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Purification and Characterization of Xanthine Oxidase from Liver of the Sheep *(Ovis Aries)*

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Abstract

Xanthine oxidase is a commercially important enzyme with wide area of medical applications to develop diagnostic kits. Xanthine oxidase was extracted, purified and characterized from sheep liver (SLXO). The purification procedure involved acetone precipitation and chromatography on DEAE-cellulose and Sephacryl S-300 columns. The sheep liver xanthine oxidase was homogeneously purified 31.8 folds with 3.5 U/mg specific activity and 24.1% recovery. SLXO native molecular weight was 150 kDa and on SDS-PAGE appeared as single major band of 75 kDa representing a homodimer protein. Isoelectric focusing of the purified SLXO resolved into two closely related isoforms with *pI* values of 5.6 and 5.8. The apparent Km for xanthine oxidase at optimum pH 7.6 was found to be 0.9 mM xanthine. $FeCl_2$ and $NiCl_2$ increased the activity of SLXO, while $CuCl_2$ and $ZnCl_2$ were found to be potent inhibitors of the purified enzyme. Allopurinol inhibits SLXO competitively with one binding site on the purified molecule and Ki value of 0.06 mM.

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Introduction

Xanthine oxidase (XO) (EC 1.2.3.22) plays a key physiological role in the metabolism of purines that catalyzes the hydroxylation reaction of hypoxanthine to xanthine, and subsequently xanthine to uric acid [1]. XO is a molybdo-flavin enzyme with a quaternary structure; each subunit contains molybdopterin, Flavin adenine dinucleotide (FAD), and iron-sulfur center [2-4]. It can be found in almost all species from bacteria to human and in the various tissues of mammals [5]. XO is widely used as a detection reagent for nucleotidases, purines, superoxide dismutases, adenosine deaminase, and inorganic phosphates [6]. XO which is mostly found in the small intestine and liver gave rise to the idea that this enzyme could be responsible for detoxifying the body from mainly nitrogen containing polar aromatic compounds believed that even poor substrates for XO would be completely metabolized by this enzyme, considering its relatively high abundance in the human body. It plays a role in iron absorption in the small intestine, by oxidizing dietary iron from the ferrous state (Fe^{2+}) to the ferric state (Fe^{3+}) within the intestinal mucosa, facilitating the absorption of iron [7]. It also mobilizes iron from the liver by promoting the release of iron ferritin [8]. It oxidizes pteridines, heterocyclic bases and participates in the oxidation of sulphydryl (thiol) groups in glutathione, fatty acids, phospholipids, amino acids and epinephrine [9, 10], catalyzes the oxidation of aldehydes to carboxylic acids [11] and plays a role in alcohol metabolism [8].

In addition, a role for xanthine oxidase in antimicrobial defence, similar to NADPH oxidase in phagocytes is possible. It has been proposed that xanthine dehydrogenase in the endothelial cell cytoplasm is released following endothelial injury by microbes, and is converted to the oxidase form in the oxygen rich environment of blood. Xanthine oxidase can oxidize substrates in the blood, leading to the formation of oxygen derived free radicals which have been suggested to provide oxidative defence [12, 13]. One of the most important xanthine oxidase competitive inhibitors is allopurinol that is a substrate analog, has similar chemical structure to hypoxanthine and used in treatment of hyperuricemia and gout [1, 14]. In the present study, we report the purification and characterization of xanthine oxidase from sheep liver as a safe source for using it in medical applications especially diagnostic kit of superoxide dismutase (SOD).

Materials and Methods

Liver Materials

Six fresh sheep liver samples were obtained from different male individuals in a local slaughter-house, Cairo, Egypt and stored at -40 °C.

Chemicals

Xanthine sodium salt, Nitroblue tetrazolium (NBT), Phenazine methosulphate (PMS), Albumin from bovine serum (BSA), Diethylaminoethyl-cellulose (DEAE-Cellulose), Sephacryl S-300 and chemicals for electrophoresis were purchased from Sigma-Aldrich Chemical Co. The other chemicals were of analytical grade.

Assay of Xanthine Oxidase Activity

The reaction mixture of XO activity assay contains 1 ml 0.05 M Tris-HCl, pH 7.6 requiring 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 [15].

Xanthine Oxidase Activity Staining on Polyacrylamide Gels

Activity staining of xanthine oxidase was carried out by submerging the gels 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 [16].

Purification of Xanthine Oxidase from Sheep Liver

Preparation of Crude Extract

All of the procedures were performed at 4° C. 10 gm of sheep liver were minced and homogenized with 0.02 M Tris-HCI buffer, pH 7.6, containing 0.1 mM EDTA on ice and mixed with one volume of n-butanol. The mixture was kept at -20°C overnight and centrifuged at 12000 x g for 30 min at 4°C. The aqueous phase containing the enzyme activity was saved and designated n-butanol fraction. One volume

prechilled acetone was added to the n-butanol fraction. The pellet was collected after centrifugation at 12000 x g for 30 min at 4°C, washed three times with acetone and dried under vacuum. The acetone powder was dissolved in 0.02 M Tris-HCI buffer, pH 7.6, containing 0.1 mM EDTA and designated acetone fraction [17].

DEAE-Cellulose Column Chromatography

The acetone fraction was applied on DEAE-cellulose column (6 x 2.4 cm i.d.) equilibrated with 0.02 M Tris-HCI buffer, pH 7.6, containing 0.1 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 60 ml / hour. 5 ml fractions were collected and the fractions containing XO activity were pooled and concentrated by lyophilization.

Sephacryl S-300 Column Chromatography

The concentrated DEAE –cellulose fractions containing XO activity were 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-HCI buffer, pH 7.6, containing 0.1 mM EDTA at a flow rate of 30 ml / hour and 2 ml fractions were collected.

Electrophoretic Analysis

Native gel electrophoresis was carried out with 7% PAGE [18]. SDS-PAGE was performed with 12% polyacrylamide gel [19]. The subunit molecular weight of the purified XO was determined by SDS-PAGE [20]. Electrofocusing was performed and the isoelectric point (*pI*) value was calculated from a calibration curve [21, 22]. Coomassie brilliant blue R-250 was used to stain the proteins.

Protein Determination

Protein content was determined by the dye (Coomassie Brilliant Blue G-250) binding assay method using BSA as a standard protein [23].

Results and Discussion

Purification of Sheep Liver Xanthine Oxidase

Xanthine oxidase was a commercially important enzyme with wide area of applications [24, 25]. Sheep liver XO was purified using a purification scheme consisting of the following steps, mixing crude extract with n-butanol, acetone precipitation, anion-exchange chromatography on DEAE-cellulose column and gel filtration chromatography on Sephacryl S-300 column. The method is relatively short and involves only two chromatographic steps that seem to be simple and convenient method. Different purification methods of XO were reported as that of rat liver XO [26, 27] and buffalo milk XO [28]. The elution profile of DEAE-cellulose column (Fig. 1a) indicated one peak containing XO activity that XO was eluted as single peak at 0.05M NaCl and designated sheep liver xanthine oxidase (SLXO) Fig. 1b). After chromatography on the Sephacryl S-300 column, SLXO was purified 31.8-fold with a specific activity of 3.5 units / mg protein and 24.1% recovery (Table 1). Buffalo milk xanthine oxidase; 10.66 unit/mg [28], bovine milk xanthine oxidase; 1.086 unit/mg [24], 0.3 unit/mg [29] and 15.8 unit/mg [30], human liver xanthine oxidase; 0.00061 unit/mg [31], and 0.036 unit/mg [32], buffalo liver xanthine oxidase; 7.2 unit/mg [33], rat liver xanthine oxidase; 14 unit/mg [27] and Arthrobacter M3 was 8.6 unit/mg [13].

Table 1. A typical purification scheme of the sheep liver xanthine oxidase (SLXO):

Purification steps	Total protein (mg)	Total Activity (unit)	Specific Activity	Yield (%)	Fold Purification
n-Butanol extract	145.8	17.4	0.11	100	1.0
Acetone fraction	105	14	0.13	80.4	1.2
DEAE-cellulose fraction	15	8.3	0.55	47.7	5.0
Sephacryl S-300 fraction	1.2	4.2	3.5	24.1	31.8

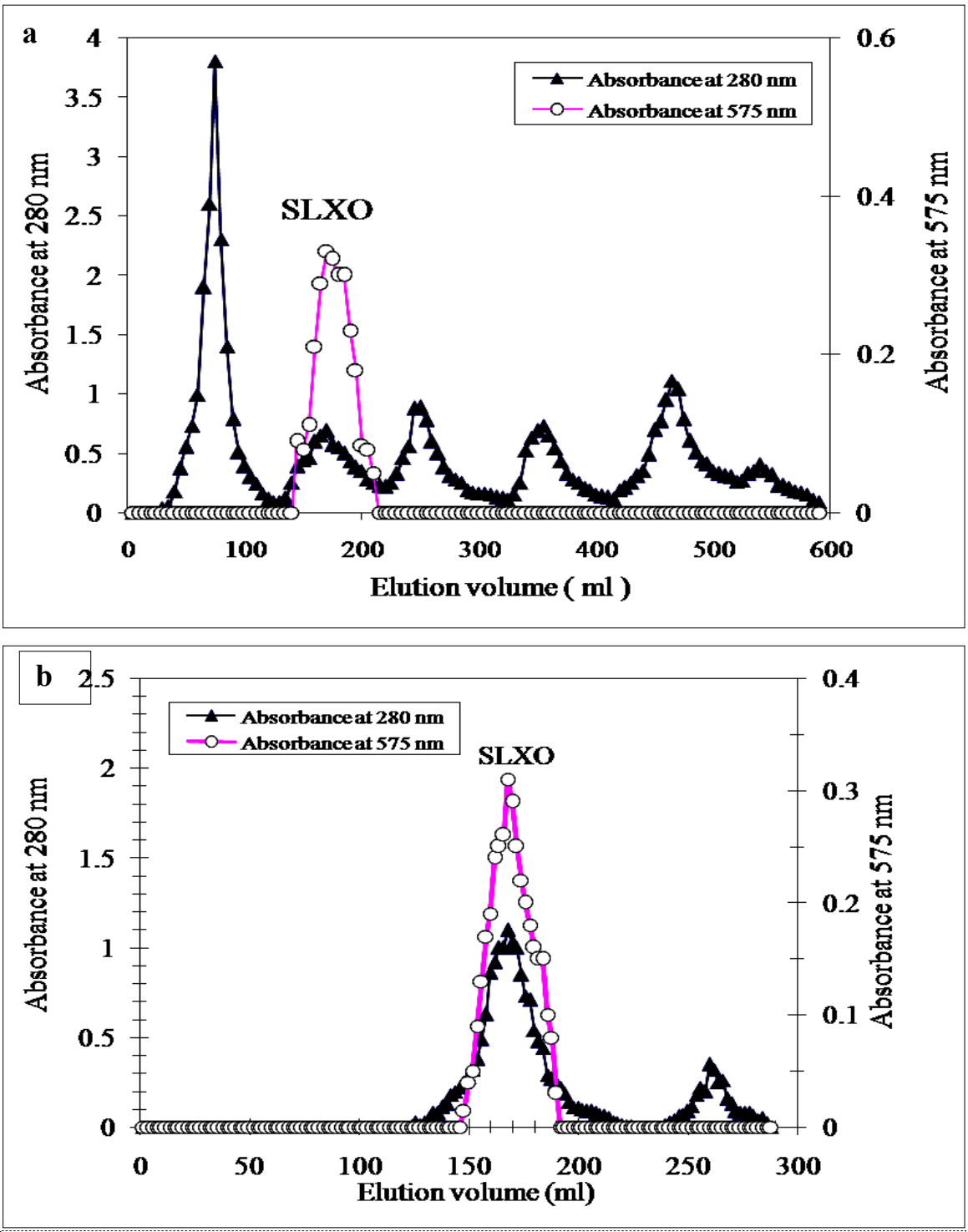

Figure 1. A typical elution profile for the chromatography of the sheep liver acetone fraction on DEAE-cellulose column (6 cm x 2.4 cm i.d.) previously equilibrated with 0.02 M Tris-HCI buffer, pH 7.6 containing 0.1 mM EDTA. The proteins were eluted by a stepwise gradient of NaCl ranging from 0 to 1 M in the equilibration buffer and 5 ml fractions were collected at a flow rate of 60 ml / h. (b) A typical elution profile for the chromatography of the sheep liver DEAE-cellulose fraction on Sephacryl S-300 column (142 cm x 2.4 cm i.d.) previously equilibrated with 0.02 M Tris-HCI buffer, pH 7.6 containing 0.1 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 Analysis of SLOX

Electrophoretic analysis of n-butanol extract, DEAE-cellulose fraction and Sephacryl S-300 purified fraction of SLXO on 7 % native PAGE revealed single protein band corresponded the enzyme activity band of the purified xanthine oxidase enzyme (Fig. 2a). Gel filtration chromatography depicted only one activity peak, with a native molecular mass of 150 kDa. The subunit molecular weight of SLXO was determined by SDS-PAGE (Fig. 2b) to be 75 kDa which revealed that the SLXO consists of two homodimer subunits. Many XO were reported to have dimeric structure such as 300 kDa for mouse liver [34], human liver [32] and rat liver [26]. The xanthine oxidase from the bacterium Arthrobacter M3 showed two polypeptides with SDS-PAGE of molecular weights of 35 kDa and 100 kDa [13]. The isoelectric point of SLXO enzyme was estimated by isoelectric focusing PAGE (Fig. 2c) as two major molecular species with a value of 5.6 and 5.8. Isoelectric pH of XO in buffalo liver were 6 and 6.2 [33], mouse liver was 6.7 [34] and rat liver were 6.13, 6.23 and 6.07 [27].

Determination of SLXO Optimum pH and Km Value

The effect of pH on the activity of sheep liver xanthine oxidase SLXO was examined in 0.05 M potassium phosphate buffer, pH (5.8-7.0) and 0.05 M Tris–HCl buffer, pH (7.2-9.0). The pH profile of SLXO displayed an optimum activity at pH 7.6 (Fig. 3a). The optimum pH of the rabbit liver XO was found at pH 8.1 [34]. The Km value was calculated from the Lineweaver-Burk plot for the reciprocal of the reaction velocity (1/v) and substrate concentration (1/[S]) (Fig. 3b) using xanthine as substrate. SLXO enzyme has a Km value of 0.9 mM (Fig. 3b) indicating the high affinity of SLXO toward xanthine. Various Km values for XO were reported as; 1.1 mM xanthine for buffalo liver [33], 3.4 µM xanthine for Mouse liver [34], 22 µM for rabbit liver [35] and 53 µM for rat liver XO was [27].

Effect of Divalent Cations and Various Inhibitors on SLXO

The effect of metal compounds on the purified SLXO activity was examined (Table 2). Divalent cations, such as $CuCl_2$, $MgCl_2$, $MnCl_2$, $CaCl_2$, $CoCl_2$ and $ZnCl_2$ inhibited the activity of sheep liver xanthine oxidase (SLXO) whereas $FeCl_2$ and $NiCl_2$ increased its activity. These results were consistent with that of cow milk xanthine oxidase which was inhibited by Cu^{+2}, Hg^{+2} and Ag^{+} ions [36].

Furthermore, the inhibition of purified SLXO activity by several inhibitors was studied (Table 3). 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 purified SLXO was not inhibited with the serine protease inhibitor PMSF indicating that the active site of this isoenzyme doesn't contain a serine residue. No inhibition of SLXO by B mercaptoethanol and dithiothretol was observed indicating that no role of -SH groups in the enzyme activity. Iodoacetamide inhibited the purified SLXO activity which indicates that methionine, cysteine and histidine residues play important role in the structure and activity of the enzyme. The inhibition of the purified SLXO isoenzyme by the metal chelator EDTA indicates that SLXO isoenzyme is metalloenzyme. The inhibition of SLXO activity with $K_2Cr_2O_7$ was probably due to strong oxidizing power of $K_2Cr_2O_7$ that may cause oxidation of metal prosthetic groups in enzyme that important to enzyme activity. Allopurinol was found to be the most potent inhibitor of the purified SLXO. The effect of allopurinol concentrations on the purified SLXO indicated that 50% inhibition (I_{50}) is caused by 0.1 mM allopurinol and the maximum inhibition of the enzyme (95.8%) was achieved by 1 mM allopurinol. However, full exploitation of these data required knowledge of the number of inhibitor molecules bound per enzyme molecule. Therefore, from the titration curve data (Fig. 4a), a linear relationship was observed by constructing the Hill plot for the inhibition of the purified SLXO by allopurinol (Fig. 4b). The slope of the Hill plot was found to be 1.19 indicating the existence of one binding site for allopurinol on the purified SLXO. The type of inhibition of the purified SLXO by allopurinol was found to be competitive type, whereas the presence of allopurinol did not alter the Vmax value but increased the Km value (Fig. 4c). For the determination of the Ki value, the slopes of the reciprocal plots lines were plotted against the allopurinol concentration. The Ki value of the SLXO inhibition by allopurinol was found to be 0.06 mM directly from the intercept of the X axis of the plot (Fig. 4d) indicating the potency of the allopurinol as inhibitor

Table 2. Effect of divalent cations on the sheep liver xanthine oxidase (SLXO):

Reagent	Final Concentration (mM)	SLXO Residual activity (%)
Control	-----	100.0
$CoCl_2$	1.0	96.6
	2.0	75.5
$MnCl_2$	1.0	82.2
	2.0	66.6
$FeCl_2$	1.0	111.0
	2.0	127.0
$ZnCl_2$	1.0	68.8
	2.0	44.4
$CuCl_2$	1.0	75.0
	2.0	46.6
$NiCl_2$	1.0	108.3
	2.0	113.5
$MgCl_2$	1.0	102.8
	2.0	88.2
$CaCl_2$	1.0	91.4
	2.0	73.6

* These values represent % of the control and the means of triplicate experiments

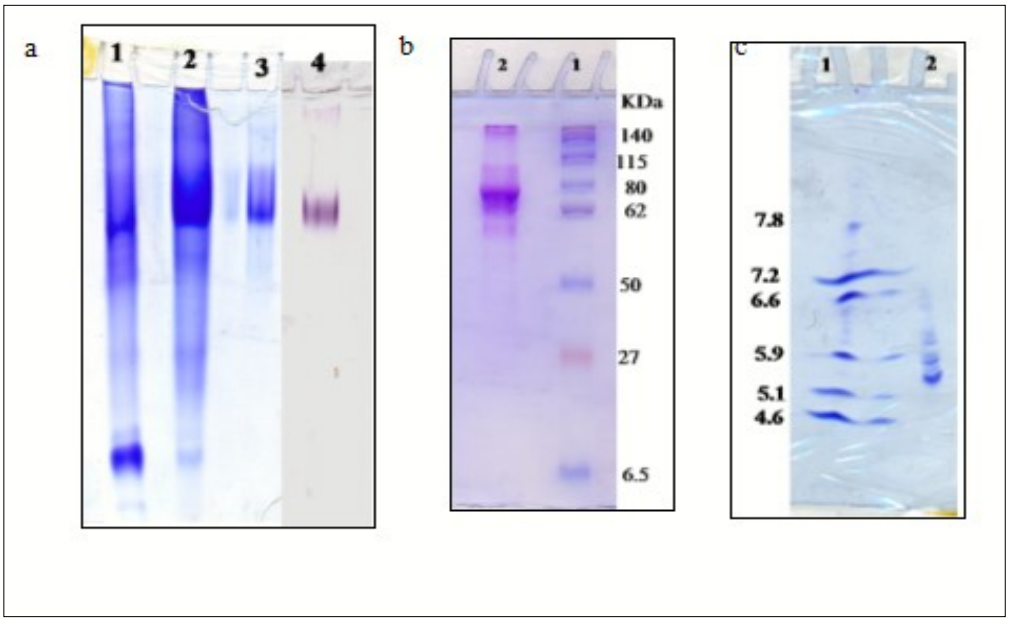

Figure 2. (a) Protein and XO isoenzyme pattern of sheep liver xanthine oxidase (SLXO) on 7 % native PAGE: (1) n-butanol extract, (2) DEAE-cellulose fraction, (3) Sephacryl S-300 purified fraction, and (4) SLXO isoenzyme pattern. (b) Subunit molecular weight determination by electrophoretic analysis of SLXO on 12 % SDS-PAGE: (1) Molecular weight marker proteins and (2) Purified SLXO. (c) Isoelectrofocusing: (1) isoelectric point (*pI*) marker proteins and (2) The purified sheep liver xanthine oxidase SLXO.

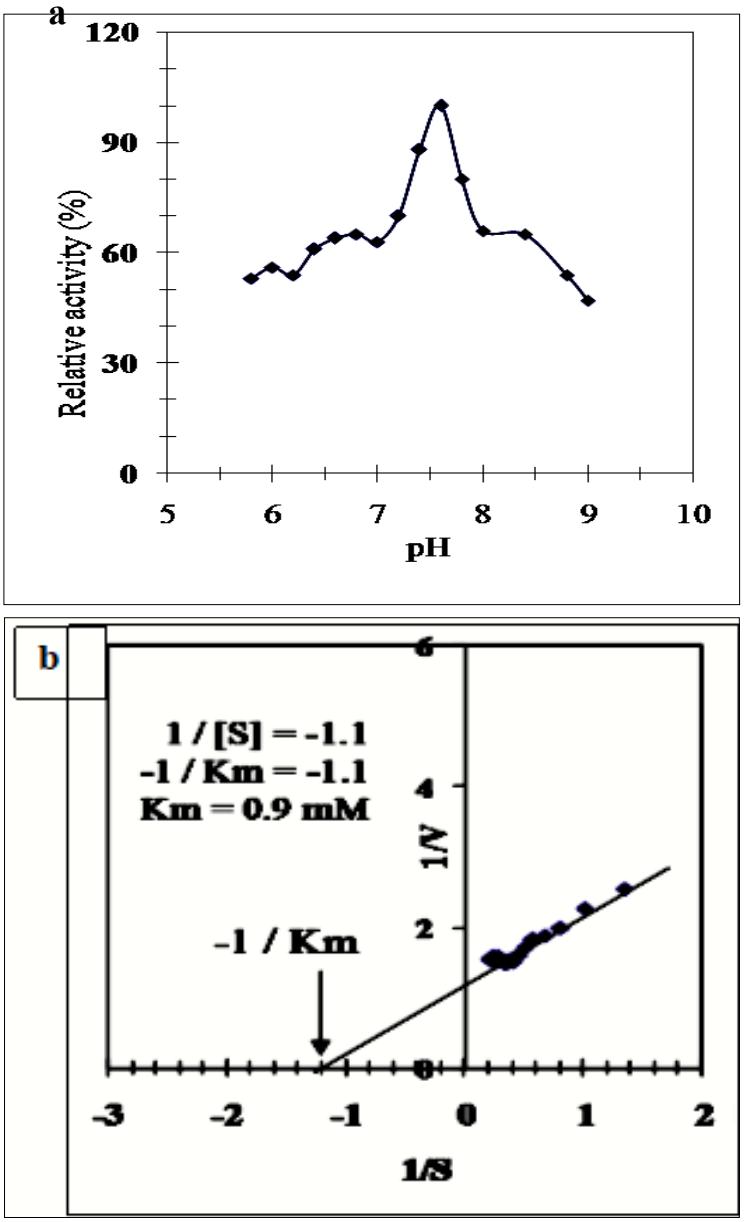

Figure 3. (a) Effect of pH on the purified sheep liver xanthine oxidase SLXO using 0.05 M potassium phosphate buffer, pH (5.8-7.0) and 0.05 M Tris–HCl buffer, pH (7.2-9.0). (b) Lineweaver-Burk plot relating the reciprocal of the reaction velocity of the purified SLXO to xanthine concentration in mM.

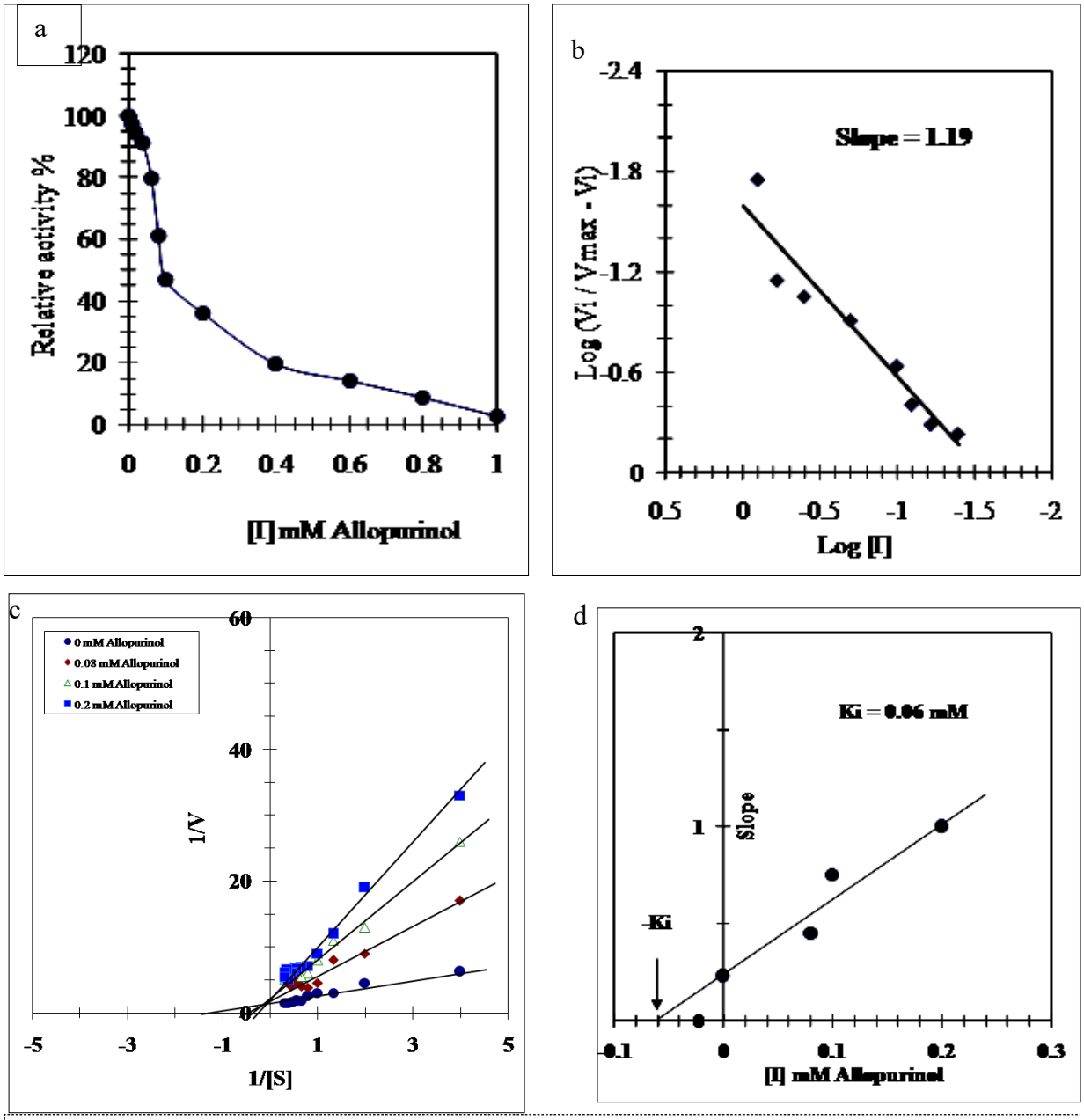

Figure 4. (a) Titration curve for inhibition of the purified SLXO by varying concentrations of allopurinol. (b) Hill plot for inhibition of the purified SLXO by varying concentrations of allopurinol. (c) Lineweaver-Burk plots showing the type of inhibition of the purified SLXO by allopurinol. (d) Determination of the inhibition constant (Ki) value for the inhibition of the purified SLXO by allopurinol.

Table 3. Effect of inhibitors on the purified sheep liver xanthine oxidase (SLXO):

Reagent	Final Concentration (mM)	SLXO Inhibition (%)
Control	-----	0.0
Allopurinol	1 mM	95.8
	2 mM	100.0
Ethylenediamine tetraacetic acid (EDTA)	2 mM	18.0
	5 mM	25.8
DL-Dithiothreitol (DTT)	2 mM	1.4
	5 mM	3.5
Iodoacetamide	2 mM	17.5
	5 mM	31.9
Hydrogen peroxide (H_2O_2)	2 mM	12.5
	5 mM	22.5
β-Mercaptoethanol	2 mM	0.0
	5 mM	0.0
1,10 Phenanthroline	2 mM	1.8
	5 mM	4.7
Phenylmethylsulfonyl-fluoride (PMSF)	2 mM	0.0
	5 mM	0.0
Potassium cyanide (KCN)	2 mM	0.0
	5 mM	0.0
Potassium dichromate ($K_2Cr_2O_7$)	2 mM	57.4
	5 mM	88.7
Sodium azide (NaN_3)	2 mM	10.0
	5 mM	27.2
Sodium dodecyl sulfate (SDS)	2 mM	14.5
	5 mM	26.3

* These values represent % of the control and the means of triplicate experiments

of XO. It is well known that, allopurinol is used as a drug for treating the elevated levels of uric acid in human by inhibiting xanthine oxidase and preventing the conversion of hypoxanthine and xanthine into uric acid.

Conclusion

In conclusions, the present study is the first study to report purification of sheep liver xanthine oxidase (SLXO). This study presents a simple, convenient and reproducible method for the purification of well characterized xanthine oxidase from sheep liver as locally available sources. Production of this enzyme on large scale will make it suitable for various medical applications as preparation of SOD diagnostic kit and a detection reagent for nucleotidase, purines, adenosine deaminase and phosphates.

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