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A cold-adapted extracellular serine proteinase of the yeast *Leucosporidium antarcticum*

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Abstract An extracellular serine proteinase, lap2, from the psychrophilic antarctic yeast *Leucosporidium antarcticum* 171 was purified to homogeneity and characterized. The enzyme is a glycoprotein with a molecular mass of 34.4 kDa and an isoelectric point of pH 5.62. The proteinase is halotolerant, and its activity and stability are dependent neither on Ca^{2+} nor on other metal ions. Lap2 is a true psychrophilic enzyme because of low optimal temperature (25°C), poor thermal stability, relatively small values of free energy, enthalpy and entropy of activation, and high catalytic efficiency at 0–25°C. The 35 N-terminal amino acid residues of lap2 have homology with subtilases of the proteinase K subfamily (clan SB, family S8, subfamily C). The proteinase lap2 is the first psychrophilic subtilase in this family.

Keywords Antarctic · *Leucosporidium antarcticum* · Psychrophile · Subtilisin-like

Introduction

Successful research developments with microorganisms indigenous to permanently cold, marine and terrestrial antarctic ecosystems, including microflora of the ectothermic zooplankton and fish of this region, have been seen recently. Collections of pure cultures of bacteria and single-cell eukaryotes, isolated from antarctic regions, have been established (Donachie 1995; Zucconi 1996; Bergero et al. 1999; Nichols et al. 1999; Chattopadhyay 2000), and biochemical and taxonomical studies on these microorganisms have revealed many novel strains (Bowman et al. 1997a, b, 1998a, b; Gosink et al. 1997, 1998; Denner et al. 2001). Bioactivity screening of antarctic microflora has yielded numerous enzymes active at low temperatures (Nichols et al. 1999; Gerday et al. 2000; Sheridan et al. 2000).

Only two psychrozymes of antarctic yeasts have been reported. The first was an aspartyl proteinase of *Candida humicola* (Ray et al. 1992), and the second was a xylanase from marine *Cryptococcus adeliae* strain (Gomes et al. 2000; Petrescu et al. 2000). This paper presents the third cold-adapted enzyme from antarctic yeast—an extracellular serine proteinase from the endemic marine yeast *Leucosporidium antarcticum* 171. *Candida*-like *L. antarcticum* is one of numerous species of antarctic marine yeasts. It was isolated for the first time by Fell in 1966 near the northern edge of the Antarctic Peninsula (Fell et al. 1969). This species, which does not exist north of 62° S, has been subjected to much longer and more strenuous selective pressure than have other, more widely disseminated psychrophilic microorganisms, leading to adaptation of its metabolism to incessantly low temperatures (the temperature of antarctic waters ranges from −2.2°C in shelf waters to 4°C in open waters, the average temperature being −1°C), and it can be considered as a representative source of cold enzymes. Furthermore, the proteinase from *L. antarcticum* 171 is the third extracellular yeast serine proteinase. Two others were derived from mesophilic yeasts (Ogrydziak 1993). Based on the 35 N-terminal amino acid residues, our enzyme may be classified into the group of subtilases of the *Tritirachium album* proteinase K subfamily (subfamily C, clan SB).

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Materials and methods

Strain and culture conditions

Leucosporidium antarcticum 171 was both isolated (from sub-glacial waters at a depth of 200 m in Admiralty Bay, Antarctica) and classified by Dr. Stuart Donachie (Donachie 1995). Agitated cultures of the strain were run at 5°C [130 rpm in a Unitron AJ207

incubator (Infors, Switzerland)] in a medium containing 3.5% marine salt (''Instant Ocean'', Aquarium Systems, France), 0.5% bactopeptone, 0.3% yeast extract, and 1% sucrose as a carbon source. The medium used in submerged cultures was inoculated with 0.5% v/v yeast cell suspension derived from static cultures of the strain in the same medium carried out for 7 days. The growth was monitored in terms of colony forming units (cfu/ml) on 2% agar after a 14-day culture.

Enzyme purification

All steps of enzyme purification were carried out at 4°C unless otherwise stated. Biomass was discarded by centrifugation (8,000 *g* for 20 min), and the proteins in the supernatant were precipitated at −20°C with cold acetone (75% of the supernatant volume) for 15 min with continuous stirring. The precipitate formed was discarded, and the second fraction of proteins was precipitated using a threefold excess of acetone. The precipitate was collected by centrifugation (10,000 *g* for 20 min at 0°C), suspended in 50 mM sodium acetate buffer at pH 5.69 and applied to a Sephadex G-75 column (2.6×100 cm) equilibrated with the same buffer. Fractions of 3 ml were collected at a flow rate of 8 ml cm^{-2} h^{-1}, and were assayed for both protein content (A_{280}) and proteolytic activity against *N*-succinyl-AAPF-*p*-nitroanilide (*N*-SucAAPF*p*NA) and urea-denatured hemoglobin (urea-Hb). Fractions containing proteolytic activity were pooled and applied to a diethylaminoethyl-Sephacel column (0.9×30 cm), equilibrated with the same buffer. Proteins adsorbed on the column were eluted initially with 0.05 M NaCl in the buffer, and next with NaCl gradient (50–80 mM, 300 ml at 25 ml cm^{-2} h^{-1}). Fractions forming one of the peaks of proteolytic activity, designated lap2, were concentrated by ultracentrifuging to a 10 kDa cutoff (AMICON) and the concentrate was purified to homogeneity by column chromatography on Sephacryl S-100 (0.9×100 cm at 8 ml cm^{-2} h^{-1}) equilibrated with 50 mM sodium acetate buffer (pH 6.5). Total activity of the homogeneous *L. antarcticum* proteinase lap2 was retained upon storage without any protectants for several months at –20°C.

Proteinase assays

The activity of lap2 was determined at 30°C with *N*-SucAAPF*p*NA as substrate (DelMar et al. 1979). The final concentration of the substrate in the reaction medium, containing 50 mM Tris-HCl buffer (pH 8.0) and 3% dimethylsulfoxide, was 0.2 mM. One unit of activity denoted 1 μmol of the substrate hydrolyzed per minute at 30°C, using a molar extinction coefficient (ϵ) of 8,480 M^{-1} cm^{-1}. Esterase activity towards *N*-benzoyl-L-tyrosine ethyl ester (BzTyrOEt) was measured spectrophotometrically at 256 nm ($\epsilon = 964$ M^{-1} cm^{-1}) at 30°C in a reaction mixture without dimethylsulfoxide with a final substrate concentration of 0.3 mM (Hummel 1959). Proteolytic activity against proteins was assayed according to Anson (1938) using urea-Hb as substrate, and was expressed as micromoles of L-tyrosine released from the substrate per minute under standard conditions (2% urea-Hb in sodium acetate buffer, pH 6.5, 30°C for 15 min, the final concentration of urea in 2% Hb solution being 20%). The same method was used for assays of proteolytic activity against native Hb (2% solution without urea) and casein (2% solution in sodium acetate buffer, pH 6.5). Activity towards elastin was estimated according to the modified method of Wagner et al. (1983), and expressed as milligrams of protein liberated from the substrate per minute under assay conditions (1% elastin solution, pH 6.5 at 30°C).

Analytical procedures

Protein content was assayed according to Lowry et al. (1951) using bovine serum albumin as a standard, and spectrophotometrically at 280 nm in eluate from chromatography columns.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in gel slabs (12.5% acrylamide; 10 cm×5.5 cm×1 mm) using the discontinuous buffer system of Laemmli (1970). The gels were stained with Coomassie Brilliant Blue R-250 for protein and with basic fuchsin for glycoprotein (Zacharius et al. 1969). Molecular mass marker proteins ranging in size from 6.5 kDa to 66 kDa (Serva, Germany) were used to determine the molecular mass of lap2. The molecular mass of the proteinase was also determined by means of matrix-assisted, laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Voyager-Elite, PerSeptive Biosystems, Framingham, Mass., USA). The wavelength of pulsating laser light used to irradiate the sample was 337 nm. The isoelectric pH was estimated by isoelectric focusing using Phast Gel IEF 3-9 in a PhastSystem (Amersham Bioscience), according to the manufacturer's instructions.

The N-terminal amino acid sequence analysis was performed on a gas-phase sequencer (model 491, Perkin Elmer, Foster City, Calif., USA) in BioCenter (Jagiellonian University, Cracow). The phenylthiohydantoin derivatives of amino acids were analyzed by on-line gradient high performance liquid chromatography on a microgradient delivery system, model 140C, equipped with programmable absorbance detector, model 785A (both from Perkin Elmer).

Determination of kinetic and thermodynamic constants

The assays were run under standard conditions (pH 8.0, 30°C). Kinetic constants in the reaction of *N*-SucAAPF*p*NA hydrolysis were determined at temperatures from 5°C to 35°C. Values of K_m and V_{max} were estimated according to the method of Lineweaver and Burk (1934), and the value of k_{cat} was calculated for the proteinase's molecular mass of 34.4 kDa. Initial velocities (v_0) in *N*-SucAAPF*p*NA hydrolysis were measured at pH 8.0 and at temperatures from 5°C to 35°C. Activation energy (E_a) for the reactions was determined from the slope of Arrhenius curves [$\ln(v_0)$ versus $1/T$]. Thermodynamic quantities such as the enthalpy of activation (ΔH^*), the entropy of activation (ΔS^*), and the free energy of activation (ΔG^*), were calculated from the following equations: $\Delta H^* = E_a - RT$; $\Delta S^* = 2.303R[\log(k) - 10.753 - \log(T) + E_a/2.303RT]$; $\Delta G^* = \Delta H^* - T\Delta S^*$, where E_a is the Arrhenius activation energy, R is the gas constant (8.31 J K^{-1} mol^{-1}), T is temperature (°K) and k is reaction velocity.

Results

Physiological and biochemical characterization of *Leucosporidium antarcticum* 171

L. antarcticum 171 is a strain of marine, obligatorily psychrophilic (T_{opt} 15°C, T_{max} 20°C), budding, non-sugar-fermenting, halotolerant (growth up to 9% NaCl) antarctic yeast. It can utilize various sugars, including maltose and sucrose, as well as tributyrin as basic carbon sources. Apart from extracellular proteolytic enzymes, *L. antarcticum* 171 synthesizes hydrolases such as extracellular α-glucosidase, lipase, and acidic phosphatase, and intracellular alkaline phosphatase and β-fructofuranosidase (M. Pazgier, unpublished data).

Biosynthesis and purification of extracellular proteases

The yield of biosynthesis of *L. antarcticum* extracellular proteolytic enzymes in submerged culture at 5°C reaches approximately 1.5 mg proteinase per liter of culture medium, and corresponds to 0.2 nanounits activity per

single yeast cell. The dynamics of the enzyme synthesis appeared to be synchronized with growth of the strain. Proteinase secretion starts at the beginning of the logarithmic phase of growth and reaches its maximum at the end of this phase, usually on the 11th or 12th day of growth. PAGE of the proteins present in the supernatant of the culture medium (12th day) revealed that only one of several protein bands (molecular mass 34–36 kDa) hydrolyzed *N*-SucAAPF*p*NA; this was completely inhibited by 1 mM phenylmethylsulphonylfluoride ($PhMeSO_2F$), thus demonstrating the presence of a serine proteinase. Further experiments showed that the biosynthesis of this proteinase was dependent on temperature, and that at 5–6°C, i.e. 10°C below optimum temperature for growth, proteolytic activity was 50% higher than at 15°C. Enzyme synthesis was also stimulated by the culture medium's salinity, and the optimum marine salt concentration was 2.5–3.5% (w/v). Proteinase synthesis was accompanied by a slight increase in pH. At the higher cardinal temperature (T_{max} 20°C) *L. antarcticum* did not produce any extracellular proteinases, and a significant decrease in pH was observed (to pH 4.0 after 9 days of growth).

Lap2 was isolated from the supernatant of the culture broth (12 days at 5°C). The procedure for enzyme purification is summarized in Table 1. Total proteolytic activity adsorbed on the anion-exchanger at pH 5.69, which was eluted neither with the starting buffer nor with 50 mM NaCl in this buffer, was eluted as two peaks (lap1 and lap2) by a narrow NaCl gradient (50–80 mM) (Fig. 1). The larger peak, lap2, was further purified to homogeneity on Sephacryl S-100. The sharp peak of active protein showed a single band in SDS-PAGE *(lane 1*, Fig. 2). Its electrophoretic mobility corresponded to a molecular mass of 34 ± 1 kDa.

General characterization of the proteinase

The homogeneous proteinase lap2 is a glycoprotein, as revealed by SDS-PAGE gels staining with basic fuchsin, with a molecular mass of 34.4 kDa, as determined by MALDI-TOF mass spectrometry, and an isoelectric point of pH 5.62. The enzyme is completely inactivated by $PhMeSO_2F$ (0.5 mM), which confirms the presence of a reactive serine residue in its active site, and is partially inhibited by chymostatin and soybean trypsin inhibitor. Partial inhibition of the proteinase lap2 by tosylphenylalanine chloromethyl ketone coincides with its activity against *N*-SucAAPF*p*NA and BzTyrOEt. EDTA, iodoacetate, and pepstatin do not affect enzyme activity. Unlike other subtilases of the proteinase K subfamily, lap2 is neither activated nor stabilized by Ca^{2+} ions. The same rate of thermal inactivation of the

Table 1 Purification of the extracellular proteinase of *Leucosporidium antarcticum* 171

Purification step[a]	Protein (mg)	Activity				Yield (%)	Purification (fold)
		Against urea-Hb[b]		Against *N*-SucAAPF*p*NA[c]			
		Specific activity (U/mg of protein)	Total activity (U)	Specific activity (U/mg of protein)	Total activity (U)		
Culture supernatant	1,150.00	0.054	62.50	0.0257	29.90	100.0	1
Acetone-precipitated material	183.30	0.268	48.80	0.128	23.35	78.1	5
Sephadex G-75	16.70	2.466	41.23	1.177	19.73	66.0	46
Diethylaminoethyl-Sephacel, pH 5.69 (eluted by 0.05–0.08 M NaCl)							
lap1	0.52	21.90	11.44	10.46	5.47	18.3	406
lap2	0.73	23.75	17.43	11.34	8.34	27.9	440
Sephacryl S-100							
lap2	0.17	84.67	14.17	40.43	6.78	22.7	1,568

[a]The enzyme was isolated from 500 ml of culture broth
[b]Urea-denatured hemoglobin
[c]*N*-Succinyl-AAPF-*p*-nitroanilide

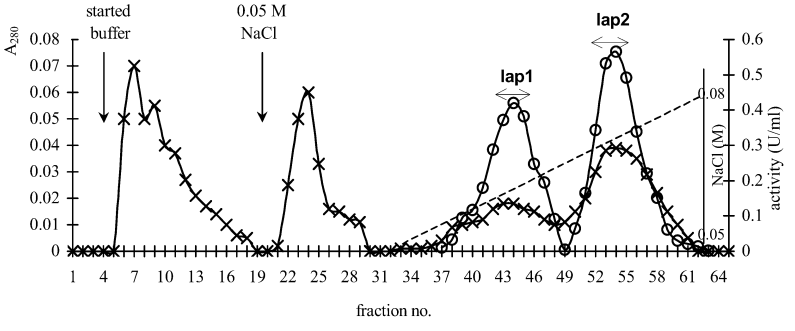

Fig. 1 Ion-exchange chromatography on diethylaminoethyl-Sephacel at pH 5.69. *Crosses* A_{280}, *open circles* proteolytic activity against urea-denatured hemoglobin (urea-Hb) under standard conditions, *double-headed arrows* pooled fractions, *dashed line* NaCl gradient

enzyme during 30 min incubation at temperatures above 40°C was observed both in the presence and in the absence of 2 mM $CaCl_2$ (results not presented). Zn^{2+}, Mg^{2+} and Mn^{2+} have no impact on hydrolytic activity of the proteinase lap2. The enzyme is highly halotolerant. It retains 100% and 50% of an initial activity in 1.5 and 2.5 M NaCl, respectively (Table 2).

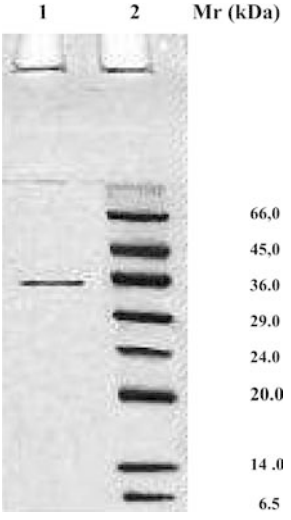

Fig. 2 Determination of molecular mass of homogeneous lap2 using sodium dodecyl sulfate polyacrylamide gel electrophoresis. *Lane 1* lap2; *lane 2* molecular mass standards: bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,000), α-lactoglobulin (14,200), aprotinin (6,500)

Table 2 Effect of selected compounds and metal ions on the activity of proteinase lap2

Compound/metal ion[a]	Concentration	Residual activity (%)
Control		100
$PhMeSO_2F$[b]	0.5 mM	0
$TosPheCH_2Cl$[c]	1 mM	75
	5 mM	55
Soybean trypsin inhibitor	0.125 μg/ml	50
	0.25 μg/ml	32
Chymostatin	1 mM	48
Iodoacetamide	5 mM	97
Pepstatin	0.022 mM	99
EDTA	1 mM	90
	5 mM	89
Ca^{2+}	1 mM	101
	2 mM	100
Mg^{2+}	1 mM	100
Mn^{2+}	1 mM	109
Zn^{2+}	1 mM	99
NaCl	1.5 M	100
	2.5 M	50
	3.5 M	2

[a]The enzyme was incubated with each of the compounds/metal ions at 0°C for 30 min, and the residual activity was determined against *N*-succinyl-AAPF-*p*-nitroanilide under standard conditions (pH 8.0, 30°C)
[b]Phenylmethylsulphonylfluoride
[c]Tosylphenylalanine chloromethyl ketone

N-Terminal sequence and initial classification

The sequence of 35 N-terminal amino acid residues of the *L. antarcticum* 171 proteinase lap2 (Fig. 3) was compared with that of enzymes regarded as typical of individual clans of serine proteases (Rawlings and Barret 1994). The comparison revealed an exclusive similarity (34–38% sequence homology) to the proteinase K subfamily of clan SB [family S8, subfamily C, according to Sietzen and Leunissen (1997)]. Furthermore, the sequenced fragment of lap2 contains the first (WGL-RI, position 8–13) of several conservative motives, characteristic only of subtilases of proteinase K subfamily (*bold line*, Fig. 3).

Substrate specificity and optimum pH

Urea-Hb is the preferred substrate of the *L. antarcticum* 171 proteinase lap2 (83.0 U per milligram of protein, pH optimum 6.7–7.1). The activity against native Hb and casein is 60–70% lower. The subtilase shows poor activity against elastin (1 mg enzyme hydrolyzes 0.1 mg elastin in 1 min at pH 6.5 and 30°C).

The enzyme displays high amidase activity (pH_{opt} in the range 8.0–8.5) against *N*-SucAAPF*p*NA and *N*-succinyl-AAPL-*p*-nitroanilide. The proteinase affinity towards both the substrates is similar ($1/K_m$ of 1.96 and 1.43 $mmol^{-1}$, respectively). However, the turnover number (k_{cat}) in the hydrolysis of *N*-SucAAPF*p*NA is more than five times higher than for the second substrate (Table 3). The antarctic subtilase also hydrolyzes amide bonds formed by apolar Ala or Leu residue adjacent to small Gly residues, but the efficiency of their hydrolysis is a few dozen times lower. The enzyme lap2 displays relatively high activity against BzTyrOEt (8.84 U per mg of protein), which is a substrate of chymotrypsin and subtilisin.

The effect of temperature on activity, stability and thermodynamic constants

The subtilase of the psychrophilic strain *L. antarcticum* 171 is most active at temperatures from 20°C to 30°C, with an optimum at 25°C (Fig. 4). The enzyme is active even at 0°C (approximately 20–25% of maximum activity against urea-Hb, and 15–20% against *N*-SucAAPF*p*NA) and below (at minus 10°C it retains as much as 18% of maximum activity against the first substrate, solutions of which contain 20% urea, protecting them from freezing at these temperatures). The substantial catalytic efficiency of the subtilase in the temperature range 5–30°C results mainly from the high affinity towards the substrate, which is roughly constant at these temperatures (Fig. 5A). The enhancement in catalytic efficiency ($K_{eff} = k_{cat}/K_m$) caused by an increase in temperature is largest in the range from 10°C to 25°C (Fig. 5B). At 5°C, values of the Arrhenius

Fig. 3 Comparison of the 35 N-terminal amino acid residue sequence of *Leucosporidium antarcticum* 171 proteinase lap2 and sequences of selected subtilases of the proteinase K subfamily: proteinase K of *Tritirachium album* Limber (Jany et al. 1986), and the proteinases of *Arthrobotrys oligospora*, *Thermus aquaticus* YT-1 (Kwon et al. 1988), and *Yarrowia lipolytica* (Davidov et al. 1987). *Bold line* Conservative motive

activation energy (E_a) and other thermodynamic constants, such as free energy of activation (ΔG^*), enthalpy of activation (ΔH^*) and entropy of activation (ΔS^*), determined for the hydrolysis of *N*-SucAAPF*p*NA by the proteinase lap2, are small (Table 4), and the energetic barrier of substrate hydrolysis is markedly reduced. The values of these quantities in the case of lap2 are lower than those of psychrophilic subtilisins from *Bacillus* TA41 [at 5°C, ΔG^* is 61.6 kJ mol^{-1}, ΔH^* is 36.0 kJ mol^{-1}, and ΔS^* is −91.5 kJ mol^{-1} (Davail et al. 1994)], and from *Shewanella* Ac10 [at 15°C, ΔG^* is 64.7 kJ mol^{-1}, ΔH^* is 39.2 kJ mol^{-1}, and ΔS^* is −88.7 kJ mol^{-1} (Kulakova et al. 1999)], which indicates that our enzyme is particularly well tuned to function in the cold.

The subtilase of *L. antarcticum* 171 displays high thermolability. It is relatively stable only at temperatures below 30°C ($\tau_{1/2}$ of about 2.5 h, Fig. 6A). The rate of its thermal inactivation is significantly increased above 30°C. At 35°C, the half time of inactivation is reduced three times in comparison to that at 30°C (50 min and 150 min, respectively), and at 55°C it is only 34 s. The pH-stability of the enzyme is dependent on the temperature. At 5°C it retains total activity for 30 min at pH 4.0–10.0 (Fig. 6B). At elevated temperatures, the pH-stability range is narrower, and above 30°C the proteinase is not stable at any pH.

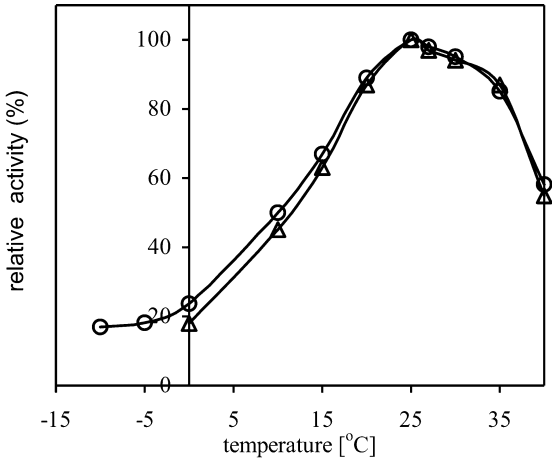

Fig. 4 The effect of temperature on lap2 activity. *Open circles* Activity against urea-Hb at pH 6.5, *open triangles* activity against *N*-succinyl-AAPF-*p*-nitroanilide (*N*-SucAAPF*p*NA) at pH 8.0. The hemoglobin solution contained 20% (w/v) urea, which facilitated the activity assays at −5°C and −10°C by lowering the freezing point of reaction mixtures

Discussion

Secretion of proteolytic enzymes by yeasts is not a common property, and the majority of extracellular yeast proteinases are acidic aspartyl enzymes, whereas production of serine proteinases is limited to few species, such as the mesophilic *Yarrowia* (formerly *Candida* or *Saccharomycopsis*) *lipolytica*, which synthesizes an alkaline serine proteinase (Tobe et al. 1976) and a neutral serine metal-dependent proteinase (Ogrydziak 1993), and *Aureobasidium pullulans*, which produces an

Table 3 Specific activity and catalytic constants in hydrolysis of synthetic substrates by lap2 (pH 8.0, 30°C)

Substrate	Specific activity (U/mg of protein)	Percentage activity	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
N-SucAAPF*p*NA[a]	40.43	100	0.51	22.95	45.10
N-SucAAPL*p*NA[b]	10.78	26.7	0.69	6.13	8.76
N-SucAAA*p*NA[c]	0.30	0.74	0.90	0.43	0.48
N-carbobenzoxyGGL*p*NA[d]	0.27	0.67	0.69	0.63	0.91
N-carbobenzoxyAA*p*NA[e]	0.23	0.57	0.95	0.48	0.51
BzTyrOEt[f]	8.84	21.9	0.16	5.99	37.4
BzArgOEt[g]	0.29	0.73	0.43	0.20	0.27

[a] *N*-Succinyl-AAPF-*p*-nitroanilide
[b] *N*-Succinyl-AAPL-*p*-nitroanilide
[c] *N*-Succinyl-AAA-*p*-nitroanilide
[d] *N*-Carbobenzoxy-GGL-*p*-nitroanilide
[e] *N*-Carbobenzoxy-AA-*p*-nitroanilide
[f] *N*-Benzoyl-L-tyrosine ethyl ester
[g] *N*-Benzoyl-L-arginine ethyl ester

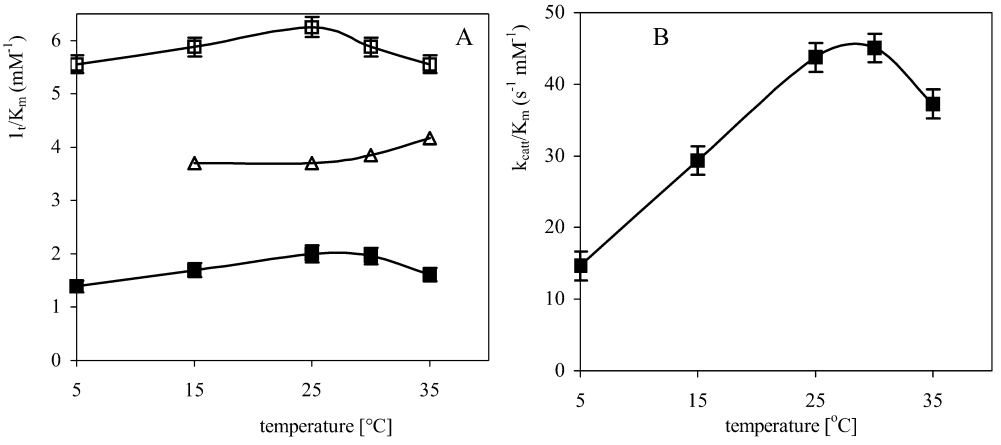

Fig. 5 A Effect of temperature on affinity ($1/K_m$) of lap2 **B** Effect of temperature on catalytic efficiency (k_{cat}/K_m) of lap2. *Open squares* *N*-Benzoyl-L-tyrosine ethyl ester (BzTyrOEt) as substrate, *solid squares* *N*-SucAAPF*p*NA as substrate), *open triangles* comparison with chymotrypsin from Atlantic cod with BzTyrOEt as substrate (Ásgeirsson and Bjarnason 1993)

Table 4 The Arrhenius activation energy (at 5–25°C) and other thermodynamic constants (at 5°C) in the hydrolysis of*N*-succinyl-AAPF-*p*-nitroanilide by lap2, as compared to those of the cold-adapted serine proteinase TA41 (Davail et al. 1994)

Proteinase	E_a (kJ mol^{-1})	ΔG^* (kJ mol^{-1} K^{-1})	ΔH^* (kJ mol^{-1} K^{-1})	ΔS^* (kJ mol^{-1} K^{-1})
Lap2	23.9	45.0	21.4	−148.5
TA41	38.5	61.6	36.0	−91.5

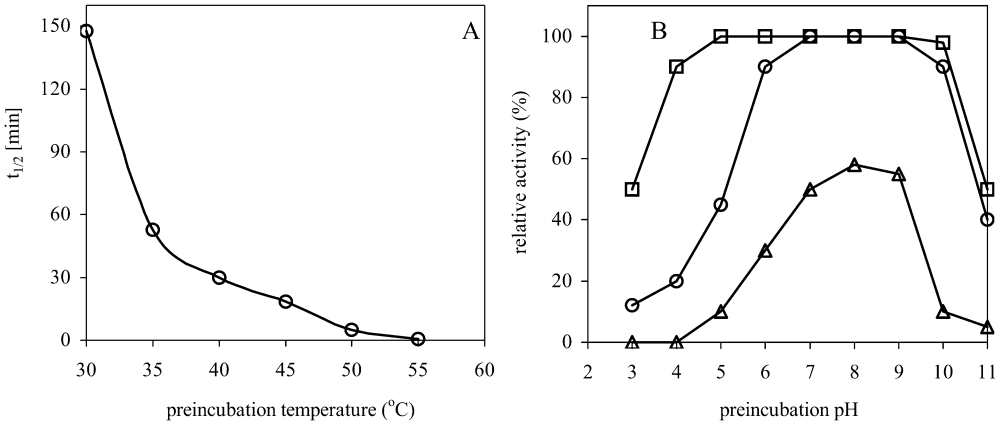

Fig. 6. A Thermal inactivation of the psychrophilic proteinase lap2—half-time of inactivation ($\tau_{1/2}$) versus temperature. The enzyme was incubated in 50 mM Tris-HCl buffer at pH 8.0 for different periods of time at temperatures from 30°C to 55°C. The residual activity was assayed against *N*-SucAAPF*p*NA under standard conditions (pH 8.0, 30°C). **B** The influence of temperature on pH-stability of the lap2 proteinase. *Open squares* 5°C, *open circles* 25°C, *open triangles* 35°C. The samples of lap2 dissolved in 0.01 M Britton-Robinson buffer solution (pH 3.0–11.0) were incubated for 60 min at given temperatures. The residual activity was assayed against urea-Hb at pH 6.5 (0.1 M sodium acetate buffer) and 30°C

alkaline serine proteinase (Ogrydziak 1993). No information on serine proteinases was included in earlier reports on extracellular proteolytic activity of *Leucosporidium* species, such as *L. scotii* [formerly *Candida scotii* (Eklund et al. 1965)], and *L. antarcticum* (Fell et al. 1969). Thus, this paper is the first report on the extracellular serine proteinase of the *Leucosporidium* strain. This enzyme was termed lap2.

The comparison of the level of homology of 35 N-terminal amino acid residues of the proteinase lap2 with that of typical representatives of evolutionary clans and families of serine peptidases (Sietzen and Leunissen 1997) indicates that the antarctic enzyme is a newly found subtilase of the clan SB (family S8 of subtilisin and subtilases, i.e. subtilisin-like proteinases). Lap2 can be classified in subfamily C, together with some enzymes of yeasts (mainly intracellular), and other fungi and gram-negative bacteria. This initial grouping of the proteinase lap2 should be verified by an estimation of this protein's complete sequence similarity to that of the typical subfamily C subtilases, such as proteinase K of *Tritirachium album*, which is the only protein of all of them with a known 3D structure. Lap2 is the first reported extracellular subtilase of a psychrophilic yeast. The subtilisin-like alkaline extracellular protease of mesophilic yeast *Yarrowia* (formerly *Candida* or *Saccharomycopsis*) *lipolytica* CX161-1B (Sietzen and

Leunissen 1997), also belonging to the proteinase K subfamily, and is an exclusive yeast secretory serine enzyme whose kinetic (Tobe et al. 1976; Ogrydziak and Scharf 1982; Bae and Kang 1985) and molecular [the enzyme of an acronym ylxpr2, and cDNA gene xpr2 (Davidow et al. 1987; Nicaud et al. 1989)] characteristics are known in detail. The extracellular neutral, metal-dependent serine proteinase of *Y. lipolytica* has, as yet, been neither characterized nor classified.

The proteinase lap2 is specific towards synthetic substrates of chymotrypsin (BzTyrOEt) and subtilisin (the substrates containing Phe and Leu residues in position P1, and Pro in position P2). This property distinguishes lap2 from the subtilase of mesophilic yeast *Y. lipolytica*, which prefers peptide bonds formed by Arg and Lys residues from the C-terminus, and cleaves that formed by Tyr markedly more slowly (Tobe et al. 1976; Ogrydziak 1993). In contrast, the activity of the homogeneous antarctic enzyme against *N*-benzoyl-L-arginine ethyl ester is 30 times lower than against BzTyrOEt (0.29 and 8.84 U mg^{-1} protein, respectively). The subtilase lap2 prefers *N*-SucAAPF*p*NA to six other substrates, and the turnover number with respect to this compound is 17.3 s^{-1} at 15°C. This value is comparable to the turnover number of the first reported antarctic subtilisin TA41, equal to 25.4 s^{-1} under the same conditions (Davail et al. 1994). The bacterial proteinase TA41 preferentially splits peptide bonds formed by Leu (an aliphatic amino acid) as compared with those formed by Phe (aromatic side group) (130% and 100% of relative activity, respectively). The subtilase lap2 from antarctic yeast *L. antarcticum* displays the opposite preferences, since it is four times more active towards linkages formed by the aromatic amino acid (Table 3). Similarly to mesophilic subtilisins, the proteinase lap2 digests *N*-succinyl-AAA-*p*-nitroanilide, which is also a typical substrate of elastase. Values of K_m in the hydrolysis of this compound are comparable with that of porcine elastase [0.73 mM, 25°C (Ásgeirsson and Bjarnason 1993)], though the efficiency of catalysis, expressed as k_{cat}/K_m, is 40 times lower in the case of the antarctic enzyme. The enzyme lap2 also digests elastin. Elastase-like activity of yeast enzyme is very rare, and this type of specificity has been mainly detected in the case of "true subtilisins" [family A of clan SB (Sietzen and Leunissen 1997)], secreted by *Bacillus* strains [subtilisins GX, BPN′ and Carlsberg (Durham 1993)].

The psychrophilic proteinase of *L. antarcticum* 171 achieved high catalytic efficiency ($K_{eff} = k_{cat}/K_m$) at low temperatures mainly via an optimization of the Michaelis constant (Table 4, Fig. 5A), small values of which are roughly the same between 5°C and 30°C, for both *N*-SucAAPF*p*NA and BzTyrOEt. Notably, the affinity of lap2 towards BzTyrOEt is 1.5 times higher than that of bovine trypsin, and similar to that of cold chymotrypsin from Atlantic cod (Ásgeirsson and Bjarnason 1993). The adaptation of molecular activity (k_{cat}), whose value at 5°C is half of that at 25–30°C (10.6 and 22.5 s^{-1} in hydrolysis of *N*-SucAAPF*p*NA, and 3.3 and 6.2 s^{-1} in digestion of BzTyrOEt, respectively), seems to be less important with respect to providing the high efficiency of the substrate's cleavage by lap2. According to Feller and Gerday (1997), such a mechanism of compensation for the reaction rate, based first of all on the "perfect optimization" of K_m, is mainly employed by cold-adapted secretory enzymes of marine microorganisms, extruded by the cells to the substrate-deficient liquid environment. One of these catalysts is lap2, produced by yeast existing in cold waters of the Southern Ocean.

We cannot yet define what substitutions of amino acid residues (if any) are involved in cold-adaptation (high activity in cold and poor thermal stability) of the *L. antarcticum* 171 subtilase lap2, taking into consideration proteinase K, whose amino acid sequence and 3D structure have been resolved, as a mesophilic counterpart. The presence of highly polar motive (residues 17–21, EKLDN, Fig. 3) within the enzyme's N-terminal fragment [the N-terminus is typically localized on the surface of subtilases (Sietzen and Leunissen 1997)] can be considered as an indirect proof of the structural flexibility of lap2, and this motive has not been detected in molecules of mesophilic representatives of the subfamily C of clan SB.

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