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# ISOLATION AND CHARACTERIZATION OF COPPER – ZINC SUPEROXIDE DISMUTASE FROM *SACCHAROMYCES CERVISIAE* EXPOSED TO COPPER

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**ABSTRACT**: ***We have isolated the enzyme Cu-Zn Superoxide dismutase (SOD) from Saccharomyces cervisiae grown in different concentrations of copper Sulphate (3, 6, 9, 12, 15 mM).We have demonstrated that Cu-Zn Superoxide dismutase activity increase with the increase of copper concentrations, this reveals that SOD act as defense in Saccharomyces cervisiae against copper toxicity. The subunit molecular mass of the enzyme was determined by denaturing gel electrophoresis, it consists of two subunits (90 KDa, 102 KDa). The isolated enzyme was pre- incubated at different pHs (3 – 10).It showed remarkable stability over pH range (5 – 9).The thermostability of the enzyme was also studied by pre-incubation of the enzyme at different temperatures and for different times at boiling point, the enzyme is thermostable where it retains 29% of its activity at temperature of 80◦C.It can also sustain boiling for 10 min with retain of 49% of its activity.***

**Key words**: ***superoxide dismutase; copper toxicity; Saccharomyces cervisiae***

## INTRODUCTION

**Although oxygen is an essential element in respiring living cells, it also causes potential problems by the generation of highly reactive oxygen species ($O_2^{\bar{\cdot}}$, $OH^{\bar{\cdot}}$, $OCL^{\bar{\cdot}}$, $H_2O_2$, etc) in the respiratory process. Living cells equipped with a family of enzymes that catalytically scavenge the super oxide free radical through the disproportionation.**

**$O_2^{\bar{\cdot}} + O_2^{\bar{\cdot}} + 2H^{+} \rightarrow H_2O_2 + O_2$ (McCord and Fridovich, (1969); therefore help to protect cells against the toxic $O_2^{\bar{\cdot}}$ species.**

**Such enzymes are denoted superoxide dismutases (SOD) (EC 1.15.1.1)**

**The yeast *Saccharomyces cervisiae* contains two species of super oxide dismutase, i.e. the copper, zinc (Cu, Zn-SOD) and the manganese – containing forms .The former one is localized in the cytosol while the second enzyme is restricted to the mitochondrial matrix. The interest of using SODs for clinical purposes and specific application in the food industry stems from the overwhelming evidence of the importance of oxygen free radicals in a variety of pathological symptoms and food spoilage (Donnelly *et al*.,1989).**

**Cu, Zn-SOD and manganese SOD behave differently in response to other stress factors such as exposure to copper (Greco, *et al*., 1990). Cu, Zn –SOD has been reported to increase its activity upon changes of the concentration in the medium, whereas manganese SOD does not (Cirolo, *et al*., 1994). The effects on Cu, Zn-SOD cannot be explained on the basis of oxygen dependent redox cycling of metal ion because a copper dependent increase of Cu, Zn-SOD is observed also under anaerobic conditions (Galiazzo, *et al.,* 1991).**

**The presence of copper at catalytic site of Cu, Zn-SOD suggests that copper availability limits the enzymatic activity thus exerting a regulation at the post – transcriptional level.**

**Copper can modulate yeast Cu, Zn-SOD at transcriptional level via ACE1 (Carri, *et al*., 1991). This is a transcription factor which, upon binding of Cu (I), activates the promoter of CUP1, the gene encoding for copper- thionein (Furst, *et al*., 1989)**

**An ACE1 – binding site has been localized in the Cu, Zn-SOD promoter (Carri, *et al*., 1991)**

**These indicate a possible role of Cu, Zn-SOD in sharing a copper sequestration function with metalothionein.**

**This report describe the isolation and characterization of Cu, Zn-SOD from *Saccharomyces cervisiae* and the effect of increase copper ion concentration upon its activity.**

## MATERIALS AND METHODS:

### Chemical

**Standard Cu, Zn -SOD from bovine serum albumin (4,520 units), standard bovine serum albumin and Comassie G250 were obtained from sigma.**

**Nitroblue tetrazolium (NBT) from ACROS, Riboflavin from Merck**

**Copper sulphate pentahydrate from ADWIC.**

**Peptone, yeast extract and Agar from Difco.**

**Organism and media*: Saccharomyces cervisiae S1 UQM49was* obtained from Cairo MIRCEN Ain Shams University.**

**YPD media consists of 1% yeast extract, 2% peptone and 2% dextrose. pH was adjusted at 5.**

### Growth conditions:

**Cultivation of *Saccharomyces cervisiae* was carried out in flasks containing media with a media volume/ flask volume ratio of 1/4. The flasks were sterilized by autoclaving at 1.2 atm for 20 min. After cooling the media were inoculated with the yeast strain .The inoculated culture flasks were agitated on rotatory shaker at 28 -30◦C and 160 rpm for 96 hrs.**

**Copper (II) was supplied as copper sulphate at different concentrations (3mM, 6mM, 9mM, 12mM and 15mM), after filtrations using Millipore bacterial**

**filter (0.45nm).A control medium was also inoculated without addition of copper sulphate .**

**After completion of the growth time the cells were harvested by centrifugation using cooling centrifuge at 12,000 rpm for 20 min. The collected cells were washed twice with phosphate buffer pH 7.8, then disrupted by grinding in liquid nitrogen and finally suspended in phosphate buffer pH 7.8, The disrupted cells were centrifuged at 12,000 rpm and the supernatant containing super oxide enzyme were collected for further analysis.**

## Protein assay: (Bradford, 1976)

**The protein content was determined according to Bradford method using bovine serum albumin as standard.**

## Determination of SOD activity:

**The method used was (Winterbourn *et al*., 1975) based on the ability of superoxide dismutase enzyme to inhibit the reduction of nitro blue tetrazolium in the presence of sensitizing agent (riboflavin).**

### For this reaction mixture containing:

**1- 0.2ml 0.1M EDTA,**
**2- 50µl 0.12mM Riboflavin,**
**3- 100 µl1.5mM Nitrobluetetrazolium (NBT),**
**4- plus 100 µl of the enzyme extract or standard enzyme**
**5- and this reaction mixture was completed with 0.067 M potassium phosphate buffer, pH 7.8 to 3ml, then was exposed to fluorescent light for 12 minutes**
**6-The optical density were measured every 3min at wave length 560 nm.**
**The enzyme volume necessary to inhibit 50% of NBT reduction was determined and defined as unit of the enzyme.**

## Electrophoretic analysis: (Davis 1964)

## i) Denatured protein electrophoresis:

**The sub-unit molecular mass of the enzyme was determined by electrophoresis on 12% polyacrylamide gel in the presence of sodium dodecyl sulphate (SDS-PAGE).Before electrophoresis, samples from yeast and serum bovine albumin were boiled for 10 min in reducing loading buffer (62.5mM Tris, pH 6.8, 5%glycerol, 2%SDS, 5%2- mercaptoethanol, 12.5 µg/ml bromophenol blue).High range molecular weight protein marker from Bio-Rad was used. Electrophoresis was carried out at about 100 volts in 1x Tris/glycine –SDS-running buffer After completion of the protein migration, the gel was stained in 50 ml of staining solution (0.125% Coomassie blue R-250, 50%methanol and 10% acetic acid).**

## ii) Non denatured protein electrophoresis: (Culotta, *et al*.,1997)

**For the analysis of SOD activity by NBT, cells were grown by shaking flask cultivation in YPD medium in the presence and absence of $CuSO_4$ (10 mM) up to an absorbance of 1.0, harvested, and washed once in cold water. The resulting cell pellet was resuspended in 0.5 ml of lysis buffer containing 10 mM $Na_2HPO_4$ (pH 7.8), 1.0 mM EDTA, 0.1% Triton, 20 µg/ml leupeptin, 10 µg/ml pepstatin and 1.0 phenylmethylsulfonyl flouride .Cells were broken with glass beads and homogenized, and glycerol was added at a final concentration of 5%. For non denaturating gel electrophoresis 50µg of the resulting cell extract was applied directly without boiling to a non-denaturing 12% polyacrylamide gel without SDS. Following electrophoresis, the gel was stained in a solution containing 50 mM $K_2HPO_4$ (pH 7.8), 275 µg/ml NBT, 65 µg/ml riboflavin, and 3.2 µg/ml TEMED.**

**After 45-min incubation in the dark, the blue NBT stain for $O_2^-$ was developed by exposure to light. Staining was absent at sites of $O_2^-$ scavenging.**

## Effects of pH and temperature on SOD activity:

**Stability of the SOD enzyme as function of pH was determined by quantifying the residual activity after 24 hrs pre-incubation at constant temperature 25 ˚C at various pH values.**

**For this a dilution of the enzyme equivalent to protein concentration of 0.4 mg/ml of *Saccharomyces cerevisiae* cell homogenate & a dilution of standard SOD enzyme (bovine serum) equivalent to 25 ng/ml were both prepared with the corresponding buffer system; Citrate phosphate buffer pH range (3 – 8)and Sod. Carbonate - Sod. Bicarbonate buffer pH range (9 – 10 ). After this, the SOD activity was determined as described by Winterbourn *et al*., (1975).**

**Thermo stability was determined both by heating the samples & standard (bovine serum) at 95 ˚C for different times (0, 2, 4, 6, 8, 10 min) and by preincubation of the enzyme at various temperatures (25 – 35 – 45 – 55 – 65 – 75 – 85)˚C for 20 min before the measurement of the enzyme activity.**

## Inhibition tests:

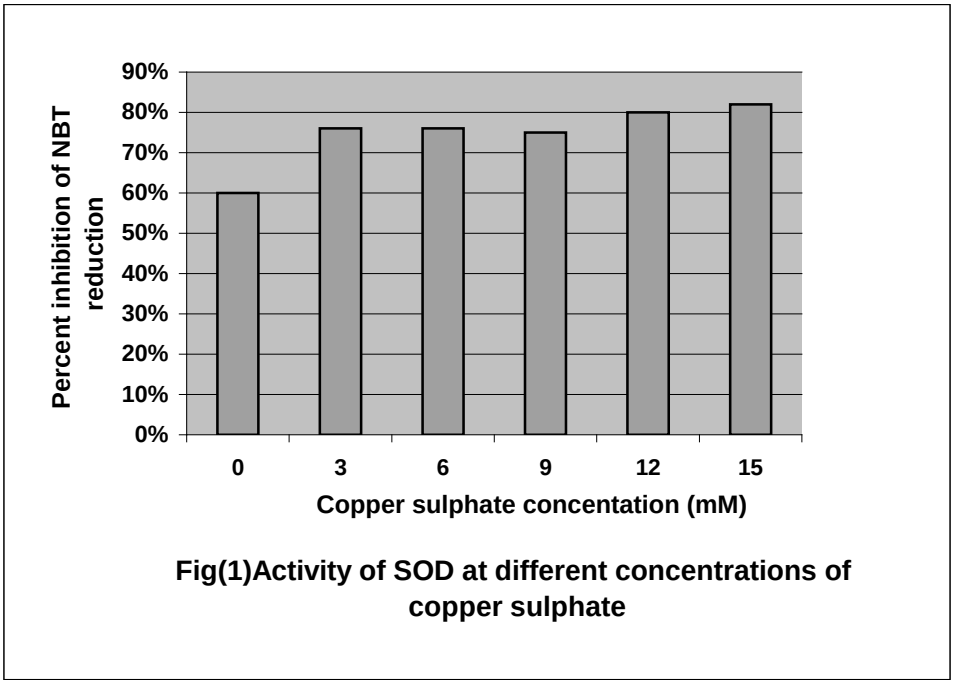
**Enzyme samples and standard SOD were pre-incubated in phosphate buffer (50 mM, pH7.8) containing 5 mM sodium cyanide, 1 mM sodium doedecyl sulphate (5 min at 20 ˚C before addition of the substrate. The residual activity is considered as the percentage of remaining activity compared to control sample.**

## RESULTS AND DISCUSSION:

## The effect of copper ions concentration on SOD activity:

**From fig (1) it was found that a positive co-relation between the increase in copper ion concentration and activity of SOD enzyme represented in terms of percentage inhibition of nitro blue tetrazolium (NBT) which increase from 60% without $CuSO_4$ to 75.5% with addition of 3mmol $CuSO_4$, 76 % with 6 mmol $CuSO_4$, 77% with 9 mmol $CuSO_4$, 80% with 12 mmol $CuSO_4$ and 82% with 15 mmol $CuSO_4$.**

**The above results agree with the results obtained by (Culotta *et. al,* 1995), since they demonstrated that copper toxicity in yeast lacking the CUP1metallothionine is suppressed by over expression of CRS4 which is equivalent to SOD1, encoding copper-zinc superoxide dismutase (SOD). While over expression of SOD1 enhanced copper resistance, a deletion of SOD1, but not SOD2 (encoding manganese SOD), conferred increased sensitivity toward copper.**




**Fig(1)Activity of SOD at different concentrations of copper sulphate**

## Electrophoretic analys
## i) Denaturated protein electrophoresis:

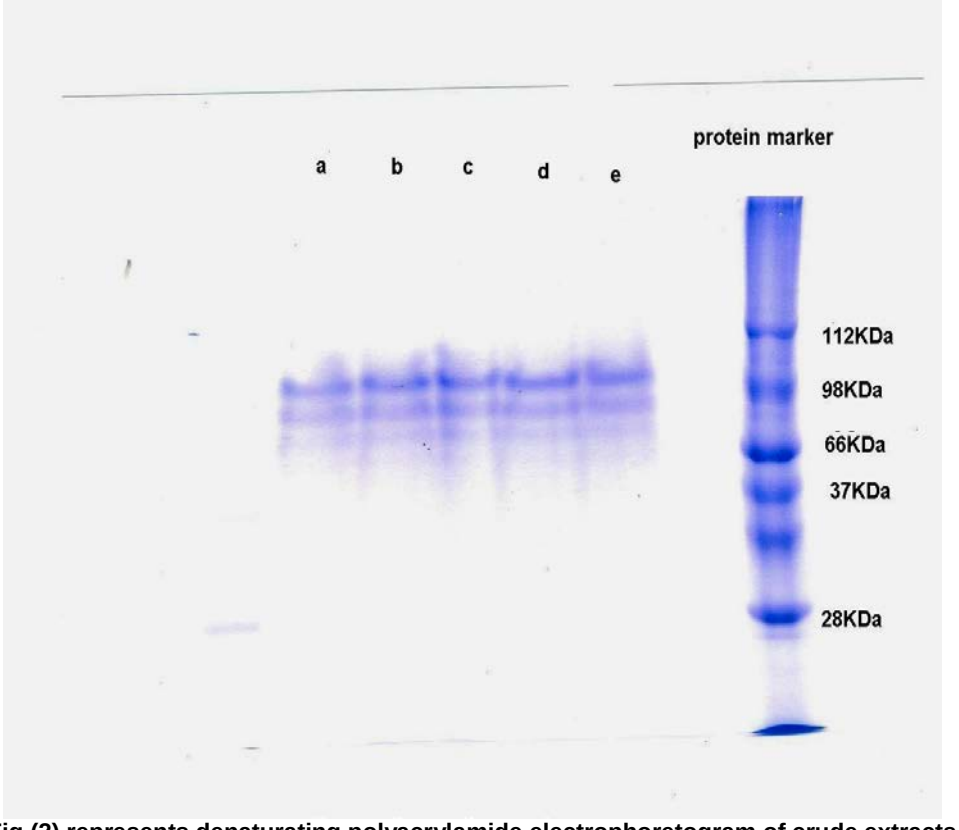



**Fig.(2) represents denaturating polyacrylamide electrophoretogram of crude extracts of *Saccharomyces cervisiae* (S1) and standard preparation of Cu-Zn SOD from bovine erythrocytes stained with coomassie blue :lanes (a , e) represent crude extract of *Saccharomyces cervisiae* (S1) treated with copper sulphate(10mM) ,lane(b) crude extract of untreated *Saccharomyces cervisiae* (S1) and lanes (c,d) represent standard Cu-ZnSOD from bovine erythrocytes.**

**It is clear from the above electrophoretogram that enzyme obtained from both treated and untreated yeast cells gave the same electrophoretic patterns which is similar to patterns resulted by Cu-ZnSOD from bovine erythrocytes.**

**The shown electrophoretogram (fig 2) indicates that Cu-Zn SOD both from *Saccharomyces cervisiae* (S1) and bovine erythrocytes consists of two subunits of 90 and 102 KDa, while the data obtained by(Hernandez *et. al* 1999) reveals that Cu-Zn SOD from *Saccharomyces cervisiae* and**

***Debaryomyces hansenii*** **consists of two identical monomer and the calculated molecular mass was 18 kDa± 0.14.**

**ii) SOD gel electrophoresis:**

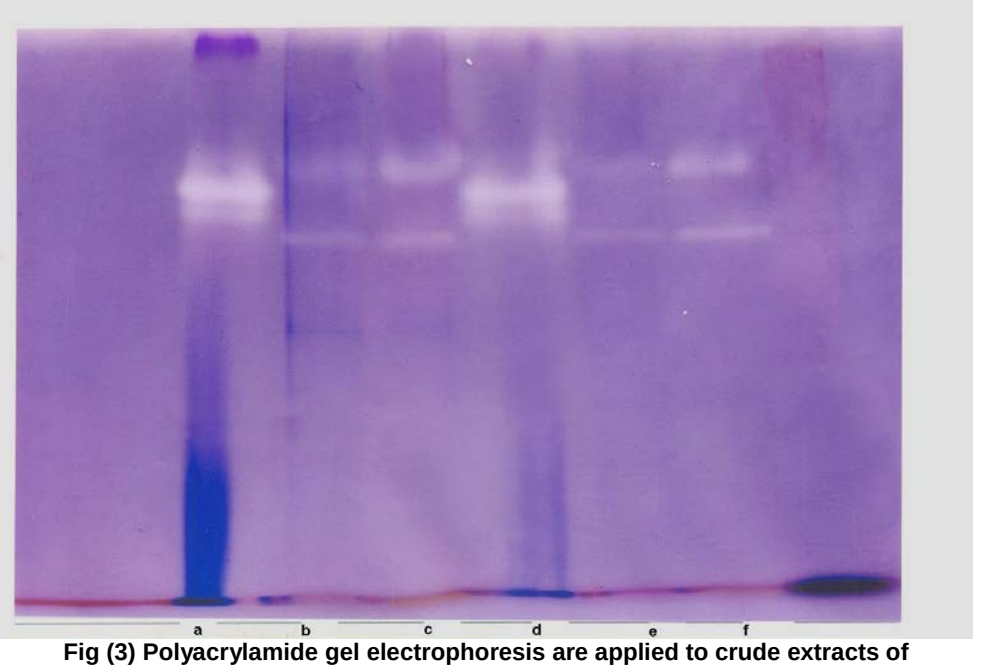



**Fig (3) Polyacrylamide gel electrophoresis are applied to crude extracts of *Saccharomyces cervisiae*(S1) and standard preparation of Cu-Zn SOD from bovine erythrocytes for SOD activity. Lanes (a , d) represents standard Cu-Zn SOD from bovine erythrocytes and (c , f) represent crude extract of *Saccharomyces cervisiae*(S1) treated with 10 mM copper sulphate ,and(b , e) crude extract of untreated *Saccharomyces cervisiae*(S1).**

**In SOD gel activity the staining was absent at sites of $O_2^-$ scavenging. i.e. at sites of SOD activity. Fig (3) indicate that *Saccharomyces cervisiae (*S1) contains two species of SOD showing two separate bands of enzyme activity one type of them is Cu-Zn SOD as it shows activity band corresponding to standard Cu-Zn SOD band. It is clear that Cu-Zn SOD show enhanced activity in lanes(c , f) i.e. enzyme from *Saccharomyces cervisiae* treated with copper sulphate and this results agree with the results obtained by (Carri *et al*.1991.Gralla *et al*., 1991) as they have been observed that an increase in concentration of divalent metals such as $Cu^{++}$ may affect the expression of Cu-ZnSOD enzymes in yeast, through its co-regulation with a metallothionine system via ACE1 factor**

## Effect of pH and temperature on SOD activity

**Fig (4), illustrate that SOD from bovine erythrocytes retains more than 85% of activity over abroad pH range (5 – 9), SOD from *Saccharomyces cerevisia*e (S1) maintained 80%of the enzyme activity at pH range (4 - 7) but at higher pHs the enzyme activity was rapidly lost.**

**Fig. (5) illustrates that bovine erythrocytes SOD lost activity rapidly after 2 minutes boiling, whereas the SOD from *S.cervisiae* (S1) retained 70% of the original activity .**

**Fig (6) illustrates that sensitivity of SOD bovine erythrocytes is higher compared with *Saccharomyces cerevisia*e (S1), while SOD from bovine erythrocytes completely lose its activity when incubated at 80˚C for 20 min, SOD from *Saccharomyces cerevisia*e (S1) retained 29% at 80˚C.**

**These results are similar to those obtained by (Hernandez *et. al* 1999)**

**Where they found that both two types of enzymes completely retained their activity when treated or stored for short periods at pH 5-8 and they mentioned that both of two kinds of the enzyme lose their activity rapidly at pH below 5 and above 9 .**

**The thermal stability of both kinds of enzyme were also studied by Hernandez et.*al* (1999), They showed that bovine erythrocyte SOD lost activity rapidly after 1 min boiling, whereas the Cu-Zn SOD from *Saccharomyces cervisiae* retained 80% of their original activity .Even after 10 min boiling, Cu-Zn SODs from yeast retain more than 30% of activity.**

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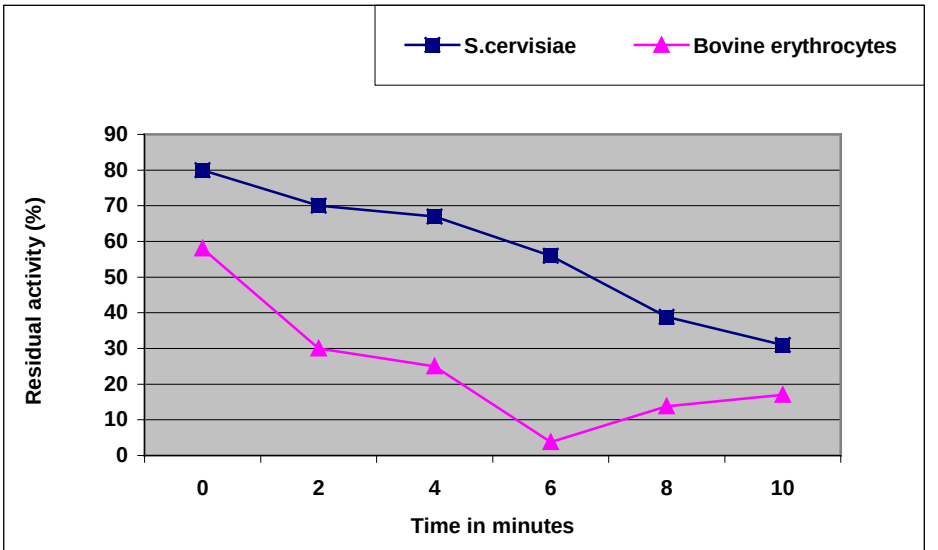


**Fig.(5): Effect of boiling time on superoxide dismutase activity from both *S.cervisiae* and bovine erythrocytes**

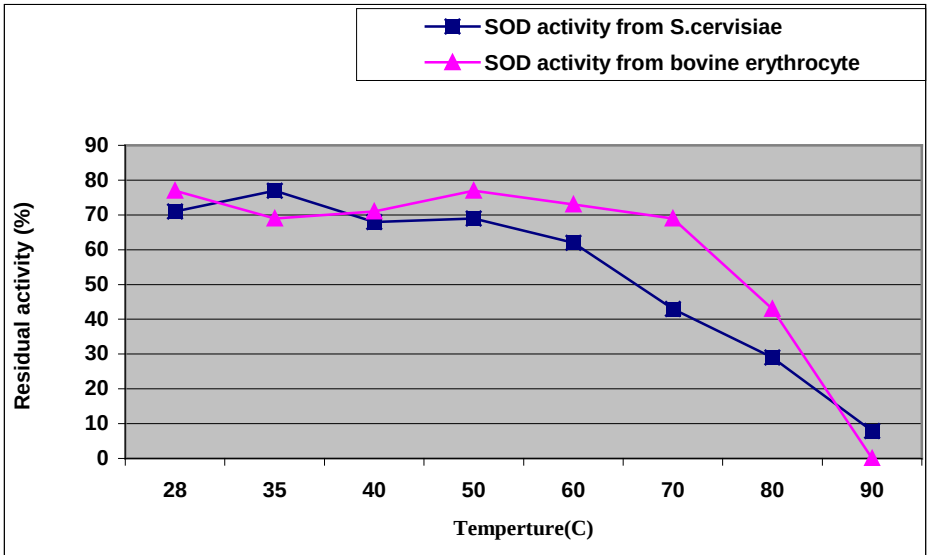


**Fig (6)Effect of temperture on superoxidedismutase activity both fom *S.cervisiae* (S1) and bovine erythrocytes**

## Inhibition tests:

**Table(1) illustrates that Cu Zn SOD activity from both *Saccharomyces cerevisiae* and bovine erythrocyte are strongly inhibited by 5mM NaCN and marginally affected by1 mM SDS.**

**These results are compatible with those obtained by (Hernandez *et. al* 1999), where they suggested that Cu-Zn SOD activity is strongly inhibited by NaCN and only marginally affected by SDS.**

**Table (1): Effect of inhibitors on SOD activity**

| | Residual SOD activity (%) | |
|---|---|---|
| **Inhibitor** | ***S.cervisiae* (S1)** | **Bovine erythrocytes** |
| **5mM NaCN** | **26%** | **8.4%** |
| **1 mM SDS** | **46%** | **47%** |

## CONCLUSION:

***Saccharomyces cervisiae* (S1) show a great tolerance to high concentrations of copper as when copper sulphate were added at concentration 10 mM to the growth media, a moderate mass of yeast cells were observed .**

***Saccharomyces cervisiae* responds to toxic level of copper by increasing the level of SOD.**

**The first defensive role of SOD is to scavenge $O2^{-}$ , and the second role of defense is to act as metal chelator to avoid poisioning by heavy metals (Cirolo *et al*, 1994, Culetta *et al*., 1995)**

**The hypothesis of metal chelator is supported because it has been observed that an increase in concentration of divalent metals such as $Cu^{++}$ may affect the expression of Cu-Zn SOD enzymes in yeast, through its corregulation with a metallothionine system via ACE1 factor (Carri *et al*.1991.Gralla *et al*., 1991).**

**The inhibition tests used to differniate between three types of SOD. In this work inhibition tests suggest strongly that this type of SOD is Cu–Zn SOD.**

**SOD is thermostable enzyme, retains its activity over a wide range of pH.**

## ABBREVIATIONS:

**The abbreviations used are: SOD, superoxide dismutase; NBT, nitroblue tetrazolium; ACE1, activator ofCUP1metallothioneins**

**expression; CRS5, copper – resistant suppressor .**

## REFERENCES

**Carri, M.T., F. Galiazzo, M. R. Cirolo and G. Rotilio (1991) Evidence for co-regulation of Cu, Zn superoxide dismutase and metalothionein gene expression in yeast through transcriptional control by copper via ACE1 factor.*FEBS Lett*. 2278: 263 – 266.**

**Cirolo, M. R., P.Civitereale, M.T. Carri, A. De Martino, F. Galliazo and G. Rotilio (1994). Purification and characterization of Ag, Zn superoxide dismutase from *Saccharomyces cervisiae* exposed to silver. *J. Biol. Chem*. 269: 25 783 – 25 787.**

**Culotta, V. C., H. D. Joh, S. J. Lin, K. H. Slekar and J. strain (1995). A physiological role of Saccharomyces cerevisiae copper/zinc superoxide dismutase in copper buffering. *J. Biol. Chem*. 270 : 29 991 – 29 997.**

**Culotta, V. C., L.W Klomp, J. Strain, R. L Casareno, B. Krems and J. D. Gitlin (1997). The Copper Chaperone for Superoxide Dismutase. *J. Biol. Chem.* 272: 23 469-23 472]**

**Donnelly, J. K. M., J. L. Walker and D. S. Robinson (1989). Copper-Zinc Superoxide Dismutase from the marine yeast Debaryomyces hansenii *Yeast* 15: 657 - 668.**

**Furst, P. and D. Hammers (1989). Copper activates metallothionein gene transcription by altering the conformation of a specific DNA binding protein*. Proc. Natl. Acad*. *Sci.U.S.A*. 86: 5267 – 5271.**

**Greco, M. A., D. I. Hrab, W. Magner and D.J Kosman (1990). Cu, Zn superoxide dismutase and copper deprivation and toxicity in *Saccharomyces cerevisiae*. *J*. Bacteriol. 172: 317- 325**

**Davis, B. J. (1964). Disc electrophoresis. II. Method and application to human serum proteins. *Ann. NY Acad. Sci*.121: 404 427.**

**Gralla, E. B., D. J. Thiele, P. Silar and J.S. Valentine (1991). ACE1 a copper – dependent transcription factor, activates the expression of the yeast copper-Zinc superoxide dismutase gene. *Proc. Natl Academy Sci. U.S.A* 88: 8558-8562**

**Galiazzo, F., M.T. Ciriolo, P. Civitareale, L. Marcocci, F. Marmocchi and G. Rotilio (1991). Activation and induction by copper of Cu/Zn superoxide dismutase in *Saccharomyces cerevisiae*. Presence of an inactive proenzyme in anaerobic yeast. *Eur. J.Biochem*. 196: 545 – 549.**

**McCord, J. M. and I. Fridovich (1969). Superoxide dismutase. An enzymatic function for erythrocuprein(hemocuprein). *J.Biol.Chem*. 244: 6049-6055.**

**Marion M. Bradford, (1976). A Rapid and sensitive method for the Quantitation of Microgram Quantities of protein utilizing the principle of protein-Dye Binding .*Analytical Biochemistry* 72: 248 -254.**

**Hernandez-Saavedra, N. Y. and J. L. Ochoa (1999). Copper – Zinc Superoxide Dismutase from the marine yeast *Debaryomyyces hansenii. Yeast* 15: 657 - 668**

**Winterbourn, C., R. Hawkins, M. Brian, and R. Carrell (1975). The Estimation of Red Cell Superoxide Dismutase Activity, *J Lab Clin Med 85*: 337**

# عزل وتمييز الانزيم المضاد لشارد السوبراكسيد من خميرة الخباز (السكارواميسز سرفيسي) المعرضة للنحاس

**السيد حسن حسناين – خليل الحلفاوي – اشرف فرج الباز –**
**ابراهيم حلمي السيد – مروة خيرت علي**
**معهد الهندسة الوراثية والتكنولوجيا الحيوية بالسادات . جامعة المنوفية**

**الملخص العربي**

**لقد تم عزل الانزيم المضاد لشارد السوبراكسيد من خميرة الخباز (السكارواميسز سرفيسي) التي تم تنميتها في وجود تركيزات مختلفة من من النحاس (٣، ٦ ،٩ ،١٢، ١٥مللي مول كبريتات النحاس) وتم اثبات ان نشاط الانزم يزيد بزيادة تركيز النحاس مما يعكس دور الانزيم في ميكانيكية الدفاع ضد التسمم بالنحاس. تم تعيين الكتلة الجزيئية للانزيم المعزول بالفصل الكهربي ووجدنا انه يتكون من تحت وحدتين كتلة كل منهما ٩٠، ١٠٢ كيلو دالتون. بتحضير الانزيم المعزول في تركيزات مختلفة من الاس الهيدروجيني اظهر ثبات ملحوظ خلال المدي من ٥ إلي٩. كما تم دراسة الثبات الحراري للانزيم عن طريق تحضينه في درجات حرارة مختلفة وايضا لمدد مختلفة عند درجة الغليان، ووجد انه ثابت حراريا حيث انه يحتفظ بحوالي ٢٩ % من نشاطه عند درجة ٨٠ درجة مئوية ، كما انه يتحمل الغليان لمدة ١٠ دقائق مع احتفاظه ب ٤٩% من نشاطه .**