

ELECTROPHORESIS AND BIOPHYSICAL CHARACTERIZATION OF SARCOSINE OXIDASE - MODIFICATION OF MAGNETIC NANOPARTICLES

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<https://doi.org/10.37904/nanocon.2019.8510>

Abstract

Sarcosine oxidase (SOX) is a flavoprotein and cleaves sarcosine to form hydrogen peroxide, glycine and formaldehyde. Sarcosine is commonly found in muscle, tissues, toothpaste and food supplements. Increased amounts have been found in patients with prostate cancer. SOX is an enzyme suitable for the enzymatic determination of sarcosine. Biotechnological applications require increased stability of used enzymes. In our study, we focused on temperature (5, 15, 20, 30, 35, 40, 50 and 60 °C) and pH-dependent changes (pH 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10 and 10.5) in SOX activity. We used native polyacrylamide gel electrophoresis (Native-PAGE) to monitor changes in SOX. Native-PAGE can be sensitive to any process that changes either the charge or conformation of the protein, so they are excellent for detecting various chemical degradations, aggregations, binding changes, acidic, alkaline or denaturing in general. In addition, SOX was bound to the surface of magnetic nanoparticles (SPIONs). When SOX was bound to SPIONs and lyophilized, SOX activity remained unchanged (100 %) after 6 months.

Keywords: Sarcosine oxidase; sarcosine; enzymatic colorimetric assay; thermostability; Native-PAGE

1. INTRODUCTION

Quantitative determination of sarcosine is important in clinical chemistry, food and fermentation industries. Increases in sarcosine levels are associated with Alzheimer, dementia, prostate cancer, colorectal cancer, stomach cancer and sarcosinemia [1,2]. Sarcosine oxidase is a FAD dependent oxidoreductase found in the cell cytoplasm which participates in oxidation-reduction biological process [3-5]. This enzyme is a flavoprotein that catalyzes the oxidative demethylation of sarcosine and forms equimolar of formaldehyde, glycine and hydrogen peroxide [6,7]. Many organisms produce intracellular sarcosine oxidase eggs, bacteria, mammals or even plants. Monomeric sarcosine oxidase (MSOX) is one of the simplest members of eukaryotic and

prokaryotic enzymes that catalyze similar oxidation reactions with different secondary or tertiary proteins. The MSOX contains 390 amino acids and has a molecular weight of 43 kDa [7,8].

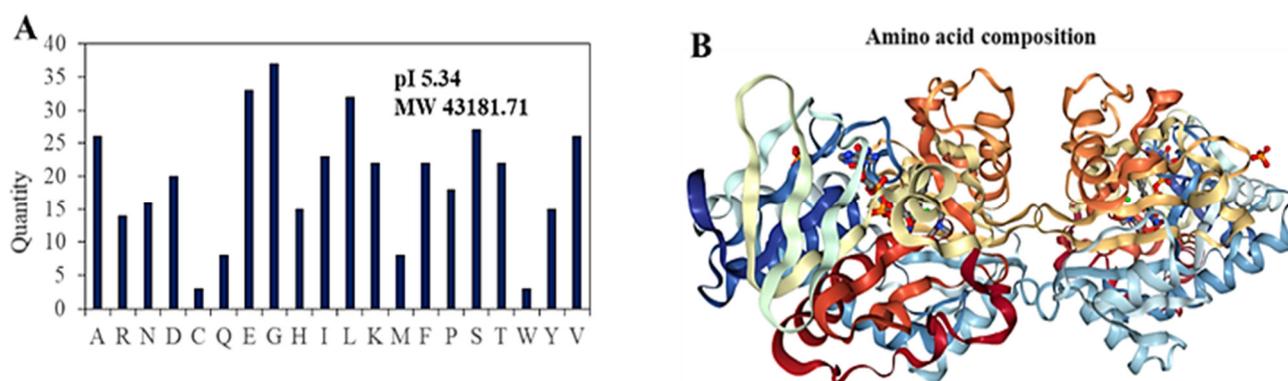


Figure 1 Sarcosin oxidase (SOX). Amino acid composition of SOX (A). MSOX structural model from Expasy database (B).

We used native polyacrylamide gel electrophoresis (Native-PAGE) to monitor changes in sarcosine oxidase. Native gel or non-denaturing electrophoresis takes place without denaturing agents. While, for example, electrophoretic mobility of proteins in SDS-PAGE depends primarily on their molecular weight, in Native-PAGE proteins migrate through the gel based on total charge, hydrodynamic size, and shape. The sensitivity of electrophoresis is determined by the size of the gel pores.

2. MATERIAL AND METHODS

Chemicals

All chemicals that were used for gel electrophoresis were purchased from VWR (USA) and other chemicals were purchased from Merck (USA) at a purity >99%. All plastic materials used (tubes, tips) in this study were purchased from Eppendorf (Germany). Working standard solutions were prepared daily by dilution of the stock solutions. Deionised water was prepared by using the reverse osmosis equipment Aqual 25 (Czech Republic), and was further purified by using an ELGA apparatus equipped with a UV lamp (United Kingdom). The resistance was 18 MΩ and the pH was measured using the pH meter WTW (Czech Republic).

Preparation of SPION nanoparticles and their modification with SOX

SPION nanoparticles were prepared according to the procedure published elsewhere [9]. Shortly, SPIONs nanoparticles were prepared from Fe(NO₃)₃ and a solution of borohydride in 3.5% NH₃. The synthesis was carried out with stirring at 100 °C for 2 hours. After 2 hours, the mixture was allowed to stir overnight at room temperature. The prepared iron nanoparticles were washed and their surface was modified by the reaction with HAuCl₄, the mixture was stirred for 3 hours. Trisodium citrate was then added and the mixture was stirred again overnight. The resulting SPIONs nanoparticles were washed, dried and ready for further use. For further modification, the SPIONs nanoparticles were weighed, washed and SOX was attached to them using chitosan and tripolyphosphate.

Spectrophotometry

A UV/VIS UV-3100PC, VWR (USA) single-beam spectrophotometer was used to record the UV-VIS spectra. The VIS spectrum was measured every 2 nm in the range of 350–700 nm in plastic cuvettes with an optical path of 1 cm. The Infinite F50 (Tecan, Switzerland) was used for measurement on a polystyrene microtiter plate (Gama Group a.s., Czech Republic). Automated spectrometric measurements: BS-300 chemical analyzer from Mindray (China), cuvettes 5 x 6 x 30 mm, optical path 5 mm, a volume of the reaction mixture in the cuvette 180 - 500 μ L. Photometric detector measuring at wavelengths: 340, 405, 450, 510, 546, 578, 630, and 670 nm. Reagents and samples were placed on the cooled sample holder (4 °C) and automatically pipetted directly into plastic cuvettes. Incubation proceeded at 37 °C. The mixture was consequently stirred. The washing steps by distilled water (18 M Ω) were done in the midst of the pipetting. Apparatus was operated using software BS-300 (Mindray, China) and Qinslab (Prevention Medicals, Czech Republic).

Temperature study

SOX (1 mU) was placed in a Eppendorf tube and sealed in a thermoblock (Mastercycler NEXUS, Eppendorf, Germany). Temperatures (5, 15, 20, 30, 35, 40, 50 and 60 °C) were set for 60 min. Thereafter, a spectrophotometric study of SOX activity was performed as a color product formation. Lyophilization of SOX to SPIONs was carried out for 48 h at -80 °C and a pressure of about 0.1 mb (LABCONCO, USA).

Gel electrophoresis

For Native PAGE, we used a 6% gel (6% acrylamide / bis-acrylamide, 0.16% TEMED and 44 μ M ammonium peroxydisulfate), using 60 mM HEPES and 40 mM imidazole as the running buffer. Sarcosine oxidase enzyme samples were prepared in Britton-Robinson buffer (0.04 M phosphoric acid, 0.04 M acetic acid, and 0.04 M boric acid) at varying pHs. Samples were maintained at pH 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10 and 10.5 for 1 hour at 37 °C and at room temperature (25 °C). Samples were then mixed with the loading buffer (2:1) and applied to the gel. 50% glycerol with 0.01% bromophenol blue was used as the loading buffer. The concentration of sarcosine oxidase per well was 10 μ g. Electrophoresis was run at 150 V for 1 hour. Gels were stained with silver to visualize the results. Subsequently, the gels were scanned and evaluated by Colortest.

3. RESULTS AND DISCUSSION

Analytical approaches to sarcosine analysis are based on its detection by HPLC or CE techniques. Trinder-reaction based enzymatic methods can be used as low-cost tools in urine detection [10]. Enzymes often change their activity depending on the surrounding conditions, so their study is important [2]. In our preliminary study, the effect of pH and temperature on SOX activity was monitored. To determine the effect of temperature on the SOX reaction, the enzyme activity was measured at various temperatures (5, 15, 20, 30, 35, 40, 50 and 60 °C). For determination of the enzymatic thermostability, the enzyme solution was incubated for 60 min. For SOX activity measurement, the concentration of SOX of 1 μ g/ μ L was used. For the reaction with substrate (sarcosine), phosphate buffer (pH = 8), 4-aminoantipyrine (4-AAP), sarcosine oxidase (SOX) and diluted horseradish peroxidase (HRP) were used. Standard solutions of sarcosine were prepared at different concentrations (90, 188, 375, 750, 1000, 1300 μ M). The reactions were performed in 96-well microtiter plates and the absorbance was measured by a microplate reader. The total volume of each reaction was 300 μ L. Reactions were performed by adding 50 μ L of sarcosine solution and 250 μ L of reaction mixture.

The rate of formation of colored product (absorbance) was immediately measured at 540 nm at laboratory temperature every minute for 60 min. The stability of SOX was up to 40 °C. At temperatures above 45 °C SOX activity was 70 - 80 % of the maximum value. At temperatures of 50 - 55 °C, SOX activity was about 10 % and

above 65 °C SOX was already inactivated. At temperatures in the range of 4 to 20 °C, SOX activity was observed between 95 - 99 % of maximum activity. In lyophilized SOX, the activity was between 95 - 110 % of maximum activity. Similar results were obtained from Tong et al. [11]. Next, SOX was incubated in reaction buffer at different pHs (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0 and 10.5) for one hour at room temperature and at 37 °C. Subsequently, a reaction with sarcosine (100 µM) was performed and individual SOX samples were loaded onto Native-PAGE. SOX activity was at a low pH (5 and 5,5) 40 - 60 %, at pH6 an increase in SOX activity to 80 - 90 % was recorded and a peak of SOX activity was between pH 7 and 8 (100 %). SOX activity was 80 - 70 % of the maximum activity above pH 8. 3 bands are visible on the gel. The most significant bands are those at the front of the gel, with shifts and color changes depending on the pH being most visible. The sample at pH 5 is very bright, but all three samples are observable. For all others, the lanes are darker with increasing pH, but from pH 8, the first band is visibly losing. Similar results were obtained at 25 °C. When SOX (100 mg/g of nanoparticles) was immobilized to the SPION surface, the converted SOX activity was about 90 - 100 % of the maximum activity. In addition, SOX on the surface of SPIONs nanoparticles could be lyophilized. Lyophilized enzyme showed long-term stability under normal conditions (after more than 6 months its activity was 95 - 100 % of maximum activity).

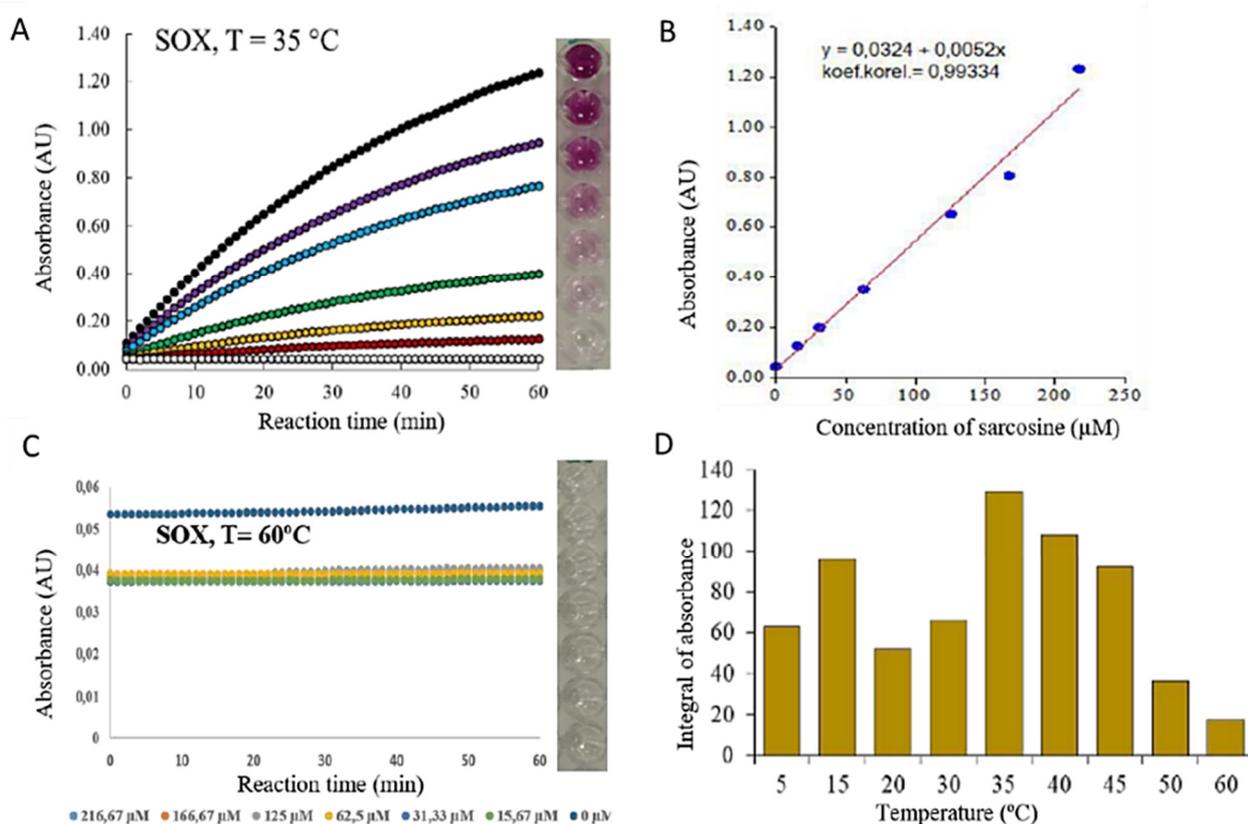


Figure 2 Tracking SOX activity after exposure to elevated temperature. Dependence between absorbance and sarcosine concentration (0, 15, 30, 60, 125, 170 and 220 µM) over time. The signal of SOX activity has increased every minute during 1 hour after SOX has been exposed to 35 °C for 60 minutes (A). Calibration curve of sarcosine - concentration ranges from 0 µM to 220 µM (B). SOX activity after incubating the enzyme at 60 °C for 60 minutes (C). Changes in the integral of SOX signal when exposed to different temperatures (5, 15, 20, 30, 35, 40, 50 and 60 °C) for 60 minutes showing that SOX is relatively unstable when exposed to temperatures up to 45 °C (D).

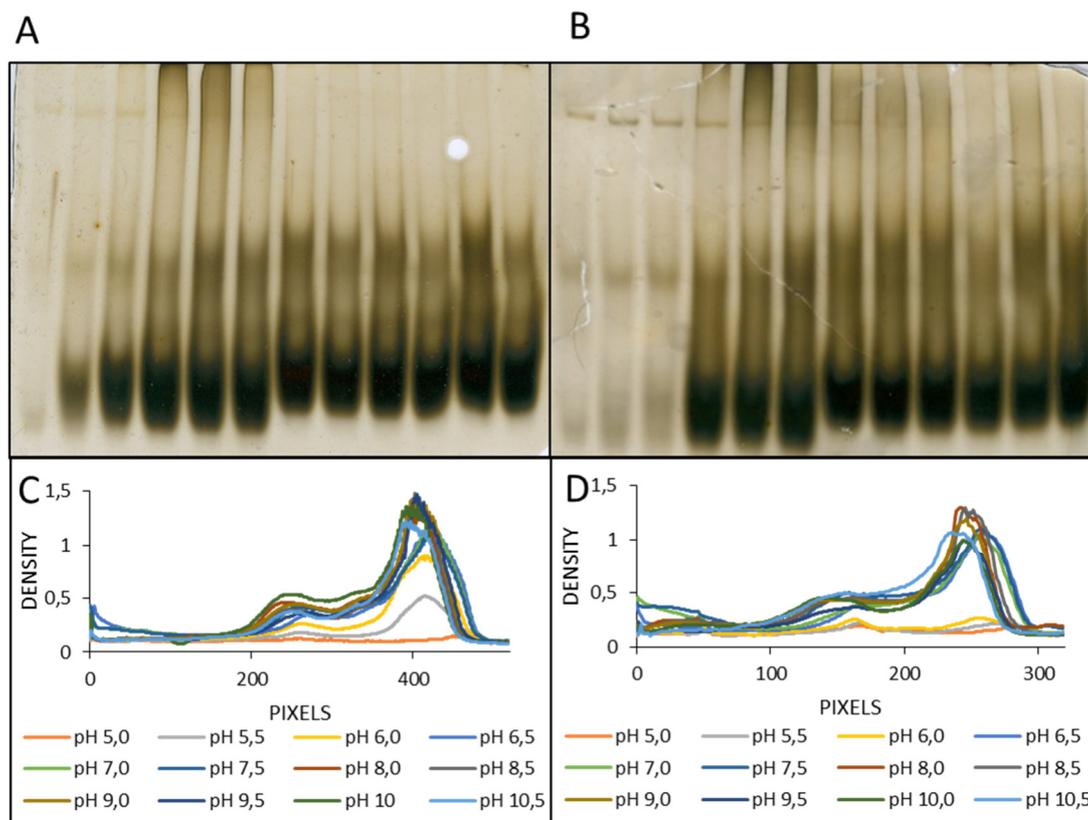


Figure 3 Native-PAGE SOX incubated for 60 min at pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0 and 10.5) A) at room temperature; B) at 37 °C; C) and D) A summary graphs of the evaluated sample paths in the gels over the graphs in the Colortest. Dependence is the distance in pixels per density. Density is defined as $\text{Log}(I_0/I)$, where $I_0 = 255$ and I is our measured density. Further conditions are given in Material and Methods.

4. CONCLUSION

Rapid diagnostics is essential for effective initiation of treatment. Sarcosine is extensively studied as a potential tumor marker for prostate cancer [12,13]. The Trinder reaction with SOX provides a very good analytical response [14,15]. The critical part of analytical determination is SOX. The work has shown that SOX is relatively stable when exposed to temperatures up to 50 °C. Another critical parameter is pH. The best pH for this enzymatic reaction is pH 8 [9]. On Native-PAGE, it is apparent that SOX at a pH lower than 8 has an altered conformation. The work has shown that SOX is relatively stable when exposed to temperatures up to 50 °C. When SOX was bound to SPIONs and lyophilized, SOX activity was about 100 % after 6 months.

ACKNOWLEDGEMENTS

The work was supported by the SARKOTEST 165/2015, H2020 ERASMUS+ and international collaboration project of The European Technology Platform for Nanomedicine.

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