A C T A U N I V E R S I T A T I S L O D Z I E N S I S
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Anna Jeziorowska, Wanda Leyko

CHARACTERIZATION OF HUMAN PLACENTAL SUPEROXIDE DISMUTASE (SOD-1) ACTIVITY AS MEASURED BY THE POLAROGRAPHIC METHOD*

Human placental superoxide dismutase (SOD-1) isolation procedure and properties are reported. The influence upon the SOD-1 enzymatic activity of the denaturating agents (urea, SDS, increased temperature, different pH values) and inhibitors (azide-N_3, diethyldithiocarbamate-DDC) was determined by means of the polarographic method which proved to be the most suitable for this kind of investigations.

INTRODUCTION

Superoxide dismutases have been isolated from numerous mammalian tissues. Human superoxide dismutase (E.C.1.15.1.1)(SOD-1) was isolated and studied as a copper protein from erythrocytes, brain and liver 22, 26, 27. Bovine Cu-Zn SOD-1 is the most studied protein among this large class of enzymes which have similar properties with respect to each other. SOD-1 from cytosol of eucaryotic organisms contains 2 atoms of copper per molecule of molecular weight 32 000 daltons [12]. Copper is essential for the activity of the enzyme, which can be summarised by the equations [5]:

$$Cu^{2+} + O_2^- \rightarrow O_2 + Cu^+; \qquad Cu^+ + O_2^- \rightarrow Cu^{2+} + H_2O_2$$

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Zinc seems to have rather a structural role [5, 7, 8, 10, 14]. The primary structure of the human erythrocytes SOD-1 has been recently reported by B a r r a et al., [2], B r i g g a, F e e [6] and H i l l et al. [20]. The human placental SOD-1 has been isolated and roughly characterised for the first time by P h a n van H i e n et al. [41]. The properties of the human enzyme are obviously interesting in view of potential applications to medical problems. This paper describes some of the properties of the human placental SOD-1 as well as the effect of some denaturating agents and inhibitors upon its activity measured by the polarographic method.

MATERIAL AND METHODS

Fresh placentae were purified from the connective and other accessory tissues and the blood was throughly washed out first with tap and then with distilled water. SOD-1 was prepared according to the method of F r i e d et al. [13]. The sample of human SOD-1 was subsequently chromatographed on Cellulose DE-32. Further purification was performed by gel filtration on Bio-gel P-60 (Polish patent P-200257). The purity of the enzyme was tested by means of analytical 7.5% polyacrylamide gel electrophoresis (PAGE) in veronal buffer pH 8.6 and 3.5 mA per gel. The SOD-1 activity on the gels was localised by the photochemical method (B e s u c h a m p and F r i d o v i c h [5]). Protein bands were stained with Coomassie Blue. Subunit molecular weight was determined by the method of W e b e r and O s b o r n [36]. Isoelectric focusing was performed according to S v e n s s o n [31]. Absorption spectra were recorded on a Beckman Spectrophotometer. The SOD-1 activity was assayed by the polarographic method of catalytic currents applied to a wave of oxygen by R i g o et al. [28] on a Radelkis OH-102 polarograph.

RESULTS AND DISCUSSION

Purification and properties of the human SOD-1

The examination of the purity of the enzyme preparations by means of PAGE revealed that the final preparations obtained from human placenta were homogenous electrophoretically. The homogeneity of the preparations was also confirmed by the method of isoelectric focusing. The value of the isoelectric point of SOD-1 isolated from human placenta is 4.7 and it is similar to the values reported elsewhere for the human enzyme [7, 16]. The method of F r i e d et al. [13] applied for the isolation of SOD-1 from human placenta with subsequent chromatography and gel filtration proved to be the proper method of obtaining a pure and homogenous preparation of SOD-1. In all cases of electrophoretic procedures a single protein band was seen which exhibited specific enzymatic activity of $3.7 \pm 0.11 \cdot 10^{-5}$ M (n = 6). Subunit molecular weight of SOD-1 from human placenta was determined by SDS-PAGE. The samples of SOD-1 were submitted to electrophoresis after incubation with different reagents in order to establish the dissociation conditions of the protein molecule into subunits.

T a b l e 1

The human placental SOD-1 subunit molecular weight determination by means of the SDS PAGE

Oznaczenie masy cząsteczkowej podjednostek SOD-1 z łożyska ludzkiego metodą elektroforezy w żelu poliakryloamidowym z SDS

Incubation conditions	1 h, 45°C 1% SDS	2 min, 100°C 1% SDS 4 M urea	5 h, 37°C 1% SDS 6 M urea 1% (2%) 2-mercaptoethanol
Molecular weight (daltons) MW ±S.D. (n = 6)	64 000 ±1 900	16 000 ±1 800	16 000 ±1 200

Table 1 summarises the results of the subunit weight estimation - for different SOD-1 samples. According to the literature data the mammalian cytosolic dismutases are composed of two identical subunits, each of the molecular weight 16 000 [3, 9, 17]. The protein bands of about 16 000 daltons obtained after the simultaneous action of SDS, urea, 2-mercaptoethanol and heat to the enzyme are identified as subunits. Similar results were reported for the bovine [1, 38] and hog enzyme [4], as well as for the placental SOD-1 obtained and purified by S u g i u r a et al. [30]. 2-mercaptoethanol seems necessary for the complete dissociation of the SOD-1 molecule into subunits. It acts probably on disulfide bridges localised within each subunit, resulting in conformational changes of the protein molecule and a total break-down into subunits, which are linked by strong non-covalent interactions. High molecular fractions (m.w. about 64 000) obtained in the experiments are probably the result of the formation of aggregated SOD-1 molecules, due to interactions between whole enzyme molecules in the presence of SDS. This kind of aggregates of the human enzyme molecules have been already reported [17].

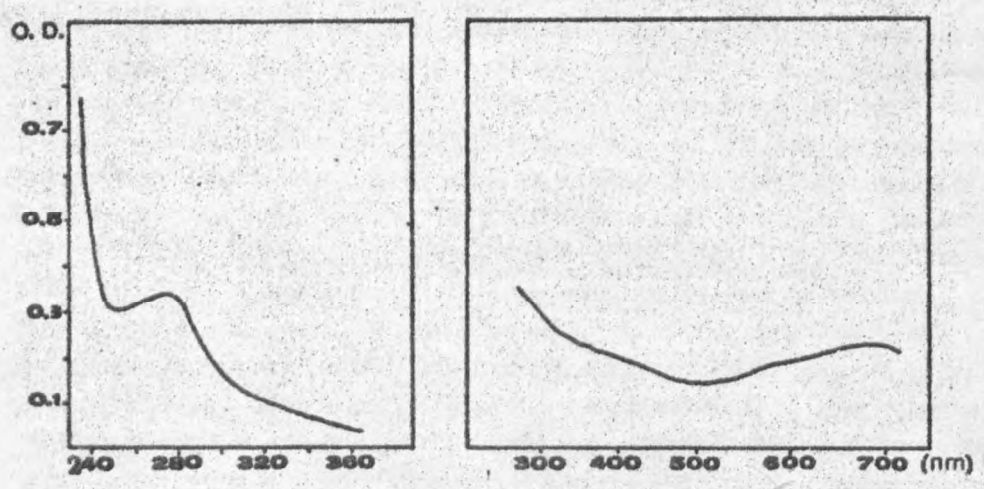

Fig. 1

Ultraviolet and visible absorption spectra of SOD-1 from human placenta. 0.5 mg/ml SOD-1 in 50 mM phosphate buffer (pH 7.4) was examined in 1 cm cell in a Beckman spectrophotometer

Widmo SOD-1 z łożyska ludzkiego w zakresie ultrafioletu i widzialnym. Widmo uzyskano dla próbki 0,5 mg/ml SOD-1 w 50 mM buforze fosforanowym (pH 7,4) w spektrofotometrze Beckman

The ultraviolet and visible range absorption spectra of the human placental SOD-1 are shown in Fig. 1. The absorption profiles are similar to those reported for SOD-1 from other human tissues [7, 16, 30, 41].

Effect of denaturating agents and inhibitors

The results of the influence of high concentrations of urea (8 M and 10 M) and the action of 1% and 4% SDS upon the enzymatic activity of SOD-1 are shown in Table 2.

Table 2

The influence of 8 M and 10 M urea and 1% and 4% SDS upon the human placental SOD-1 activity and dissociation of its molecule into subunits

Wpływ 8 M i 10 M mocznika oraz 1% i 4% SDS na aktywność SOD-1 z łożyska ludzkiego i dysocjację cząsteczki enzymu na podjednostki

Native SOD-1 activity	SOD-1 activity after incubation	
$1.4 \cdot 10^{-6}$ M	with 8 M urea $1.06 \cdot 10^{-6}$ M (75%)	with 10 M urea $0.92 \cdot 10^{-6}$ M (65%)
	with 1% SDS $1.4 \cdot 10^{-6}$ M (100%)	with 4% SDS $0.84 \cdot 10^{-6}$ M (60%)

The loss of activity after the treatment with 8 M and 10 M urea, 25% and 35% respectively was immediately reversed after diluting of the incubated sample with phosphate buffer pH 7.4. The inactivation caused by the action of 4% SDS was also reversible. After precipitation with concentrated KCl and centrifugation after 48 h in room temperature the enzyme gained its 100% activity. In both cases the SDS PAGE of the incubated samples revealed two protein bands of molecular weighlar weights 64 000 and 16 000 with the proportion of 75% and 25% respectively.

Tests of SOD-1 stability were carried out according to the procedure of T e g e l s t r ö m [34]. The results of the in-

fluence of the increased temperature and different pH values upon human placental SOD-1 activity are given in Fig. 2. The activity of the enzyme after incubation was measured polarographically and calculated as a percentage of the initial activity of the intact enzyme. Superoxide dismutases are extremely stable enzymes

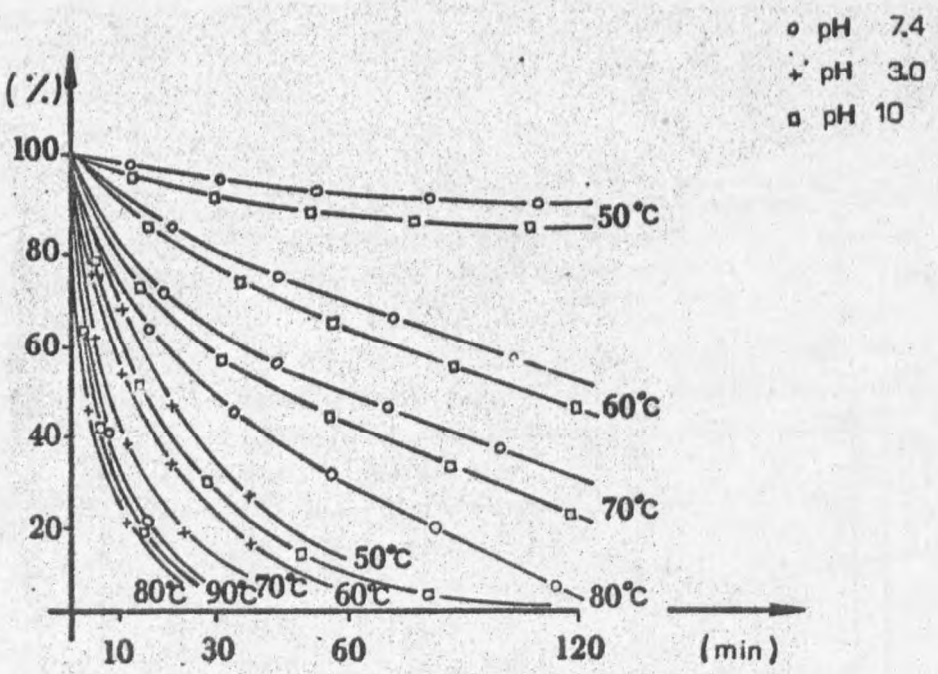

Fig. 2

Temperature stability of human placental SOD-1 at different pH values of the incubation media. Aliquots were withdrawn at intervals and assayed for the remaining activity

Test stabilności temperaturowej SOD-1 z łożyska ludzkiego w różnych wartościach pH. Próbki pobierano w różnych przedziałach czasowych i oznaczano pozostałą aktywność enzymatyczną

[34, 37]. At pH 7.4 SOD was most stable - 50% inactivation was observed after 50 min incubation at 70°C. Total loss of activity was found at the same pH value after 30 min incubation at 90°C. However, at pH lo the enzyme retains 50% of the native activity

after 60 min incubation at 70°C, but at the low pH values it is readily inactivated. The activity was found unaffected by buffering the protein for 12 h within the range of pH 4.8-10.. The enzymatic activity decreases significantly with the increased values of pH above 10. Also after incubation of the protein at pH 3 the activity was reduced by 40%.

The results above indicate that the human placental SOD-1 is essentially independent of pH over a wide range. This property has been established before for the bovine [33], hog [4] and human placental SOD-1 [30]. The observations performed by V a l e n t i n e et al. [40] of the pH dependence of the visible spectra and EPR spectra indicated that the pH dependent changes between 3 and 4.5 could be accounted for by conformational changes at the copper binding site. It is clear from the results above and previous studies that the metal binding properties of copper-zinc SOD-1 are strongly pH dependent.

In the studies of the inhibitors action upon the human placental activity two different inhibitors were chosen in the present work. These are: azide (N_3^-) and diethyldithiocarbamate (DDC). The polarographic method of the SOD activity assay - appeared especially suitable in this kind of determinations, because it was not affected by N_3^- nor by DDC, in contrary to the spectrophotometric assay systems [15, 35]. It has been recently reported that azide [29] combines with the prosthetic metal of the iron containing SOD of E. coli as well as with the Cu-Zn SOD-1 and Mn-SOD from human liver [23]. The iron SOD is most sensitive toward inhibition by azide. The Cu-Zn SOD-1 is least sensitive while the Mn-SOD exhibits moderate susceptibility. Thus inhibition by azide, which is impaired and reversed rapidly, appears to provide the useful criterion for distinguishing among classes of these enzymes.

In this work 50% inhibition of the SOD-1 activity was obtained at the concentration equal 30 mM of N_3^-. The amount of 10 mM of azide inhibited the enzyme by 10% (Fig. 3). Reversibility of the SOD-1 inhibition was gained by dialysis against phosphate buffer pH 7.4. The copper chelating agent diethyldithiocarbamate (DDC) has been shown to cause marked inactivation of SOD-1 both in vitro and in vivo [18, 19, 24]. In the present work to-

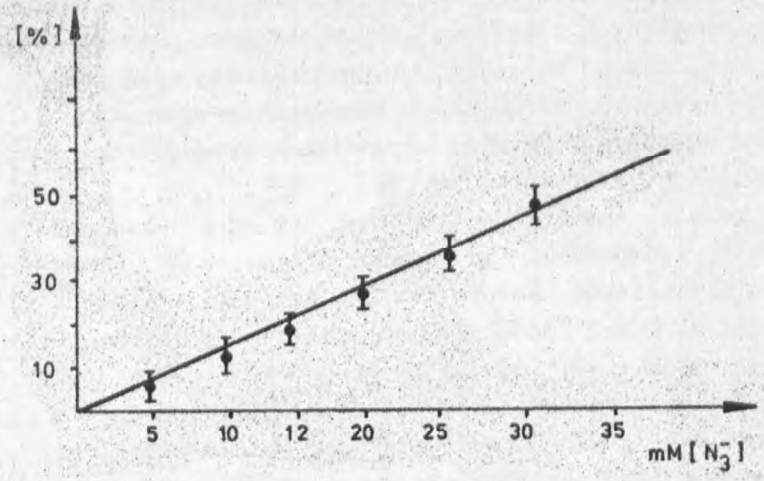

Fig. 3

Inhibition of the human placental SOD-1 activity by azide (N_3^-) at the indicated concentrations

Hamowanie aktywności SOD-1 z łożyska ludzkiego przez azydek (N_3^-) o oznaczonych stężeniach

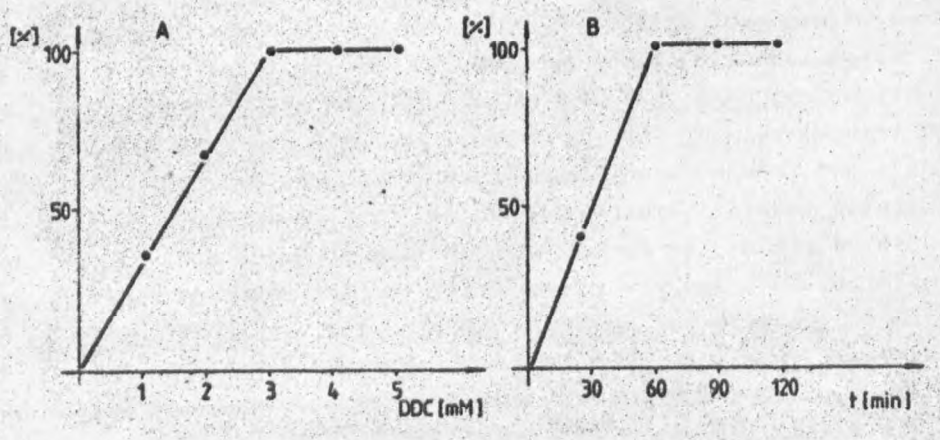

Fig. 4

Inhibition of human placental SOD-1 activity by diethyldithiocarbamate (DDC): A - as a function of the DDC concentration; B - as a function of the incubation time with 3 mM of DDC

Hamowanie aktywności SOD-1 z łożyska ludzkiego przez dwuetylodwutiokarbaminian (DDC): A - jako funkcja stężenia DDC; B - jako funkcja czasu inkubacji z 3 mM DDC

tal inhibition of the human placental superoxide dismutase was observed at the concentration of 3 mM of DDC and after 60 min of incubation (Fig. 4). The loss of enzymatic activity was accompanied by the appearance of a coloured (yellow to orange) enzyme--inhibitor complex. As it has been observed earlier [19] the loss in enzymatic activity could not be reversed by dialysis against distilled water (500 vol) in 4°C for 24 h, but after dialysis, the native activity was restored by incubation with $5 \cdot 10^{-4}$ M $CuSO_4$ during 1 h at 37°C.

DDC by inhibiting the Cu containing form of SOD may be a useful pharmacological tool in establishing the vital protective role of this enzyme. It may be used in order to evaluate the importance of SOD as a protective enzyme in eucaryotes during exposure to high content of O_2 [11]. DDC may also be a potentially powerful sensitizing agent in tumor therapy, since there is increasing evidence that tumor cells have lower levels of SOD [21, 25, 32, 39].

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Laboratory of Genetics
Institute of Endocrinology
Medical Academy of Łódź

Department of Biophysics
Institute of Biochemistry
and Biophysics
University of Łódź

Anna Jeziorowska, Wanda Leyko

CHARAKTERYSTYKA AKTYWNOŚCI DYSMUTAZY PONADTLENKOWEJ (SOD-1) Z ŁOŻYSKA LUDZKIEGO METODĄ POLAROGRAFICZNĄ

Przedstawiono sposób izolowania i oczyszczania dysmutazy ponadtlenkowej EC 1.15.1.1 (SOD-1) z łożyska ludzkiego. Badano wpływ czynników denaturują-

cych białko (mocznika, SDS, podwyższonej temperatury, zmian pH) oraz inhibitorów (azydku, dwuetylodwutiokarbaminianu-DDC) stosując do oznaczania zmian aktywności enzymu metodą polarograficzną, która okazała się być najbardziej odpowiednią do tego typu badań.