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Purification and partial characterization of Cu/Zn superoxide dismutase from *Kluyveromyces marxianus* yeast

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ABSTRACT

A new thermostable Cu/Zn SOD from a thermotolerant yeast strain *Kluyveromyces marxianus* NBIMCC 1984 has been purified and characterized. The purification procedure comprises thermal treatment and dialysis, ion-exchange chromatography and chromatofocusing. The methodology is a rapid, efficient and highly specific, generating pure preparation (specific activity 996 U mg of protein^{-1}) with a yield of 53%. The purified enzyme is a homodimer with Mw of 34 034 Da and has high N-terminal homology with other yeasts' Cu/Zn SOD enzymes. The protein is characterized with some unique features such as—thermostability ($t_{1/2}$ at 70 °C = 30 min), pH stability in the alkaline range (7.5–8.5) and resistance to inhibitors and variety of chemicals. These characteristics reveal possibilities for wide practical application of *K. marxianus* Cu/Zn SOD enzyme.

1. Introduction

Superoxide dismutase [(SOD) EC 1.15.1.1.] is a metalloenzyme that catalyses the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen. The enzyme plays an essential role in protecting the cell against harmful effects of oxygen radicals. Depending on their metal co-factor, four types of SODs are distinguished: copper/zinc, manganese, iron and nickel SODs [1–3]. Cu/Zn SODs are widely used in medicine, pharmaceutical and food industry [4,5]. They have been isolated and purified from various plant and mammalian tissues [6–9]. However, the procedures' efficiency is strongly restricted by the specificity of the raw material. Some methods for obtaining SODs from microorganisms, including yeasts are also available [10–13]. The main disadvantage of these procedures is their duration and multistage approach: in general, they include several steps of dialysis, ultrafiltration, lyophilisation, saturation with ammonium sulfate, ion-exchange chromatography, gel-filtration, etc. Recently, a recombinant *Kluyveromyces marxianus* strain overexpressing its Cu/Zn superoxide dismutase (*KmSOD1*) gene has been constructed and the production of the enzyme depending on cultural medium composition was studied [14].

The potential role of yeasts as SOD producers is well recognized [15–17]. Their application in food biotechnology creates demands for enzymes with advanced technological properties. A major advantage of commercial enzyme preparations is their resistance to elevated temperatures, since thermal denaturation is a common reason for their inactivation. Thus, thermostable SODs are useful due to their high stability.

The aim of this study was to purify and characterize a new thermostable Cu/Zn SOD from a thermotolerant yeast strain *K. marxianus* NBIMCC 1984.

2. Experimental

2.1. Microorganisms and biomass accumulation

As producer of superoxide dismutase lactose utilizing thermotolerant yeast strain *K. marxianus* NBIMCC 1984 was cultivated batch-wise in a 15 L New Brunswick bioreactor (New Brunswick Scientific, NJ, USA). During the cultivation process the following parameters were maintained: t° – 38 °C; pO_2 – 30%, aeration L L^{-1} min^{-1}; agitation – 300 rpm. The strain was cultivated on Rider nutrient medium [18] with lactose as a sole carbon source. The superoxide dismutase-enriched biomass was obtained at late exponential growth phase (18 h), when the carbon source was entirely consumed.

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2.2. Cell-free extract preparation

The collected biomass was washed, resuspended in an equal volume of 0.05 M potassium-phosphate buffer, pH 7.8, and disrupted with glass beads (0.5 mm, Sigma–Aldrich, Steinheim, Germany) on ice (4 x 10 min) with a vibration homogeniser VHG1 (B Brown Melsungen AG, Melsungen, Germany). The resulting suspension was centrifuged for 30 min at 21 000 × *g* at 4 °C and the obtained supernatant was used as cell-free extract. The cell-free extract was incubated at 57 °C (water bath) for 10 min and rapidly cooled to 4 °C. The denatured protein precipitates were removed by centrifugation at 15 000 × *g* for 10 min at room temperature and the supernatant obtained was dialyzed (via dialysis membrane with molecular weight cut off of 6–8 kD) against 100 volumes of 0.025 M potassium-phosphate buffer, pH 7.1 for 12–14 h.

2.3. Chromatographic purification of Cu/Zn SOD of *Kluyveromyces marxianus* NBIMCC 1984 (Cu/Zn SOD Km)

The dialyzed sample was applied to a Toyopearl 650 M (Tosoh Bioscience GmbH, Stuttgart, Germany) column (1.6 cm × 35 cm), equilibrated with 0.05 M potassium-phosphate buffer, pH 7.8, at a flow rate of 21 ml h^{-1}. The column was washed with 500 ml of the same buffer with a gradient of 0.005–0.050 M of NaCl at the same flow rate. 3.2 ml fractions were collected and those exhibiting SOD activity were pooled and subjected to additional purification procedure, as follows: the pooled fractions were applied to a column (1.0 cm × 40 cm) of PBE 94 (Amersham Pharmacia Biotech AB, Uppsala, Sweden), equilibrated with 0.05 M imidazole HCl buffer, pH 7.4, at a flow rate of 52 ml h^{-1}. A washing with 50 ml of the same buffer followed by elution with imidazole HCl buffer, pH 4.0 in a total volume of 300 ml was applied. During these procedures 3.8 ml fractions were collected.

After each purification step the protein content [19] and superoxide dismutase activity were measured [20].

2.4. Electrophoretic procedure

PAGE and specific staining for SOD were performed as described previously [21]. To test SOD inhibition, each sample was separated by electrophoresis on 10% polyacrylamide gel and then incubated with 5 mM KCN or 5 mM H_2O_2 for 30 min prior to the determination of the enzyme activity.

2.5. Visible and ultraviolet absorption spectra

The UV and visible spectra of the pure enzyme preparation were performed on a UV-VIS Jenway 6105 (T/As Jenway, Dunmow, Essex, UK) UV/Vis spectrophotometer.

2.6. Amino acid sequence determination

The PBE 94 elution fractions exhibiting SOD activity were pooled, lyophilised and loaded onto a HPLC Hypersil column (250 mm × 4.6 mm; 5 μl HyPURITY C_{18}, Thermo Quest), eluted with the eluents A (0.1% TFA in water) and B (80% acetonitrile in A), using a gradient program from 0% B for 5 min to 0–100% B for 60 min; the flow rate was 0.7 ml min^{-1}. The UV absorbance of the elution was monitored at 214 nm.

The purified on HPLC system protein was subjected to automated Edman degradation (Procise 494A Pulsed Liquid Protein Sequencer, Applied Biosystems GmbH, Weiterstadt, Germany) after dissolving the samples in 40% methanol, 1% formic acid.

2.7. Spectrometry analyses of purified SOD

The molecular mass of the native protein was determined by electrospray ionization mass spectrometry and the mass spectrum was acquired on the (ESI-MS) Q-TOF mass spectrometer (Micromass, Manchester, UK). The protein sample was prepared by diluting the protein stock solution in 10 mM ammonium acetate buffer, pH 6.8 and 6 μl of the sample were injected. ESI source settings were kept constant throughout all measurements to avoid changes in the ion desorption and transmission. The Q-TOF spectra were acquired at a rate of 5 s. To ensure a high signal-to-noise ratio, typically 180–280 scans were averaged to generate each spectrum.

Mass spectrum of the native SOD was measured by MALDI mass spectrometry (Voyager, PerSeptive Biosystems, Wiesbaden, Germany). The protein sample (10–50 pmol), obtained using anion-exchange chromatography, was dissolved in 0.1% (v/v) TFA, dialyzed against water, and applied to the target. Analysis was carried out using a-cyano-4-hydroxycinnamic acid as a matrix. Chicken egg ovalbumin (44 400 Da) and bovine serum albumin (66 430 Da) were used for mass scale calibration.

2.8. Characterization of the oligosaccharide residue of the purified SOD enzyme

The analysis of the polysaccharide residue associated with the protein moiety of the purified Cu/Zn SOD was performed through gas chromatography following the method of Chaplin [22]. Ten micrograms of the purified enzyme as well as 1 mg of the following monosugars: D-Arabinose, D-Galactose, N-Acetyl-glucosamine, D-Xylose, D-Mannose, D-Rhamnose and D-Ribose (Merk, Darmstadt, Germany) were placed in different glass ampoules. To each sample 1 ml of a 1 mg ml^{-1} mannitol solution (in 80% (v/v) ethanol) was added. The samples were subjected to vacuum evaporation and the dry products were kept for 24 h in a desiccator with P_2O_5. Then 1 ml of 0.64 M HCl in absolute methanol was added to each sample and the ampoules were sealed and subjected to methanolysis through thermal treatment at 80 °C for 16 h. After methanolysis the ampoules were opened and their content was evaporated to dry matter, followed by addition of 2 ml of diethyl ether per ampoule and evaporated again. The last procedure was performed twice and the resultant samples were placed in a desiccator with P_2O_5 for 24 h. Finally, to each sample 0.5 ml of bis-(trimethylsylil) acetamide (BSA) was added. The ampoules were sealed again and placed at 110 °C for 30 min. The samples thus pretreated were subjected to gas chromatographic analysis on a Carlo Erba (Milan, Italy) gas chromatograph with a capillary column (25 m) of fused silica, an inner diameter of 0.22 mm, and filled with DB5 silicon wax. The following gradient program was used: initial temperature: 120 °C, initial hold: 5 min, temperature program: 3 °C min^{-1}, final temperature: 220 °C, final retention: 5 min, gas: nitrogen, flow rate: 4 ml min^{-1}. The identification of the unknown carbohydrates was made through comparison of their retention times with those of the standard monosugars, listed above. For the quantification of the monosugars found the Internal Standard (D-Mannitol) method was used following the formulas:

$$C_s = \frac{C_{is}.A_s}{F_{ms}.A_{is}},$$

where C_s: sample concentration; C_{is}: internal standard concentration; A_s: sample area; A_{is}: internal standard area, A_{ms}: standard monosugar area, F_{ms}: relative response factor for a defined monosugar, calculated as A_{ms}/A_{is} ratio.

Approximately 100 μg of Cu/Zn SOD Km enzyme were treated with PNGase F (1 unit) (Roche Diagnostics GmbH, Mannheim, Germany) and incubated for 24 h at 37 °C. One microlitre of the protein sample before and after treatment with PNGase F was applied onto

Table 1
Purification steps of Cu/Zn SOD from *Kluyveromyces marxianus* NBIMCC 1984.

Step	Volume (ml)	Protein (mg ml^{-1})	Total protein (mg)	Specific enzyme activity (U mg^{-1} of protein)	Total enzyme activity (U)	Yield (%)	Purification (fold)
Cell-free extract	33	25.0	820.0	35.0	28 700	100	1
Thermal treatment and dialysis	25	10.4	259.0	72.0	18 641	65	2.1
	25	7.8	194.0	100.0	19 350	67	2.9
Ion-exchange chromatography	16	1.7	27.1	566.0	15 339	53	16.2
Chromatofocusing	15	1.0	15.3	996.2[a]	15 242	53	28.5

[a] According to the method of McCord and Fridovich [26] this value corresponds to 2988 U/mg of protein.

the MALDI target. A total of 1500 shots were acquired in the MS mode. Spectra from m/z 8000 to 20 000 were recorded.

2.9. Effect of temperature and pH on SOD activity

The effect of the temperature on SOD activity was studied by incubation of the purified enzyme at 60, 65 and 70 °C in 0.05 M potassium-phosphate buffer, pH 7.8, for a period of 30 min. The residual SOD activity was measured in aliquots removed at various time intervals (each 5th minute) and compared with controls incubated at 25 °C for the same periods. The enzyme operation stability expressed in terms of half-life ($t_{1/2}$) was determined.

The effect of pH on the pure enzyme activity was examined by incubation of the enzyme for 10 min in 0.05 M buffers with different pH values as follows: acetate for pH 5.0–6.0; potassium phosphate for pH 6.5–8.0; Tris–HCl for pH 8.5–9.5 and carbonate for pH 10.0. The activity of the samples was assayed under standard conditions.

The influence of temperature on the enzyme stability at different pH values was studied by circular dichroism. Circular dichroism (CD) spectra were recorded on a J-720 spectropolarimeter (Jasco, Tokyo, Japan). Cylindrical temperature-controlled quartz cells with a path length of 10 mm were used in all experiments. CD spectra were recorded in the range between 200 and 250 nm at 0.2 nm intervals with a bandwidth of 1 nm, a scan speed of 50 nm min^{-1}, and a time constant of 8.0 s. Ten microlitre of the protein solution in 50 mM Tris/HCl buffer, pH 7.0 were diluted in 50 mM buffers (sodium phosphate, Tris–HCl, HEPES, and citrate) with different pH values (from 1.5 to 12.0). The samples were thermostatically controlled using a NESLAB thermostat model RTE-110, connected to a digital programming controller and a thermocouple placed inside the optical cell. Temperature denaturation studies for the samples at different pH values (from 1.5 to 12.0) were measured after 20 min incubation, from 15 up to 95 °C. The $[\theta]_{222}$ values were recorded in intervals of 5 ± 0.2 °C. Thereafter, temperature was decreased at the same rate down to 25 °C. The thermal equilibrium of samples was confirmed at each temperature by the constancy of their ellipticity. Each experimental spectrum was obtained by averaging two or three separate scans and was corrected for baseline, recorded with buffer as blank. The CD spectrum of a protein can be considered as the sum of the CD spectra of each secondary structure component of the protein.

2.10. Effect of chemicals and inhibitors on SOD activity

The influence of various metal ions (Na^+, Mg^{2+}, Ca^{2+}, Zn^{2+}; chlorides), metal-chelating agents (EDTA) and detergents (SDS) on pure enzyme SOD activity was determined by adding each compound to the reaction mixture at final concentrations of 0.1 or 1 mM. It was evaluated through calculation of the activity of each sample as a percentage of a non-treated control samples. Using the same approach, the effect of various specific (KCN, H_2O_2, NaN_3) and nonspecific (p-chlorpmercuribenzoate [p-CMB], 2-iodacetamide [2-IAA]) inhibitors with reverse action on the enzyme activity, was studied.

3. Results and discussion

3.1. Chemical purification of Cu/Zn SOD Km

Recently, *K. marxianus* yeast strains are demonstrating significant biotechnological potential due to their interesting physiological and biochemical properties, such as utilization of a wide variety of substrates, high growth rates at elevated temperatures, no ethanol production when exposed to sugar excess, and Generally Recognized As Safe (GRAS) status [5,23]. In this study, the thermotolerant yeast strain *K. marxianus* NBIMCC 1984 cultivated on nutrient medium with lactose was used for isolation and purification of Cu/Zn SOD. This strain-producer has been chosen due to its thermotolerance, high growth yield ($Y_{x/c}$ = 0.41) and maximal growth rate (μ^{max} = 0.35 h^{-1}) and potentially valuable properties for producing Cu/Zn SOD with increased thermostability. It exhibits an increased operation temperature expressed in terms of half-life ($t_{1/2}$): 22 min at 75 °C [18].

The current study proposes a procedure for an efficient isolation of Cu/Zn SOD Km, in high purity including three steps: thermal treatment and dialysis, ion-exchange chromatography and chro-

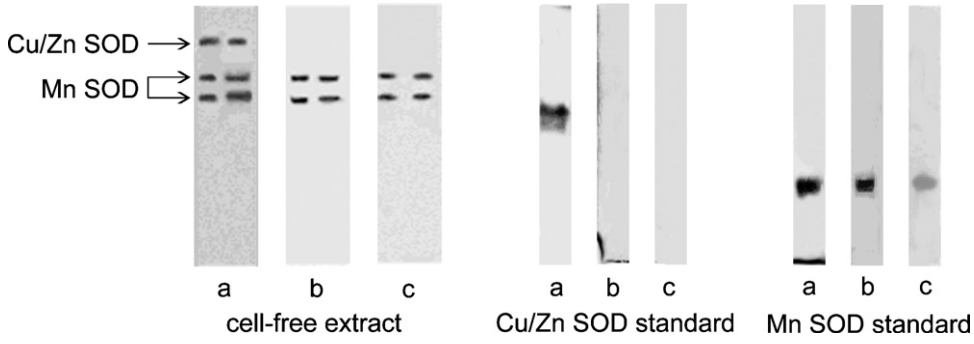

Fig. 1. Electrophoretical profile of SOD activity in *K. marxianus* NBIMCC 1984. (a) Total cellular extract; (b) inhibitory assay with 5 mM KCN; (c) inhibitory assay with 5 mM H_2O_2. The standard Cu/Zn SOD is from bovine erythrocytes and the Mn SOD—from *E. coli*.

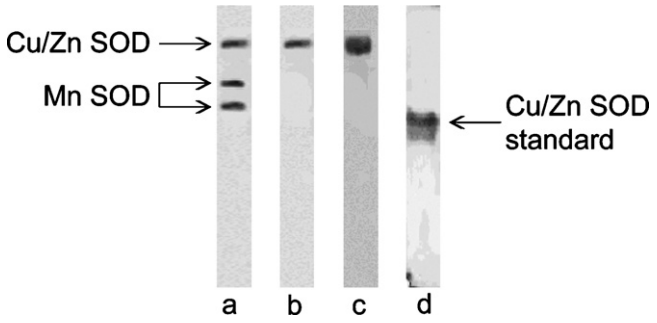

Fig. 2. Native electrophoretical analysis of thermostable Cu/Zn SOD Km during purification procedure. (a) Cell-free extract; (b) after thermal treatment and dialysis step; (c) after chromatofocusing step; (d) purified Cu/Zn SOD from bovine erythrocytes (SERVA).

matofocusing. The summary of the purification procedure is given in Table 1.

The strain-specific Cu/Zn SOD thermostability [18] allows the performance of a rapid and efficient first step of the purification scheme, i.e. the thermotreatment of the cell-free extract. It results in precipitation of the considerable part of the protein fraction, which concentration in the treated sample decreases 2.4-fold. Consequently, the specific activity of SOD enzyme increases 2.1-fold, representing 65% of the total SOD activity of the cell-free extract and is due to Cu/Zn homodimer type. This fact is proven by the inhibition analysis peformed with total cell-free extract subjected to PAGE (Fig. 1) and the specific staining for SOD activity of the sample after thermal treatment. As it can be seen in Fig. 2, the thermolabile Mn SOD is inactivated during heat treatment. Thus, the values for the specific SOD activity determined during the following steps are due only to the Cu/Zn enzyme.

The Cu/Zn SOD Km is eluted specifically from the DEAE Toyopearl 650 M at $I = 0.023$ M (the concentration was experimentally selected; data not shown). During the dialysis the sample ion strength was changed from 0.05 to 0.025 M, which allows the elution of the Cu/Zn SOD at the very beginning of the buffer gradient (step 2). In this way the second step of the purification procedure is additionally simplified: since at $I < 0.023$ M no proteins exhibiting SOD activity were registered it was not necessary to apply the gradient buffer in a total volume of 500 ml. As a result of the dialysis, an additional decrease in the protein content (1.3-folds) reflecting in the corresponding increase of the specific enzyme activity is observed.

The ion-exchange chromatography resulted in collection of 5 fractions exhibiting specific SOD activity of 566 U/mg of protein. They were pooled and loaded to the PBE 94 column.

To create a better pH gradient (from 7.4 to 5.3), the proteins' elution with imidazole HCl buffer, pH 4.0 was preceeded by application of the equilibrating buffer (see Section 2). Cu/Zn SOD Km did not absorb on the PBE 94 and was specifically eluted during the washing with the equilibrating buffer as a single peak (Fig. 3) with a protein content of 15.3 mg and specific activity of 996[1] U mg of protein^{-1}. The reduced waste protein is 11.8 mg and the yield of Cu/Zn SOD Km is 56.5%. As a consequence eluting buffer gradient can be omitted given that in the protein fractions obtained after the gradient application, no SOD activity was registered.

The proposed three-step procedure is a rapid, easy to perform and efficient one (53% yield of Cu/Zn SOD Km enzyme) as compared to the already known schemes for Cu/Zn SOD purification from various fungal sources (*Aspergillus* sp. [24], *Thermoascus aurantiacus* [25], *Cordyceps militaris* [10], etc.).

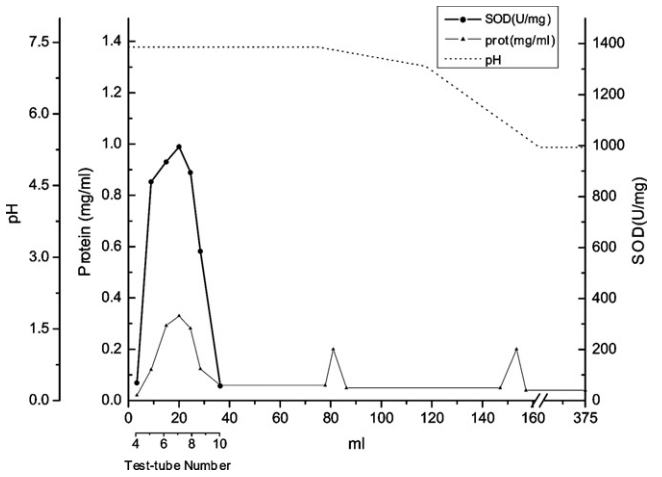

Fig. 3. Chromatofocusing on PBE 94 of the active fractions obtained from the ion-exchange chromatography step. The PBE 94 column (1.0 cm × 40 cm) was equilibrated with 0.05 M imidazole HCl buffer, pH 7.4, at a flow rate of 52 ml h^{-1}, followed by washing with the same buffer (50 ml) and elution with imidazole HCl buffer, pH 4.0 (300 ml).

3.2. Characterization of the purified Cu/Zn SOD Km enzyme

3.2.1. Molecular mass, carbohydrate content, amino acid sequence, visible and UV absorption spectra

3.2.1.1. Molecular mass. To determine the molecular mass, the purified enzyme was subjected to a HPLC Hypersil column and one fraction was eluted, using a gradient program as described in Section 2.

The native protein was subjected to ESI-MS analysis and the mass spectrum obtained is presented in Fig. 4A. Using this spectrum the molecular mass of Cu/Zn-SOD Km was calculated to be 34 034 Da was, which confirms that the enzyme is a homodimer. Under the acidic pH of 0.1% (v/v) TFA the homodimer Cu/Zn SOD Km dissociated into monomers as was measured by MALDI-TOF. In the mass spectrum, shown in Fig. 4B, the m/z ion at 17 096.63 corresponds to the molecular mass of one subunit of Cu/Zn SOD Km.

When stained for specific Cu/Zn SOD activity after native PAGE, the purified sample represents also a singe band (Fig. 2c). However, the native Cu/Zn SOD Km exhibits an atypical behaviour in PAGE in comparison with Cu/Zn SOD from bovine erythrocytes (SERVA GmbH, Heidelberg, Germany) (Fig. 2d). It expresses a slower relative mobility (Rm = 0.14) as compared with the bovine erythrocytes standard (Rm = 0.42). It is very likely that this is due to the enlarged hydrodynamic volume and higher molecular mass of the native enzyme.

3.2.1.2. Carbohydrate content. The gas chromatographic analysis of the purified enzyme has shown its glycoprotein nature. The protein moiety of the enzyme is associated with carbohydrate components comprising at least 4 monosugars (D-Xylose, D-Mannose, D-Galactose and N-Acetyl-glucosamine) (Fig. 5).

Based of the differences in the masses, determined by MALDI before and after treatment of the enzyme with the specific glycosidase PNGase F, the mass of the carbohydrate chain was determined to be about 1200 Da (Fig. 4B).

The glycopeptide nature of the enzyme provides consistent explanations for its peculiar properties. The size, number, localization and eventually branching of the carbohydrate chain are manifested through their influence on the hydrodynamic volume

[1] According to the method of McCord and Fridovich [26] this value corresponds to 2989 U mg of protein^{-1}.

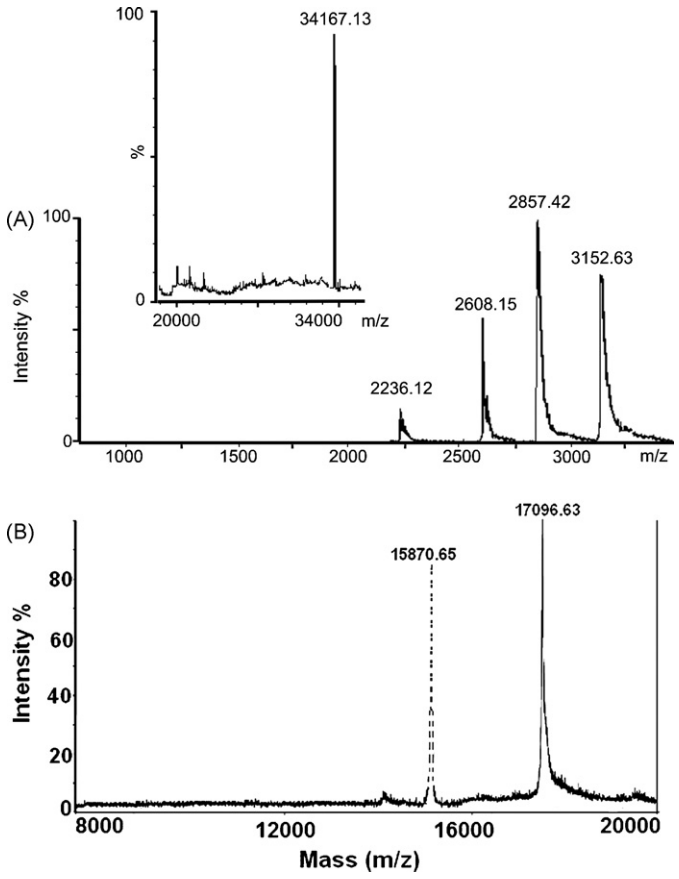

Fig. 4. (A) ESI mass spectrum of the purified enzyme in 10 mM ammonium acetate buffer, pH 6.8. The Q-TOF spectra were acquired at a rate of 5 s and 180–280 scans were averaged to generate each spectrum. (B) MALDI-MS spectrum of Cu/Zn SOD Km enzyme before (—) and after treatment with PNGase F (-----). Solutions of human albumin (66,347.7 Da) and rabbit actin (43 kDa) were used to calibrate the mass scale. (Insert) Orsinol/H_2SO_4 method on: (1) native Cu/Zn-KmSOD; (2) Cu/Zn SOD Km after treatment with PNGase F; (3) buffer.

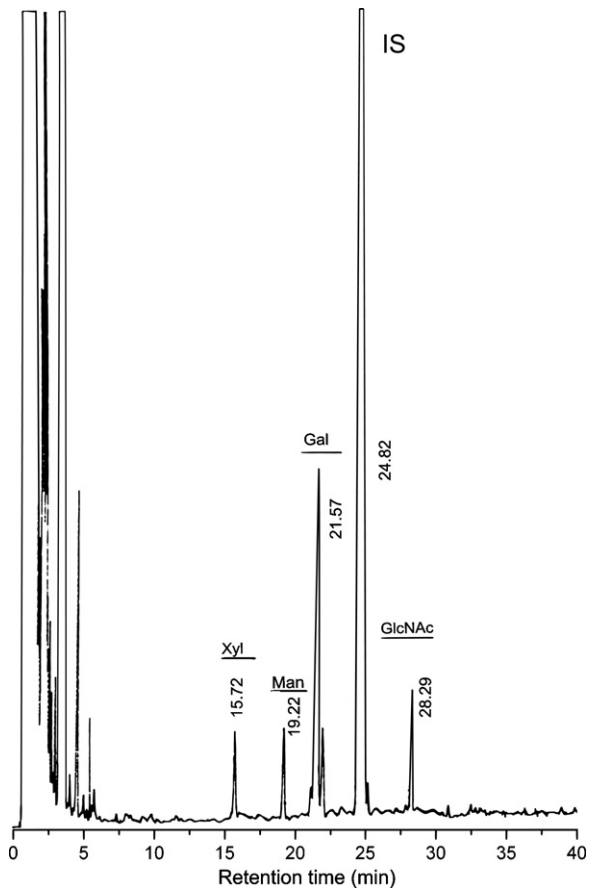

Fig. 5. Gas chromatogram of Cu/Zn SOD Km: qualitative and quantitative composition of the monosugars comprising the oligosaccharide moiety of the enzyme. A capillary column (25 m) of fused silica and inner diameter of 0.22 mm and filled with DB5 silicon wax was used. The experimental conditions comprised initial temperature: 120 °C, initial hold: 5 min, temperature program: 3 °C min^{-1}, final temperature: 220 °C, final retention: 5 min, gas: nitrogen, flow rate: 4 ml min^{-1}. IS – Internal Standard; Xyl – D-Xylose, Man – D-Mannose, Gal – D-Galactose, GlcNAc – N-acetyl-glucosamine.

of the glycopeptide in PAGE. This, together with the higher molecular mass of Cu/Zn SOD Km explains the atypical electrophoretical behaviour of the enzyme. The oligosaccharide residues probably influence as well the Cu/Zn SOD Km binding capacity during PBE 94 chromatography.

3.2.1.3. Amino acid sequence. A comparison of the Cu/Zn SOD Km amino acid sequence with those of other yeast species, belonging to genera *Criptococcus* and *Humicula* is shown in Fig. 6. The alignment shows a very high homology of Cu/Zn SOD Km in the conserved regions 2–10, 14, 18, 20 and 28 with Cu/Zn SODs isolated from *Cryptococcus liquefaciens* strain N6 (CliqN6; BAF42028), *C. gattii* (Cgat; AAK31918), *C. neoformans var. neoformans* (Cneo; AAK31916), *C. neoformans var. grubii* (Cgru; AAK31914) and *Humicola lutea* 103 (P83684).

3.2.1.4. Absorption spectra. The purified enzyme (2.5 mg ml^{-1} aqueous solution) is colourless and the lyophilised powder is light green. The visible absorption spectrum shows a broad maximum at λ = 600–700 nm (an area where the Cu ions absorb) and a typical UV maximum for Cu/Zn SOD proteins at λ = 270 nm. The spectral characteristics of the preparation together with the defined molecular mass of the protein, confirms that it is a Cu/Zn containing enzyme. The specific staining and inhibition analysis of the pure preparation validate this conclusion.

3.3. Influence of the temperature and pH on SOD activity and stability

The purified Cu/Zn SOD enzyme shows good stability at 60, 65 and 70 °C (see Section 2) (Fig. 7A). Its values were comparable with those of the commercial preparation of bovine erythrocytes (Fig. 7B), with $t_{1/2}$ at 70 °C = 30 min. This considerable heat resistance is probably due to the glycoprotein nature of the enzyme,

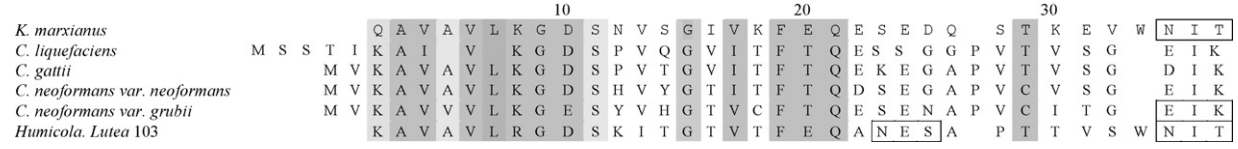

Fig. 6. Alignment of the N-terminal sequences of Cu/Zn SODs of different sources: *Kluyveromyces marxianus* NBIMCC 1984, *Cryptococcus liquefaciens* N6 (CliqN6; BAF42028), *C. gattii* (Cgat; AAK31918), *C. neoformans var. neoformans* (Cneo; AAK31916), *C. neoformans var. grubii* (Cgru; AAK31914), *Humicola lutea* 103 (P83684).

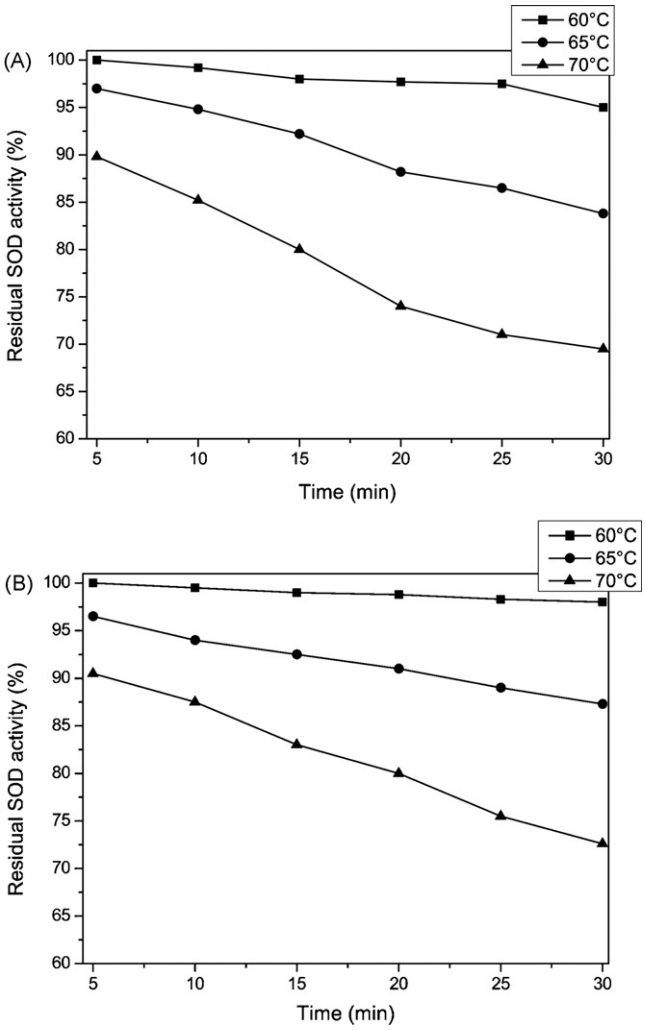

Fig. 7. Effect of temperature on the stability of Cu/Zn SODs. The purified enzyme was incubated at 60, 65 and 70 °C in 0.05 M potassium-phosphate buffer, pH 7.8, for 30 min. The residual SOD activity was measured in aliquots removed each 5th minute and compared with controls incubated at 25 °C for the same periods. (A) Purified Cu/Zn SOD Km; (B) purified Cu/Zn SOD from bovine erythrocytes (SERVA).

since glycosylation is one of the possible reasons for enzymes' thermostability [24].

The effect of pH on the purified enzyme is shown in Fig. 8. The profile of the enzyme activity was outlined at different pH values at 25 °C, for 10 min. The pH optimum was registered to be at pH 7.8, and the enzyme shows stability in the alkaline range (7.5–8.5) retaining about 65% of its activity. In the acid pH range it was rapidly inactivated, over 80% at pH 6.5 and less.

The combined action of the above mentioned parameters—temperature and pH, on the enzyme stability was studied by circular dichroism. All data collected from the CD spectra represent values of $[\theta]_{222}$, obtained for the native enzyme. An intrinsic feature is the presence of T-induced changes within a wide temperature interval (15–90 °C). The CD spectra of the native enzyme in 50 mM Tris/HCl buffer, pH 7.0 at different temperatures are shown in Fig. 9A. A maximum at 208 nm of the spectra exposed the β-barrel fold of the enzyme. The amplitude $\Delta[\theta]_N - \Delta[\theta]_D$ for the curves at different pH values slightly decreases on moving to extreme pH values. For some of the curves, it is clear that they are composed by two or more components and represent complex temperature transitions (Fig. 9B). Even smooth pH changes influence specific T-dependent stability, as shown in a number of curves with unique and diverse features in relation to others of the same

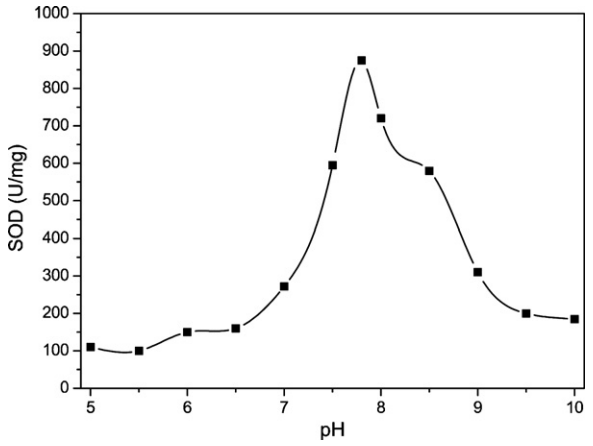

Fig. 8. Effect of pH on the stability of purified Cu/Zn SOD Km. The enzyme was incubated for 10 min in buffers with pH values in the range 5–10 and the samples' activity was assayed under standard conditions as described in Section 2.

family. The relatively small changes of the initial $[\theta]_{222}$ at high temperatures indicate that many secondary structural elements are preserved, especially at neutral pH and even at extreme high temperatures. Thus, T-dependent unfolding was not detected and probably even at temperatures above 90 °C the proteins retain a "globule state". These data are in compliance with the increased thermal stability of the purified enzyme. Another thermostable enzyme is BSOD with a conformational melting temperature greater than 80 °C [27–29].

The range of the $[\theta]_{222}$ T-changes are too wide to be a single transition, as shown in Fig. 9B, inserted. Tm values for the Cu/Zn SOD Km enzyme at different pH were calculated from the amplitude $\Delta[\theta]_N - \Delta[\theta]_D$ of the curves. Unfolding of the protein was observed in two pH regions (pH 6.5–1.5 and 8.7–10). In the pH interval 6.5 and 8.9 the enzyme is stable and unfolding of the enzyme was observed in the basic region >pH 9 and in the acidic region <pH 6.

Three steps of transition were observed in the acidic region in interval I (pH 6.6–5.7), II (pH 5.7–5.0) and III (pH 5.00–4.00). The transition in pH interval 6.6–5.7 could be explained with the dissociation of the dimeric form of SOD Km in monomeric. As it is known, the unfolding of the monomeric enzyme, unlike dimeric Cu/Zn superoxide dismutases, is highly reversible, a behaviour that may be explained by the absence of free cysteines and the highly polar nature of its molecular surface. The thermal stability of the enzymes is dependent of the active-site metals. Apo-superoxide dismutase from *E. coli* was published to be nearly as stable as the bovine apoenzyme, whose holo form is much more stable and less sensitive to pH variations [30]. The melting temperature of the *E. coli* enzyme was found to be also pH-dependent with the holoenzyme transition centered at 66 °C at pH 7.8 and at 79.3 °C at pH 6.0. Previous results on SOD and other proteins suggest a major role for thiols in the irreversible protein denaturation. In bovine and human SOD [31], the removal of free cysteines by sitedirected mutagenesis decreased irreversible denaturation as measured by thermal inactivation at elevated temperatures.

3.4. Influence of different organic compounds, metal ions and enzyme inhibitors on Cu/Zn SOD Km activity

The results of these studies are shown in Table 2. The enzyme is stable at increased concentrations of various metal ions. 1.0 mM EDTA inhibits the enzyme activity at about 66%. Similar is the effect of the detergent SDS. The specific enzyme inhibitors influence its activity as follows: 2.0 mM KCN inhibits the Cu/Zn SOD with about 30%, while H_2O_2 and NaN_3 did not show any inhibitory effect. Thiol

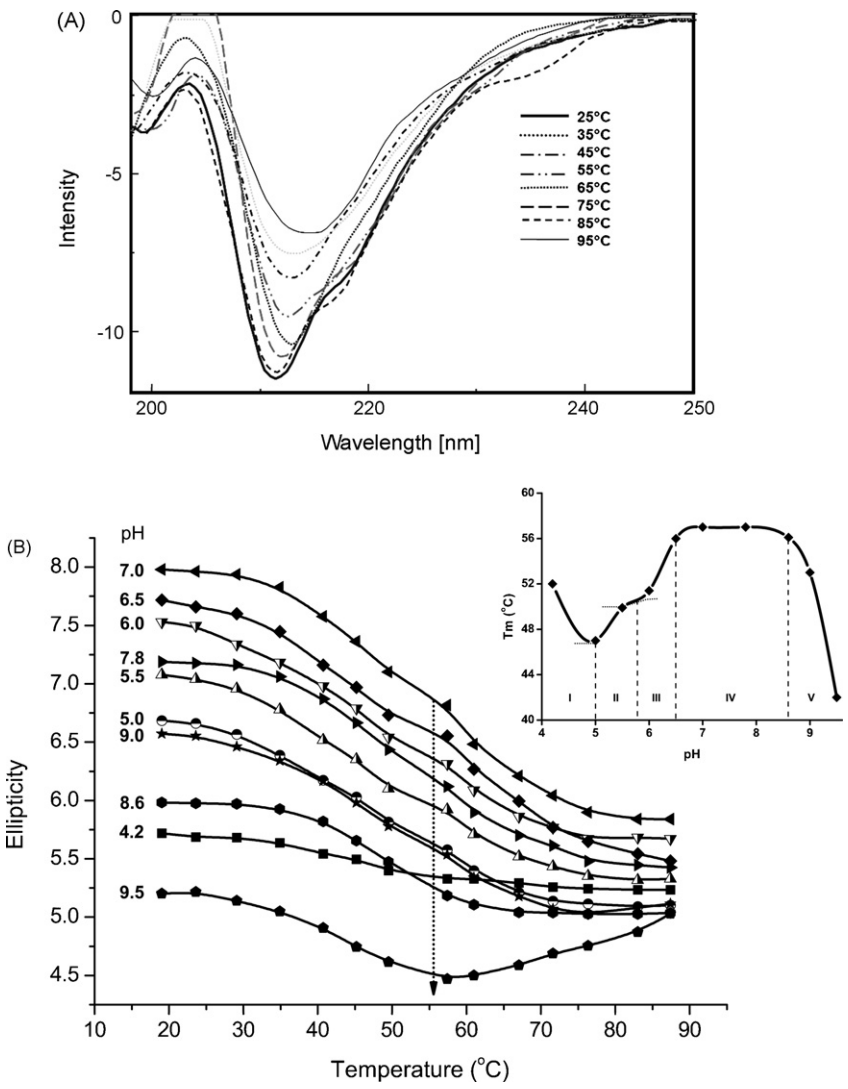

Fig. 9. (A) The CD spectra of the native enzyme in 50 mM Tris/HCl buffer, pH 7.0 at different temperatures; (B) circular dichroism parameters of Cu/Zn SOD Km in 20 mM Tris/HCl buffer at different pH values (from 1.5 to 12.0) were measured from 15 up to 95 °C on a J-720 spectropolarimeter (Jasco, Tokyo, Japan) after 20 min incubation. The $[\theta]_{222}$ values were recorded in intervals of 5 ± 0.2 °C.

Table 2
Comparative characteristics of the effect of metal ions, organic compounds and enzyme inhibitors on the activity of purified Cu/Zn SOD Km (A) and a commercial Cu/Zn SOD preparation from bovine erythrocytes (SERVA) (B). Each compound was added to the reaction mixture at final concentrations of 0.1 or 1 mM. The effect was evaluated through calculation of the activity of each sample as a percentage of a non-treated control samples.

	Residual specific enzyme activity (%)			
	A		B	
	0.1 mM	1.0 mM	0.1 mM	1.0 mM
H_2O_2	100[a]	88[b]	100[a]	90[b]
KCN	100	75 (73[a])	100	78 (70[a])
NaN_3	100	100	100	100
NaCl	100	85.3	100	90.1
$MgCl_2$	100	100	97.3	69.2
$CaCl_2$	99.7	99	100	98.1
$ZnCl_2$	100	91.6	100	89.4
EDTA	59	34.3	73	29
SDS	48.3	–	45.3	–
P-CMB	100	73.4	100	77.8
2-IAA	100	76.5	100	69.6

(–): the enzyme activity cannot be determined due to masking of the colour reaction.
[a] H_2O_2: 5 mM; KCN: 2 mM.
[b] H_2O_2: 10 mM.

enzyme inhibitors, p-CMB and 2-IAA, did not influence the enzyme activity at concentration of 0.1 mM and inactivated the enzyme by 30% at 1.0 mM. A comparative analysis of these data with those of a commercial preparation of Cu/Zn SOD from bovine erythrocytes demonstrates the good properties of our purified enzyme.

4. Conclusion

As a result of the three-step procedure described, 15 mg pure enzyme with a specific activity of 996 U mg protein^{-1} were obtained from 820 mg cell-free protein. The product was purified 28.5-fold and in terms of total enzyme activy a yield of 53% was achieved. It can be summarized that the proposed procedure for the purification of Cu/Zn SOD Km is rapid, highly specific and efficient. The purified Cu/Zn SOD Km is a thermostable one ($t_{1/2}$ at 70 °C = 30 min). This specific characteristic is most probably due to the intrinsic stability of the β-barrel fold, the dimeric structure and metal binding. It can be speculated that the glucosilation observed also contributes to the better resistance to heat inactivation of Cu/Zn SOD Km. Similar findings in this respect are reported by Holdom et al. [24] for *Asperigillus fumigatus* Cu/Zn SOD enzyme. In addition to its thermal stability Cu/Zn SOD Km represents pH

stability in the alkaline range (7.5–8.5) and resistance to inhibitors and variety of metal ions, detergents and inhibitors.

Due to the above mentioned characteristics the newly isolated and characterized Cu/Zn SOD Km enzyme from *K. marxianus* NBIMCC 1984 strain contributes to the wide range of applications of this microorganism in pharmacy and food & beverage industries.

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