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Partial Purification and Kinetic Study of Xanthine Oxidase from Serum of Hyperuricemia Patients

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Abstract:
The research included Xanthine oxidase isolation and purification from serum of patients with hyperuricemia and estimation its approximate molecular weight by using two techniques; gel filtration chromatography by using Sephadex G-200 and by electrophoresis by using SDS. Sixteen ml of serum as collected for isolation from patients with hyperuricemia from Al- Salam Hospital in Mosul, Iraq, for period from September to December 2021, their ages ranged from (25 years and above). The results showed that there are a significant increase in the activity of XO in serum of patients with hyperuricemia (29.23 ± 2.21) U/L compared to control group (8.38 ± 0.92) U/L at the probability level ($p \leq 0.05$). XO was isolated by different biotechnologies. One proteinous band was isolated from ammonium sulfate solution using gel filtration chromatography with specific activity (5.2) U/L, and the recovery percentage was (89.8%), and the number of purification times was equal to (7.1). The molecular weight was estimated using gel filtration and it was in the limits of 218776 Daltons approximately, While by using SDS-PAGE it was equal to 221513 Daltons approximately. Also the study determined to find the optimal conditions for the activity of XO, Where the maximum activity was obtained by using (150 µL) of the partially purified enzyme and pH (8.2), at temperature (40 ºC) with a rection time 5 minute and at concentration of (0.35 mM) of the substrate (xanthine). The maximum velocity (Vmax) and Michaelis-Menten constant (km) value were determined using Linweaver- Burk plot and it was equal to was (41.6) U/L and (0.0952) mM, respectively.

Keywords: Xanthine oxidase, Isolation, Hyperuricemia

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Introduction

Xanthine oxidase (XO) is an important enzyme that catalyzes the hydroxylation of hypoxanthine to xanthine and then xanthine to uric acid, which is excreted by the kidneys. Excessive production and/or inadequate excretion of uric acid leads to hyperuricemia [1]. It is found in milk, heart, lung, kidney, and lining of blood vessels. In humans, xanthine oxidase activity is highest in the liver and intestines [2]. Xanthine oxidase EC (1.17.3.2) is a homodimer with molecule weight of 290 kilo Dalton. Xanthine oxidase is from the molybdenum-protein family, which contain one molybdenum, one flavin adenine dinucleotide (FAD), and two iron-sulfur (2Fe-2S) centers of the ferredoxin type in each of its two independent subunits. The enzyme contains two separated substrate-binding sites [3, 4]. XO delivers electrons directly to molecular oxygen (O_2) and thus generates reactive oxygen species (ROS), superoxide anions ($O2^{\cdot-}$) and hydrogen peroxide (H_2O_2), by reducing one and two electrons respectively, resulting in the production of a hydroxyl radical ($HO\cdot$) in the presence of iron through (Hyper-Weiss and Fenton) reactions [5]. Uric acid itself, as well as the ROS that are released during the enzymatic reaction, can have harmful effects on the body. Gout is a common condition that occurs due to the increased production of uric acid by this mechanism [6].

Isolation of XO, being one of the most common enzymes among different species, involves extraction from a wide range of materials (milk, bacteria, various animal organs, etc.) and purification from the crude extract [4]. (Beyaztaş & Arslan, 2015) were purified xanthine oxidase from cow's milk by making a new affinity gel. They prepared the gel on Sepharose 4B matrix in which a spacer arm based on L-tyrosine was covalently linked via activation of CNBr, followed by reaction with the XO inhibitor p-aminobenzamidine [7]. Baghiani et al. [8] were purified xanthine oxidase also from mammalian milk (bovine), by using (10 mM) of dithiothreitol, by ammonium sulphate fractionation, followed by affinity chromatography on heparin agarose. Xanthine oxidase was also purified from serum of renal failure patients by using ammonium sulfate and dialysis process [9].

Material and methods

Blood samples collection and isolation of serum:

Blood samples were collected from control subjects and patients with hyperuricemia where their ages are ranged from (25 and above) from Al- Salam Teaching Hospital in Mosul, Iraq. The serum is isolated by putting the tubes of blood in water bath at (37 ºC) for 15 minutes, then centrifuged at (3000 rpm) for 15 minutes. The serum was taken and kept in clean and covered tubes at a temperature of (-20 ºC) [10].

Quantitative determination of protein:

Folin-Lowry method modified by researchers (Schacterle and Pollack, 1973) used to determine the total concentrations of protein [11, 12].

Xanthine oxidase assay:

Xanthine oxidase activity was determined by the method [13]. This method depends on the enzymatic oxidation of xanthine, which followed spectrophotometrically by measuring uric acid formation at (293 nm).

Partial purification of Xanthine oxidase (XO):

Precipitation of enzyme: Enzyme was precipitated from the serum of patients by gradually addition of ammonium sulphate at 65 % saturation at 4 ºC with stirring and left overnight in the refrigerator, then separated by refrigerated centrifuge at (13000xg) for 30 minute [10]

Dialysis: The protein precipitate resulted from refrigerated centrifugation was dialyzed for about 48 hours against ammonium bicarbonate solution (0.1 M) , and insuring to replacing the dialysate solution every 2.5 hours at 4 ºC [14].

Estimation of approximate molecular weight of xanthine oxidase:

By Gel Filtration Chromatography: The solution obtained from dialysis was fractionated using gel filtration technique, Sephadex (G-200) was used in packing the column with dimensions (1× 52) cm with a height of (42) cm, Distilled water was used as elution solution. The protein content was followed up by reading the intensity of absorption at 280 nm. The fractions that showed xanthine oxidase activity were collected together and lyophilized, then preserved at 20 ºC to be used in the study of some properties of xanthine oxidase. The enzyme restoration volume was compared with the restoration volumes of known molecular weight compounds to determine the approximate molecular weight of the enzyme.

By SDS-PAGE Gel Electrophoresis: According to the method that followed by the researcher [15], SDS was utilized in electrophoresis technique to isolate the charged particles under an electrical field.

Statistical analysis:

The data analysis is performed using SPSS 26. All results are expressed as the mean ± standard error (SE). The results have been analyzed statistically using T-Test to find the significant differences between the study groups and the probability level $P \leq 0.05$ is considered significantly difference [16].

Results and discussion

A Significant increase was noticed at the probability level ($p \leq 0.05$) in the activity of XO in the serum of patients with hyperuricemia (29.23 ± 2.21) U/L compared to its activity in the healthy group (8.38 ± 0.92) U/L. This is consistent with what was stated by the researchers [17], where they found that an increase in the level of uric acid

in the blood is accompanied by an increase in the activity of xanthine oxidase. The reason for the high activity of xanthine oxidase is due to its main role in the metabolism of purines, as it works to oxidize hypoxanthine to xanthine, and then oxidize the latter to uric acid. Increasing the activity of the enzyme leads to increased uric acid production and hyperuricemia [18].

Table 1 Xanthine oxidase activity in serum of patients with hyperuricemia compared to control group

Xanthine oxidase activity (U/L)	Mean ± Standard error	Increase %
Patient group	29.23 ±2.21	248.8 %
Control group	8.38 ±0.92	

Table 2 shows the purification steps of xanthine oxidase. The specific activity of the enzyme in the obtained band became (5.2) U/L after it was (0.74) U/L in the crude serum, and the recovery percentage was (89.8%), and the number of purification times was equal to (7.1). Figure 1 explains the elution profile of purified xanthine oxidase by gel filtration chromatography, as shown we obtained one band at elution volume (8-24) ml.

Table 2 Xanthine oxidase purification steps from serum of patients with hyperuricemia

Purification steps	Volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Total activity (*)(U/L)	Specific activity (**) (U/L)	Number of purification folds	% of restoration
Crude	16	8737.5	139.800	103.36	0.74	1	100
Ammonium sulfate suspension	5	22450	112.250	38.5	0.34	0.46	37.25
Dialysis	7	24600	172.200	54.18	0.31	0.42	52.42
Gel purification by using Sephadex G-200	16	1108.75	17.740	92.8	5.23	7.1	89.8

(*) Total activity: enzyme's amount which one free one-micromole substrate in minute. (**) Specific activity: number of present enzyme units /mg of protein.

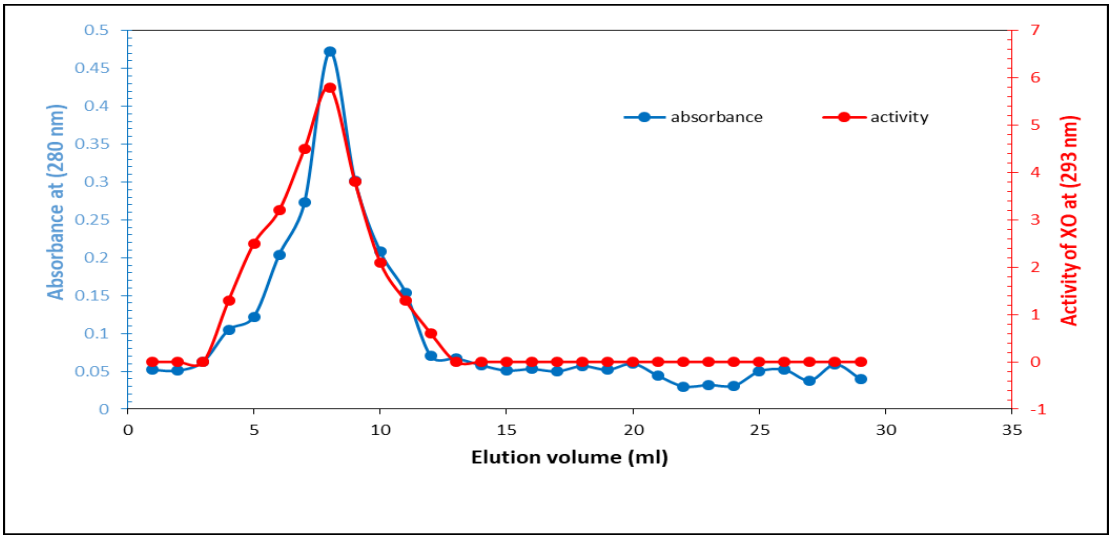

Figure 1: Purification of Xanthine oxidase by Gel filtration chromatography using Sephadex G-200

Estimation approximate molecular weight:

Gel filtration chromatography used for separation and estimation of approximate molecular weight. As shown in Figure 1, the elution volume of the xanthine oxidase solution collected by the gel filtration column was 16 mL, which corresponds to a molecular weight of 218776 when using the standard curve shown in Figure 2.

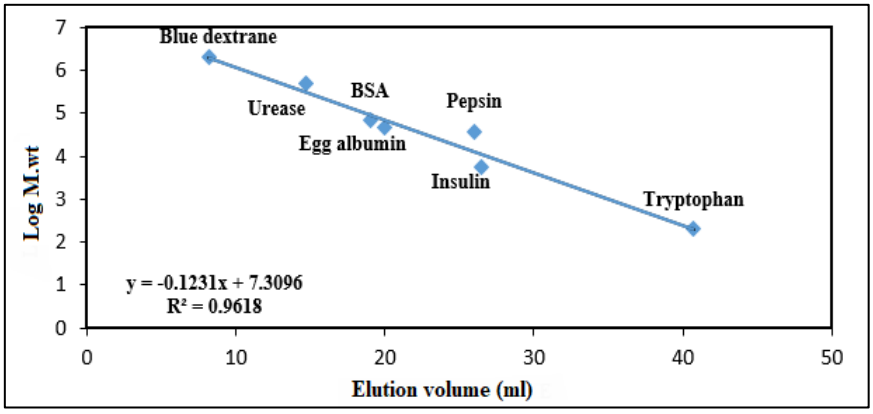

Figure 2: Standard curve used to estimate the approximate molecular weight of xanthine oxidase using gel filtration technique

The approximate molecular weight of xanthine oxidase, which is partially purified from the serum of patients with hyperuricemia was estimated also by the SPS-PAGE electrophoresis. On sodium dodecyl sulphate electrophoresis the purified enzyme obtained showed a single band. As shown in Figure 3 the relative distance of the XO is (2.4) cm, This distance was relied upon to estimate the approximate molecular weight of the enzyme, which was found to be equal to (221513) Dalton using of the standard curve shown in the Figure 4

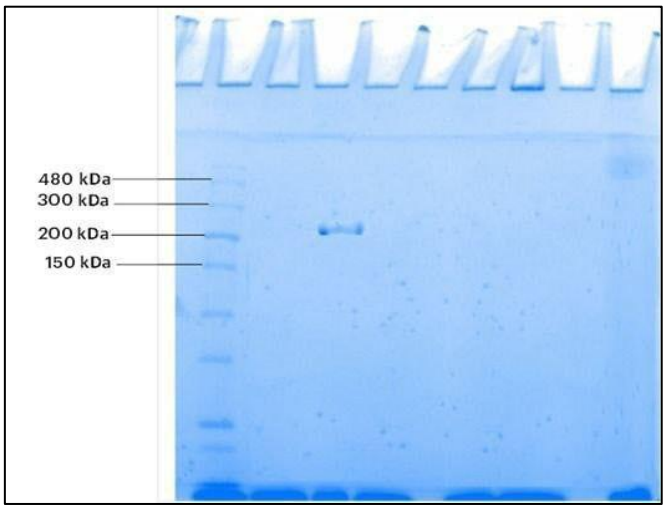

Figure 3: SDS-PAGE electrophoresis pattern of xanthine oxidase

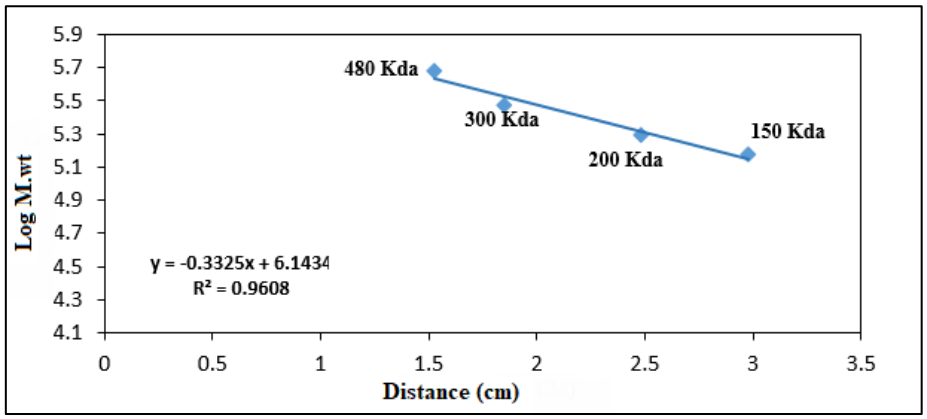

Figure 4: Standard curve for SDS-PAGE used in determination of xanthine oxidase molecular weight

Xanthine oxidase purified from human cytosol was shown as a single band with a molecular weight 300 kDa on polyacrylamide gel electrophoresis [19].

Study of some factor affecting the activity of the partially purified xanthine oxidase from serum of hyperuricemia patients:

Effect of enzyme concentration: The enzyme activity of xanthine oxidase was measured using different volumes (50-150 µL) of the protein bundle solution and other fixation factors. It was observed, as shown in Figure 5 that the activity of the enzyme increased with an increase in the concentration of the enzyme. The reason for this increase is due to the increased availability of active sites that are associated with the substrate. The results showed that the highest activity of the enzyme was at a volume of (150 µL).

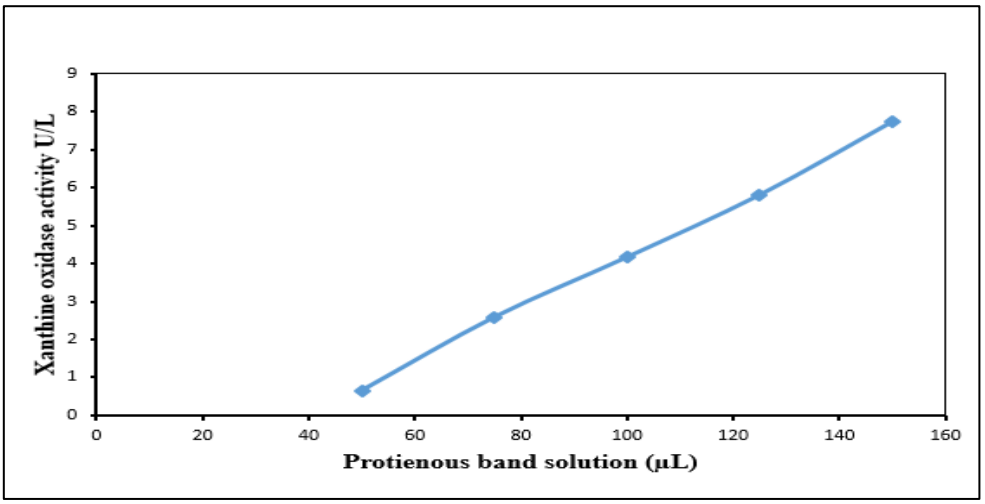

Figure 5: The effect of different concentrations on the activity of partially purified xanthine oxidase from hyperuricemia patients' serum

Effect of pH: Each enzyme has a special pH that shows the highest activity at which it is called the optimal pH. The enzyme activity was measured using sodium phosphate buffer solution (0.1 M) with a range between (7.6 pH -8.6 pH), where it was found that the highest activity of the enzyme was at the pH 8.2 As shown in Figure 6.

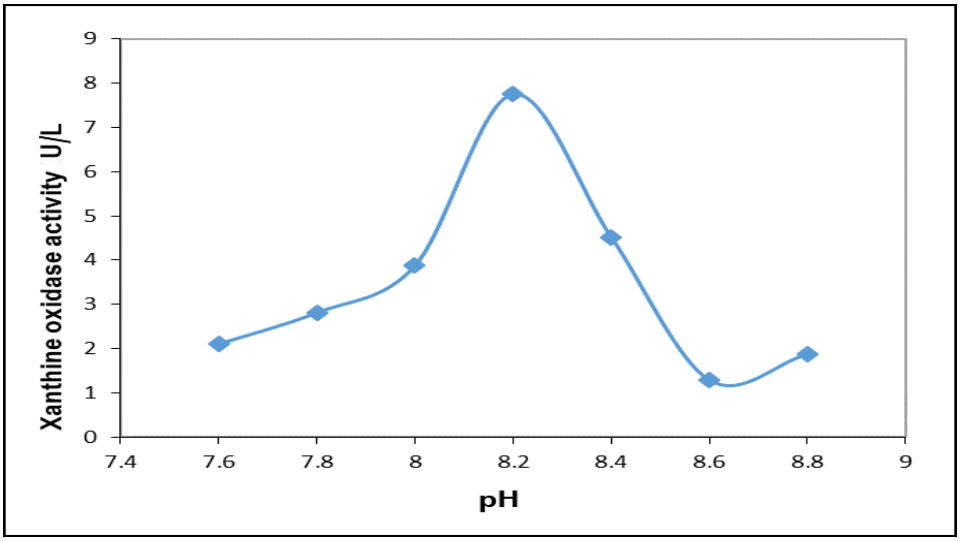

Figure 6: The effect of pH on the activity of partially purified xanthine oxidase from hyperuricemia patients' serum

Effect of temperature: The enzyme showed a highest activity at 40 ºC, which is the optimum temperature as shown in Figure 7.

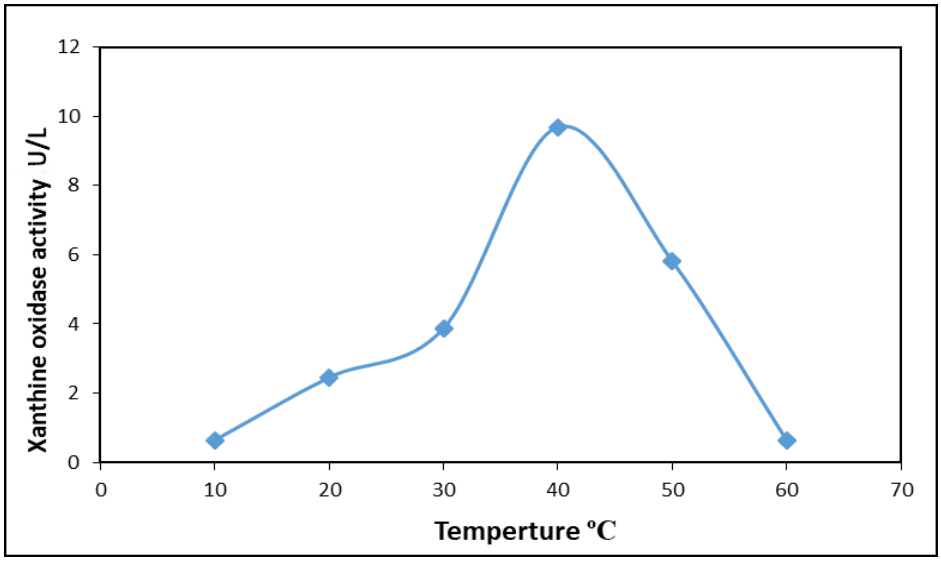

Figure 7: The effect of temperature on the activity of partially purified xanthine oxidase from hyperuricemia patients' serum

Effect of reaction time: The reaction time was studied to find out the optimal period for the activity of the xanthine oxidase enzyme. The results shown in Figure 8 indicate that the best activity of the XO was at the fifth minute, and then the activity began to decline, as it was Conduct several experiments using the reaction time (3-7 minutes).

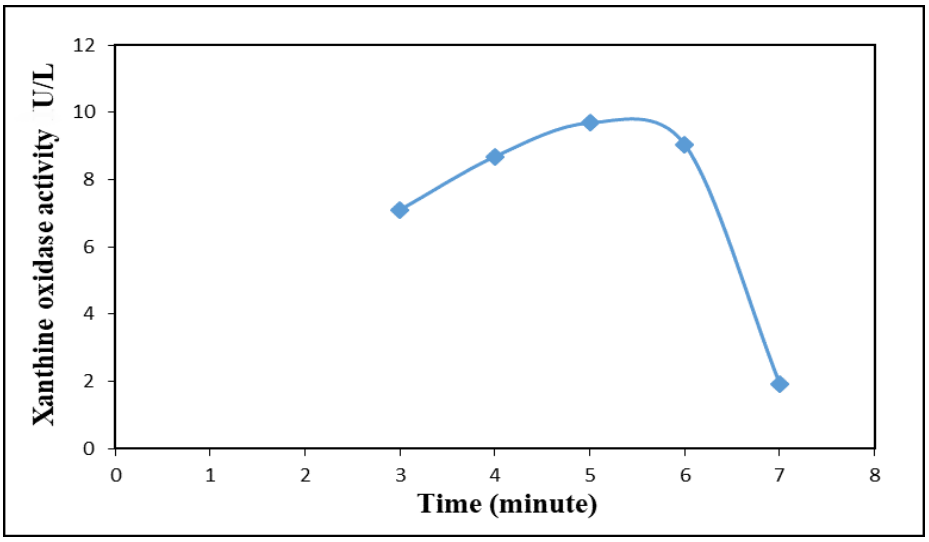

Figure 8: The effect of reaction time on the activity of partially purified xanthine oxidase from hyperuricemia patients' serum

Effect of Substrate concentration: The activity of the xanthine oxidase enzyme was measured by taking different concentrations of the substrate (Xanthine), ranging from (0.05-0.35) mM, Figure 9 shows that an increase in the concentration of the substrate leads to an increase in the rate of enzymatic reaction velocity. The results showed that the concentration of the substrate (xanthine), which led to the middle of the maximum speed, was (0.095) mM. , and represents the value Michaelis-Menten constant (km), and the value of maximum velocity was (V_{max}) was (38) IU/L. When relying on the Lineweaver-Burk plot, it was found that the value of V_{max} and Km was (41.6) IU/L and (0.0952) mM, respectively, as shown in the Figure10

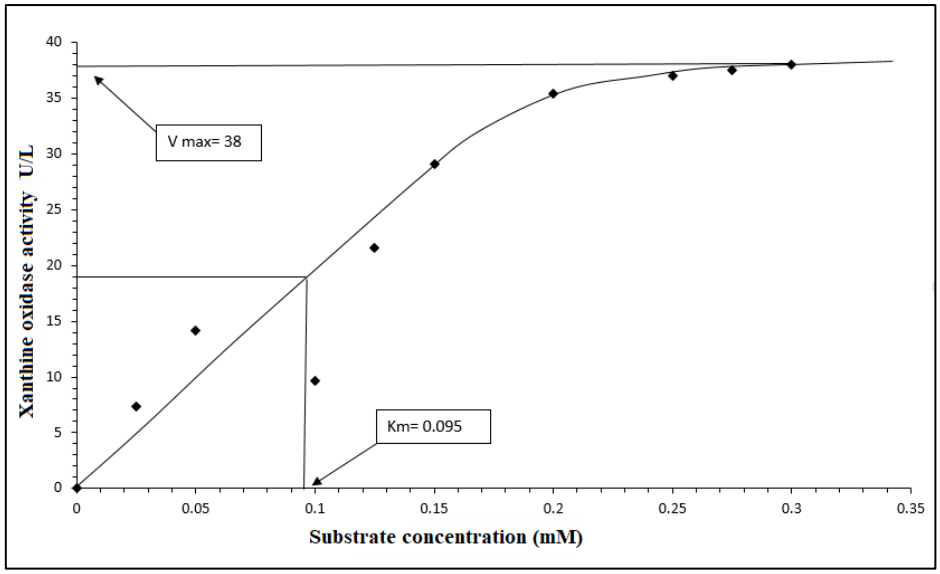

Figure 9: Effect of xanthine concentration on the activity of partially purified xanthine oxidase from hyperuricemia patients' serum

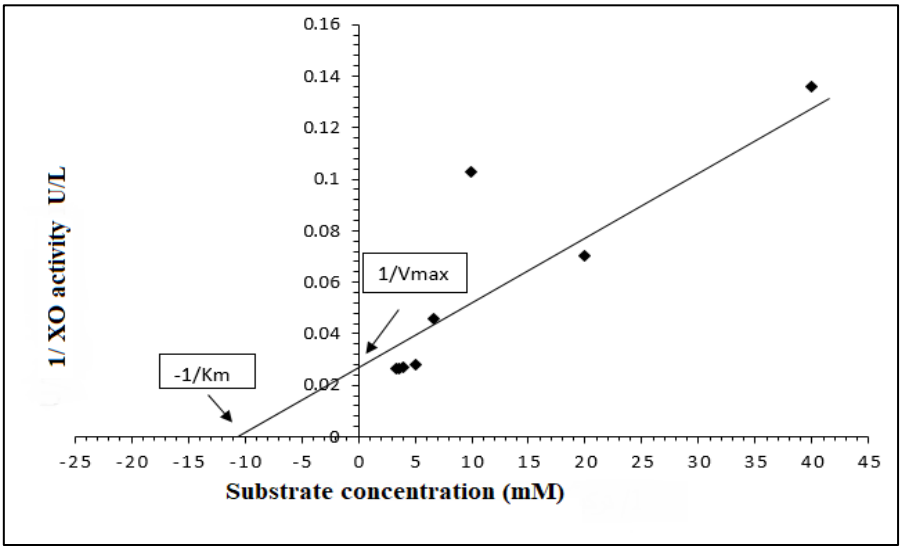

Figure 10: Lineweaver- Burk plot of partially purified xanthine oxidase from hyperuricemia patients' serum

Conclusion

A significant increase was found in the activity of Xanthine oxidase in patients with hyperuricemia. The molecular weight of XO estimated by gel filtration chromatography was 218776 Daltons, and by using electrophoresis it was found to be equal 221513 Daltons. The optimal conditions were determined in which the highest activity was at (150 µL) of partial purified xanthine oxidase, pH (8.2), temperature (40 ºC), reaction time 5 minute and at concentration of (0.35 mM) of the substrate (xanthine).

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