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Purification of Xanthine Oxidase Enzyme and Investigation of Its Immobilization with Glutaraldehyde*

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Abstract: In this study, the xanthine oxidase (XO) enzyme was purified by affinity chromatography technique using Sepharose-4B-L-tyrosine-4-aminobenzamidine gel and its immobilization with glutaraldehyde was investigated. Using ammonium sulfate precipitation and affinity gel, xanthine oxidase was purified 643.04-fold in an 11.5% yield. The purity of the enzyme was checked by SDS polyacrylamide gel electrophoresis and a single band around 150 kDa was observed. K_M (the Michaelis constant) and V_{Max} (the asymptotic reaction velocity at infinite substrate concentration) of the enzyme were determined at $1.67x10^{-4}$ M and 0.56 U/mL.min respectively by using a xanthine compound as a substrate. The *in vitro* effects of NH_4F, NH_4Cl, $CaCl_2$, $ZnCl_2$, $HgCl_2$, $Hg(NO_3)_2.H_2O$ compounds and commercially named colchicum dispert, commonly used in the treatment of gout disease in the clinic, were investigated. The IC_{50} values of compounds showing inhibition effects were determined. Afterward, XO was immobilized with glutaraldehyde. The highest XO activity was observed in the sample of the immobilized enzyme at a rate of 6% glutaraldehyde. The kinetic constants (K_M and V_{Max}) of the immobilized enzyme were determined as $5.18x10^{-4}$ M and 0.73 U mL^{-1} min^{-1} respectively. These values revealed that the catalytic activity of the free enzyme was higher than the immobilized enzyme.

Keywords: Xanthine oxidase, affinity chromatography, inhibition, enzyme immobilization, glutaraldehyde

1. Introduction

Xanthine oxidoreductase (XOR) enzyme is a complex flavoenzyme containing molybdenum in the fat globule membrane. With a molecular weight of 300 kDa, xanthine oxidase (XO; EC: 1.2.3.22) and xanthine dehydrogenase (XDH; EC: 1.1.3.204) has two different forms that can be transformed into each other (Nelson and Handler, 1968; Hart et al., 1970; Bray, 1975). It has been determined that both forms can reduce oxygen, but only the xanthine dehydrogenase form can reduce nicotinamide adenine dinucleotide (NAD^+). It is known that both XO and XDH are produced by the same gene, and subunit compositions and cofactor requests are similar (McManaman and Bain, 2002). Each subunit of the enzyme contains one molybdenum molecule, one flavin adenine dinucleotide (FAD) molecule, and two ferredoxin (Fe_2-S_2) groups. In addition, each subunit contains 1330 amino acid residues (1332 amino acid residues in cow's milk) (Hart et al., 1970; Bray, 1975). In general, the enzyme xanthine is synthesized as dehydrogenase and can be easily converted into XO form by oxidation of sulfhydryl groups or by proteolysis. Although all forms of the enzyme XO, and XDH are known and used as XOR, it is reported that it is more accurate to use as XOR (Harrison, 2006). Although it is found in all mammalian milks investigated to date, it also shows a wide distribution in mammalian tissues. It is most common in the small intestine mucosa, liver, and kidneys. The enzyme in question is most commonly found in milk. Therefore, milk is the most preferred source in purification (Massey et al., 1969; Page et al., 1998; Murray et al., 2004; Fox and Kelly, 2006). Xanthine oxidoreductase enzyme is the speed-

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restricting enzyme of purine catabolism and catalyzes the last two stages of purine degradation in purine metabolism.

The enzyme generates free radicals (reactive oxygen species such as superoxide radicals and hydrogen peroxide) when it catalyzes the oxidation of hypoxanthine to xanthine, and can further catalyze the oxidation of xanthine to uric acid. Uric acid is the end product of the reaction because there is no urea oxidase enzyme in humans (Granger et al., 1986; Metinyurt, 2003). It has long been known that xanthine oxidoreductase in the presence of hypoxanthine has bactericidal activity and is activated *in vivo* in response to bacterial infections. The bactericidal role of the enzyme also provides an explanation for its presence in breast milk. In this way, the XOR enzyme has a protective activity of the newborn's sterile digestive system from pathogenic bacteria (Tubaro et al., 1980; Kenan and Patton, 1995; Metinyurt, 2003). This activity in breast milk is identical to XOR enzyme activity in the digestive system epithelia and complementary to each other. The main pathologies affecting the biological functions of Xanthine oxidoreductase are the jury of hereditary xanthinuria, hyperuricemia, gout, and ischemia-reperfusion. Hereditary xanthinuria is a congenital disorder with autosomal recessive transition scanning with deficiencies of these enzymes (Metinyurt, 2003). In ischemia-reperfusion (IR), cellular energetic load reduction due to adenosine triphosphate (ATP) degradation in ischemia tissue increases cytosolic calcium ion (Ca^{2+}) concentration. Intracellular Ca^{2+} increase activates Ca^{2+} or Ca^{2+} calmodulin-dependent proteases. These proteases cause XDH, the main XOR form found in tissues under physiological conditions, to XO with partial proteolysis (Granger et al., 1986; Metinyurt, 2003). At the same time, hypoxanthine accumulates in the cells following ATP degradation. In the reperfusion phase, increased hypoxanthine, molecular oxygen (O_2), and XO in tissues together produce abundant superoxide and hydrogen peroxide. These free radicals also cause tissue damage (Granger et al., 1986; Coetzee et al., 1990). Gout is a type of arthritis that happens when uric acid gets too high in the blood (hyperuricemia). Hyperuricemia may eventually lead to permanent bone, joint, and tissue damage, kidney disease, and heart disease in humans, and animals. The biochemical mechanism of gout is not yet understood. It is reported that a genetic defect that causes the overproduction of urate plays a role, especially in primary gout. Hypouricemia is a rare disorder associated with decreased or increased excretion of urate synthesis. Hypouricemia may occur in hereditary XO deficiency, severe liver disease, and renal tubular disorders such as Fanconi syndrome (Onat et al., 2006). It is technically difficult to recover the active enzyme from the reaction mixture for reuse after use and the fact that many enzymes are unstable that they require a very high fee for purification (Polaina and MacCabe, 2007). However, thanks to immobilization, the protection of catalytic activity as well as the physical binding or imprisonment of enzymes, so that it can be repeated and applied continuously, it is known in the literature that it is very useful in solving (Cabral and Kennedy, 2000; Dekker, 2000).

The superoxide radical is a primary free radical that at hyperproduction can trigger a metabolic cascade leading to oxidative stress. Xanthine oxidase belongs to the chief system that shapes the formation of active oxygen metabolites exerting a damaging effect at tissue and cellular levels. Strong xanthine oxidase functions stimulating the induction of superoxide, carcinogenesis, and cell apoptosis have been established (Dement'ev, 2013).

XO enzyme is very important in human diseases, animal breeding, and feeding. Reactive oxygen species (ROS) are very active in some diseases such as ischemia-reperfusion in animals and humans. For this reason, in the current study, the activities of free and immobilized XO will be calculated and potential inhibitors for the XO enzyme, which has a restrictive role in purine metabolism, will be determined.

2. Materials and Methods

2.1. Materials

Sepharose 4B, L-tyrosine, acrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), N, N-methylenebisacrylamide, β- mercaptoethanol, sodium dodecyl sulfate (SDS), glycine, Coomassie Brilliant Blue G-250, phenol red, bromothymol blue, xanthine, ethylenediaminetetraacetic acid (EDTA), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), benzamidine, and ammonium sulfate were obtained from Sigma-Aldrich (Taufkirchen, Germany). All other chemicals used were of analytical grade and obtained from either Sigma-Aldrich or Merck (Darmstadt, Germany). Fresh raw bovine milk was purchased from regional milk producers.

2.2. Preparation of affinity gel

Four grams of cyanogen bromide (CNBr) was added to 20 mL Sepharose 4B and with 4 M NaOH (sodium hydroxide), the mixture's pH was kept at 11. The reaction was stopped by filtering the gel on a Buchner funnel and washing it with cold 0.1 M $NaHCO_3$ (pH= 10) buffer. L-Tyrosine by using

saturated L-tyrosine solution in the same buffer was coupled to Sepharose-4B activated with CNBr. The reaction was completed by stirring with a magnet for 90 min. To remove excess L-tyrosine from the Sepharose-4B-L-tyrosine gel, the mixture was washed with distilled water. The affinity gel was obtained by diazotization of p-aminobenzamidine and coupling of this compound to the Sepharose-4B-L-tyrosine. The pH was adjusted to 9.5 with 1.0 M NaOH and, after gentle stirring for 3 h at room temperature; the coupled red Sepharose derivative was washed with 1 L of water and then 200 mL of 0.05 M Tris-sulfate (pH= 7.5) buffer.

2.3. Enzyme purification

Fresh bovine milk was cooled down to 4 °C overnight without adding preservative. Ethylenediaminetetraacetic acid (EDTA) and toluene were then added to give final concentrations of 2 mM and 3% (v/v), respectively. The milk was churned with a blender at maximum speed for 30 min at room temperature. This sample was brought to 38% saturation by the addition of solid ammonium sulfate (Ozer et al., 1999; Beyaztaş and Arslan, 2011). The suspension was centrifuged at 15000 rpm for 30 min and the precipitate formed was discarded. The supernatant was brought to 50% saturation with solid ammonium sulfate. The precipitate formed was collected by centrifugation at 15 000 rpm for 60 min and dissolved in 0.1 M Tris-HCl (pH= 7.6) buffer. The pooled precipitate obtained from bovine milk by using ammonium sulfate precipitation was subjected to affinity chromatography. The sample was applied to the Sepharose-4B-L-tyrosine-p-aminobenzamidine affinity column equilibrated with 0.1 M glycine/0.1 M Na_2SO_4 (pH= 9.0) buffer. The sample was applied to the affinity gel and was washed with 0.1 M glycine (pH= 9.0) buffer. XO was then eluted with 25 mM benzamidine in 0.1 M glycine/0.1 M Na_2SO_4 (pH= 9.0) buffer fractions of 1. 5 mL were collected and their absorbance was measured at 280 nm (Beyaztaş, 2010).

2.4. Xanthine oxidase (XO) activity measurements

Xanthine oxidase activity was determined by the modified method of Massey (Massey et al., 1969). The *in vitro* conversion of xanthine uric acid was followed by monitoring the change in absorbance at 292 nm, using UV-Visible Spectrophotometer (ε_{292}= 9.5 mM^{-1} cm^{-1}). The reaction mixture contained 50 mM Tris-HCl (pH= 7.6) buffer and 0.15 mM xanthine at 37 °C. The assay was initiated by the addition of the enzyme. One unit of enzyme activity was defined as the amount of enzyme that converts 1 μmol of xanthine to uric acid per minute under defined conditions (McManaman et al., 1999; Beyaztaş and Arslan, 2015)

2.5. Total protein determination

After the elution step, the enzyme was determined spectrophotometrically at 280 nm and protein during the purification steps was determined spectrophotometrically at 595 nm according to the Bradford method, using bovine serum albumin as the standard (Bradford, 1976).

2.6. Control of enzyme purity with SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)

To control the enzyme purity, sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli's method (Laemelli, 1970). The acrylamide concentrations of the stacking and the separating gels were 3% and 10%, respectively, and they contained 0.1% SDS.

2.7. Determination of kinetic constants of XO enzyme

To determine K_M (the Michaelis constant) and V_{Max} (the asymptotic reaction velocity at infinite substrate concentration) values, enzyme activity measurements were made at varying concentrations of xanthine substrate under optimum conditions. Each measurement was made twice, and the average of the values found was taken. The activity values measured at 295 nm are taken as the reaction rate (U mL^{-1} min^{-1}). 1/V versus 1/[S], known as the Linewear-Burk chart (Laemelli, 1970; Arslan, 2001).

2.8. Determination of IC_{50} values

Enzyme activity was measured in constant substrate and concentrations of different NH_4F, NH_4Cl, $CaCl_2$, $ZnCl_2$, $HgCl_2$, $Hg(NO_3)_2.H_2O$, and colchicum dispert to determine IC_{50} values. The activity was accepted as 100% by using it as tube control that does not contain inhibitors. %Activity-[inhibitor] graph for inhibitors is drawn and IC_{50} values are calculated by this chart.

2.9. Immobilization of purified enzyme with glutaraldehyde

Glutaraldehyde (GA) with a density of 0.265 g mL^{-1} of 25% has been added to the pure enzyme in different percentages. Glutaraldehyde/free enzyme mixture, at 25 °C, 65-70 rpm churn rate of one-day incubation was left to enzyme aggregations. While the free enzyme is colorless, it has been observed that there is a brown change in the color of the immobilized enzyme. After one day, the

immobilized enzyme (5000 rpm, 4 °C, 5 minutes) was centrifuged and the solid and liquid phase was separated. Since the XO enzyme remains in the precipitated solid part, enzyme activity was compared with spectrophotometric measurement for both free and immobilized enzymes after the solid phase was washed with cold water at a minimum volume.

3. Results

3.1. Results for free XO enzyme

The XO enzyme was obtained with 11.5% efficiency and 694.04 purification degree. Kinetic data on the enzyme in question, protein quantities, % purification yield, and finally purification degree are given in Table 1. To determine K_M and V_{Max} values, enzyme activity measurements were made at varying concentrations of xanthine substrate under optimum conditions (Table 2) and a Linewear-Burk graph was drawn (Figure 1). Using the graph, the K_M value was $1.667x10^{-4}$ M and the V_{Max} value was 0.56 U mL^{-1} min^{-1}.

The purity of the enzyme purified by affinity chromatography was controlled by SDS-PAGE and a single band of around 150 kDa was observed (Figure 2).

In vitro effects of NH_4F, NH_4Cl, $CaCl_2$, $ZnCl_2$, and colchicum dispert, used to treat gout, were investigated and IC_{50} values were found $1.42x10^{-4}$, $1.25x10^{-4}$, $0.99x10^{-4}$, $1.07x10^{-4}$, and $0.27x10^{-4}$ respectively as seen in Table 3.

Table 1. Purification table of XO enzyme

Step	Volume (mL)	Activity (U mL^{-1})	Total activity (U)	Protein amount (mg mL^{-1})	Total protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Purification degree
Milk	180	70.8	12742.2	3.19	574.2	22.2	100	-
Ammonium sulfate precipitation	12	394.2	4730.4	3.44	41.3	114.6	37.1	5.16
Affinity chromatography	2	736.9	1473.8	0.01	0.012	73692.1	11.5	643.04

Table 2. The volumes, activity, 1/V, and 1/[S] values of the solutions used to determine the K_M and V_{Max} values using the xanthine substrate of the XO enzyme

pH= 7.6 50 mM Tris buffer (µL)	Volume of enzyme solution (µL)	Volume of substrate solution (µL)	Total volume of cuvette (µL)	Substrate concentration in cuvette [S]×10^{-3}(mM)	ΔOD (295nm)	Activity (U $mL^{-1}min^{-1}$)	1/Vx10^{-3}	1/[S]
835	70	100	1005	9.95	0.138	216.92	4.6	10510
735		200		19.90	0.198	312.51	3.2	5255
635		300		29.85	0.218	344.83	2.9	3503
535		400		39.80	0.244	384.61	2.6	2627
435		500		49.75	0.276	434.78	2.3	2102
335		600		59.70	0.288	454.55	2.2	1767

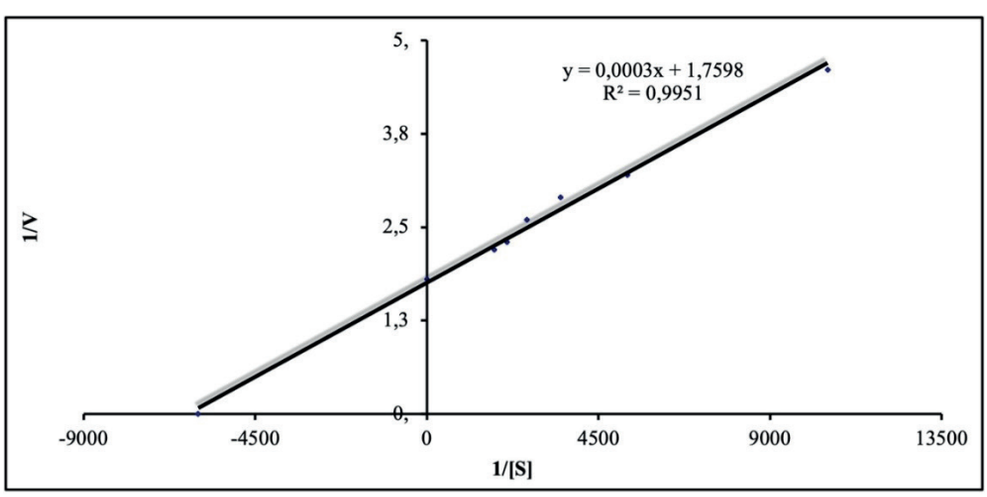

Figure 1. 1/V-1/[S] plot of the purified XO enzyme

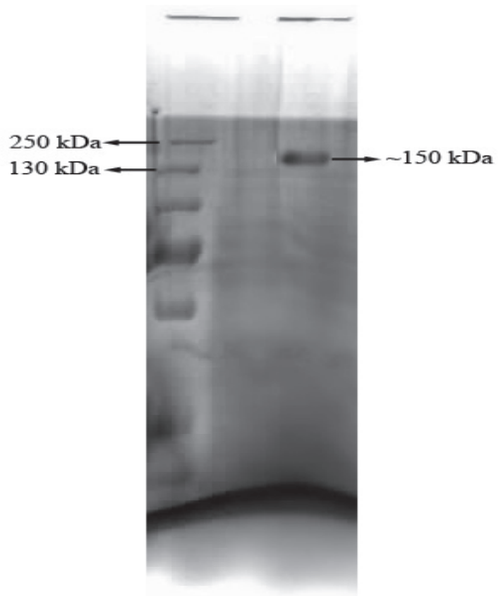

Figure 2. SDS-PAGE image of XO enzyme purified by affinity chromatography

Table 3. IC_{50} values of NH_4F, NH_4Cl, $CaCl_2$, $ZnCl_2$, $HgCl_2$, $Hg(NO_3)_2.H_2O$ and colchicum dispert

Compound	IC_{50} (mM)
NH_4F	$1.42x10^{-4}$
NH_4Cl	$1.25x10^{-4}$
$CaCl_2$	$0.99x10^{-4}$
$ZnCl_2$	$1.07x10^{-4}$
$HgCl_2$	Not detected
$Hg(NO_3)_2.H_2O$	Not detected
Colchicum dispert	$0.27x10^{-4}$

3.2. Results for glutaraldehyde immobilized XO enzyme

Different percentages of glutaraldehyde (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14%) were added to the pure enzyme and enzyme immobilization was investigated and enzyme activity was observed in the enzyme with a maximum of 6% glutaraldehyde. Therefore, immobilized enzyme was used in experimental steps taking into account the ratio of 6% (GA/pure enzyme).

3.3. Activity determination for immobilized enzyme by mixing 6% with glutaraldehyde

Since the highest activity for the immobilized XO enzyme was observed with the addition of 6% GA, the highest activity was in tube 6 with the determination of activity at 295 nm (Table 4-7). Moreover, the activity of the enzyme immobilized with 6% glutaraldehyde was measured regularly for 2 months and it was observed that it lost its activity by 50% at the end.

3.4. Calculation of catalytic activity, V_{Max}, and K_M values for immobilized and free XO enzymes

Based on different substrate concentrations, K_M and V_{Max} values were calculated using the graph in Figure 3. Catalytic activity, $V_{MAX,}$ and K_M values were determined for immobilized enzymes as 14.09×10^2 EU M^{-1}, 0.73 EU, 5.18×10^{-4} M and for free enzymes as 33.53×10^2 EU M^{-1}, 0.56 EU, 1.667×10^{-4} M respectively as shown in Table 8.

Table 4. Free enzyme, different percentages of GA and immobilized enzyme activities

Glutaraldehyde/Purified enzyme	GA	GA	Free enzyme	Change of absorbance (solid phase)	Activity of immobilized enzyme
% Ratio	Mass (g)	Volume (µL)	Mass (g)	|A2 – A1|	EU (mol min^{-1})
1	0.0038	3.58	0.3793	**	**
2	0.0077	7.23	0.3832	0.021	32180
3	0.0111	10.46	0.3699	0.023	37007
4	0.0262	13.74	0.3641	0.045	48270
5	0.0192	18.15	0.3847	0.082	19308
6	0.0227	21.40	0.3782	0.153	127111
7	0.0262	24.68	0.3737	0.083	28962
8	0.0301	28.31	0.3771	0.032	51488
9	0.0333	31.42	0.3702	0.047	75623
10	0.0385	36.36	0.3854	0.006	9654
11	0.0404	38.04	0.3741	0.023	37007
12	0.0422	40.11	0.3718	0.034	54706
13	0.0456	43.21	0.3812	0.008	12872
14	0.0480	46.12	0.3863	0.002	3218

**: EU calculation could not be made since precipitation could not be observed

Table 5. GA and free enzyme amounts for the immobilized enzyme by mixing with 6% GA

Glutaraldehyde/Purified enzyme	Glutaraldehyde	Glutaraldehyde	Free enzyme
% Ratio	Mass (g)	Volume (µL)	Mass (g)
6	0.0227	21.4	0.3782

Table 6. Activity assay schedule for immobilized enzyme

pH= 7.6 Activity buffer (µL)	Substrate (Xanthine) (µL)	Immobilized enzyme (µL)	Change of absorbance |A2 - A1|
835	100	70	0.009
735	200		0.121
635	300		0.181
535	400		0.208
435	500		0.252
335	600		0.273

Table 7. EU and substrate concentration values for the immobilized enzyme by mixing with 6% GA

Change of absorbance |A2 - A-1|	Enzyme unit (EU) (mol min^{-1})	1 / $Vx10^{-3}$	Substrate concentration [S] ($M\times10^{-3}$)	1 / [S]
0.009	140.840	7.100	10.010	9980.230
0.121	191.900	5.210	18.170	5501.120
0.180	284.900	3.510	29.530	3386.140
0.208	328.900	3.040	40.060	2496.070
0.252	399.360	2.504	50.040	1998.600
0.273	432.520	2.312	59.520	1680.120

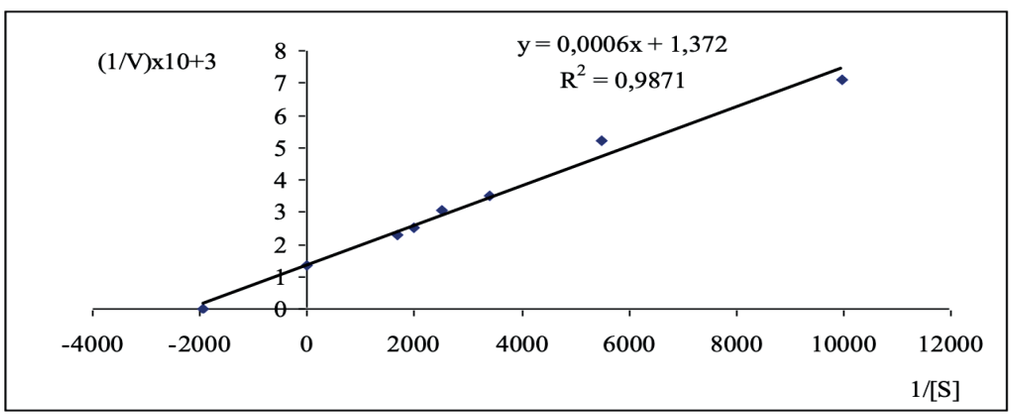

Figure 3. 1/V-1/[S] plot for immobilized enzyme with 6% GA

Table 8. Catalytic efficiency, V_{Max} and K_M values for immobilized and free enzyme

	V_{Max} (EU)	K_M (10^{-4} M)	V_{Max}/K_M (10^2 EU M^{-1})
Free enzyme	0.56	1.667	33.53
Immobilized enzyme	0.73	5.180	14.09

4. Discussion and Conclusion

XO was purified using affinity gel with a chemical structure of Sepharose 4B-L-tyrosine-4-aminobenzamidine and immobilized on glutaraldehyde. *In vitro* effects of NH_4F, NH_4Cl, $CaCl_2$, $ZnCl_2$, $HgCl_2$, $Hg(NO_3)_2.H_2O$, and colchicum dispert compounds were investigated on the pure enzyme.

XO needs to be purified quickly and in more economical ways. Because the enzyme has a broad substrate specificity, it is important for preparative organic chemistry. The enzyme XO is known to be used in many organic syntheses. Furthermore, the industrial importance of this enzyme demonstrates the necessity of more practical purification methods (Pauff et al., 2007). XO was detected in the liver, small bowel mucosa, milk-secreting mammary glands, heart, kidney, brain, aorta, lungs, skeletal muscle, and intestines in the endothelial cells of small vessels and breast milk (Fridovich, 1964; Schoutsen et al., 1983; Parks and Granger, 1986; Werns and Lucchesi, 1990; Moriwaki et al., 1993; Linder et al., 1999; Metinyurt, 2003).

The XO enzyme amount is quite high in cattle milk, for this reason, cattle milk is preferred as a source of enzymes. Before the technique of affinity chromatography, precipitating was applied to cattle milk with neutral salts, which is one of the pre-purification techniques. Ammonium sulfate was preferred as neutral salt. The ammonium sulfate precipitation range has been reported in the

literature as 38-50% for XO (Ozer et al., 1999). SDS-PAGE has been applied to control the purity of the enzyme. The purified enzyme was found to weigh 150 kDa and the estimated XO enzyme was observed as a single band in the SDS-PAGE gel. This value is consistent with the literature. XO's molecular weight was determined by SDS-PAGE and McManaman et al. about 150 kDa, and 300 kDa with natural electrophoresis (McManaman et al., 1996). Because the structure of the enzyme has been confirmed by experimental studies (Maia and Mira, 2002).

Some of the compounds used in this study are known as heavy metal contents. The most important reason for the selection of these compounds is their frequent use in the medical field and industry. As detailed in the introduction, we think it is important to determine how these compounds are effective on XO, which has important physiological functions. Furthermore, the fact that there is no information in the literature about the effects of colchicum dispert active ingredient on XO enzyme further increases the authenticity of the study.

The inhibition effect of these compounds causing inhibition were given by finding IC_{50} values. IC_{50} values and substrate concentrations were kept constant, different concentrations of these compounds and percentage activities were determined, and then the compound concentration which causes 50% inhibition was calculated by graphs.

It is reported that it slows the formation of uric acid, the active ingredient of colchicum dispert, which is used treatment of gout (drops) and FMF (Familial Mediterranean Fever) disease, and Behçet syndrome. In addition, nausea, vomiting, aqueous and bloody diarrhea, and abdominal pain can be caused by side effects of the active substance. Considering these side effects, necessary precautions should be taken if diarrhea is continuous and severe and should be used with caution in elderly and sluggish patients, especially those with kidney, stomach, bowel, or heart disease. Since it can cause fetal damage in pregnancy, it should be given considering the benefit/harm ratio of the drug in the necessity of precise use.

Inhibition constants (IC_{50}) of NH_4F, NH_4Cl, $CaCl_2$, $ZnCl_2$, $HgCl_2$, $Hg(NO_3)_2.H_2O$ and colchicum dispert were investigated as $1.42x10^{-4}$ mg mL^{-1}, $1.25x10^{-4}$ mg mL^{-1}, $0.99x10^{-4}$ mg mL^{-1}, $1.07x10^{-4}$ mg mL^{-1}, and $0.27x10^{-4}$ mg mL^{-1}, respectively. When investigating the *in vitro* effects of heavy metal compounds ($HgCl_2$ and $Hg(NO_3)_2.H_2O$) an activity could not be detected, because a large amount of white sediment was formed. For this reason, IC_{50} values for $HgCl_2$ and $Hg(NO_3)_2.H_2O$ compounds could not be determined as shown in Table 3.

K_M and V_{Max} (Kinetic constants) of the XO enzyme, purified using sepharose-4B-L-tyrosine-4-aminobenzamidine structured affinity gel, were determined using the xanthine compound as a substrate. K_M and V_{Max} values obtained from Lineweaver-Burk graphs were found to be $1.667x10^{-4}$ M and 0.56 U mL^{-1} min^{-1}, respectively, when used as a xanthine substrate. In the literature, V_{Max} and K_M values against the xanthine substrate of XO are similar to the V_{Max} and K_M values determined by us (McCord and Fridovich, 1968).

The basic principle of immobilization with glutaraldehyde; Cross-linked enzyme aggregations are created using glutaraldehyde homo cross-binding reagent with free enzyme molecules. The importance of the glutaraldehyde reactant is that both ends are reactive. Thus, by the coexistence of many enzyme molecules, the soluble structure is insoluble and enzyme molecules bind strongly.

Immobilization of the enzyme XO was the primary goal of our research. The highest immobilized XO enzyme activity was found at the rate of 6% glutaraldehyde. K_M and V_{Max} values obtained from the Lineweaver-Burk graph of the XO enzyme immobilized with 6% glutaraldehyde were found to be $5.18x10^{-4}$ M and 0.73 U mL^{-1} min^{-1}, respectively. Since there is no such study in the literature review, K_M and V_{Max} values could not be compared with the literature.

Enzyme unit and substrate concentration values were calculated for the enzyme immobilized at 6% glutaraldehyde ratio and catalytic activity, and K_M and V_{Max} values were obtained. The same processes were tried for the free enzyme and catalytic activity was observed greater than the free enzyme. Although the K_M value of the immobilized enzyme increases, it can be used repeatedly to work at a high concentration. This can be interpreted as the advantage of the immobilized enzyme.

Declaration of Author Contributions

Conceptualization, Material, Methodology, Investigation, Data Curation, Formal Analysis, Visualization, Writing-Original Draft Preparation, Writing-Review & Editing, *Y. KAYA*; Conceptualization, Material, Methodology, Investigation, Formal Analysis, Supervision, Writing-Original Draft Preparation, Writing-Review & Editing, *S. IŞIK*; Material, Methodology, Formal Analysis, Writing-Original Draft Preparation, Writing-Review & Editing, *S. BEYAZTAŞ UZUNOĞLU*; Material, Formal Analysis, Visualization, Writing-Original Draft

Preparation, Writing-Review & Editing, *M.O. KAYA*. All authors declare that they have seen/read and approved the final version of the article ready for publication.

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Declaration of Conflicts of Interest

All authors declare that there is no conflict of interest related to this article.

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