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Xanthine oxidase from milk of the water buffalo (*Bubalus bubalis):* Purification, characterization and application in SOD assay diagnostic kit.

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ABSTRACT

Xanthine oxidase enzyme has broad medical applications as it is a significant component in SOD diagnostic kit. Here, buffalo milk xanthine oxidase (BMXO) was extracted and purified to homogeneity using toluene, ammonium sulfate precipitation, affinity chromatography on Heparin-agarose column and gel filtration chromatography on Sephacryl S-300 column. BMXO is purified 10.66 units / mg protein specific activity which represent 12.4 fold purification and 19.1% yield. The native molecular weight of the purified BMXO enzyme is 270 kDa homodimer with subunit molecular weight of 135 kDa as determined by SDS-PAGE. The isoelectric point (*p*I) value of BMXO showed a single molecular species at pH 6.2. BMXO showed its optimum activity at pH 8 and the value of Km is 1 mM xanthine. $MnCl_2$ and $FeCl_2$ increased the activity of BMXO while $NiCl_2$, $MgCl_2$, $ZnCl_2$ and $CuCl_2$ were found to be inhibitors of the purified enzyme. Allopurinol inhibits BMXO competitively and has one binding site on the enzyme with Ki value of 0.3 mM. The purified BMXO is used in the construction of SOD diagnostic kit which is found sensitive and comparable with the commercially available from Sigma-Aldrich.

Keywords: Xanthine oxidase; Purification; Characterization; Buffalo milk; SOD Diagnostic kit

Abbreviations: BSA, Bovine serum albumin, BMXO, Buffalo milk xanthine oxidase, XO, Xanthine oxidase, NBT, Nitroblue tetrazolium, PMS, Phenazine methosulphate, SOD, Superoxide dismutase

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INTRODUCTION

Xanthine oxidase (XO) is a metallo-flavo protein containing flavin adenine dinucleotide (FAD), molybdenum and iron, in a ratio of 2:2:8 [1, 2, 3]. Xanthine oxidase enzyme catalyzes the metabolism of hypoxanthine to xanthine and finally converts xanthine to uric acid in the presence of molecular oxygen to yield superoxide anion and hydrogen peroxide that causes the oxidative injury to living tissues [4, 5]. Purines or other substrates react with xanthine oxidase at the site containing molybdenum, and the electron acceptors (oxygen or nicotinamide adenine dinucleotide) react at the FAD site [6]. The enzyme can oxidize a range of substrates including purines, pyrimidines, pteridines, azopurines, cytochrome C, heterocyclic compounds, Oxygen, NAD^+ and ferricyanide [7]. Milk is the richest source of xanthine oxidase that a possible role of this enzyme is antibacterial activity by mean of its capability to make reactive oxygen species [8]. XO is concentrated in the milk fat globule membrane in which it is the second major protein, after butyrophilin [9].

Xanthine oxidase (XO) enzyme is a main ingredient of the diagnostic kit for the estimation of superoxide dismutase (SOD). This kit is very sensitive in the measurement of SOD activity in blood, serum and plasma which is mainly used in diagnosis, monitoring and treatment of oxidative stress. Oxidative stress is involved in pathogenesis of cardiovascular pulmonary diseases, cerebral infarction, brain trauma, hypertension, mutagenesis, atherosclerosis, thrombosis, ischemia-reperfusion, diabetes, pulmonary edema, hypoxia, hyperoxia, inflammations, cystic fibrosis, Parkinson's disease, Alzheimer disease, cancer and acquired immunodeficiency syndrome [10]. Investigation of SOD activity in human cerebrospinal fluid is a useful index of central nervous system aging [11]. Plasma SOD activity may serve as a helpful parameter to estimate aserosclerosis progression and microvascular changes in ischemia [12]. So, the target of this study is purification and characterization of XO enzyme from the buffalo milk as a locally available rich source. As a direct application, the purified XO enzyme is used in the preparation of SOD diagnostic kit. A comparison of the constructed kit with the commercially available Sigma-Aldrich SOD diagnostic kit is carried out.

MATERIALS AND METHODS

Materials

Fresh buffalo milk was obtained from healthy dairy water buffalo. Bovine serum albumin (BSA), Xanthine sodium salt, Nitroblue tetrazolium chloride (NBT), Phenazine methosulphate (PMS), Heparin-agarose, Sephacryl S-300, Protein molecular weight standards and chemicals for electrophoresis were purchased from Sigma-Aldrich Chemical Co. The other chemicals were of analytical grade.

Assay of xanthine oxidase activity

The XO activity assay reaction mixture contained in 1 ml of 0.05 M Tris-HCl, pH 7.6 containing 2 mM xanthine, 0.5 mM NBT and the xanthine oxidase solution. The reaction mixture was incubated for 5 minutes at 37 °C, centrifuged at 2000 rpm for 2 minutes and the absorbance was measured at 575 nm. To calculate XO units, a control reaction was done with 0.02 unit commercially available bovine milk xanthine oxidase [13].

Xanthine oxidase activity staining on polyacrylamide gels

Activity staining of xanthine oxidase was determined as described by [14]. The gels were submerged in 50 mM Tris-HCl, pH 7.6, 0.5 mM xanthine, 0.25 mM nitroblue tetrazolium and 630 mM TEMED. Staining of the gels was continued till the activity bands appear on the gels (10 sec–15 min).

Extraction and purification of buffalo milk xanthine oxidase (BMXO)

Xanthine oxidase was purified from 2 liters of fresh raw buffalo milk. The milk was cooled down overnight to 4°C then mixed with EDTA and toluene to a final concentrations of 2 mM and 3% (v /v), respectively. The milk was churned vigorously with shaking at 250 rpm for 30 min at 40 °C. After cooling the churned milk to about 4 °C, the churning process was repeated and the sample was filtered through six cheesecloth layers. The opalescent filtrate was brought to 38% ammonium sulfate saturation. The suspension was centrifuged at 8000 *x g* for 15 min and the pellet formed was discarded. The supernatant was brought to 50% ammonium sulfate saturation and precipitate formed was collected by centrifugation at 10400 *x g* for 30

min and dissolved in 10 mM Tris-HCl pH 7.6, 5 mM 2-mercaptoethanol and 0.1 mM EDTA. The suspension was dialyzed against this buffer followed by dialysis against the same buffer but containing only 0.03 mM EDTA. The dialyzed fraction was centrifuged at 40000 *x g* for 30 min and the supernatant was applied to a Heparin-agarose column (8 x 1.5 cm i.d.) pre-equilibrated with 0.02 M Tris-HCl buffer, pH 7.6, containing 0.03 mM EDTA. The protein fractions were eluted with stepwise NaCl gradient ranging from 0 to 1 M prepared in the equilibration buffer at a flow rate of 30 ml / hr. and 4 ml fractions were collected. The fractions containing XO activity were pooled and concentrated by lyophilization. The concentrated fraction containing XO activity was applied to a Sephacryl S-300 column (142 cm X 1.75 cm i.d.). The column was equilibrated and run with 0.02 M Tris-HCl buffer, pH 7.6, containing 0.03 mM EDTA at a flow rate of 30 ml / h and 2 ml fractions were collected [15].

Electrophoretic analysis

Native gel electrophoresis was carried out with 7% PAGE [16]. SDS-PAGE was performed with 12% polyacrylamide gel [17]. The subunit molecular weight of the purified XO enzyme was determined by SDS-PAGE [18]. Electrofocusing was performed and the isoelectric point (*pI*) value was calculated from a calibration curve [19, 20]. Coomassie brilliant blue R-250 was used to stain the proteins.

Protein determination

Protein content was determined by the dye binding assay method [21] using BSA as a standard protein.

Construction of superoxide dismutase (SOD) diagnostic kit

The purified buffalo milk xanthine oxidase (BMXO) is used in the construction of SOD diagnostic kit [22]. The kit is composed of two reagents: (R1: 50 Mm Potassium phosphate buffer pH 7, 100 mM EDTA and 50 mM Sodium xanthine) and (R2:100 mM Cytochrome C). In the control test, 900 µl R1 was mixed with 100 µl R2 and the reaction was started by adding 21 m unit BMXO enzyme and follows the absorbance at 550 nm for three minutes. In sample test, 800 µl R1 was mixed with 100 µl R2 and 100 µl sample then the reaction was started by adding 21 m unit BMXO enzyme and follow the absorbance at 550 nm for three minutes. One unit of SOD activity is defined as the amount of enzyme giving 50% inhibition of cytochrome C reduction at 550 nm.

RESULTS

Purification of buffalo milk xanthine oxidase (BMXO)

The purification procedure of XO from buffalo milk is summarized in Table (1). The purification procedure involved mixing crude milk with toluene, ammonium sulfate precipitation, affinity chromatography on Heparin-agarose column and gel filtration chromatography on Sephacryl S-300 column. The purified xanthine oxidase was found to be stable at basic pH above 7.0; hence alkaline Tris-HCl buffer, pH 7.6 was used during all purification steps of this enzyme. The elution profile of Heparin-agarose column (Fig. 1a) indicated the existence of one peak containing XO activity that designated BMXO and eluted with 0.1 M NaCl. The fractions of this peak were collected, lyophilized and applied onto a Sephacryl S-300 column (Fig. 1b) which revealed the presence of one peak of BMXO enzyme activity. The purified BMXO enzyme from the gel filtration column has 10.66 units / mg protein specific activity and 12.4 folds purification (Table 1).

Table (1): A typical purification scheme of the buffalo milk xanthine oxidase (BMXO):

Purification steps	Total protein (mg)	Total Activity (unit)	Specific Activity	Yield (%)	Fold Purification
38-50 % $(NH_4)_2SO_4$ Fration	77.5	67	0.86	100	1.0
Heparin-agarose fraction	12.5	30.5	2.44	45.5	2.8
Sephacryl S-300 fraction	1.2	12.8	10.66	19.1	12.4

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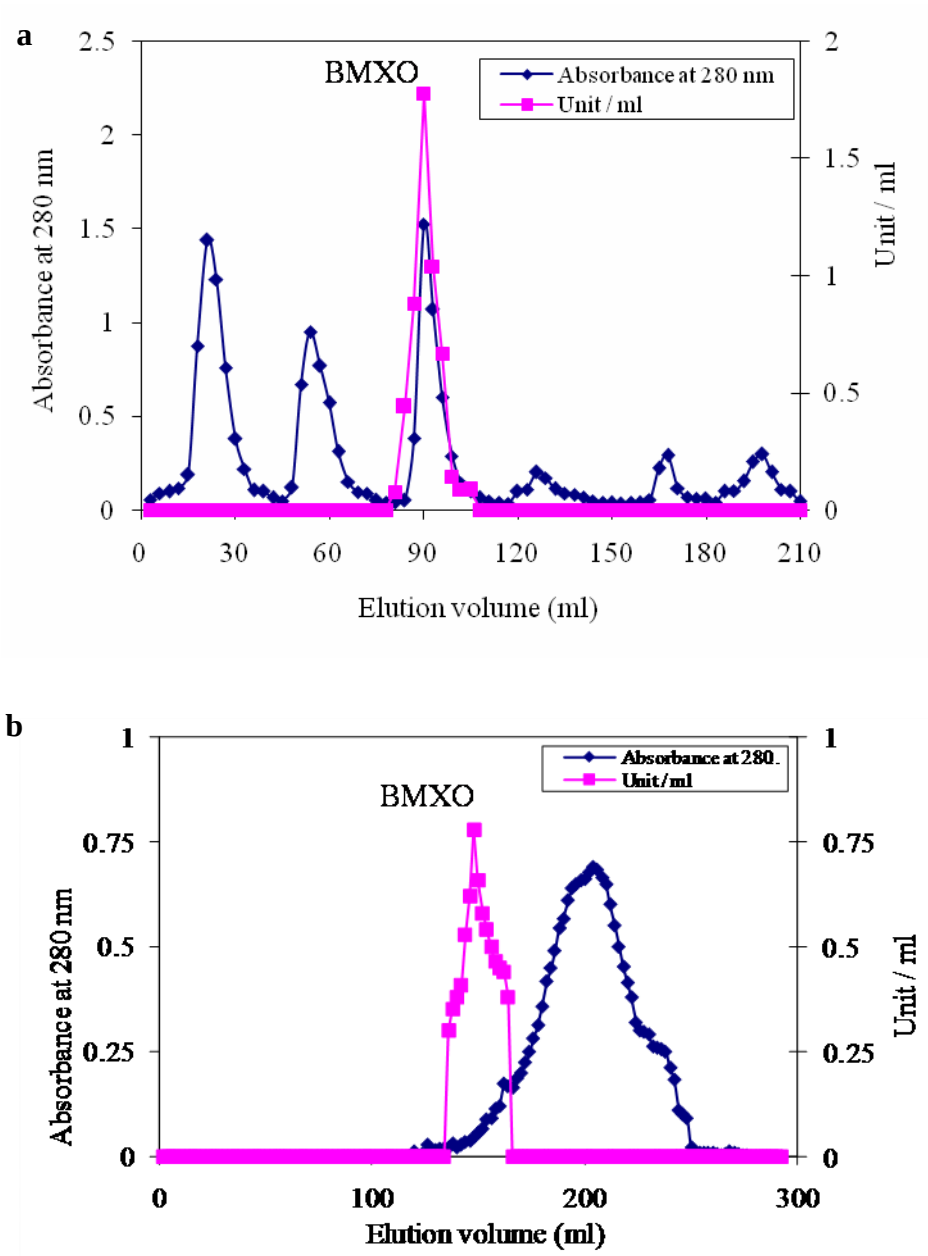

Fig 1: (a) A typical elution profile for the chromatography of the buffalo milk ammonium sulfate fraction on Heparin-agarose column (8 cm x 1.5 cm i.d.) previously equilibrated with 0.02 M Tris-HCl buffer, pH 7.6 containing 0.03 mM EDTA. The proteins were eluted by a stepwise gradient of NaCl ranging from 0 to 1 M in the equilibration buffer and 4 ml fractions were collected at a flow rate of 30 ml / h. (b) A typical elution profile for the chromatography of BMXO on Sephacryl S-300 column (142 cm x 2.4 cm i.d.) previously equilibrated with 0.02 M Tris-HCl buffer, pH 7.6 containing 0.03 mM EDTA. The proteins were eluted by the same buffer and 2 ml fractions were collected at a flow rate of 30 ml / h.

Molecular weight determination and electrophoretic analyses of BMXO

Electrophoretic analysis of ammonium sulfate, heparin-agarose and Sephacryl S-300 fractions of BMXO on 7 % native PAGE revealed single protein band corresponded the enzyme activity band of the purified xanthine oxidase enzyme (Fig. 2a). The native molecular weight of the prepared BMXO resulted from Sephacryl S-300 column was calculated from a standard curve to be 270 kDa. SDS-PAGE of denatured purified BMXO enzyme showed a major protein band of molecular weight of 135 kDa and three other minor bands of approximately 17 kDa, 41 kDa and 77 kDa (Fig. 2b). The p*I* value of BMXO was visualized on isoelectrofocusing PAGE as single species at pH 6.2 (Fig. 2c).

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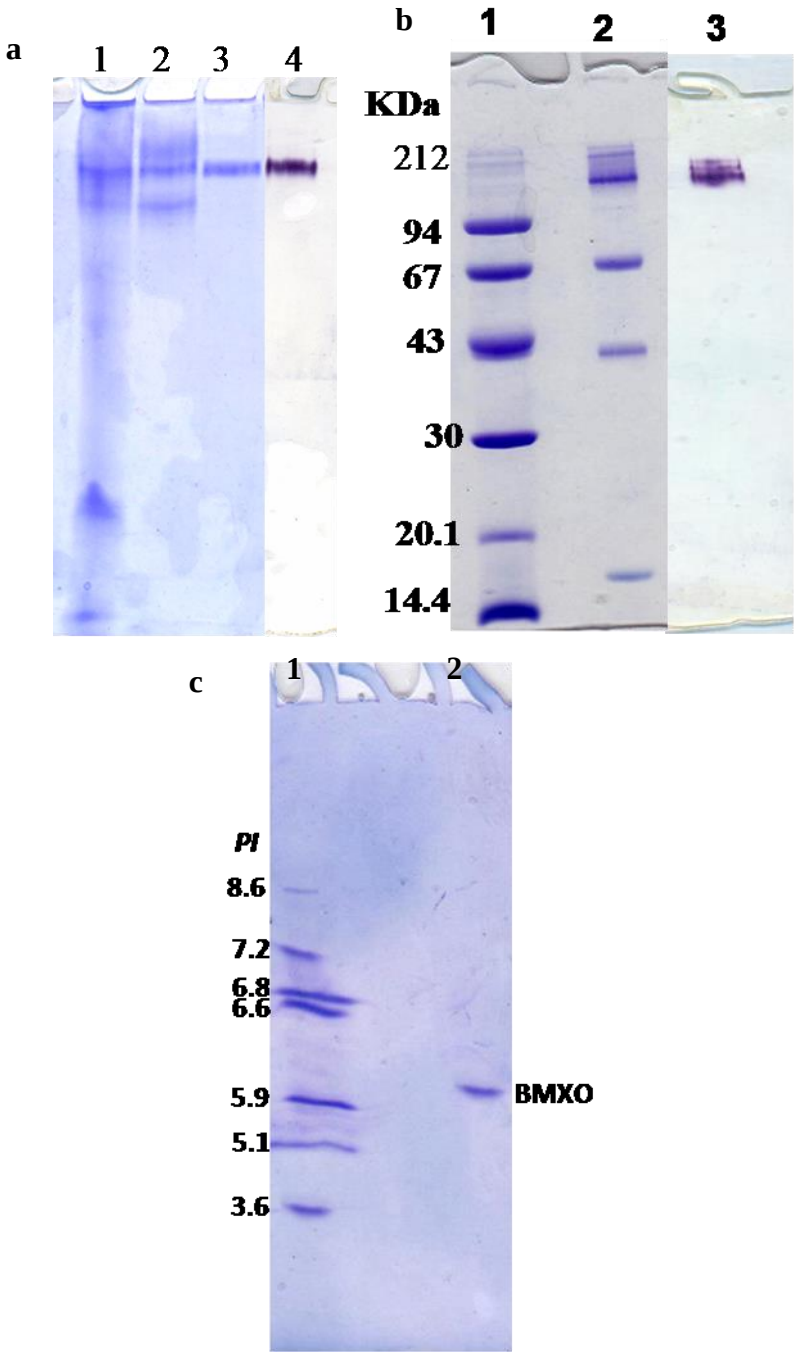

Fig 2: (a) Electrophoretic analysis of protein pattern of BMXO on 7 % native PAGE: (1) ammonium sulfate fraction, (2) Heparin-agarose fraction, (3) Sephacryl S-300 purified fraction and (4) Isoenzyme pattern of purified BMXO. (b) Molecular weight determination of BMXO subunit by 12% SDS-PAGE: (1) Molecular weight marker proteins, (2) Purified BMXO and (3) BMXO Isoenzyme pattern. (c) Isoelectrofocusing: (1) Isoelectric point (*pI*) marker proteins and (2) The purified BMXO.

Determination of BMXO optimum pH and Km value

The effect of pH on BMXO activity was measured and the most stable pH range for the enzyme activity was found between pH 7.6 – 8.4 at 37°C. The optimum activity of BMXO enzyme was found at pH 8.0 (Fig. 3a). The value of Km was calculated from the Lineweaver-Burk plot for the reverse of the reaction velocity (1/v) and substrate concentration (1/[S]) to be 1 mM xanthine (Fig. 3b).

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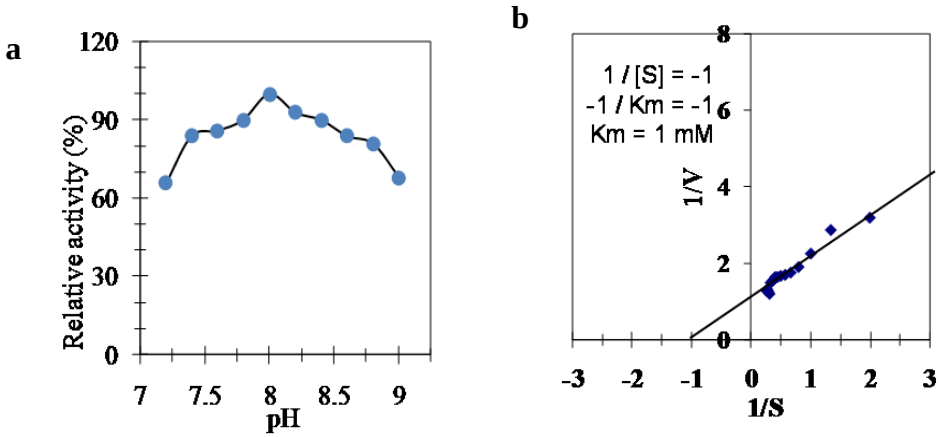
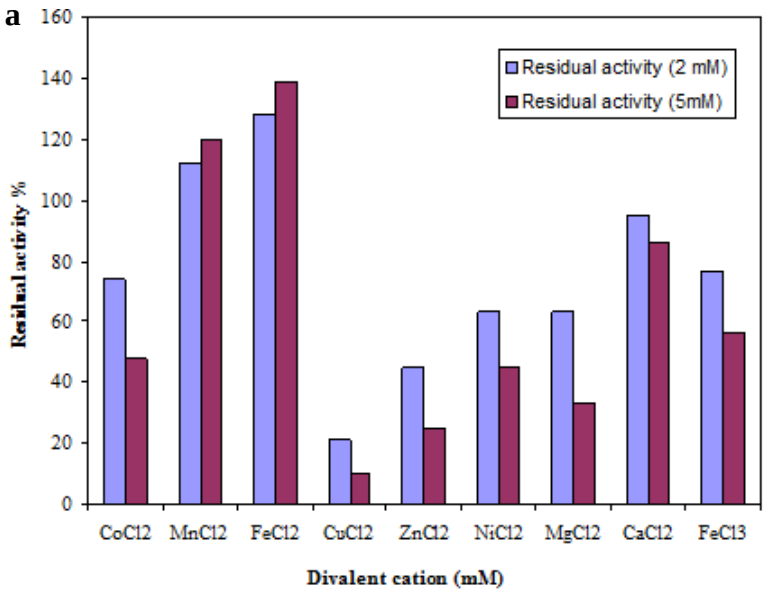


Fig 3: (a) Effect of pH on the purified BMXO using 0.05 M Tris-HCl buffer, pH (7.0-9.0). (b) Lineweaver-Burk plot relating the reciprocal of the reaction velocity of the purified BMXO to xanthine concentration in mM.

Effect of divalent cations and various inhibitors on BMXO

The effect of metal compounds on the purified BMXO activity was examined (Fig. 4a). The BMXO was inhibited with $NiCl_2$, $MgCl_2$, $ZnCl_2$ and $CuCl_2$. However, it was activated by $MnCl_2$ and $FeCl_2$. Also, the inhibition of purified BMXO activity by several inhibitors was studied (Fig 4b). Pre-incubation of the inhibitors for 5 min at 37˚C were carried out and the inhibition % was concluded as a proportion of a non-inhibited control. The ability of each compound to inhibit BMXO at different concentration revealed that allopurinol is the strongest inhibitor of BMXO enzyme since 2 mM allopurinol inhibited 82.1 % of the purified BMXO activity and 98.5 % maximum inhibition was achieved by 5 mM. A titration curve of allopurinol concentrations on the purified BMXO activity is carried out (Fig. 5a). The Hill plot slope was found to be 1 (Fig. 5b). Allopurinol inhibited BMXO enzyme activity competitively (Fig. 5c) and the Ki value of the BMXO inhibition by allopurinol is determined to be 0.3 mM (Fig. 5d).



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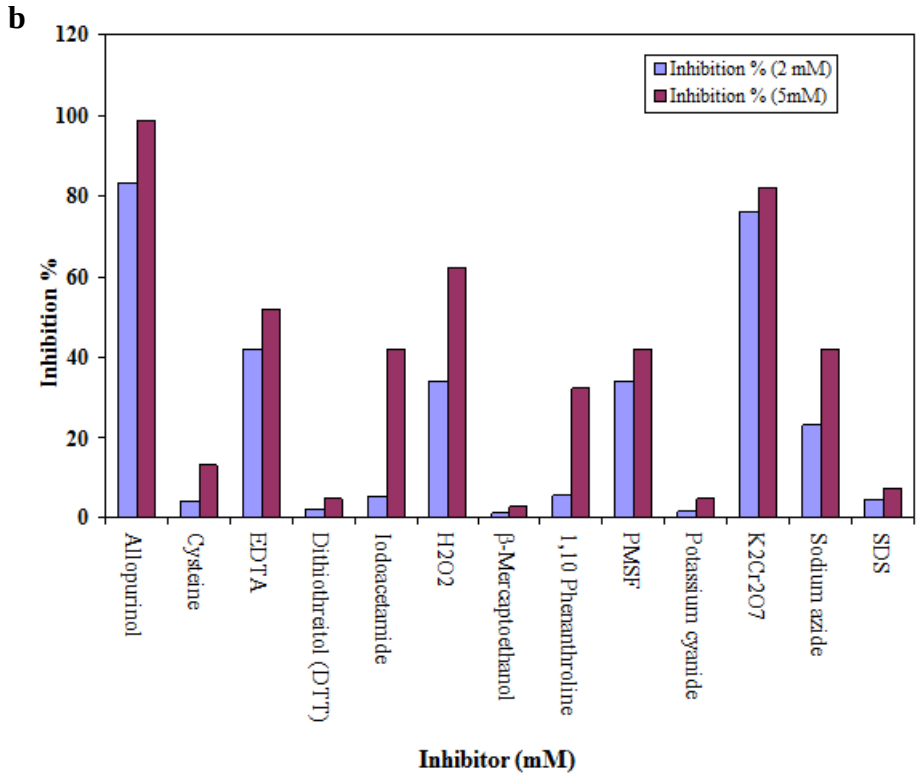
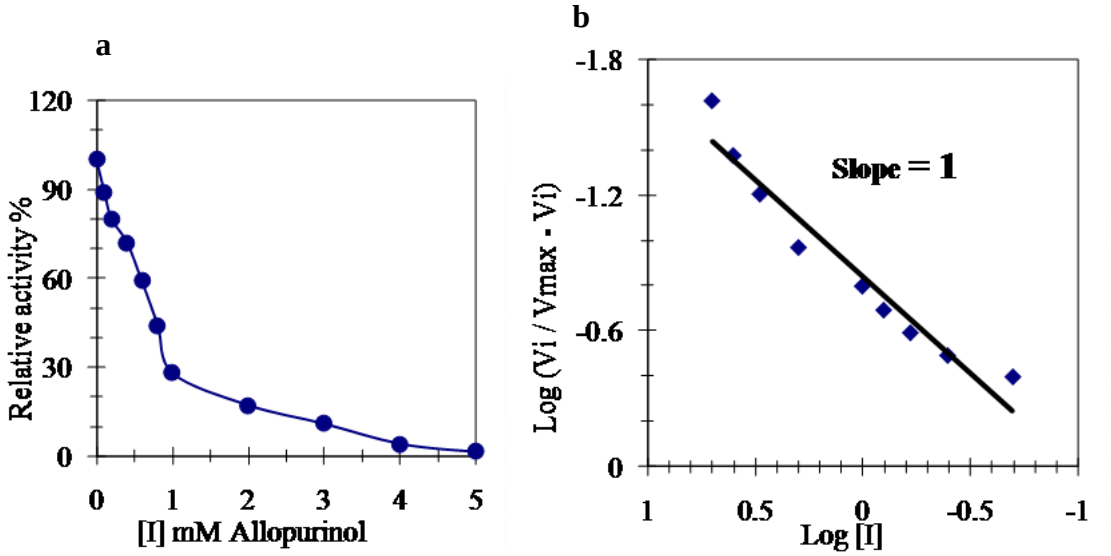


Fig 4: (a) Effect of divalent cations on the buffalo milk xanthine oxidase (BMXO). (b) Effect of inhibitors on the purified buffalo milk xanthine oxidase (BMXO). The values I both figures represent % of the control and the means of triplicate experiments.



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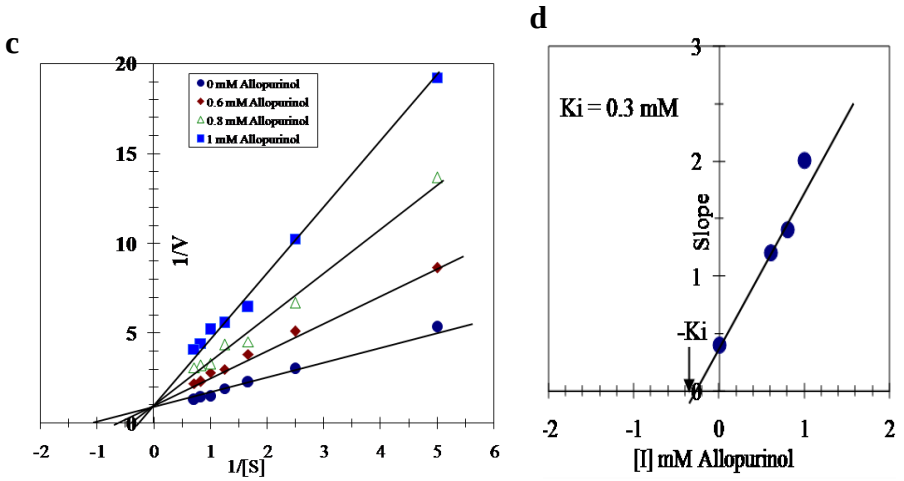

Fig 5: (a) Inhibition of BMXO by varying concentrations of allopurinol. (b) Hill plot for inhibition of BMXO by allopurinol. (c) Lineweaver-Burk plots showing the type of inhibition of BMXO by allopurinol. (d) Determination of the inhibition constant (Ki) value for the inhibition of the BMXO by allopurinol.

Comparison of the constructed kit with the commercially available kits

The constructed kit (prepared SOD diagnostic kit with the purified BMXO enzyme) has been compared with commercially available kit (Sigma-Aldrich SOD diagnostic kit) using twenty five patient samples (Table 2).

Table (2): Comparison of the constructed kit with commercially available Sigma-Aldrich SOD diagnostic kit:

Serum samples	SOD Units	
	Constructed SOD Diagnostic Kit	Sigma-Aldrich SOD Diagnostic Kit
1	70.2	71.0
2	53.3	60.7
3	67.1	74.8
4	57.3	66.1
5	48.2	52.1
6	73.7	74.8
7	66.1	54.8
8	51.9	63.5
9	51.7	48.6
10	59.1	59.5
11	54.7	59.9
12	63.4	68.5
13	82.2	84.1
14	57.6	66.2
15	57.9	67.5
16	52.8	60.1
17	58.6	54.6
18	69.8	53.3
19	58.2	59.5
20	57.2	58.2
21	59.2	62.4
22	60.1	57.2
23	68.4	62.9
24	67.1	61.8
25	47.6	49.1

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DISCUSSION

Determination of the content and activity of xanthine oxidase enzyme can be utilized for diagnosis applications. Xanthine oxidase participates in the catabolic pathway for the breakdown of purine nucleotides to xanthine and uric acid in most animal species [7]. It also performs a significant role in liver diseases [23], cardiovascular diseases [24] and employed as antimicrobial agents [25, 26, 27]. In this study, xanthine oxidase is purified from buffalo milk that designated BMXO. The purification procedure involved mixing crude milk with toluene, deposition using ammonium sulfate, Heparin-agarose affinity column and Sephacryl S-300 gel filtration column. This purification procedure involves only two phases of column chromatography and therefore, it seems to be simple and convenient method. Various purification procedures of xanthine oxidase from milk were reported for bovine milk [14, 15, 28, 29, 30, 31, 32] and human milk [33]. The BMXO was purified about 12.4-fold over the ammonium sulfate dialyzed fraction with 10.66 units / mg protein specific activity and 19.1 % yield (Table 1). Bovine milk xanthine oxidase was prepared from milk with 69 % yield and 10.16 units / mg protein specific activity [15], bovine milk with 20 % yield and 2.5 units / mg protein specific activity [31] and human milk with 18 % yield and 4.3 units / mg protein specific activity [34].

BMXO is homogeneously purified as revealed on 7 % native PAGE that a singular protein band matched the BMXO isozyme band indicating that the xanthine oxidase is tentatively purified (Fig. 2a). The intact molecular mass of prepared BMXO is deduced by gel filtration as 270 kDa. The subunit molecular weight is determined by SDS-PAGE of denatured BMXO enzyme as 135 kDa indicating the homodimer structure of the purified enzyme. Three minor bands of 17 kDa, 41 kDa and 77 kDa were seen on SDS-PAGE that may be due to proteolytic cleavage of subunit band during purification [35] or it may be cleaved by denaturing factors on the SDS-PAGE. These minor bands consists the three domains forming the 135 kDa subunit [the N-terminus iron-sulfur centers domain, the middle FAD domain, and the C-terminus molybdopterin domain] [36, 37]. Many XO were reported to have a dimeric structure consists of two protein subunits such as; 290 kDa human milk XO [34], 283 kDa bovine milk XO [15], 300 kDa bovine milk XO [30; 32], 300 kDa mouse liver XO [38, 39] and 300 kDa human liver XO [33]. The BMXO enzyme has p*I* value of 6.2 similar to buffalo liver XO that has p*I* value between 6.0 -6.2 [40].

The effect of pH on BMXO activity was measured and the most stable pH range for the enzyme activity was found between pH 7.6 – 8.4. The optimum activity of BMXO enzyme was found at pH 8.0 (Fig. 3a). Different pH optima were reported for xanthine oxidase, bovine milk pH 8.6 [41] and pH 7.5 [42], goat milk pH 7.2 – 7.4 [42], the rabbit liver pH 8.1 [43] and buffalo liver pH 7.6 [40]. The Km value was calculated from the Lineweaver-Burk plot for the reverse of the reaction velocity (1/V) and substrate concentration (1/[S]) to be 1 mM xanthine (Fig. 3b). Therefore, BMXO enzyme seems to have higher affinity towards xanthine in comparison to cow milk 8.6 mM xanthine and goat milk 43.6 mM xanthine [42]. The BMXO was inhibited with $NiCl_2$, $MgCl_2$, $ZnCl_2$ and $CuCl_2$. However, it was activated by $MnCl_2$ and $FeCl_2$ (Fig. 4a). These results were consistent with that of xanthine oxidase from bovine milk which was inhibited by Cu^{+2}, Hg^{+2} and Ag^{+} ions [36].

Allopurinol is found to be the most powerful inhibitor of BMXO enzyme since 5 mM almost inhibited the enzyme completely (Fig. 4b). PMSF inhibited BMXO which indicates that, a serine residue is contained in the enzyme active site. Also, EDTA and 1, 10 phenanthroline inhibited BMXO activity indicating that BMXO is metalloenzyme. $K_2Cr_2O_7$ inhibited BMXO activity possibly due to the powerful oxidation capacity of $K_2Cr_2O_7$ that oxidizes the metal groups which are substantial for enzyme efficiency. A titration curve of allopurinol concentrations on the purified BMXO activity (Fig. 5a) indicated that 50% inhibition (I_{50}) = 0.8 mM allopurinol. The Hill plot slope was found to be 1 (Fig. 5b) deciding the presence of one bounding site for allopurinol on the purified BMXO molecule. Allopurinol inhibited BMXO enzyme activity competitively (Fig. 5c) since the presence of allopurinol increased the value of Km and did not alter the Vmax value. The inhibition of BMXO by allopurinol gives Ki value of 0.3 mM (Fig. 5d) indicating the potency of the allopurinol as inhibitor of xanthine oxidases. The purified BMXO is used in the construction of SOD diagnostic kit. The prepared kit was found to be sensitive similar to the commercial available kit from Sigma-Aldrich. The variance between the constructed and commercial kit was found more or less within the experimental error. In conclusion, the present research provides an easy, convenient and reproducible purification method for the xanthine oxidase from buffalo milk. The purified BMXO enzyme is well characterized and applied in SOD assay diagnostic kit preparation and compared with a commercial kit.

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