

Purification and characterization of xanthine oxidase from liver of the water buffalo *Bubalus bubalis*

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ABSTRACT

Xanthine oxidase (XO) is an important enzyme with broad medical applications as detecting reagent in many diagnostic kits. In this study, buffalo liver xanthine oxidase (BLXO) was purified to homogeneity by acetone precipitation and chromatography on DEAE-cellulose and Sephacryl S-300 columns with a specific activity of 7.2 units / mg protein which represent 31.3 folds. The native molecular weight of the purified enzyme is 200 kDa and its subunit molecular weight was determined by SDS-PAGE to be 67 kDa. The isoelectric point (pI) value of BLXO isoenzyme is at pH 6.0 – 6.2. It displayed its pH optima at 7.6 and the Km value is 1.1 mM xanthine. FeCl₂ increased the activity of BLXO while CuCl₂, MnCl₂ and ZnCl₂ were found to be inhibitors of the purified enzyme. Allopurinol inhibits BLXO competitively and has one binding site on it with Ki value of 0.025mM.

Abbreviations: BSA, Bovine serum albumin, XO, Xanthine oxidase, NBT, Nitroblue tetrazolium, PAGE, Polyacrylamide gel electrophoresis, PMS, Phenazine methosulphate, BLXO, Buffalo liver xanthine oxidase

INTRODUCTION

Xanthine oxidase (XO) (EC 1.2.3.22) catalyzes the oxidation of xanthine and hypoxanthine into uric acid (Nguyen *et al.*, 2005). XO has a quaternary structure in which each subunit contains molybdopterin, flavin adenine dinucleotide and iron-sulfur center (Kuwabara *et al.*, 2003). This enzyme is widely used as a detector for nucleotidases, purines, superoxide dismutases, adenosine deaminase, phosphates of blood serum and in the determination of liver diseases (Heinz *et al.*, 1980; Groot and Noll, 1985). It had also been reported as playing vital roles in innate immune system (Vorbach *et al.*, 2003), diseases of cardiovascular system (Berry and Hare, 2004) and employed as antimicrobial agent (Martin *et al.*, 2004, Zhang *et al.*, 2012). XO plays important roles in hyperuricemia and gout since XO inhibitors; allopurinol, oxypurinol and febuxostat have been used widely for the treatment of such disorders. The inhibition of XO reduces the vascular oxidative stress and circulating levels of uric

acid. In addition, the superoxide anion radicals generated by XO are involved in various pathological processes such as liver diseases, inflammatory diseases, aging, and carcinogenesis. Thus, XO inhibitors may be useful for treatment of many other diseases (Nguyen *et al.*, 2005; Higgins *et al.*, 2011). Xanthine oxidase has been purified from several sources including human liver (Krenitsky *et al.*, 1986; Moriwaki *et al.*, 1993), rabbit liver (Catignani *et al.*, 1975), mouse liver (Carpani *et al.*, 1990; McManaman *et al.*, 1996; Maia and Mira, 2002), rat skeletal muscle (Lalanne and Willemot, 1975), human milk (Abadeh *et al.*, 1992), cow milk (Waud *et al.*, 1975; Nishino *et al.*, 1981; Spitsberg and Gorewit, 1998; Ozer *et al.*, 1999; Belattar, 2009), *Arthrobacter sp* bacteria (Xin *et al.*, 2012; Zhang *et al.*, 2012). In this study, we purify and characterize XO from the buffalo liver for using it in preparation of superoxide dismutase diagnostic kit which has wide use in medical applications.

MATERIALS AND METHODS

Liver material

Six fresh buffalo liver samples were obtained from different individuals in a local slaughter-house and stored at -40 °C.

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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 XO activity assay reaction 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 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 (Agarwal and Banerjee, 2009).

Xanthine oxidase activity staining on polyacrylamide gels

Activity staining of xanthine oxidase was determined as described by (Ozer *et al.*, 1998). 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.

Purification of xanthine oxidase from buffalo liver

Preparation of crude extract

All of the procedures were performed at 4° C. 100 gm of liver were minced and homogenized with 0.02 M Tris/HCl 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 \times 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 \times 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/HCl buffer, pH 7.6, containing 0.1 mM EDTA and designated acetone fraction (Oida *et al.*, 1984).

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/HCl 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 / hr. 5 ml fractions were collected and the fractions containing XO activity were pooled and concentrated by lyophilization.

Sephacryl S-300 column Chromatography

The concentrated material 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 0.1 mM EDTA at a flow rate of 30 ml / h and 2 ml fractions were collected.

Electrophoretic analysis

Native gel electrophoresis was carried out with 7% PAGE (Smith, 1969). SDS-PAGE was performed with 12% polyacrylamide gel (Laemmli, 1970). The subunit molecular weight of the purified XO enzyme was determined by SDS-PAGE (Weber and Osborn, 1969). Electrofocusing was performed and the isoelectric point (pI) value was calculated from a calibration curve (O'Farrell, 1975; Ubuka *et al.* 1987). Coomassie brilliant blue R-250 was used to stain the proteins.

Protein determination

Protein content was determined by the dye binding assay method (Bradford, 1976) using BSA as a standard protein.

RESULTS

Purification of XO enzyme from buffalo liver

The XO specific activity of the buffalo liver was found to be 0.23 units/mg protein. A typical purification scheme of XO from the buffalo liver is presented in table (1). The DEAE-cellulose elution profile (Fig. 1a) revealed the presence of one peak containing XO activity designated BLXO and eluted with 0.05 M NaCl. The DEAE-cellulose fractions of this peak were pooled, concentrated by lyophilization and applied onto a Sephacryl S-300 column. Sephacryl S-300 column (Fig. 1b) revealed the presence of one peak of BLXO enzyme activity. The BLXO was purified with a specific activity of 7.2 units / mg protein and 31.3 folds purification over the crude extract with 33.8% yield (Table 1).

Table 1: A typical purification scheme of the buffalo liver xanthine oxidase BLXO:

| Purification steps | Total protein (mg) | Total Activity (unit) | Specific Activity | Yield (%) | Fold Purification |
|--------------------------|--------------------|-----------------------|-------------------|-----------|-------------------|
| Acetone fraction | 84.4 | 19.5 | 0.23 | 100 | 1.0 |
| DEAE-Cellulose fraction | 10.4 | 11.8 | 1.13 | 60.5 | 4.9 |
| Sephacryl S-300 fraction | 0.92 | 6.6 | 7.2 | 33.8 | 31.3 |

Molecular weight determination by gel filtration

The native molecular weight of BLXO eluted from Sephacryl S-300 column was deduced from a calibration curve to be 200 kDa.

Electrophoretic analyses of BLXO

Samples from crude extract, DEAE-cellulose and Sephacryl S-300 fractions of BLXO were analyzed electrophoretically on 7 % native PAGE (Fig. 2a). Single protein band coincided with the enzyme activity band of the purified xanthine oxidase indicating the tentative purity of the preparation. SDS-PAGE of denatured purified BLXO enzyme calculated the subunit molecular weight to be 67 kDa (Fig. 2b). The pI value of BLXO was calculated from a calibration curve to be at pH 6.0-6.2 (Fig. 2c).

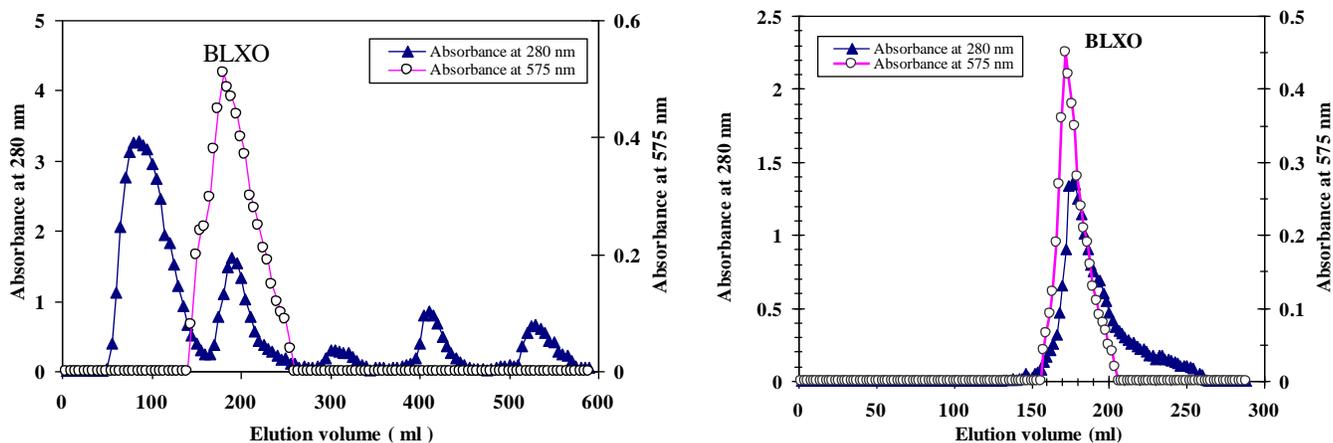


Fig. 1: (a) A typical elution profile for the chromatography of the buffalo liver on DEAE-cellulose column (6 cm x 2.4 cm i.d.) previously equilibrated with 0.02 M Tris-HCl buffer, pH 7.6 containing 0.1 mM EDTA. The proteins were eluted by a stepwise gradient of NaCl ranging from 0 to 1 M in the equilibration buffer and 5 ml fractions were collected at a flow rate of 60 ml / h. (b) A typical elution profile for the chromatography of BLXO 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 0.1 mM EDTA. The proteins were eluted by the same buffer and 2 ml fractions were collected at a flow rate of 30 ml / h.

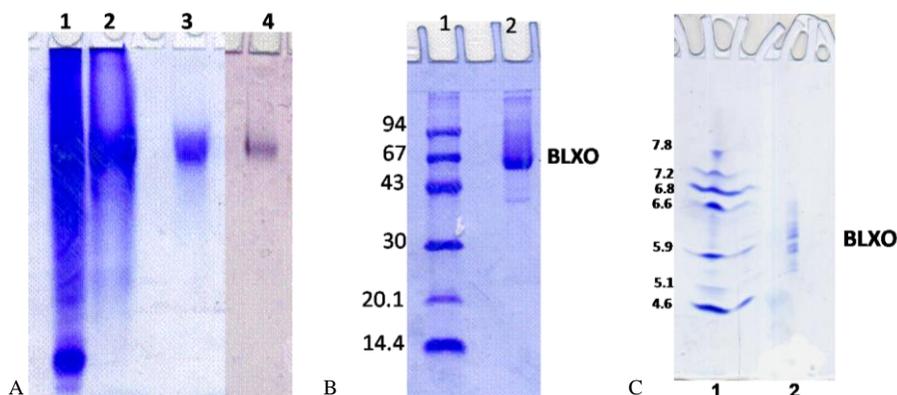


Fig. 2: (a) Electrophoretic analysis of protein pattern of BLXO on 7 % native PAGE: (1) crude extract, (2) DEAE-cellulose fraction, (3) Sephacryl S-300 purified fraction and (4) Isoenzyme pattern of purified BLXO. (b) Molecular weight determination of BLXO subunit by 12% SDS-PAGE: (1) Low molecular weight marker proteins and (2) Purified BLXO. (c) Isoelectric focusing: (1) Isoelectric point (*pI*) marker proteins, (2) The purified BLXO.

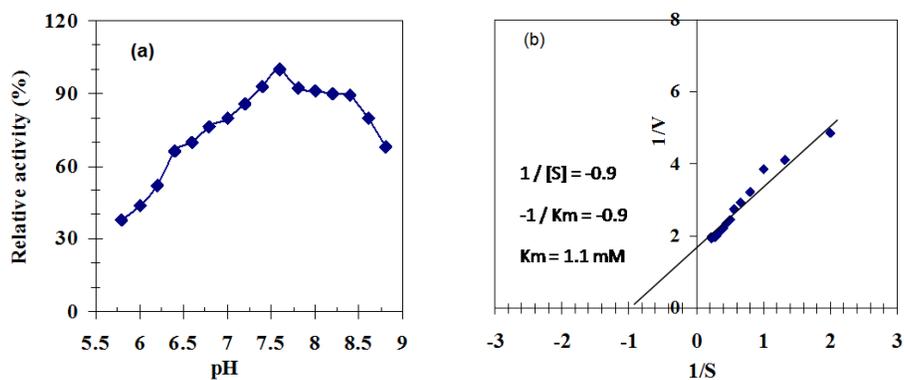


Fig. 3: (a) Effect of pH on the purified BLXO using 0.05 M potassium phosphate buffer, pH (5.8-8.0) and 0.05 M Tris-HCl buffer, pH (8.0-9.0). (b) Lineweaver-Burk plot relating the reciprocal of the reaction velocity of the purified BLXO to xanthine concentration in mM.

Determination of BLXO optimum pH and Km value

The effect of pH on the activity of BLXO was set in potassium phosphate and Tris-HCl buffers. The pH profile of BLXO displayed its optimum activity at pH 7.6 (Fig. 3a). The Km value was calculated from the Lineweaver-Burk plot for the reciprocal of the reaction velocity (1/v) and substrate concentration (1/[S]) to be 1.1 mM xanthine (Fig. 3b).

Effect of divalent cations

The purified buffalo liver xanthine oxidase was preincubated with 1 mM and 2 mM of each cation at 37°C and the activity was assayed. The data presented in Table (2) show the activity of BLXO in the presence of various cations. A control test without any cation was taken as 100 % relative activity. FeCl₂ activated the BLXO enzyme while CuCl₂, MnCl₂ and ZnCl₂ were

found to be potent inhibitors of buffalo liver xanthine oxidase BLXO activity.

Table 2: Effect of divalent cations on the buffalo liver xanthine oxidase BLXO:

| Reagent | Final Concentration (mM) | BLXO Residual activity (%) |
|-------------------|--------------------------|----------------------------|
| Control | ---- | 100.0 |
| CoCl ₂ | 1.0 | 88.2 |
| | 2.0 | 85.1 |
| MnCl ₂ | 1.0 | 48.8 |
| | 2.0 | 41.8 |
| FeCl ₂ | 1.0 | 121.6 |
| | 2.0 | 138.4 |
| ZnCl ₂ | 1.0 | 22.8 |
| | 2.0 | 16.6 |
| CuCl ₂ | 1.0 | 31.3 |
| | 2.0 | 30.0 |
| NiCl ₂ | 1.0 | 77.3 |
| | 2.0 | 101.5 |
| MgCl ₂ | 1.0 | 78.8 |
| | 2.0 | 66.2 |
| CaCl ₂ | 1.0 | 92.1 |
| | 2.0 | 66.2 |

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

Effect of various inhibitors

The purified buffalo liver xanthine oxidase was preincubated with each inhibitor for 5 min at 37°C and the inhibition % was calculated as a ratio of a control without inhibitor. Allopurinol is the strongest inhibitor of BLXO enzyme (Table 3).

Table 3: Effect of inhibitors on the purified buffalo liver xanthine oxidase BLXO:

| Reagent | Final Concentration (mM) | BLXO Inhibition (%) |
|---|--------------------------|---------------------|
| Control | ---- | 0.0 |
| Allopurinol | 1 mM | 98.8 |
| | 2 mM | 100.0 |
| Ethylenediamine tetraacetic acid (EDTA) | 2 mM | 4.5 |
| | 5 mM | 17.9 |
| DL-Dithiothreitol (DTT) | 2 mM | 1.4 |
| | 5 mM | 3.5 |
| Iodoacetamide | 2 mM | 12.2 |
| | 5 mM | 37.6 |
| Hydrogen peroxide (H ₂ O ₂) | 2 mM | 37.3 |
| | 5 mM | 55.8 |
| β-Mercaptoethanol | 2 mM | 2.2 |
| | 5 mM | 8.5 |
| 1,10 Phenanthroline | 2 mM | 0.0 |
| | 5 mM | 2.5 |
| Phenylmethylsulfonyl-fluoride (PMSF) | 2 mM | 1.2 |
| | 5 mM | 10.4 |
| Potassium cyanide (KCN) | 2 mM | 1.5 |
| | 5 mM | 4.8 |
| Potassium dichromate (K ₂ Cr ₂ O ₇) | 2 mM | 44.5 |
| | 5 mM | 78.4 |
| Sodium azide (NaN ₃) | 2 mM | 2.8 |
| | 5 mM | 20.9 |
| Sodium dodecyl sulfate (SDS) | 2 mM | 69.9 |
| | 5 mM | 92.5 |

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

Kinetics of BLXO inhibition by Allopurinol

Allopurinol inhibited BLXO enzyme activity vigorously. A titration curve of allopurinol concentrations on the purified BLXO activity (Fig. 4a) indicated that 50% inhibition (I_{50}) = 0.05 mM allopurinol. The maximum inhibition of the enzyme (98.8 %) was achieved by 1 mM allopurinol. A linear relationship was observed by constructing the Hill plot for the inhibition of BLXO by allopurinol. The slope of the Hill plot is 0.8 (Fig. 4b), and allopurinol inhibited the BLXO activity competitively (Fig. 4c). The K_i value of the BLXO inhibition by allopurinol is determined to be 0.025 mM (Fig. 4d).

DISCUSSION

Xanthine oxidase is a commercially important enzyme with a wide area of applications (Ozer *et al.*, 1999). This study aims at purification of XO from buffalo liver to be used in preparation of SOD diagnostic kit. In this study, buffalo liver XO was purified by acetone fractionation, anion-exchange and gel filtration columns. Various purification procedures of XO were reported like that of mouse skeletal muscle; purified by ammonium sulfate fractionation, anion-exchange and affinity chromatographies (Lalanne and Willemot, 1975). XO from rat liver was purified by heat denaturation, ammonium sulphate precipitation, and chromatography on hydroxylapatite and Q-Sepharose Fast Flow columns (Maia and Mira, 2002). Also, rat liver XO was purified by heat denaturation, ammonium sulphate precipitation and Chromatography on benzamidine-Sepharose column (McManaman *et al.*, 1996). The chromatography of buffalo liver XO (BLXO) on DEAE-cellulose column revealed the presence of one XO activity peak eluted with 0.05 M NaCl (Fig. 1a). BLXO was purified from Sephacryl S-300 column (Fig. 1b) with a specific activity of 7.2 units / mg protein, 31.3 folds purification over the crude extract and 33.8% yield (Table 1). XO was purified from mouse liver 302-fold with 3.6% yield (Carpani *et al.*, 1990), from rabbit liver 330-fold with 16% yield (Catignani *et al.*, 1975) and from rat liver 1200-fold with 20% yield (Maia and Mira, 2002).

The purity of BLXO was investigated by analysis on 7% native-PAGE. BLXO turned out the presence of a major protein band which coincided with the enzyme activity band confirming that the single protein band is the enzyme band (Fig. 2a). The gel filtration chromatography calculated BLXO molecular weight to be 200 kDa (Fig. 1b). The subunit molecular weight of BLXO isoenzyme was determined by SDS-PAGE to be 67 kDa (Fig. 2b). In comparison with the native molecular weight, it was found that BLXO is homotrimer protein composed of three subunits. Many XO were reported to have a dimeric structure such as; human liver XO (Krenitsky *et al.*, 1986), mouse liver XO (Carpani *et al.*, 1990) and rat liver XO (Maia and Mira, 2002). The isoelectric point (pI) value is estimated for the purified BLXO isoenzyme from buffalo liver at pH 6.0 – 6.2 (Fig. 2c). Various isoelectric points (pI) values were reported as XO from mouse liver with pI value of 6.7 (Carpani *et al.*, 1990) and XO from rat liver with pI values of 6.13,

6.23 and 6.07 (McManaman *et al.*, 1996). The purified BLXO displayed its pH optima at 7.6 (Fig. 3a). Similarly, rabbit liver XO optimum pH was pH 8.1 (Catignani *et al.*, 1975). BLXO enzyme has a Km value of 1.1 mM xanthine (Fig. 3b) indicating the high affinity of BLXO toward xanthine. Mouse liver XO had Km value of 3.4 μ M xanthine (Carpani *et al.*, 1990), of rabbit liver XO was 22 μ M (Catignani *et al.*, 1975) and of rat liver XO was 53 μ M (McManaman *et al.*, 1996). FeCl₂ increased the activity of BLXO while CuCl₂, MnCl₂ and ZnCl₂ inhibited the activity of it (Table 2). This was similar to that of cow milk xanthine oxidase which was inhibited by Cu⁺², Hg⁺² and Ag⁺ ions (Mondal *et al.*, 2000). Iodoacetamide inhibited the purified BLXO isoenzyme activity which indicates that the active site of this enzyme has methionine, cysteine and histidine residues that have important effects on the structure and activity of the enzyme. The inhibition of the purified BLXO isoenzyme by the metal chelator EDTA indicates that BLXO isoenzyme is metalloenzyme. BLXO activity was inhibited with K₂Cr₂O₇ which may be due to strong oxidizing power of

K₂Cr₂O₇ that may cause oxidation of metal prosthetic groups in enzyme that important to enzyme activity (Table 3). Allopurinol was found to be the most potent inhibitor of the purified BLXO (Table 3). 0.06 mM allopurinol inhibited the purified BLXO 50% and the maximum inhibition of the enzyme was achieved by 1 mM (Fig. 4a). The slope of the Hill plot was found to be 0.8 (Fig. 4b) indicating the existence of one binding site for allopurinol on the purified BLXO. Allopurinol inhibited BLXO enzyme activity competitively (Fig. 4c) since the presence of allopurinol increased the Km value and did not alter the Vmax value. The Ki value of the BLXO inhibition by allopurinol is determined to be 0.025 mM directly from the intercept of the X axis of the plot (Fig. 4d). In conclusions, the present study reported buffalo liver xanthine oxidase (BLXO) and introduced a simple, convenient and reproducible method for the purification of well characterized xanthine oxidase from buffalo liver as a locally available source. Production of this enzyme on large scale will make it suitable for preparation of SOD diagnostic kit.

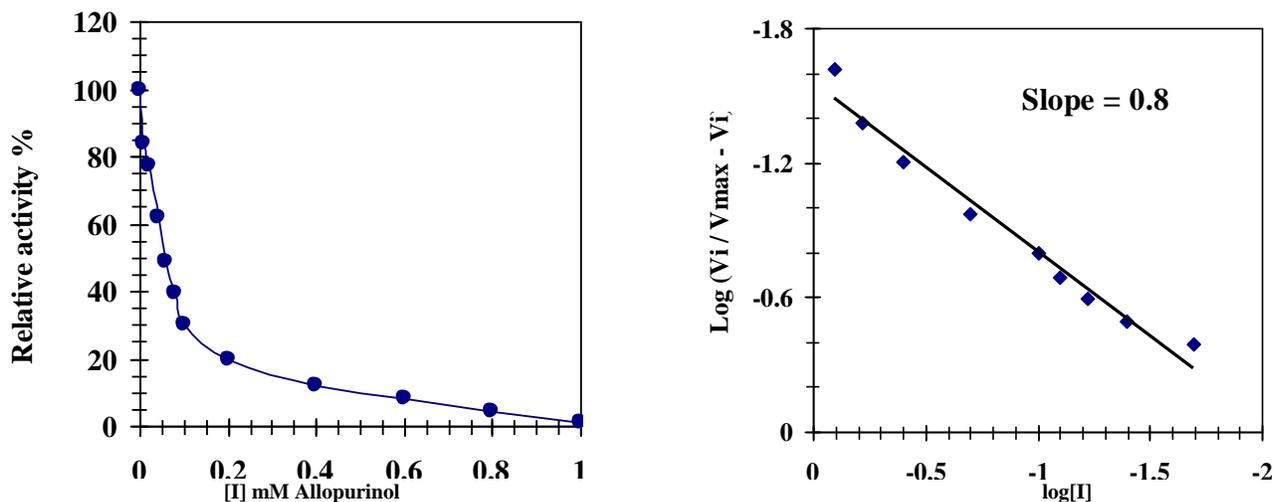


Fig. 4: (a) Inhibition of BLXO by varying concentrations of allopurinol. (b) Hill plot for inhibition of BLXO by allopurinol.

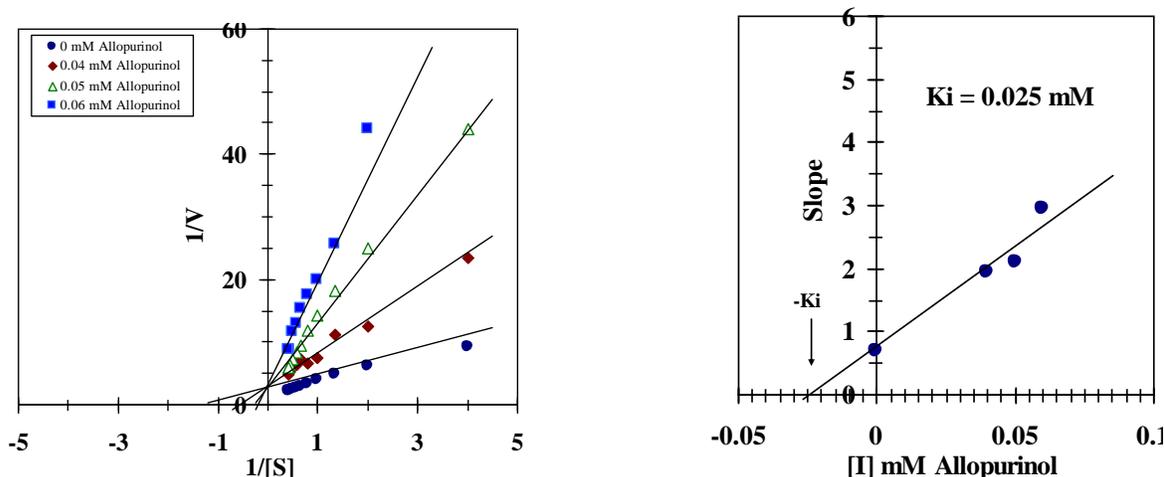


Fig. 4: (c) Lineweaver-Burk plots showing the type of inhibition of BLXO by allopurinol. (d) Determination of the inhibition constant (Ki) value for the inhibition of the BLXO by allopurinol.

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