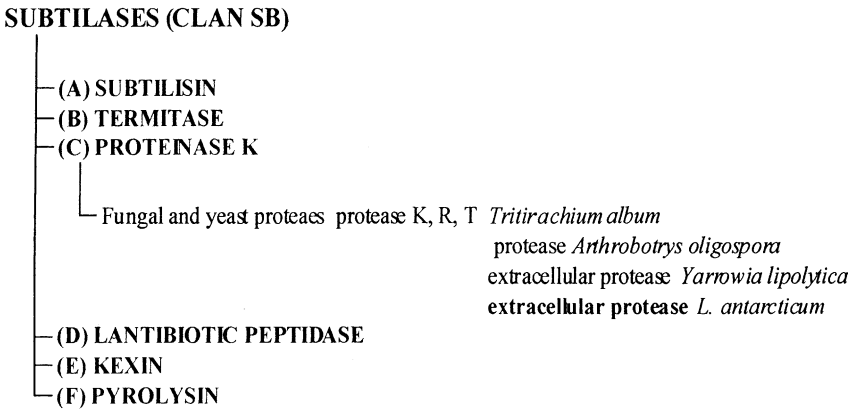

Fig. 2. Proposed position of the extracellular serine proteinase from *L. antarcticum* in the subfamilies A–F of clan SB.

Perkin-Elmer Applied Biosystems, USA) and aligned with the corresponding sequences of the proteinase K from *T. album*, which is a typical subtilisin-like proteinase from the subfamily C of clan SB (evolutionary family S8, subtilisin and subtilases, [7]), and of the subtilase from the mould *Arthrobotiys oligospora* (Table 2). The Antarctic enzyme shows 31% homology of its N-terminus to the proteinase K and 37% to the enzyme from *A. oligospora*. It also contains a conserved sequence, typical exclusively of subtilases of this subfamily (TWGL.RI.). Searching in databases revealed no N-terminal sequence homology between the *L. antarcticum* proteinase and serine proteinases from other clans and families.

Based on these results, the *L. antarcticum* serine proteinase can be initially classified into the subfamily C of clan SB (Fig. 2). According to the state of art, our enzyme is the first psychrophilic subtilase in this subfamily, which mainly comprises mesophilic enzymes of filamentous fungi and yeast, and one thermophilic serine proteinase—aqualisin I from *Thermus aquaticus* YT-1 ([7]). Further research on the *L. antarcticum* subtilase, which are mainly aimed at determination of its complete amino acid sequence and structural characteristics that confer cold adaptation, are in progress.

Acknowledgements

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References

[1] G. Feller, C. Gerday, Cell Mol. Life Sci. 53 (1997) 830.
[2] C. Gerday, M. Aittaleb, J.L. Arpigny, E. Bais, J.P. Chessa, G. Garsoux, I. Petrescu, G. Feller, Biochim. Biophys. Acta 1342 (1997) 119.
[3] M.K. Ray, et al., Appl. Environ. Microbiol. 58 (1992) 1918.
[4] I. Petrescu, et al., Extremophiles 4 (2000) 137.
[5] J.W. Fell, A.C. Statzell, I.L. Hunter, L. Phaff, Antonie Laeuwenhoek 35 (1969) 433.
[6] D.M. Ogrydziak, Crit. Rev. Biotechnol. 13 (1993) 1.
[7] R.J. Sietzen, J.A.M. Leunissen, Protein Sci. 6 (1997) 501.
[8] S. Donachie, Ecophysiological description of marine bacteria from Admiralty Bay (Antarctica) and the digestive tracts of selected *Euphausiidae*, Ph. D, Thesis, Department of Antarctic Biology, Polish Academy of Sciences, Warsaw, 1995.
[9] S. Davail, et al., J. Biol. Chem. 269 (1994) 17448.