A novel glycosylated Cu/Zn-containing superoxide dismutase: production and potential therapeutic effect

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The fungal strain *Humicola lutea* 103 produces a naturally glycosylated Cu/Zn SOD. To improve its yield, the effect of an increased concentration of dissolved oxygen (DO) on growth and enzyme biosynthesis by the producer, cultivated in a 3 l bioreactor, was examined. Exposure to a 20% DO level caused a 1.7-fold increase of SOD activity compared to the DO-uncontrolled culture. Maximum enzyme productivity of SOD was approximately 300×10^3 U (kg wet biomass)⁻¹. The novel enzyme was purified to electrophoretic homogeneity. The presence of Cu and Zn were confirmed by atomic absorption spectrometry. The molecular mass of *H. lutea* Cu/Zn SOD was calculated to be 31870 Da for the whole molecule and 15936 Da for the structural subunits. The N-terminal sequence revealed a high degree of structural homology with Cu/Zn SOD from other prokaryotic and eukaryotic sources. *H. lutea* Cu/Zn SOD was used in an *in vivo* model for the demonstration of its protective effect against myeloid Graffi tumour in hamsters. Comparative studies revealed that the enzyme (i) elongated the latent time for tumour appearance, (ii) inhibited tumour growth in the early stage of tumour progression (73–75% at day 10) and (iii) increased the mean survival time of Graffi-tumour-bearing hamsters. Moreover, the fungal Cu/Zn SOD exhibited a strong protective effect on experimental influenza virus infection in mice. The survival rate increased markedly, the time of survival rose by 5.2 d and the protective index reached 86%. The *H. lutea* SOD protected mice from mortality more efficiently compared to the selective antiviral drug ribavirin and to commercial bovine SOD. In conclusion, our results suggest that appropriate use of the novel fungal SOD, applied as such or in combination with selective inhibitors, could outline a promising strategy for the treatment of myeloid Graffi tumour and influenza virus infection.

Keywords: protein sequencing, myeloid Graffi tumour, influenza infection, protective effect

INTRODUCTION

Superoxide dismutases (SODs, EC 1.15.1.1) are metalloenzymes involved in the physiological response

Abbreviations: DO, dissolved oxygen; MST, mean survival time; SOD, superoxide dismutase; TBH, tumour-bearing hamsters.

to oxygen toxicity by their ability to catalyse the dismutation of the superoxide anion (O_2^-) to molecular oxygen and H_2O_2 (Fridovich, 1995). Three classes of SODs have been reported, based on their active site metals: Cu/Zn SOD, Fe SOD and Mn SOD (Fridovich, 1995). At present, there is evidence that Ni SOD occurs in prokaryotes as well (Matès & Sánchez-Jimenez,

1999). The recent interest in this enzyme is determined by its important physiological role in primary cellular antioxidant defence and its potential therapeutic use. Numerous elucidations of structures and functions of SOD from prokaryotes and eukaryotes contribute to the understanding of the molecular evolution of this enzyme family and the therapeutic application of SOD as an antioxidant. Considerable experimental evidence has been accumulated that the use of SOD might be beneficial in the treatment of postischaemic reperfusion injury (Rios *et al.*, 1999), rheumatoid and osteoarthritis (Kakimoto *et al.*, 1993), brain trauma (Yunoki *et al.*, 1997) and influenza-induced lung pneumonitis (Sidwell *et al.*, 1996). SODs have been proposed to be clinically useful for a wide variety of applications, including prevention of oncogenesis, tumour promotion, tumour invasiveness (Matès & Sánchez-Jiménez, 2000) and reduction of the cytotoxic and cardiotoxic effects of anticancer drugs (Trotti, 1997; Sabitha & Shyamaladevi, 1999).

The large scientific and practical interest in SODs has resulted in intensive developments of new technologies for enzyme production from various sources, including animal and human erythrocytes, plants and micro-organisms. Filamentous fungi are especially suitable for SOD production because of their potential advantages, i.e. rich abundant mycelium, intensive respiration and high level of cyanide-resistant respiration (Sakajo *et al.*, 1993). Microbial technology would also be more effective and inexpensive compared to the production of this enzyme from bovine erythrocytes. Moreover, despite great efforts, efficient production of recombinant human SOD in prokaryotic systems or simple eukaryotes has failed. This lack of success has greatly complicated large-scale production and genetic engineering of the protein (Stenlund & Tibell, 1999).

To improve the *in vivo* pharmacological activity of SOD, the enzyme has been conjugated with mono- and polysaccharides (Fujita *et al.*, 1992), haemoglobin (D'Agnillo & Chang, 1998) and lecithin (Nakauchi *et al.*, 1996), etc. A major advantage of such modified SODs is their longer half life in plasma and blood (Maksimenko *et al.*, 1993). Our preliminary investigations have shown that the fungal strain *Humicola lutea* 103 mainly produces the Cu/Zn enzyme. Moreover, *H. lutea* SOD is a naturally glycosylated enzyme (unpublished data) which could be isolated in very few other cases. The secretory tetrameric extracellular mammalian SOD is the only glycosylated SOD, besides the *H. lutea* enzyme, described so far (Edlund *et al.*, 1992). Glycoenzymes of this kind do not need additional processing for conjugation and modification as do non-glycosylated enzymes.

Here, we present the conditions for the production of a novel, naturally glycosylated Cu/Zn SOD from the fungal strain *H. lutea* 103. We report also on the characterization of the purified enzyme, as well as on its protective effect in experimental influenza virus infection and on myeloid Graffi tumour.

METHODS

Micro-organism. The fungal strain, *Humicola lutea* 103, from the Mycological Collection of the Institute of Microbiology, Sofia, was used throughout and maintained at 4 °C on beer agar, pH 6.3.

Cultivation, equipment and conditions. Cultivation was performed in a 3 l bioreactor, ABR-09, developed and constructed by the former Central Laboratory for Bio-instrumentation and Automatisatation (CLBA) of the Bulgarian Academy of Sciences. The bioreactor was equipped with pH and automatic dissolved oxygen (DO) monitoring equipment and a control system.

The composition of the culture medium has been described previously (Angelova *et al.*, 1996). For the inoculum, 80 ml seed medium was inoculated with 5 ml spore suspension at a concentration of 2×10^8 spores ml⁻¹ in 500 ml Erlenmeyer flasks. The cultivation was performed on a shaker (220 r.p.m.) at 30 °C for 24 h. For bioreactor cultures, 160 ml of the seed culture was brought into the 3 l bioreactor, containing 1850 ml of the production medium. The cultures were grown at 30 °C for 120 h. The fermentation parameters under DO-uncontrolled conditions were: impeller speed, 600 r.p.m.; air flow, 1 v.v.m. [1 vol. air (1 vol. liquid)⁻¹ (min)⁻¹]. In this case, only the changes in DO level during fermentation were measured. For the DO-controlled culture system, aeration and impeller speed were regulated in such a way as to produce 20 % oxygen saturation in the liquid. The results obtained in this investigation were evaluated from experiments repeated using three or five parallel runs.

Analytical methods. The cell-free extract was prepared as described previously (Angelova *et al.*, 1995). SOD activity was measured by the nitro blue tetrazolium (NBT) reduction method of Beauchamp & Fridovich (1971). One unit of SOD activity was defined as the amount of SOD required for inhibition of the reduction of NBT by 50 % (A_{560}) and was expressed as U (mg protein)⁻¹. Cyanide (2 mM) was used to distinguish between the cyanide-sensitive isoenzyme Cu/Zn SOD and the cyanide-resistant Mn SOD. Cu/Zn SOD activity was obtained as total activity minus the activity in the presence of 2 mM cyanide. Protein was estimated by the Lowry procedure, using crystalline bovine albumin as standard. Soluble reducing sugars were determined by the Somogyi-Nelson method (Somogyi, 1952). Dry weight determination was performed on samples of mycelia harvested throughout the culture period. The culture fluid was filtered through a Whatman (Clifton, USA) no. 4 filter. The separated mycelia were washed twice with distilled water and dried to a constant weight at 105 °C.

Purification of *H. lutea* Cu/Zn SOD. The frozen mycelium was partially thawed and then suspended in 4 vols 25 mM potassium phosphate buffer, pH 7.8, containing 1 mM PMSF. The suspended material was disrupted by an Ultra-Turrax-IKA-Werk homogenizer. Cell debris was removed by filtration through a Buchner funnel and the mixture was clarified by centrifugation at 13000 g for 20 min at 4 °C. All subsequent steps were performed at 4 °C. The supernatant was concentrated approximately three times by membrane ultrafiltration (Amicon PM-10). The concentrated crude extract was further fractionated using acetone, chilled to -20 °C. Cooled acetone was slowly added to the culture filtrates to a concentration of 0.4 vols with vigorous stirring for 30 min. The mixture was incubated for 2 h at -20 °C and the precipitate was collected, centrifuged at 13000 g for 20 min and discarded, while the supernatant was treated with 1:1 vols of chilled acetone. The

mixture was held for 12 h at -20°C ; then the precipitate was collected by centrifugation (13000 g for 20 min) and suspended in distilled H_2O . The clear viscous supernatant was loaded on a Sephadex G-75 column (2×50 cm; Pharmacia), previously equilibrated with 50 mM potassium phosphate buffer, pH 7.8, and the column was eluted with the same buffer.

Further purification was achieved by ion exchange chromatography on a DEAE cellulose-52 column (3.5×10 cm; Serva), equilibrated in 50 mM KH_2PO_4 buffer, pH 7.8. The Cu/Zn SOD was eluted with an NaCl gradient (0–0.1 M). The active fractions were loaded for additional purification onto an FPLC system, equipped with a 10/10 Mono Q anion exchange column. The column was previously equilibrated with 50 mM potassium phosphate buffer, pH 7.8, and the elution was effected employing stepwise increases in NaCl (0–0.1 M). Fractions with $3100 \text{ U (mg protein)}^{-1}$ were further purified and desalted on a Nucleosil RP C18 column (250×10 mm; Macherey-Nagel) to allow determination of the N-terminal sequence and amino acid composition. The conditions used for HPLC separation were as follows: eluant A, 0.058% trifluoroacetic acid (TFA); eluant B, 80% acetonitrile in A. The gradient was run from 5 to 100% B within 60 min at a flow rate of 1 ml min^{-1} .

Characterization of *H. lutea* Cu/Zn SOD. Flameless atomic absorption spectroscopy of Cu, Zn and Mn was performed on a Perkin-Elmer 400 S spectrometer, equipped with a HGA 76B graphite furnace.

Purity control of the enzyme and measurement of molecular mass were performed on 10% polyacrylamide gels, as described by Laemmli (1970), and the gels, stained for protein detection, were compared with duplicate gels stained for SOD activity as described by Misra & Fridovich, (1977). The following molecular mass standards were used: trypsinogen (24 kDa), egg albumin (45 kDa), bovine albumin (66 kDa) and *Limulus polyphemus* haemocyanin (monomer, 70 kDa).

N-terminal amino acid sequence analysis was performed using an Applied Biosystems sequencer, model 473A.

Mass spectra were obtained by matrix-assisted laser desorption ionization MS (MALDI 1-MS) (Kratos, MALDI III equipment; Shimadzu). The sample (10 pmol) was dissolved in 0.1% (v/v) TFA and applied onto the target. Analysis was carried out in α -cyano-4-hydroxycinnamic acid. Solutions of chicken egg ovalbumin (44400 kDa) and bovine serum albumin (66430 Da) were used to calibrate the molecular mass scale.

The protein was analysed for carbohydrates with the orcinol/ H_2SO_4 test (Francois *et al.*, 1962).

Experiments concerning the effect of *H. lutea* SOD on myeloid Graffi tumour. The protective effect was examined by the following parameters: mean latent time (d) for tumour appearance; mean survival time (MST, d), estimated at day 45 after tumour transplantation; and inhibition (%) of tumour growth, estimated according to Toshkova (1995)

$$\text{Inhibition (\%)} = \frac{\text{diameter of tumours of control hamsters} - \text{diameter of tumours of } H. lutea \text{ SOD-treated hamsters}}{\text{diameter of tumours of control hamsters}} \times 100$$

Experimental animals. Forty, 2-month-old Golden Siberian hamsters of both sexes, weighing 80 to 100 g, were used for the experiments. For the examination of the SOD-protective

effect, the animals were separated into four experimental groups.

Group 1. Animals were treated intraperitoneally (i.p.) with a 65 U *H. lutea* SOD single dose per animal, twice a week, 1 week before and 2 weeks after tumour transplantation.

Group 2. Animals were treated with a 65 U *H. lutea* SOD single dose per animal, twice a week, 2 weeks after tumour transplantation, starting on the day of transplantation.

Group 3. Animals were treated with a 125 U *H. lutea* SOD single dose per animal, twice a week, 2 weeks after tumour transplantation, starting on the day of transplantation.

Group 4. Control animals with tumours without treatment.

Tumour. The myeloid tumour was induced by the mouse Graffi virus, adapted for hamsters by Yakimov *et al.* (1979). The tumour was maintained *in vivo* by subcutaneous (s.c.) inoculation of 2×10^4 viable cells in the interscapular region. The tumour was 100% transplantable and 100% lethal for hamsters (Yakimov *et al.*, 1979; Toshkova, 1995).

Experiments concerning the effect of *H. lutea* SOD in experimental influenza infection. Male and female inbred ICR mice (16–18 g) were obtained from the Experimental Animal Station of the Bulgarian Academy of Sciences in Slivnitsa, Sofia, Bulgaria. They were quarantined 24 h prior to use and maintained under standard laboratory conditions with tap water *ad libitum* for the duration of the studies. The experimental groups were of 10 animals each. At the end of the experiments, surviving mice were sacrificed by cervical dislocation.

Virus infection. In mice, the infection was induced under ether anaesthesia by intranasal inoculation of A/Aichi/2/68 (H3N2), adapted to the mouse lung. This virus causes haemorrhagic pneumonia in mice. The strain was from the collection of the Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria. To cause lethal infection, mice were infected by passages in the lungs with 5–10 LD_{50} of the virus in a 0.05 ml volume of physiological saline per mouse.

Experimental design. Mice were treated with *H. lutea* Cu/Zn SOD (500 U per mouse d^{-1}) in 0.1 ml saline administered intravenously (i.v.) 4–7 d after virus exposure and were observed for death daily for 21 d. All experiments were done in parallel with the selective antiviral drug ribavirin (Sigma-Aldrich) and SOD from bovine erythrocytes (Sigma-Aldrich). Ribavirin was inoculated i.p. in a dose of 125 mg per mouse d^{-1} ; bovine SOD was inoculated i.v. in a dose of 500 U per mouse d^{-1} .

The protective effect was estimated by the increase in the rate of survival (%), protective index (PI, %) and prolongation of MST (d) as described by Serkedjieva & Ivanova (1997). PI was determined from the equation $(\text{PR} - 1)/\text{PR} \times 100$, where PR (protective ratio) is $M_{\text{control}}/M_{\text{experiment}}$ (M is mortality).

The results are the mean values from three or four independent experiments. Reduction of mortality rates compared to placebo controls were evaluated using χ^2 analysis. Student's *t*-test was employed to analyse differences in survival times. Standard deviations were determined within 95% confidence intervals.

Ethical aspects. Experiments with animals are inevitable and indispensable in investigations concerning the treatment of influenza infection and tumour growth. Only in this way could a matter-of-fact characterization of the protective effect of *H. lutea* SOD be obtained. That is why the experiments with

animals cannot be replaced by experiments with tissue culture cell lines. The number of experimental animals was reduced as much as possible, depending on statistical significance. The animals were sacrificed using ether narcosis and cervical dislocation to minimize the amount of suffering. Refinement of the tests with animals was achieved by careful planning of multifactor experiments.

The animals were bred under standard conditions, accepted by the Bulgarian Veterinary Health Service. Specialized personnel took care of the welfare of the animals. In implementing this study we have adhered most strictly to all national and international ethical provisions applicable to Bulgaria where the investigations with experimental animals were carried out.

RESULTS AND DISCUSSION

Effect of DO concentration on Cu/Zn SOD production

It is well known that SOD expression is markedly enhanced by augmenting intracellular production of $\cdot\text{O}_2^-$ under conditions of increased levels of DO in the

Fig. 1. Time courses of (a) mycelia growth (■, □) and dissolved oxygen concentration (●, ○), and (b) SOD production (●, ○) and glucose consumption (■, □) in *H. lutea* 103 culture, grown under DO-uncontrolled (■, ●) and 20% DO-controlled (□, ○) conditions.

Fig. 2. Isoenzyme profiles of SOD produced by *H. lutea* upon cultivation at different DO levels. (a) Uncontrolled DO; (b) controlled DO (20%). ■, Mn SOD; □, Cu/Zn SOD.

growth medium (Hassan, 1983). To produce SOD in large quantities, we examined growth, glucose uptake and formation of SOD by *H. lutea* 103 in a bioreactor under DO-uncontrolled and 20% DO-controlled conditions (Fig. 1a, b).

Under DO-uncontrolled conditions, the culture showed a typical profile of fungal growth (Fig. 1a). The biomass increased rapidly (exponential phase) when glucose uptake was maximal, without a lag period (Fig. 1b). The culture period from 36 to 84 h (stationary phase) showed decreased fungal growth and the carbohydrate source was almost exhausted. Moreover, the DO level decreased sharply and oscillated around zero. The sudden drop of oxygen concentration might be attributed to the oxygen required for cell growth and metabolism (Petrucchioli *et al.*, 1995).

Table 1. Comparison of SOD productivity at the activity maxima

Cultivation system*	Cultivation time (h)	Wet cell weight (g l ⁻¹)†	Protein yield (mg l ⁻¹)‡	Specific SOD activity [U (mg protein) ⁻¹]§	10 ⁻³ × total SOD activity (U l ⁻¹)	10 ⁻³ × SOD productivity (U kg ⁻¹)¶	Cu/Zn SOD (%)#
Uncontrolled DO							
1st maximum	48	89.67	468.75	65.39	30.7	341.8	87
2nd maximum	102	64.25	305.40	71.34	21.8	339.0	89
Controlled DO (20 %)							
1st maximum	48	81.24	282.30	112.09	31.6	389.5	53
2nd maximum	84	42.56	84.42	123.61	10.4	245.1	45

* *H. lutea* was cultivated under conditions of uncontrolled DO and controlled DO (20 %; see Methods).

† Wet biomass with 75 % humidity.

‡ Calculated as mg intracellular protein produced in the wet biomass (litre culture medium)⁻¹.

§ Activities at the time of harvest (see Fig. 1b).

|| Calculated as the amount of enzyme (litre culture medium)⁻¹.

¶ Calculated as the amount of enzyme (kg wet biomass)⁻¹.

Expressed as a percentage of total SOD activity using the data from Figs 1(b) and 2.

Irrespective of accelerated glucose uptake (Fig. 1b), 20 % DO-controlled conditions led to a reduction in duration of the stationary phase as well as to a decrease in the maximum level of biomass (Fig. 1a) and intracellular protein content (data not shown). Petruccioli *et al.* (1995) and Guidot *et al.* (1993) demonstrated similar data for *Penicillium variable* and *Saccharomyces cerevisiae*, respectively, due to increasing DO level.

The effects of two different levels of DO (uncontrolled DO and DO at 20 %) on SOD production were evaluated (Fig. 1b). Increasing enzyme activity in *H. lutea* cells, cultivated under both conditions, was detected in the early exponential phase and an active synthesis was observed during a period of intensive oxygen consumption. Moreover, the time courses of SOD production show two maxima. A similar phenomenon, a secondary increase in SOD activity during the late stationary phase, has been observed for SOD production by filamentous fungi and yeasts (Shilova *et al.*, 1989; Angelova *et al.*, 1996). It can be explained by an intensification of the process of $\cdot\text{O}_2^-$ generation when the cells utilize endogenous sources of carbon and nitrogen (organic or amino acids).

The results demonstrated that the oxygen level had a significant influence on SOD activity. Under high DO conditions, higher levels of SOD were produced at 1st and 2nd maxima, reaching activities of about 112 and 123 U (mg protein)⁻¹, respectively. Considering enzyme formation in both experiments, DO-controlled cultures produce approximately 1.7-fold higher SOD activity than DO-uncontrolled cultures. Exposure to high DO levels has been reported to cause both enhancement (Vercellone *et al.*, 1990) and decrease (Shilova *et al.*, 1989) in SOD activity for microbial cells.

The isoenzyme profiles of SOD, produced by *H. lutea*

upon cultivation at different DO levels, are illustrated in Fig. 2. The fungal strain produces two isoforms, namely Mn- and Cu/Zn-containing SOD. Cu/Zn SOD activity was obtained as total activity minus the activity in the presence of 2 mM cyanide, and the conclusion from these results is that this isoenzyme was responsible for approximately 90 % of total SOD activity in DO-uncontrolled cultures. The numbers of isoforms did not change under hyperoxic conditions when *H. lutea* was exposed to an atmosphere of 20 % O₂. There was no significant change in Cu/Zn SOD activity, whereas Mn SOD expression increased 10–12-fold by elevated DO. So, it appears that Mn SOD is the primary defence in *H. lutea* cells against oxygen toxicity. Similar findings about the lack of response of Cu/Zn SOD to oxidative stress have been reported for a variety of cells (Stralin & Marklund, 1994). The dependence of Cu/Zn SOD on oxygen was found to be related to the availability of copper to *H. lutea* (M. Angelova, unpublished data), yeasts (Howlett & Avery, 1999) and mammalian cells (Roughead *et al.*, 1999).

The results of DO-controlled and DO-uncontrolled SOD production are summarized in Table 1. The highest efficiency of SOD biosynthesis was achieved at the 1st maximum of activity for both kinds of cultures. Although specific SOD activity was higher at the 2nd maximum, total enzyme activity was only 77 and 33 % of that of the first maximum for DO-uncontrolled and DO-controlled cultures, respectively. Because of the differences in the ratio of isoenzymes, the conditions for achieving the 1st maximum in DO-uncontrolled cultures appear to be the most appropriate for Cu/Zn SOD production by *H. lutea* 103. The productivity of *H. lutea* 103 Cu/Zn SOD is comparable to the maximum enzyme productivity by micro-organisms [300 × 10³ U (kg wet biomass)⁻¹] (Kazanina & Selezneva, 1992).

Table 2. Purification steps for Cu/Zn SOD from *H. lutea* 103

Purification step	Total protein (mg)	$10^{-3} \times$ total SOD activity (U)	Specific SOD activity (U mg ⁻¹)	Yield (%)	Purification (-fold)
Crude extract (after concentration)	7089	884	124	100	1.0
Acetone fraction (0.4 vols)	4343	852	196	96	1.6
Acetone fraction (1.1 vols)	443	329	743	37	6.0
Sephadex G-75	100	191	1894	21	15.2
DEAE cellulose 52	56	122	2167	13	17.4
Mono Q 10/10 (FPLC)	36	111	3100	12	25.6

Fig. 3. PAGE (10% gel) of purified Cu/Zn SOD. Lanes: 1, gel stained for enzymic activity; 2, gel stained for protein; 3, standard mixture: a, trypsinogen (24 kDa); b, egg albumin (45 kDa); c, bovine albumin (66 kDa); d, *Limulus polyphemus* haemocyanin (monomer, 70 kDa).

Purification and characterization of *H. lutea* Cu/Zn SOD

The results of the purification procedure are shown in Table 2. We found that fast-performance anion-exchange chromatography (FPLC) on a Mono Q 10/10 column is a highly efficient purification method for Cu/Zn SOD. After FPLC purification, the enzyme presents as a single band in PAGE with an activity of 3100 U (mg protein)⁻¹ (Fig. 3, lane 1). The presence of 59 ng-atom copper and 52 ng-atom zinc (mg enzyme)⁻¹ in the purified *H. lutea* Cu/Zn SOD was confirmed by atomic absorption spectrometry. No manganese or iron could be detected.

The molecular mass of *H. lutea* Cu/Zn SOD was determined by several methods. The sample was subjected to 12% SDS-PAGE, producing one band at 32 kDa (Fig. 3, lane 2). The same results were confirmed by means of matrix-assisted laser desorption ionization

MS (Fig. 4). The mass was calculated to be 31690 Da for the whole molecule and 15845 Da for the structural subunits. From these results it is suggested that the investigated enzyme is a dimer, composed of two identical structural subunits.

The *H. lutea* Cu/Zn SOD is a glycosylated enzyme, as determined by the orcinol-sulphuric acid assay, with a carbohydrate-binding site, Asn-Xxx-Ser/Thr, typical for N-glycosylation (Francois *et al.*, 1962).

The N-terminal sequence of Cu/Zn SOD from *H. lutea* 103 was compared with corresponding regions of Cu/Zn SODs isolated from other organisms (Bordo *et al.*, 1994; Forest *et al.*, 2000; Fig. 5). The first 35 residues showed more or less the same percentage (42–71%) of identity with the enzymes from eukaryotes. The highest homology was established with the Cu/Zn SODs from fungi and yeasts (71.4 and 62.9% with *Neurospora crassa* and *Saccharomyces cerevisiae*, respectively). Comparison with enzymes from higher eukaryotes reveals a level of homology of about 48.5% with human and 42.4% with bovine Cu/Zn SOD. As already mentioned, the enzyme from *H. lutea* 103 shows a low level of homology (about 20%) with SODs from prokaryotic organisms such as *Actinobacillus pleuropneumoniae*, *Haemophilus ducreyi*, *Haemophilus influenzae* and *Haemophilus parainfluenzae*.

Effect of *H. lutea* Cu/Zn SOD on the survival of hamsters with transplanted myeloid tumours

During recent years, clinical investigations have indicated that the oxidant/antioxidant balance is variable during the oncogenic process (Kahlos *et al.*, 1999). Several authors have demonstrated that antioxidant levels in most human and animal cancers are low (Ambrosone *et al.*, 1999). These literature data suggest a new concept for cancer prevention and treatment by systematic modulation of the antioxidant defence system. Moreover, modern cancer therapy produces substantial acute and chronic toxicity that impairs quality of life and limits the effectiveness of treatment (Trotti, 1997). One of the goals of the present experiments was to determine whether inoculation of *H. lutea* SOD would be of therapeutic benefit for hamsters with myeloid Graffi tumour.

The results of the latent time of tumour appearance and

Fig. 4. Matrix-assisted laser desorption ionization mass spectrum of the functional unit of Cu/Zn SOD. The sample (10 pmol) was dissolved in 0.1% TFA and applied onto the target. Analysis was carried out in α -cyano-4-hydroxycinnamic acid. Solutions of chicken egg ovalbumin (44400 Da) and bovine serum albumin (66430 Da) were used to calibrate the mass scale.

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Fig. 5. N-terminal amino acid sequence alignment of *H. lutea* (*H. l.*) Cu/Zn SOD and Cu/Zn SODs from eukaryotic and prokaryotic organisms. SOD amino acid sequences are taken from the following sources: human (*H.*), bovine (*B.*), *Neurospora crassa* (*N. c.*), *Saccharomyces cerevisiae* (*S. c.*) (Bordo *et al.*, 1994), *Actinobacillus pleuropneumoniae* (*A. p.*), *Haemophilus ducreyi* (*H. d.*), *Haemophilus influenzae* (*H. i.*) and *Haemophilus parainfluenzae* (*H. p.*) (Forest *et al.*, 2000).

Table 3. Mean latent time for tumour appearance and MST with transplanted myeloid Graffi tumours of hamsters treated i.p. by *H. lutea* SOD

Experimental group*	Mean latent time (d)	MST (d)
1	10.50 ± 0.6	36.5 ± 1.5
2	13.80 ± 0.8	36.0 ± 1.4
3	10.75 ± 0.5	39.6 ± 1.8
4	10.10 ± 0.9	32.1 ± 1.2

* See Methods.

the MST are shown in Table 3. The latent time of tumour appearance was elongated in hamsters of all experimental groups treated with *H. lutea* SOD simultaneously or before tumour transplantation compared to the control group. Furthermore, the application of *H. lutea* SOD enhanced the MST of Graffi-tumour-bearing hamsters. The effect was better expressed in the 3rd group treated with 125 U *H. lutea* SOD (39.6 d), compared to the untreated control animals (32.1 d). Though groups 1 and 2 were treated with a lower dose of SOD, a comparable value of MST was established (36.5 and 36 d for groups 1 and 2, respectively).

The results obtained are not surprising because of the scavenging activity of SOD. It has been reported that tumour appearance is accelerated by inflammation-induced active oxygen species, including $\cdot\text{O}_2^-$, and accompanied by decreased levels of intracellular antioxidant enzymes in the cells (Okada *et al.*, 1999; Seidman *et al.*, 1999). Addition of SOD leads to a reduction of

$\cdot\text{O}_2^-$ flux and prevents the cells from phenotype alterations (Okada *et al.*, 1999; Ambrosone *et al.*, 1999). Li *et al.* (1998) demonstrated a protective effect of several antioxidants on tumour transformation, both *in vivo* and *in vitro*. Oberley & Buettner (1979) have also shown similar results that i.v. or intra-muscular (i.m.) injection of Cu/ZnSOD markedly increased the life span of animals carrying Ehrlich ascites tumour cells or Sarcoma 180 tumour cells in solid form. According to these authors and others (Seidman *et al.*, 1999), the effect must be due to $\cdot\text{O}_2^-$ -scavenging outside the cell.

On the other hand, it is worthwhile mentioning that groups 2 and 3 show a different response in both determinations (Table 3). Whereas the higher dose (125 U; 3rd group) caused the highest value of MTS, the lower dose (65 U; 2nd group) was more suitable for the prevention of tumour appearance. These data suggest that the effect of exogenous added *H. lutea* SOD is stage-dependent. In the first stage of carcinogenesis, the levels of antioxidant enzymes (SOD and catalase) in tumour-bearing hamsters (TBH) were almost equal with those in the control animals (E. Ivanova, unpublished data) and a single dose of 65 U SOD was sufficient to restore the oxidant/antioxidant balance in the host organism. In contrast, because of the interference of the endogenous SOD with the higher dose of injected *H. lutea* SOD (125 U; 3rd group), a significant increase in H_2O_2 accumulation was established (E. Ivanova, unpublished data). Moreover, the treatment of TBH with *H. lutea* SOD caused an insignificant increase in catalase activity in liver and tumour cells (E. Ivanova, unpublished data) and consequently enhances the cell's ability to generate the highly toxic hydroxyl radical (Fenton reaction).

Fig. 6. Inhibition of tumour growth (%) in Graffi-tumour-bearing hamsters treated by *H. lutea* SOD. The values are calculated as the percentage decrease in diameter of tumours of *H. lutea* SOD-treated hamsters [groups 1 (■), 2 (○) and 3 (●)] relative to the diameter of tumours of hamsters without treatment (group 4). Experimental groups are described in Methods.

Regarding the MST, a possible interpretation of the data includes our previous results obtained during the tumour progression stage: (i) the superoxide production by phagocytes was four- to fivefold higher in TBH compared to the control animals (Dimitrova *et al.*, 2000); (ii) the levels of SOD and catalase decreased in TBH compared to the healthy animals (E. Ivanova, unpublished data). Probably, this modification of the antioxidant defence during tumour growth resulted in a serious disturbance of the oxidant/antioxidant balance in the cells. It is possible that the higher dose of injected *H. lutea* SOD (125 U) restores the redox balance to a higher degree compared to the dose of 65 U.

The *H. lutea* SOD treatment furthermore inhibited tumour growth in the early stage of tumour progression (73–75% at day 10 and 48–50% at day 15 for the experimental animals in groups 1–3; Fig. 6). During the following days the most expressed inhibition of tumour growth was established for group 1. It has been reported that reduction of the radicals and oxidants with antioxidants, including SOD, antagonizes tumour promotion activity (Kong & Lillehei, 1998). Our results supported this concept that SOD has a protective effect during the promotional phases of cancer development (Matès & Sánchez-Jimenez, 2000).

Protective effect of the *H. lutea* SOD in murine experimental influenza virus infection

Because recently available vaccines are not completely protective against influenza virus infection and no specific therapy of proven value currently exists for

Fig. 7. Protective effect of fungal Cu/Zn SOD on experimental influenza infection with A/Aichi (H3N2) in mice. ◆, Virus control; ●, fungal SOD (4×500 U per mouse d^{-1} , i.v.); ▲, bovine SOD (4×500 U per mouse d^{-1} , i.v.); ■, ribavirin (4×125 mg per mouse d^{-1} , i.p.). Inoculations were performed on days 4–7 each after viral exposure (day 0). Values were taken from 10 animal experiments for each group.

severe forms of the infection, there is great interest in the development of new strategies for its control.

The preliminary results from toxicity studies showed that the fungal SOD did not cause either acute or chronic toxicity in experimental animals, treated four-fold with doses of 500 U per mouse d^{-1} . It is significant that no cytotoxicity was observed in cell culture studies (results not shown).

The design of the treatment was evaluated after a series of preliminary experiments. We found that the optimal schedule for treatment included four consecutive i.v. inoculations of 500 U per mouse d^{-1} on days 4–7 after viral exposure that caused 80% mortality in the control group. The protective effect of SOD treatment during the critical period of influenza-induced pneumonitis on the survival of mice is presented in Fig. 7. Our results indicate that treatment with *H. lutea* SOD exhibits a strong protective effect on experimental influenza virus infection in mice and that this effect occurs, if the virus challenge is moderate and when the therapy is initiated late during infection. The lateness of therapy initiation particularly relates well to the time when one would anticipate starting therapy in the human patient. The survival rate increases markedly with respect to placebo control: the time of survival rises within 5.2 d and the protective index reaches 86.1%. The effect of the fungal SOD is comparable to that of the selective antiviral drug ribavirin. Bovine SOD at a dose of 500 U per mouse d^{-1} also protects mice from mortality significantly, though less efficiently. The higher efficiency of *H. lutea* SOD could be explained by the presence of a carbohydrate chain attached to the glycosylation site of the fungal enzyme. Human, bovine and recombinant Cu/Zn SOD are metalloproteins lacking oligosaccharides. For all of these a protective effect against influenza infection has been reported which is, however, less than that with the

fungal *H. lutea* SOD glycoprotein. Human SOD, inoculated at a dose of 1000 U per mouse at a late stage of infection caused 60–80% survival (Dolganova & Sharonov, 1997; Sharonov *et al.*, 1991), bovine Cu/Zn SOD at a dose of 1000 U per mouse resulted in 60% survival of the infected mice (Oda *et al.*, 1989) and treatment with recombinant Cu/Zn SOD (1 mg per mouse d⁻¹) exhibited low protective activity only (40% survival) (Nimrod *et al.*, 1994).

Recently, the effect of reactive oxygen species was suggested to play a role in influenza infection pathogenesis (Oda *et al.*, 1989). In murine models, influenza-virus-induced pneumonitis leads to a remarkable increase in intracellular and extracellular oxygen radicals (Akaike *et al.*, 1996). It has been suggested that the main cause of mortality is the cytotoxicity which is determined by the substantially increased levels of $\cdot\text{O}_2^-$ rather than by the viral replication *per se* in bronchial epithelial cells (Akaike *et al.*, 1996). This hypothesis has been confirmed by the fact that inoculation of the reactive oxygen species scavenger Cu/Zn SOD (conjugated with a pyran polymer) reduced the mortality rate dramatically in experimental animals (Oda *et al.*, 1989). Significant improvement of survival in lethal influenza virus infection has been achieved also by the application of native human SOD (Sharonov *et al.*, 1991) and recombinant Mn SOD (Sidwell *et al.*, 1996). The therapeutic effect of SOD seems to be connected with its ability to scavenge $\cdot\text{O}_2^-$ radicals which are the main factor in pathological changes (Oda *et al.*, 1989). It is apparent from the lack of *in vitro* antiviral activity observed after SOD treatment, that the disease-inhibitory effect of the metalloproteins is not the result of a direct antiviral effect (data not shown; Sidwell *et al.*, 1996).

The results from the present investigations on the protective effect of the novel fungal Cu/Zn SOD on experimental influenza infection can be considered as encouraging. They support the findings that the appropriate use of reactive oxygen species scavengers, applied alone or in combination with selective viral inhibitors, is a promising approach for the treatment of influenza virus infection.

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