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Purification, Characterization of Glutathion Reductase Enzyme From Sheep Spleen Tissue and Investigation of the Effects of Some Antibiotics on Enzyme Activity

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Abstract: In this study, glutathione reductase (EC 1.8.1.7; GR, Glutathione: $NADP^+$ oxidoreductase), which is the key enzyme of antioxidant metabolism, was purified from sheep spleen using ammonium sulfate precipitation and 2', 5'-ADP Sepharose-4B affinity chromatography. As a result of purification, the GR enzyme was purified, with 20.03 EU/mg of specific activity, 1564.8 times a yield of 40.61%. The purity of the enzyme was checked by SDS-PAGE. In the characterization studies, optimum pH, optimum ionic strength, stable pH, optimum temperature and subunit molecular mass of the enzyme were determined. In addition, K_M and Vmax values were found to determine the enzyme's affinity for GSSG and NADPH substrates. It was determined as K_M constant 0.0061 mM and V_{max} value 0.259 EU/mL, for NADPH, K_M constant was determined as 0.351 mM and Vmax value was determined as 0.604 EU/mL for GSSG. In addition, the effects of ampicillin, streptomycin sulfate, gentamicin, cefoperazone sodium and precort-lyo on enzyme activity were investigated. It was determined that these drugs showed an inhibitory effect on GR enzyme activity purified from sheep spleen tissue. The IC_{50} values for precort-lyo, ampicillin, streptomycin sulfate, cefoperazone sodium, and gentamicin were 1.27, 3.22, 7.95, 16.97, and 17.20 mM, respectively; and Ki constants were calculated as 0.466±0.387 (competitive), 1.057±0.110 (non-competitive), 3.386±1.305 (competitive), 4.910±0.960 (competitive), and 20.770±8.169 mM (non-competitive), respectively.

Koyun Dalak Dokusundan Glutatyon Redüktaz Enziminin Saflaştırılması, Karakterizasyonu Ve Bazı Antibiyotiklerin Enzim Aktivitesi Üzerine Etkilerinin Araştırılması

Anahtar Kelimeler
Glutatyon redüktaz,
Koyun dalak,
Saflaştırma,
Antibiyotik,
İnhibisyon

Öz: Bu çalışmada, antioksidan metabolizmanın anahtar enzimi olan glutatyon redüktaz (EC 1.8.1.7; GR, Glutatyon: $NADP^+$ oksidoredüktaz), koyun dalak dokusundan amonyum sülfat çöktürmesi ve 2', 5'-ADP Sefaroz-4B afinite kromatografisi kullanılarak, 20.03 EÜ/mg spesifik aktivite ile %40.61 verimle 1564.8 kat saflaştırıldı. Enzimin saflığı SDS-PAGE ile kontrol edildi. Enzime ait karakterizasyon çalışmalarında, optimum pH, optimum iyonik şiddet, stabil pH, optimum sıcaklık ve alt birim molekül kütlesi belirlendi. Ayrıca enzimin GSSG ve NADPH substratlarına ait olan K_M ve Vmax değerleri bulundu. NADPH için K_M sabiti 0,0061 mM ve Vmax değeri 0,259 EU/mL, GSSG için K_M sabiti 0,351 mM ve Vmax değeri 0,604 EU/mL olarak belirlendi. Buna ilaveten ampisilin, streptomisin sülfat, gentamisin, sefoperazon sodyum ve prekort-lyo'nun enzim aktivitesi üzerine etkileri araştırıldı. Bu ilaçların koyun dalak dokusundan saflaştırılan GR enzim aktivitesi üzerinde inhibitör etki gösterdiği belirlendi. Prekort-liyo, ampisilin, streptomisin sülfat, sefoperazon sodyum ve gentamisin ilaçları için IC_{50} değerleri sırasıyla 1.27, 3.22, 7.95, 16.97 ve 17.20 mM ve Ki sabitleri sırasıyla 0,466±0,387 (yarışmalı), 1,057±0,110 (yarışmasız), 3,386±1,305 (yarışmalı), 4,910±0,960 (yarışmalı) ve 20,770±8,169 mM (yarışmasız) olarak hesaplanmıştır.

1. INTRODUCTION

Glutathione (GSH, γ-L-glutamyl-L-cysteinyl-glycine) is a low molecular weight thiol that is responsible for protecting organisms from the harmful effects of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [1]. GSH is highly reactive and often conjugates with other molecules via its sulfhydryl moiety. GSH, detoxification of intracellular free radicals, xenobiotics, some antineoplastic drugs and some metabolic end products by conjugation, protection of thiol groups of some proteins such as hemoglobin and various enzymes, DNA and protein synthesis, amino acid transport, breaking of disulfide bonds of some proteins such as insulin, intracellular cysteine. It plays a role in many vital reactions inside the cell. GSH metabolism may play both a protective and pathogenic role in cancer. It is very important in the removal and detoxification of carcinogens, and reactions in this pathway can have a significant impact on cell survival [2,3].

Glutathione reductase (E.C. 1.8.1.7; GR) is in the group of oxidoreductases [4]. GR is an important enzyme that catalyzes the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) according to the following reaction and keeps the GSH/GSSG ratio at a certain level.

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

The GSH/GSSG ratio in erythrocyte cells is approximately 500/1. A decrease in this ratio in erythrocyte cells causes hemolysis [5]. For the reaction catalyzed by GR to occur, NADPH supplied from the pentose phosphate pathway is needed [6].

GR is found in prokaryotes in the periplasmic space in relation to the inner membrane facing the cytoplasm, in the cytoplasm in eukaryotes, and in organelles including the nucleus and mitochondria [7]. It was determined that the enzyme, whose structure was first determined by Meldrum and Tarr in 1935, carried out the reduction reaction of GSSG in the blood and NADPH was a cofactor in this reaction [8]. In previous studies, GR enzyme, porcine erythrocyte, bovine erythrocyte, rat liver, bovine liver, sheep brain, mammalian tissues such as sheep liver, rainbow trout and turkey liver tissue, fungi, microorganisms such as cyanobacteria, vegetable plants such as wheat, corn, pea and spinach. It has been characterized by purification from many prokaryotic and eukaryotic sources [9,10].

Antibiotics are drugs that treat infectious diseases as well as enable many modern medical procedures, including cancer treatment, organ transplants, and open heart surgery. However, the misuse of these drugs causes the patient to suffer from drug side effects, as well as financial losses and a rapid increase in antimicrobial resistance (AMR), where some infections can no longer be effectively treated [11]. In kinetic studies on enzymes, it has been determined that drugs, including antibiotics, interact with enzymes and affect enzymes in vivo and in vitro [12, 13]. In studies carried out to date, it has been determined that the GR enzyme has not been purified from sheep spleen.

The aim of this study is to purify and characterize the glutathione reductase enzyme, which has an important effect on the regulation of glutathione metabolism in the cell and accordingly the functioning of the antioxidant system, from sheep spleen, and amoxicillin, tylosin, ampicillin, streptomycin sulfate, gentamicin, cefuroxime sodium, cefazolin sodium, cefaperazone sodium, lincomycin, and clindamycin. To investigate the in vitro effects of novamizole, ketogenic and precort-lyo drugs on enzyme activity.

2. MATERIAL AND METHOD

2.1. Material and Method Subheading

2.1.1. Material

NADPH, $NADP^+$, GSH, GSSG, bovine serum albumin (BSA), N,N,N′,N′-tetramethyl ethylenediamine TEMED, sodium bicarbonate, ethylenediaminetetraacetic acid (EDTA), Coomessie Brillant Blue G-250, sodium dodecylsulfate (SDS), ammonium sulfate, trihydroxymethylaminomethane (Tris), Acryamide, N,N′-methylene bisacrylamide, 2′, 5′-ADP Sepharose-4B were obtained from Sigma Chemical Comp. and E.Merc AG.

2.1.2. Supply of sheep spleen tissue and preparation of homogenate

The sheep spleen used in the study was obtained from the Meat and Milk Institution of Bingöl and brought to the laboratory according to the cold chain rules. 15 g of fresh spleen tissue was suspended in 45 mL of 50 mM KH_2PO_4 (pH: 7.5) buffer. Then it was centrifuged at 13.000 g for 1 hour and homogenate was formed by discarding the precipitate. All operations were carried out at +4 °C [9, 14].

2.1.3. Measuring enzyme activity

The activity measurement of the GR enzyme purified from sheep spleen tissue was carried out according to the method described by Carlberg and Mannervik [15]. This method is based on the determination of the amount of NADPH at 340 nm, which decreases due to the oxidation of NADPH in the presence of oxidized glutathione (GSSG).

2.1.4. Ammonium sulphate precipitation and dialysis

Ammonium sulfate precipitation was performed for the prepared homogenate. For this purpose, precipitation was performed in the ranges of 0% - 20%, 20% - 30%, 30% - 40%, 40% - 50%, 50% - 60%, 60% and 70%, respectively, and the interval in which the enzyme precipitated was determined [16].

2.1.5. Enzyme purification by 2', 5'-ADP sepharose-4B affinity chromatography

The supernatant obtained as a result of homogenate preparation and ammonium sulfate precipitation was applied to the 2', 5'-ADP Sepharose-4B affinity chromatography column. For this; 2',5'-ADP sepharose-4B gel was weighed 2 g dry. Afterwards, this gel was washed several times with 400 mL of distilled water to remove impurities, and the gel was swollen during this time. The air formed due to inflation was removed by vacuuming method using a water trumpet. The prepared gel was packed into a cooled column consisting of a 1x10 cm closed system. The column is 0.1 M K-acetate/0.1 M K-phosphate (pH=7.85), 0.1 M K-phosphate/0.1 M KCl (pH=7.85) and 50 mM KH_2PO_4 washed with /1 mM EDTA (pH=7) buffers. After washing the column, the enzyme was eluted with elution buffer (50 mM KH_2PO_4/1 mM EDTA, 1mM GSH and 0.5 mM NADPH, pH 7.3). The equilibration of the column was understood from the approximate equalization of the absorbance at 280 nm with the buffer passed through the column. Elutions were taken into 1.5 mL eppendorf tubes and activity measurements were made [14, 17, 18].

2.1.6. Control of enzyme purity and determination of molecular mass by SDS-PAGE

The degree of purity of the GR enzyme purified from sheep spleen tissue and the molecular mass of the possible subunit of the enzyme were determined by SDS-PAGE based on the Laemmli method [19].

2.1.7. Protein determination

The protein amounts in the supernatants obtained as a result of homogenate, ammonium sulfate precipitate and 2', 5'-ADP Sepharose-4B affinity chromatography were determined by Bradford method. Standard bovine albumin solution (containing 1 mg protein in 1 mL) was used to create a standard graph, and protein amounts were determined by using the standard graph.

2.1.8. Optimum pH studies

Tris-HCl with pH values of 7, 7.5, 8.0, 8.5 and 9.0 for the determination of the optimum pH of the GR enzyme purified from sheep spleen tissue and pH of 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 KH_2PO_4 buffers were used.

2.1.9. Optimum ionic strength studies

To determine the optimum ionic strength of the GR enzyme purified from sheep spleen tissue, activity measurements were made using 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.5, 0.8 and 1 M KH_2PO_4 solutions at optimum pH.

2.1.10. Stable pH studies

For the determination of stable pH value of GR enzyme purified from sheep spleen tissue, KH_2PO_4 with pHs of 5, 5.5, 6, 6.5, 7.0, 7.5 and 8.0 and pH of 7, 7.5, 8.0, 8.5 and 9.0 Tris-HCl buffers were used. Measurements were made for 7 days and the stable pH of the enzyme was determined.

2.1.11. Effect of optimum temperature on enzyme activity

In order to determine the effect of optimum temperature on the GR enzyme purified from sheep spleen tissue, activity measurements were made between 0°C and 90°C at 10°C intervals.

2.1.12. Kinetic studies

In order to determine the K_M constant and V_{max} value of the substrates of the GR enzyme purified from sheep spleen tissue, activity measurements were made at 5 different concentrations, which are the substrates of the enzyme, GSSG and NADPH. Lineweaver-Burk graphs were drawn and K_M and V_{max} values were determined for NADPH and GSSG substrates with the help of these graphs [20].

2.1.13. *In vitro* inhibition studies

In order to examine the effects of some drugs on the GR enzyme activity purified from sheep spleen tissue, drugs at different concentrations were taken and added to the cuvette medium. IC_{50} values for drugs that cause enzyme inhibition, such as prekort-lyo, ampicillin, streptomycin sulfate, cefoperazone sodium and gentamicin, were calculated using the Activity%-[I] graphs drawn. In order to determine the Ki constants of these drugs showing inhibitory effect, enzyme activities were measured at 3 different fixed concentrations and 5 different substrate concentrations. Lineweaver-Burk graphs were created by converting each activity value to enzyme unit and calculating 1/V and 1/[S] values. Using these graphs, Ki constants and inhibition types were determined.

3. RESULTS

In this study, the GR enzyme was purified with a specific activity of 20.03 EU/mg, 40.61% yield and 1564.8-fold from sheep spleen tissue using homogenate preparation, ammonium sulfate precipitation and 2', 5',-ADP Sepharose-4B affinity chromatography. Purification results of GR enzyme purified from sheep spleen tissue aregiven in Table 3.1.

Table 3.1. Purification steps of glutathione reductase enzyme

Purification step	Total Volume (mL)	Activity (EU/mL)	Protein (mg/mL)	Total Protein (mg)	Total Activity (EU)	Specific Activity (EU/mg)	Yield %	Pur. fold
Homogenate	25	0.523	40.85	1021	13.075	0.0128	100	1
Ammonium sulfate precipitation (40-70%)	13	0.689	52.94	688	8.957	0.0130	68.50	1.015
Affinity Chromatog.	9	0.590	0.029	0.265	May.31	20.Mar	40.61	1564.8

3-8% SDS-PAGE was performed to determine the purity of the GR enzyme purified from sheep spleen tissue. The SDS-PAGE photograph is shown in Figure 3.1. The logMK-Rf graph was drawn for the determination of the molecular mass of the possible subunit of the enzyme. The molar mass of sheep spleen GR enzyme was calculated as approximately 91 kDa by using the graphic equation.

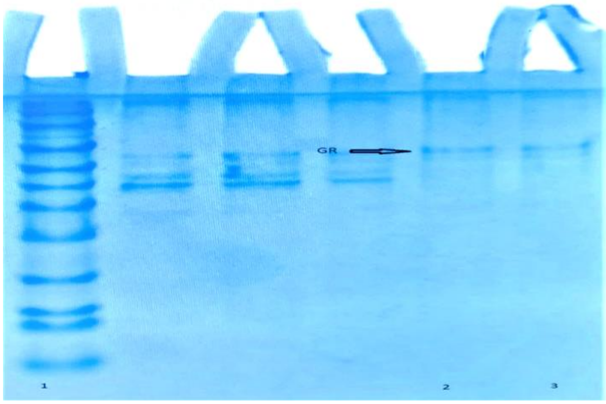

Figure 3.1. SDS-PAGE photograph obtained for the GR enzyme. Well 1: standard protein (molecular size of the markers; 175, 130, 95, 70, 62, 51, 42, 29, 22, 14 and 10.5 kDa), wells 2 and 3: pure GR enzyme from the affinity column.

The optimum pH of GR enzyme purified from sheep spleen tissue was determined as 8.0 (KH_2PO_4 buffer), optimum ionic strength was 0.1 M (KH_2PO_4 buffer), stable pH was 7.0 (KH_2PO_4 buffer) and figures 3.2, 3.3 and figures shown in 3.4. In addition, in studies conducted to determine the effect of temperature on the GR enzyme, the temperature with the highest enzyme activities was determined as 40ºC and is shown in figure 3.6.

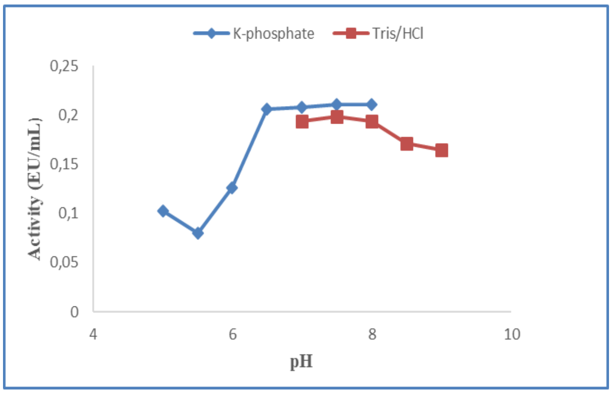

Figure 3.2. pH-Activity graph showing the result of the optimum pH study for sheep spleen tissue GR enzyme.

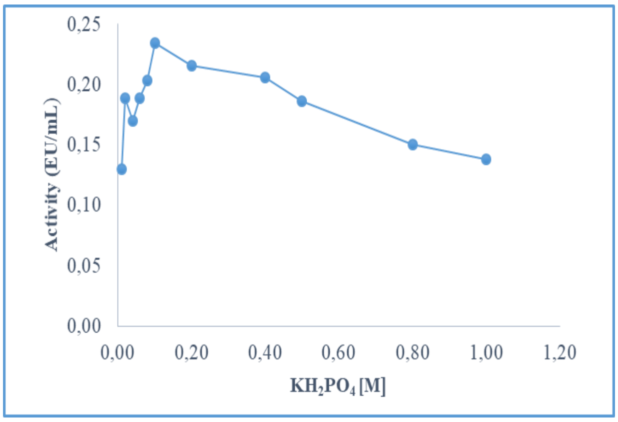

Figure 3.3. pH-Activity graph showing the result of the optimum pH study for sheep spleen tissue GR enzyme.

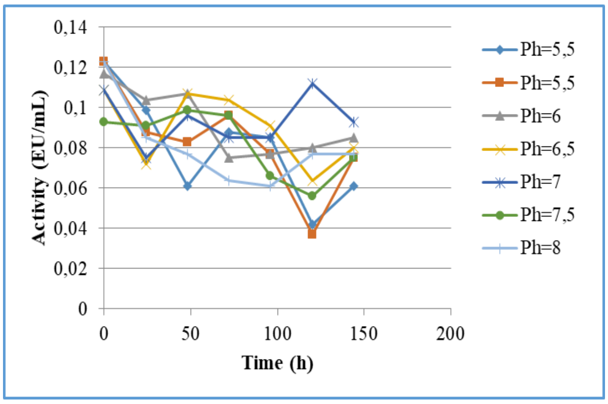

Figure 3.4. Stable pH graph for sheep spleen tissue GR enzyme using KH_2PO_4 buffer solution at different pHs

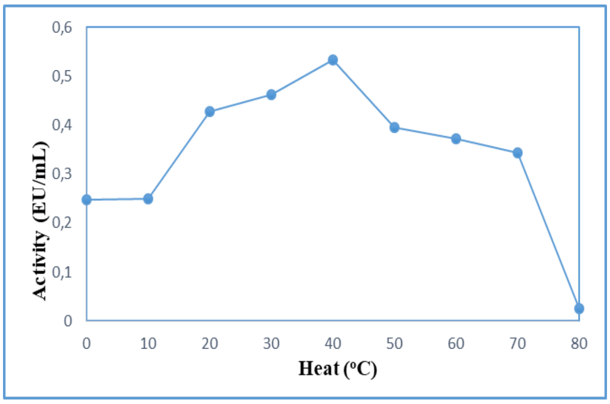

Figure 3.5. Sheep spleen tissue GR enzyme, activity-temperature change graph.

Lineweaver-Burk plot was drawn to determine the K_M constant and V_{max} value for GSSG and NADPH, which are the substrates of the GR enzyme purified from sheep spleen tissue. Using the graph, the K_M constant was 0.0061 mM and the V_{max} value was 0.259 EU/mL for NADPH, and the K_M constant was 0.351 mM and the V_{max} value was 0.604 EU/mL for GSSG, it was shown in Figures 3.6 and 3.7.

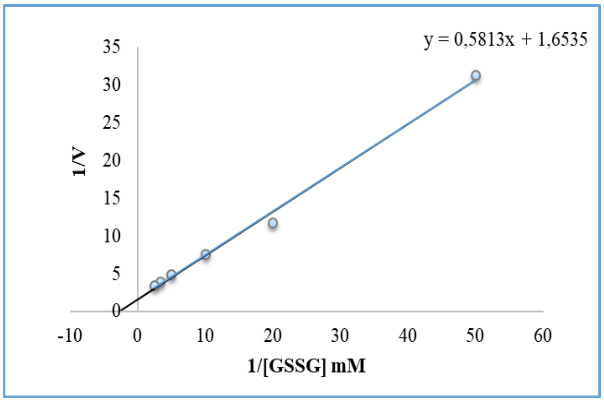

Figure 3.6. Plot 1/V-1/[S] plotted to determine the K_M constant and V_{max} of the GSSG substrate

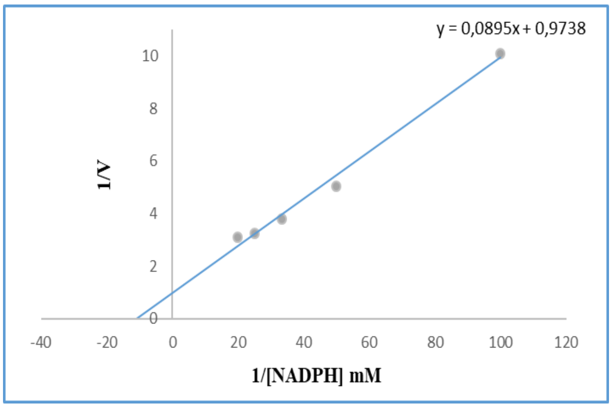

Figure 3.7. Plot 1/V-1/[S] plotted to determine the K_M constant and V_{max} of the NADPH substrate

As a result of the kinetic studies, ampicillin, streptomycin sulfate, gentamicin, cefoperazone sodium and precort-lyo showed an inhibitory effect on the GR enzyme activity. It was determined that amoxicillin, tylosin, cefuroxime sodium, cefazolin sodium, lincomycin, novamizole, ketogenic and clindamycin drugs did not show any activation or inhibition effect on the GR enzyme activity. For ampicillin, streptomycin sulphate, gentamicin, cefoperazone sodium and precort-lyo drugs showing inhibitory effects on the enzyme, Activity%-[I] graphs were drawn and IC_{50} values were calculated with the help of these graphs (Figure 3.8-3.9 and Table 3.2).

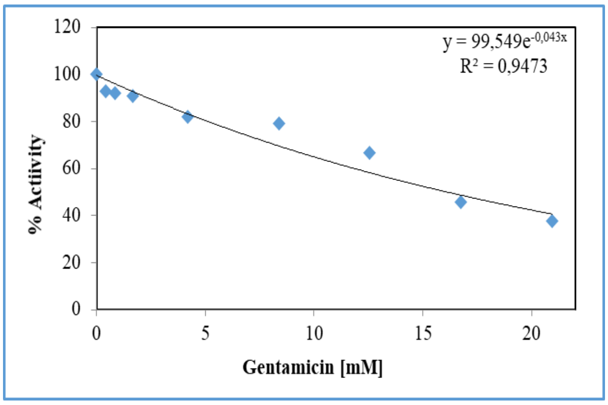

Figure 3.8. Effect of ampicillin on sheep spleen GR enzyme

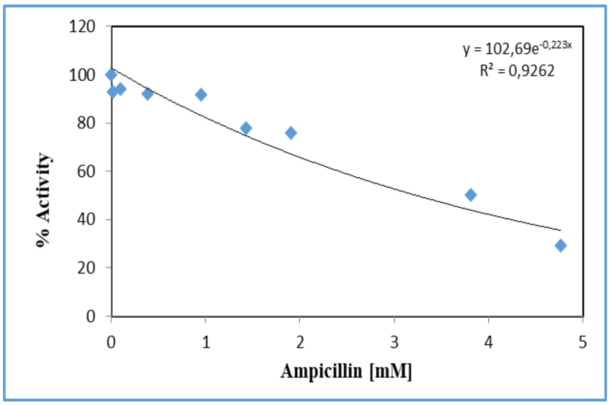

Figure 3.9. Effect of gentamicin on sheep spleen GR enzyme

Table 3.2. IC_{50} values found for some drugs

Drug	**IC_{50} (mM)**
Precort-lyo	1.27
Ampicillin	3.22
Streptomycin sulfate	7.95
Cefoperazon sodium	16.97
Gentamicin	17.20

Lineweaver-Burk graphs were drawn for ampicillin, streptomycin sulfate, gentamicin, Cefoperazone sodium and precort-lyo drugs, which had an inhibitory effect on GR enzyme activity purified from sheep spleen tissue, and Ki constants and inhibition types were determined with the help of these graphs (Figure 3.10-3.11 and Table 3.3).

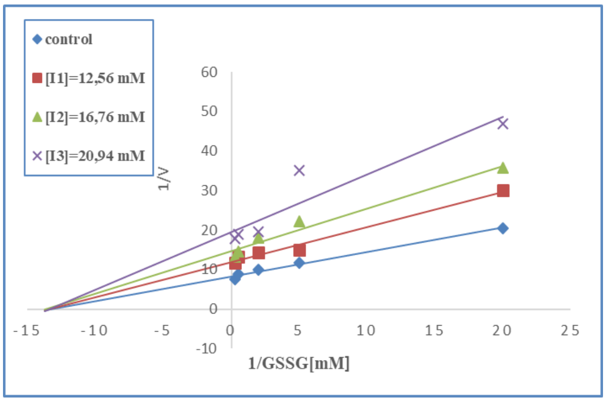

Figure 3.10. Lineweaver-Burk plot for determination of Ki constant for gentamicin sulfate plotted on five different substrates and three different inhibitor concentrations

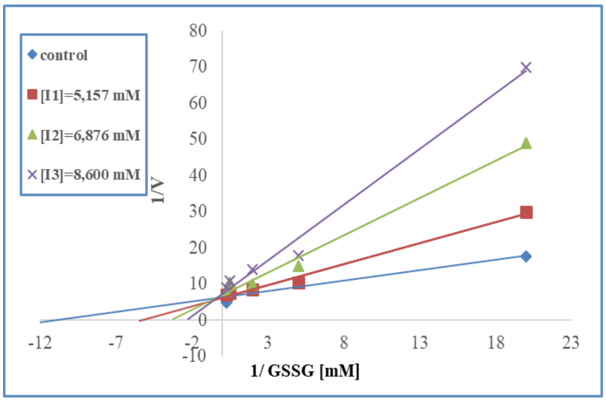

Figure 3.11. Lineweaver-Burk plot for determination of Ki constant for streptomycin sulfate plotted on five different substrates and three different inhibitor concentrations

Table 3.3. Ki values and inhibition types calculated from Lineweaver-Burk plots plotted for sheep spleen GR enzyme at five different substrates and three different concentrations of constant inhibitör

Inhibitor	[I] (mM)	K_i (mM)	Average Ki (mM)	Type of inhibition
Ampicillin	[I1] = 2.862 mM [I2] = 3.816 mM [I3] = 4.770 mM	[I1] = 1.172 mM [I2] = 1.048 mM [I3] = 0.951 mM	1.057 ± 0.11	non-competitive
Gentamicin sulfate	[I1] = 12.56 mM [I2] = 16.76 mM [I3] = 20.94 mM	[I1] = 28.55 mM [I2] = 21.50 mM [I3] = 12.26 mM	20.770 ± 8.17	non-competitive
Streptomicin sulfate	[I1] = 5.157 mM [I2] = 6.876 mM [I3] = 8,600 mM	[I1] = 4.83 mM [I2] = 3.04 mM [I3] = 2.29 mM	3.386 ± 1.31	competitive
Cefoperazon sodium	[I1] = 4.992 mM, [I2] = 9.984 mM, [I3] = 19.968 mM.	[I1] = 3.88 mM [I2] = 5.07 mM [I3] = 5.78 mM	4.910 ± 0.96	competitive
Precort-lyo	[I1] = 0.089 mM, [I2] = 0.356 mM, [I3] = 1.780 mM	[I1] = 0.19 mM [I2] = 0.30 mM [I3] = 0.91 mM	0.466 ± 0.39	competitive

4. DISCUSSION AND CONCLUSION

The GR enzyme (E.C. 1.8.1.7; GR), which belongs to the oxidoreductases ($NADP^+$ oxidoreductase) enzyme group, catalyzes electron transfer between reduced pyridine nucleotides and disulfide substrates. GR enzyme is a very important enzyme for glutathione metabolism, which plays a major role in the balanced and regular conduct of biochemical events in the cell by converting more than 99% of oxidized glutathione (GSSG) into reduced glutathione (GSH) in the reaction it catalyzes [21].

In this study, the GR enzyme, whose importance was explained above, was first purified and characterized from sheep spleen tissue using ammonium sulfate precipitation and 2', 5'-ADP Sepharose-4B affinity chromatography, and the effects of some drugs on enzyme activity were also investigated. In this method, there is an advantage in terms of using less chemicals in a shorter time and reuse of the used materials, purification at once and applying a high volume of substance. When the literature studies are examined, GR enzyme was purified 5 456 times from bovine liver with 38.4% yield by 2', 5'-ADP Sepharose-4B affinity chromatography [10]. GR enzyme was purified from rainbow trout liver 1654 times with 41% efficiency [22]. In another study, it was purified 5.823 times from human erythrocytes with 24% yield [23].

SDS-polyacrylamide gel electrophoresis method was performed according to Laemmli (1970) procedure in order to control the purity of the enzyme and to determine the molecular mass of its possible subunit. The subunit molecular mass of the GR enzyme was calculated as approximately 91 kDa. This value found in the study shows similarities with mouse kidney and liver, sheep liver [24, 25, 26].

In our study, the optimum pH for the GR enzyme was determined as 8.0 (in 0.1 M KH_2PO_4 buffer). In the literature search, it was seen that the optimum pH of the GRs purified from different sources was between 7 and 8.5 [4, 10, 22, 27, 28].

Sheep spleen GR enzyme was determined as optimum ionic strength 100 mM KH_2PO_4 buffer. This value was found to be close to the values found in studies conducted in various sources before [4, 22, 25, 27]. Considering the activities measured for 7 days, it is seen that the stable pH is 7.0 K-phosphate buffer. It was determined that this pH value is close to stable pH values of GR enzyme purified from bovine erythrocyte , rainbow trout liver and turkey liver [4, 22, 27]. By examining the effect of temperature on the enzyme, the temperature at which the enzyme showed the highest activity was determined as 40ºC. In previous studies, these values were determined as 55°C for bovine erythrocytes, 60°C for sheep liver, 55°C for beef liver, 10°C for trout liver, 40ºC for turkey liver, 35-50°C for sheep spleen [4, 22, 25, 27, 29].

In studies conducted to determine the K_M constant and V_{max} value for GSSG and NADPH, which are the substrates of sheep spleen GR enzyme, the K_M constant is 0.0061 mM and the V_{max} value is 0.259 EU/mL for NADPH, and the K_M constant is 0.351 mM and the V_{max} value is 0.604 EU/mL for GSSG was determined. According to these results; It was determined that the affinity of the GR enzyme to the NADPH substrate was higher than its affinity to the GSSG substrate. This result is in agreement with the values found for sheep liver and bovine erythrocyte GR enzymes [10,27].

In our study, the effects of some drugs on enzyme activity were also investigated. In inhibition studies, it was determined that ampicillin, streptomycin sulfate, gentamicin, cefoperazone sodium and precort-lyo substances inhibited the enzyme, but amoxicillin, tylosin, cefuroxime sodium, cefazolin sodium, lincomycin, novamizole, ketogenic and clindamycin substances did not affect the enzyme activity. Precort-lyo, ampicillin, streptomycin sulfate, cefoperazone sodium and gentamicin showing inhibitory effects found IC_{50} values of 1.27, 3.22, 7.95, 16.97 mM and 17.20 mM, respectively, and K_i constants 0.466 ± 0.387 (competitive), 1.057 ± 0.110 (non-competitive), 3.386 ± 1.305 (competitive), 4.910 ± 0.960 (competitive), 20.770 ± 8.169 (non-competitive) mM. According to these results, it was determined that the drug with the most effective inhibitory effect on sheep spleen GR enzyme activity was precort-lyo, and the drug with the lowest inhibitory effect was gentamicin.

Today, many of the drugs that are used intensively for treatment in human and veterinary medicine act on regulatory enzymes that have important roles in metabolism [28-33]. An inhibition that may occur in the activity of these enzymes may play a role in eliminating any problem in cell metabolism or in correcting the situation caused by a pathogenic microorganism in the cell. The results of this study show that ampicillin, gentamicin, streptomycin sulfate, cefoperazone sodium and precort-lyo drugs have inhibitory effects on GR enzyme purified from sheep spleen. From this perspective, it may be a guide for treatments in which the GR enzyme is used as a target in human and veterinary medicine and for future studies on this enzyme.

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