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INDUCTION, PURIFICATION AND MOLECULAR CHARACTERIZATION OF SULFHYDRYL OXIDASE FROM AN EGYPTIAN ISOLATES OF *Aspergillus niger*

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The conditions for the sulfhydryl oxidase (SOX) production and activity from an Egyptian isolate of *Aspergillus niger* were optimized. Purification and determination of the kinetic properties (K_m and V_{max}) of the purified enzyme have been done. The possibility for the SOX induction using L-Cys (as a natural substrate) was studied to determine whether SOX could be produced as an inducible enzyme in addition to being a constitutive one (i.e. whether induction leads to increase SOX production and activity or not). The optimum temperature and pH for its activity were found to be 60°C and 5.5, respectively. The activity of the induced intracellular SOX, was measured according to Ellman's method using the standard GSH oxidation where it reached 94% while that of non-induced one reached only 27.6%. This wide difference in activity between the induced and non-induced SOX indicates the successful L-Cys-induction of the SOX production (i.e. SOX from *A. niger* AUMC 4947 is an inducible enzyme). Molecular characterization of the pure SOX revealed that it is constituted of two 50–55 KDa subunits. K_m and V_{max} were found to be 6.0 mM and 100 μM/min/mg respectively.

Sulfhydryl oxidase (SOX, EC 1.8.3.2) has an important and essential vital role in the generation of disulphide (S–S) bonds in proteins by the oxidation of the free thiol (–SH) groups of Cys residues in their polypeptide chains [1–3]:

$$2R{-}SH + O_2 \longrightarrow R{-}S{-}S{-}R + H_2O_2$$

These disulphide bonds (or bridges) maintaining the tertiary and quaternary structures of proteins which are essential for any protein to perform its specific function (i.e. structure-function relationship). SOX is known to be present in several mammalian systems including bovine milk and kidney and human skin [1], white avian eggs [4], rat immune, reproductive, respiratory, and digestive systems along with the retina and skin and various secretory endocrine glands (e.g. hypothalamus, pituitary, pineal, and adrenal) and the pancreatic islets of Langerhans [5]. *Aspergillus sojae, Aspergillus niger, Aspergillus oryzae, Bacillus subtilis, and Penicillium lilacinum* were found to produce SOX activity at levels high enough for its potential recovery but its biochemical role in microorganisms is not known [1]. Microbial sources of SOX enzymes have the advantage, over mammalian sources, of being available in large quantities and economic prices. SOX from microbial sources appear to be a constitutive enzyme, so, it does require induction. SOX is of interest in applications where oxidation of free sulfhydryls to disulphide linkages is sought, particularly instead of the non-specific oxidants such as hydrogen peroxide, peracids, borates, bromates, etc. having unwanted side reactions. While enzyme-catalyzed reactions provide the selectivity desired, thereby avoiding side reactions [1]. One of the industrial applications of SOX is the removal of a burnt flavor from ultra-high temperature (UHT)-sterilized milk. This burnt flavor is evolved as a result of the increased level of SH-groups produced due to the breakage of cysteine disulphide bonds of this product. Treatment of the UHT-sterilized milk with SOX will oxidize these free thiol groups and lead to the restoration of the normal flavor of milk [1]. Biosensing approach for glutathione detection using the glutathione reductase and SOX bienzymatic system was made by [6].

The aim of this investigation was to study the effect of some cultural conditions on the production and activity of SOX yielded by an Egyptian soil isolate of *A. niger,* in addition to its molecular characterization.

MATERIALS AND METHODS

Four isolates of black *Aspergillus* (Section Nigri) were isolated from soil and air in Egypt and assayed for the production of SOX. *A. niger* AUMC 4947 was isolated from soil was selected for this study as a high producer of this enzyme and deposited in Assiut University Mycological Centre (Egypt).

Culture media. Czapek´s medium of the following composition (g/l): sucrose – 20.0; $NaNO_3$ – 2.0; KH_2PO_4 – 1.0; $MgSO_4$ – 0.5, with or without yeast extract, was used for the growth of fungus. The com-

position of the medium was changed according to the requirements of the experiment.

Effect of nitrogen and sulphur sources on the production of SOX. Two sources were used, yeast extract, 1.0 g/l or urea 1.0 g/l, to replace $NaNO_3$ in liquid medium. Two sources were employed using liquid Czapek's medium + yeast extract, $MgSO_4$ (0, 0.5, 1 and 1.5 g/l) and L-Cys-HCl (0, 0.1, 0.2, and 0.3 mM) in the absence of $MgSO_4$. The intracellular and extracellular SOX were extracted and enzyme activities were determined.

Fungal inoculum. The test organism was inoculated into liquid Czapek's medium with yeast extract and allowed to grow for 5 days at 28°C, then the culture was shaken vigorously to have a homogenous spore suspension. 0.5 ml of the suspension was dispensed into each of triplicate 250 ml flask containing 100 ml of liquid Czapek's medium with yeast extract and incubated in the dark at 28°C and 150 rpm.

Crude SOX extraction. The crude SOX was extracted from *A. niger* AUMC 4947 as intracellularly and extracellularly. The mycelia were harvested by filtration using Whatman filter paper No.1, lyophilized and then ground in a mortar for 3 min using liquid nitrogen. The total proteins were extracted using extraction (E) buffer, 50 mM sodium acetate buffer pH 5.5. Cell debris was removed by centrifugation at 12,000 *g* for 10 min at 4°C. Protein of the supernatant was gradually precipitated by incubation with chilled 2× acetone at 4°C overnight, followed by the same centrifugation. The supernatant was decanted and acetone was removed from the precipitated protein using desiccator under vacuum at room temperature. The protein was dissolved in 1.0 ml of E buffer and the enzyme activity was determined using Ellman's method [7].The enzyme sample was incubated with 2.0 mM L-GSH at pH 5.5 and 25°C. At different time intervals, 100 μl of reaction mixture was added to 900 μl of 1.0 mM 5,5'-dithiobis(2-nitrobenzoic acid), DTNB, mixed well and incubated at 37°C for 5 min. OD_{412} was measured against blank using Genway 6305 spectrophotometer (USA). The blank was prepared by adding 100 μl of E buffer to 900 μl 1mM DTNB. The remaining [–SH] was calculated from:

$$\text{SH concentration in the sample M} = \\ = (\text{total volume/sample volume}) \times OD_{412}/13600.$$

In another experiment, the *A. niger* spore suspension was first cultured in liquid Czapek´s media with 0.1% yeast extract and 0.05% $MgSO_4$ for 3 days in the dark at 28°C and 150 rpm. Then, mycelia were collected by filtration, washed three times with sterile distilled water, and transferred into different concentrations of pure L-Cys (1.0 or 5 mM Cys in 50 mM E buffer). The control (non-induced) sample was prepared by transferring mycelia into E buffer without L-Cys. The induced and control mycelia were incubated for further 3 days in the same conditions, and the decrease of L-Cys concentrations in the extracellular media was monitored using Ellman's method [7] indicating a successful induction of the SOX production.

Enzyme assay was carried out by incubating 65 μl of SOX with 260 μl of 2.0 mM GSH in E buffer pH 5.5 and at different temperatures (4, 15, 25, 37, 50, 60, 70, or 80°C). After 15, 30, and 60 min, 100 μl of reaction mixture was added to 900 μl of 1.0 mM DTNB, mixed well, and incubated at 37°C for 5 min. Then OD_{412} was measured and the concentrations of oxidized SH-groups and the SOX activities were determined. Assay was carried out at 60°C and different pH values.

Purification of SOX. Sephadex G-100 in E buffer was used for packing the column (50 × 2 cm). The crude protein sample, dissolved in the same buffer, was applied to the column, and 1.5 ml-fractions (54) were collected. To determine the protein concentration OD_{280} was measured and an assay of SOX was carried out in every fraction. Fractions that gave the positive SOX assay were collected and concentrated using incubation with chilled 2× acetone at 4°C overnight. Then centrifugation was carried out at 12,000 *g* for 10 min, the supernatant was decanted and acetone was removed from the partially purified precipitated protein. Then it was dissolved in E buffer (pH 5.5) and applied to 5 ml Q Sepharose column (GE Healthcare, UK) using peristaltic pump (HBI, multistaltic pump, USA), with a flow rate of 1.0 ml/min. 20 mM Tris HCl buffer (pH 7.0) was used as a mobile phase with increasing gradient of NaCl from 100 mM to 1M. The purified precipitated protein was then dissolved in E buffer and analyzed by SDS-PAGE.

10% SDS-PAGE was performed using the method of [8], where unstained protein molecular weight markers (Fermentas Life Sciences, Germany). The current was adjusted to 22 mA. Protein bands were visualized by the double staining of the gel using Coomassie Brilliant Blue R 250 and ProteoSilver Plus Silver Stain kit (Sigma, USA).

The pure SOX dissolved in E buffer (pH 5.5), was incubated with increasing concentrations of substrate (2.0–5.0 mM GSH in the same buffer) at 25°C and the different activities of SOX were determined as described above. Lineweaver-Burk plot was constructed and the Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) of substrate oxidation were calculated.

RESULTS AND DISCUSSION

Effect of nitrogen and sulfur sources on the production of SOX. *A. niger* AUMC 4947 showed weak growth on liquid Czapek's medium containing sodium nitrate as inorganic nitrogen source after two weeks of the dark incubation at 28°C, and thus, there was no enzyme production. On the other hand, when 0.1% yeast extract was used as organic nitrogen source in the growth medium the fungus showed a good mycelial

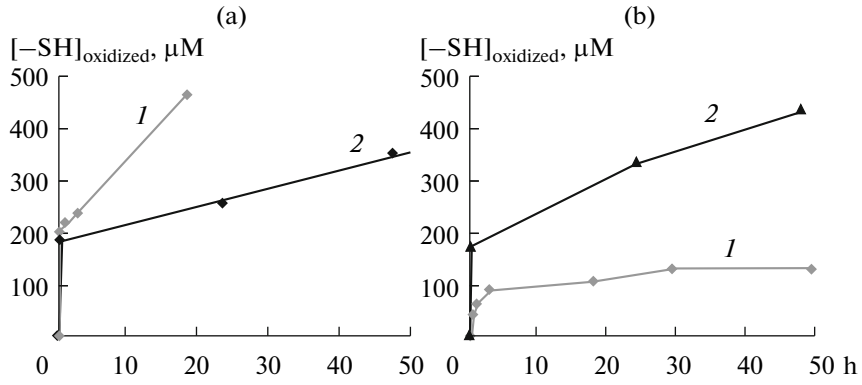

Fig. 1. Comparison between the intracellular and extracellular SOX production in the presence of yeast extract (a) and urea (b) as a nitrogen source in growth medium of *A. niger* AUMC 4947. *1* – intracellular SOX; *2* – extracellular SOX.

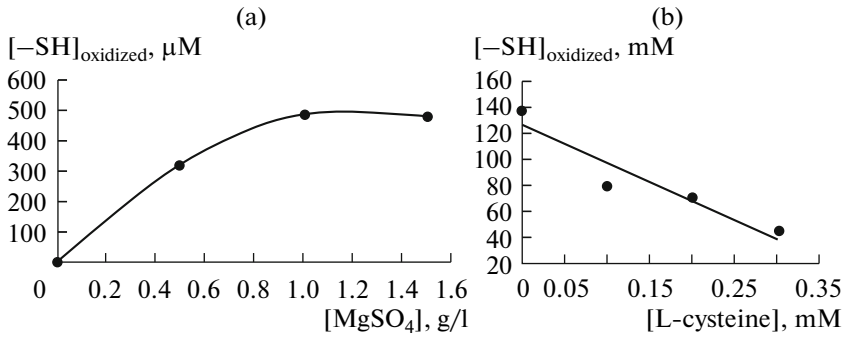

Fig. 2. Intracellular SOX production at different $MgSO_4$ (a) and L-cysteine (b) concentrations in growth medium of *A. niger* AUMC 4947.

growth and SOX was expressed both intracellularly and extracellularly. These results are in agreement with data of Oy [9] who reported that *A. niger* generates sufficient quantities of SOX both intracellularly and extracellularly. The intracellular purified SOX, however, was found to have higher activity than the extracellular one as shown in Fig. 1a. On the contrary, when urea was used as a nitrogen source, the activity of the extracellular enzyme was much higher than that of the intracellular SOX as shown in Fig.1b. This study demonstrates the importance of organic nitrogen sources (yeast extract and urea) for both the mycelial growth of *A. niger* AUMC 4947 and the SOX enzyme production, in comparison with the inorganic nitrogen source (sodium nitrate) supported weak mycelial growth. These results are in agreement with other authors [9–11] who reported that for *A. niger* growth, the best nitrogen sources were found to be yeast extract, casein and other sources of the protein nitrogen, comparatively to the mineral nitrogen. Also it was reported [11–13] that organic nitrogen sources were better supplements for the fungus than inorganic nitrogen sources.

It was noticed that in the absence of $MgSO_4$ the mycelial growth of *A. niger* AUMC 4947 was markedly weak and there was no enzyme production and the SOX activity. The enzyme activity increased with the increasing of $MgSO_4$ concentration until it reached 0.1% (Fig. 2a). On the other hand, the results obtained revealed a very weak mycelial growth, and the SOX activity in the presence of L-Cys (Fig. 2b). These results proved the importance of oxidized sulphate ($MgSO_4$) for both mycelial growth and the SOX production and inability of the fungus to use L-Cys as a sulfur source instead of sulfate. Some microorganisms other than *A. niger* also preferred oxidized sulfur sources over reduced ones. For example, Rothwell [14] investigated the nutritional requirements of *Penicillium variabilie* and found that its isolate utilized inorganic sulfur ($MgSO_4$). Thus, it could be concluded that *A. niger* AUMC 4947 prefers the oxidized inorganic sulfur compounds much better than the reduced organic sulfur compounds for both mycelial growth and the SOX production.

Induction of SOX enzyme using L-Cys-HCl. After mycelial growth for 3 days on Czapek's medium in the presence of yeast extract and $MgSO_4$, the mycelial mate was transferred to pure L-Cys taken in different concentrations (Fig. 3) for 1 h. In these conditions the fungus *A. niger* AUMC 4947 produced an excessive

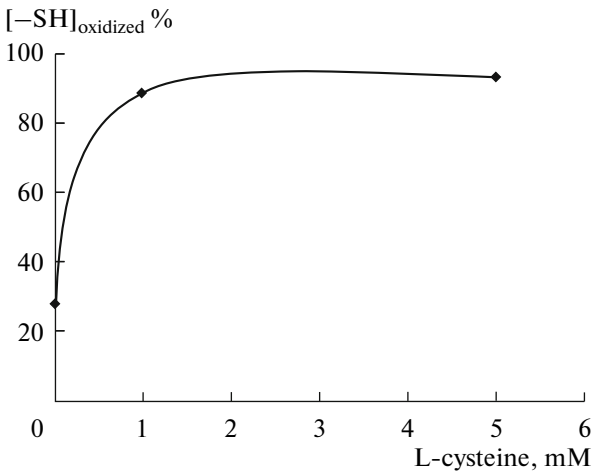

Fig. 3. The intracellular SOX induction after incubation of 3-days mycelial mat of *A. niger* AUMC 4947 in the presence of pure L-cysteine.

amount of both intracellular and extracellular SOX (i.e. L-Cys-induced SOX) compared to the control grown in the absence of L-Cys. The $[SH]_{oxidized}$%, reflecting the activity of the induced intracellular SOX, was measured according to Ellman's method [7] using the standard GSH oxidation where it reached 94% while that of non-induced one reached only 27.6%. This wide difference in activity between the induced and non-induced SOX indicates the successful L-Cys-induction of the SOX production (i.e. SOX *A. niger* AUMC 4947 is an inducible enzyme). Starnes et al. [1] reported that the microbial SOX was constitutive and did not need induction. So, it could be concluded that L-Cys, being a natural substrate of SOX enzyme, when added to the media of *A. niger* AUMC would induce the fungus to produce an increasing amount of both intracellular and extracellular SOX. Thus, this is the first report of an excessive induction of this enzyme by L-Cys after mycelial growth for three days on Czapek's media in the presence of yeast extract and $MgSO_4$.

The results presented in Fig. 4a showed that the enzyme activity was performed at a wide range of temperatures (15–70°C) and the optimal temperature was found to be 60°C (using GSH as a substrate). Oy [9] reported that the optimum temperature for the *A. niger* SOX is in the range of about 30 to about 45°C in the acetate buffer. Also, the optimum temperature for the *Aspergillus sojae* SOX is about 42°C [1] and for SOX from chicken white eggs is 25°C [4]. From that, it could be concluded that the temperature optima of SOX activity differ with the different sources of SOX enzyme and also with the geographical distribution.

As shown in Fig. 4b, the *A. niger* AUMC 4947 SOX exhibited activity over a range of pH values (4.5–7.5), i.e. from acidic range to slightly alkaline, where the optimum pH was found to be pH 5.5. This result is in agreement with the fact that the optimum pH for the *A. niger* SOX is slightly acidic of about pH 5.0–5.6 when the substrate is GSH dissolved in sodium acetate buffer [9, 15, 16] Also, the optimum pH for the *A. sojae* SOX is about 6.5 [1]. On the other hand, SOX can exhibit its optimum activity at a neutral or slightly alkaline pH as in the case of a *Penicillium* species having an optimum pH of the enzyme about pH 7.0–7.8 using GSH as a substrate [17], rat seminal vesicle having its optimum pH of about 7.0 [18] and chicken white eggs having an optimum pH its of about pH 7.0–8.0 [4]. Thus, it can be concluded that the optimum pH for the SOX activity is quite different among different organisms, genera and species.

Purification of SOX. Purification of the *A. niger* AUMC 4947 SOX was performed in two steps by gel-filtration (Sephadex G-100) and anion-exchange chromatography (Q-Sepharose) where the purified

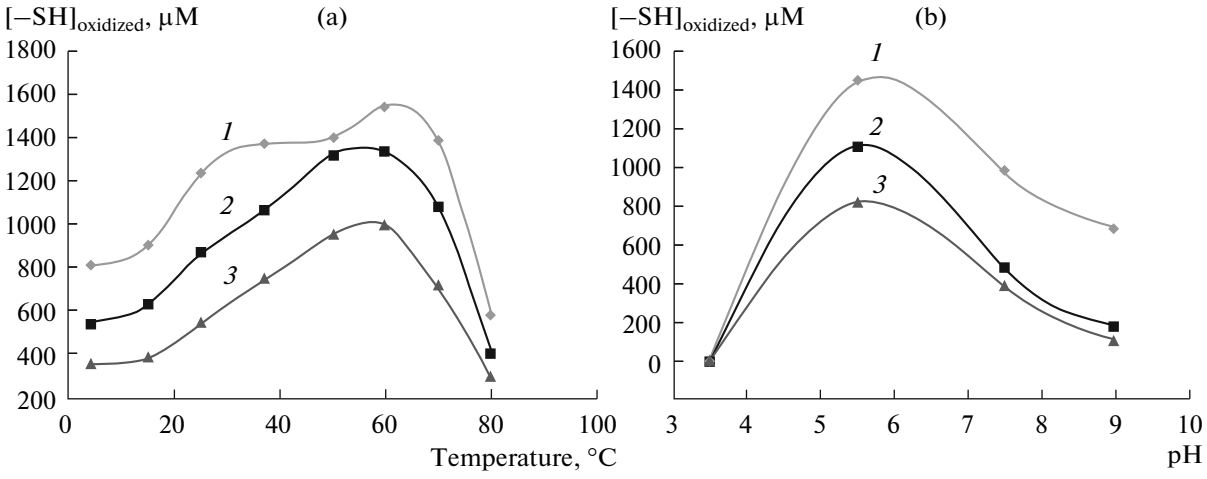

Fig. 4. Effect of temperature (a) and pH (b) on the SOX activity of *A. niger* AUMC 4947 after different periods of incubation: *1* –15 min, *2* – 30 min, *3* – 75 min (for a) and 60 min (for b).

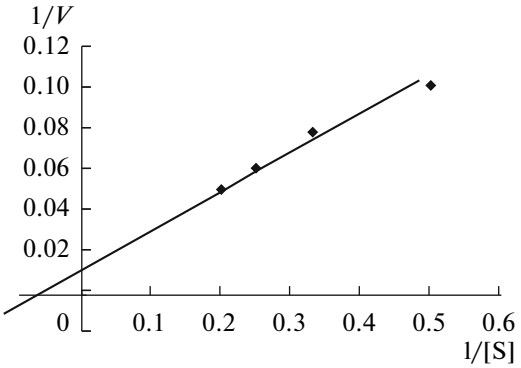

Fig. 5. Lineweaver-Burk plot for the purified SOX of *A. niger* AUMC 4947.

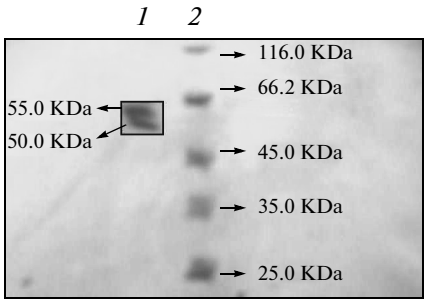

Fig. 6. SDS-PAGE of the purified SOX from *A. niger* AUMC 4947 (lane *1*), and protein markers (lane *2*) stained by Coomassie Brilliant Blue R-250.

SOX enzyme was eluted between 100-300 mM NaCl. Lineweaver-Burk plot was constructed, (Fig. 5) and the Michaelis-Menten constant (K_m = 6.7 mM) and maximum velocity (V_{max} = 100 μmol/min/mg) of substrate oxidation were calculated for the purified SOX of *A. niger* AUMC 4947. This enzyme was found to be a dimer enzyme, with two 50–55 kDa subunits visualized as two bands by the double staining of the gel after SDS-PAGE using Coomassie Brilliant Blue R 250 (Fig. 6). Oy [9] recorded that the *A. niger* SOX had a molecular weight of about 106 kDa and it was constituted by two subunits with a molecular weight of about 50 to 55 kDa as determined by SDS-PAGE. Vignaud et al. [16] reported that molecular weight of the *A. niger* SOX was 110 kDa. These data are in agreement with our results.

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