Purification of Glutathione S-Transferase Enzyme from Rainbow Trout Erythrocytes and Examination of the Effects of Certain Antibiotics on Enzyme Activity

Gökkuşağı Alabalık Eritrositlerinden Glutatyon S-Transferas Enziminin Saflaştırılması ve Bazı Antibiyotiklerin Enzim Aktivitesi Üzerine Etkilerinin İncelenmesi

Research Article

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ABSTRACT

The present study investigated the effects of certain antibiotics on the enzyme activity of glutathione-S-transferase (EC 2.5.1.18) obtained from rainbow trout erythrocytes. For this purpose, erythrocyte glutathione S-transferase enzyme was purified 8714-fold by glutathione–agarose affinity chromatography with a yield of 90%. Temperature was kept under control (4°C) during purification. Enzyme purification was checked by performing SDS-PAGE. A single band was obtained approximately at 23 kDa. GST enzyme activity was determined by spectroscopic monitoring of the formation of 1-chloro-2,4-dinitrobenzene-glutathione (CDNB-GSH) conjugate. The same method was used for all kinetic studies. Furthermore, by examining the inhibitory effects upon enzyme activity of antibiotics such as gentamicin, amikacin, cefuroxime sodium, ampicillin, ornidazole, and metranidazole, the IC_{50} values and Ki constants were calculated and inhibition types were identified for gentamicin, amikacin, and cefuroxime sodium, medications that displayed high inhibitory effects.

Key Words
Glutathione-S-transferase (EC 2.5.1.18), rainbow trout erythrocytes, antibiotics

ÖZET

Bu çalışmada bazı antibiyotiklerin gökkuşağı alabalık eritrositlerinden elde edilen glutatyon-S-transferaz (EC 2.5.1.18) enzim aktivitesi üzerine etkileri araştırıldı. Bu amaçla eritrosit glutatyon S- transferaz enzimi glutatyon–agaroz afinite kromatografisi tekniği ile %90 verimle 8714 kat saflaştırıldı. Saflaştırma esnasında sıcaklık kontrol altında tutuldu (+4°C). Enzim saflığı SDS-PAGE yapılarak kontrol edildi. Yaklaşık olarak 23 kDa'da tek bant elde edildi. GST enzim aktivitesi, 1-kloro-2,4-dinitrobenzenglutatyon (CDNB-GSH) konjugatının oluşumunun spektroskopik olarak izlenmesi ile belirlendi. Kinetik çalışmaların tamamında bu metot kullanıldı. Ayrıca enzim aktivitesi üzerine gentamisin, amikasin, sefuroksim sodyum, ampisilin, ornidazol ve metranidazol gibi antibiyotiklerin inhibisyon etkileri incelenerek yüksek inhibisyon etkisi gösteren gentamisin, amikasin, sefuroksim sodyum ilaçlarının IC_{50} değerleri ve Ki sabitleri hesaplanarak inhibisyon tipleri belirlendi.

Anahtar Kelimeler
Glutatyon-S-transferaz (EC 2.5.1.18), gökkuşağı alabalık eritrositleri, antibiyotikler

Article History: Received September14, 2011; Revised October 19, 2011; Accepted November 03, 2011; Avaliable Online: December 02, 2011.

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INTRODUCTION

Glutathione S-transferase plays a crucial role by detoxifying xenobiotics in combination with several mechanisms in the metabolism. This enzyme inactivates electrophilic xenobiotics by conjugating them with reduced glutathione (GSH) and eliminates them from the body [1]. Glutathione S-transferase isoenzymes are homodimeric and heterodimeric proteins synthesized from a multitude of gene loci with a molecular mass of about 25 kDa. Each subunit has an active position involving two different functional sites, which are the G region that binds the physiological substrate and hydrophobic H region that binds the structurally different electrophilic substrates. The aminoacid composition of GST isozymes differs in the hydrophobic H-region, which the reason behind the substrate diversity of the enzyme family. Glutathione S-transferases are a member of the family of phase-II detoxification enzymes which catalyze the conjugation of glutathione with xenobiotics, to which living organisms are exposed voluntarily or involuntarily, and convert them into less toxic metabolites [2-4].

Aminoglycoside antibiotics (amikacin, netilmicin, gentamicin etc.) are known to have nephrotoxic, neurotoxic, and autotoxic effects. With an important role in the metabolism and detoxification of many medications, glutathione has a tripeptide structure with cysteine [5]. It neutralizes medications through the sulfhydryl (-SH) group in its molecules. This process often makes use of Glutathione S-transferase enzyme [6]. This study aimed to examine the in vitro effects of antibiotics used to treat both humans and fish upon the enzyme activity of GST purified from rainbow trout erythrocytes.

MATERIAL and METHOD

Glutathione-Agarose, GSH, 1-chloro-2, 4-dinitrobenzene (CDNB) substrates, protein measurement reagents, and the chemicals used for electrophoresis were obtained from Sigma Chem. Com. All other chemicals we used for analytical measurements in our study were purchased from Sigma- Aldrich and Merck.

Hemolysate Preparation

Fresh fish blood samples were taken into tubes containing adequate amounts of EDTA. Next, the plasma and leukocyte layers were removed by centrifuging at 2500xg for 15 minutes. The erythrocyte sediment was washed thrice with 0.154 M NaCl solution and then hemolysed in five times its volume of ice-cold water. Centrifugation was performed at 15.000xg for 30 min. to remove cell membranes [7].

Glutathione-Agarose Affinity Chromatography

1 g lyophilized powder of glutathione-agarose was weighed for 10 ml of bed volume and washed several times with 200 ml distilled water to remove solid particles. Washing swelled the gel, which was deaerated by using a water trompe and suspended by adding stabilization buffer (0.05 M K-phosphate (pH 7.4), 1 mM EDTA, and 1 mM DTT). The suspended gel was packed in a cooled column with a 1x10 cm closed system. After precipitation, the gel was washed using a peristaltic pump with stabilization buffer. Stabilization of the column was clear from the fact that the absorbance and pH of the eluate and buffer were identical. The affinity column was thus prepared. The prepared hemolysate was directly applied to the glutathione- agarose affinity column. Subsequently, the column was washed by passing through 10 mM KH_2PO_4 and 0.1 M KCl (pH 8.0). The washing procedure was monitored on a spectrophotometer through equal-to-blind absorbance values. After the column was stabilized, the enzyme was purified by gradient elution. Elution solvent was prepared from a solvent gradient containing 50 mM Tris-HCl and (1.25-10 mM GSH, pH 9.5). The eluates were taken into tubes of 1.5 ml and their absorbance was examined at 340 nm. The column flow rate was set to 20 ml/hr using a peristaltic pump. The purification procedure was carried out at 4°C [8].

Calculation of Enzyme Activity

Among its many other substrates, the aromatic electrophile 1-chloro-2,4-dinitrobenzene is one that is most often used to determine GST enzyme activity. As the product obtained by the use of this substrate, dinitrobenzene S-glutathione (DNB-SG) displays maximum absorbance at 340 nm.

Activity measurements were thus carried out by using the absorbance increase at this wavelength. After activity measurements, to the cuvettes were

added 0.5 M 200 µl potassium phosphate buffer (K_2HPO_4/KH_2PO_4 pH 7.4) and 25 mM 20 µl 95% CDNB (in ethanol). Following incubation at 37°C for 3 min, 20 mM 50 µl GSH was added to cuvettes, which were thoroughly stirred. After a quick addition of 50 µl enzyme sample, the cuvettes were placed in the spectrophotometer and reading was initiated [1].

Protein determination

Bradford's method was followed in quantitative protein determination. This method is based on the binding of Coomassie Brilliant Blue G-250 to protein. The complex formed spectro-photometrically shows maximum absorbance at 595 nm. We used the standard bovine serum albumin solution [9].

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

To control enzyme purity, Laemmli's procedure was performed in 3% and 8% acrylamide concentrations for stacking and running gels, respectively. 1% SDS was added to this acrylamide solution. The obtained SDS-PAGE gel was first stabilized for 30 min in a solution containing 50% propanol, 10% TCA, and 40% distilled water. Then, the gel was stained for 2 h in a solution containing 0.1% Coomassie Brilliant Blue R-250, 50% methanol, and 10% acetic acid. Finally, washing was performed with a solvent containing 50% methanol, 10% acetic acid, and 40% distilled water until the protein bands were clear [10].

Kinetic Studies

The inhibitory effects of the antibiotics, gentamicin, amikacin, cefuroxime, ampicillin, ornidazole, and metranidazole, were studied by examining their enzyme activity at different concentrations. Enzyme activities were calculated for at least 5 different inhibitor concentrations in order to determine the IC50 values. The activity in the cuvettes containing no medication was taken as 100%. Inhibitor concentrations causing 50% inhibition (IC_{50}) were determined by drawing Activity% - [I] mM graph.

To determine K_i constants, activity measurements were performed at 5 different GSH concentrations (0.02 mM, 0.04 mM, 0.06 mM, 0.08 mM, 0.1 mM) and at constant inhibitor concentration. Lineweaver - Burk plots [11] were drawn using the data obtained to determine the Ki constants and inhibition types.

RESULTS AND DISCUSSION

As is well known, medications used for treatment have numerous adverse effects for metabolism. The metabolism contains various enzymatic and non-enzymatic defense systems to eliminate or minimize such side effects. Enzymatic defense is provided by several enzyme systems such as glutathione S-transferase, glutathione reductase, glutathione peroxidase, superoxide dismutase, and catalase [12]. With a specific role in the metabolism of a wide group of electrophilic substrate that include antibiotic, analgesic, and anticancer medications, glutathione S- transferases [13-15] constitute a group of multifunctional enzymes which are involved in conjugating both endogenous and exogenous electrophilic compounds with reduced glutathione (GSH) and thus catalyzing their conversion into less toxic metabolites [16-18]. Our aim in this study was to purify glutathione S-transferase enzyme, whose metabolic importance has long been acknowledged, from rainbow trout erythrocytes and to examine the in vitro effects of certain antibiotics upon enzyme activity.

In the study, rainbow trout erythrocyte GST enzyme with 12.2 EU/mg protein specific activity was purified 8.714-fold with a yield of 90% (Table 1). The purification procedure involved two steps, which were hemolysate preparation and glutathione-agarose affinity chromatography [8]. Since it is effectively used in purifying GSTs, glutathione-agarose affinity column was used during purification. This method has certain advantages such as short purification duration and low activity loss during purification. Following purification, SDS-PAGE was performed to control enzyme purity and a single band was obtained (Figure 1). The method involving a single band, specific activity, and purification coefficient was seen to be a suitable method for researchers.

Many studies have been conducted to purify the glutathione S-transferase enzyme with vital metabolic importance, to identify its three-dimensional structure, and to examine its kinetic and characteristic properties [19,20]. Yet, our literature review detected no study carried out to purify the enzyme from rainbow trout erythrocytes and to examine the effects upon enzyme activity of certain

Table 1. Purification steps of GST.

Purification step	Activity (EU/ml)	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification factor
Homogenate	0.065	30	46.1	1383	0.195	0.0014	100	1
Glutathione-Agarose affinity chromatography	0.220	8	0.018	0.144	0.176	12.2	90	8.714

antibiotics such as gentamicin, amikacin, cefuroxime sodium, ampicillin, ornidazole, and metranidazole. Many medications and antibiotics used to treat fish diseases are known to have adverse effects on the organism [21]. This will directly or indirectly affect humans, who are at the top of the food chain and this fact has now been recognized by many researchers. Therefore, our study is highly significant.

As a result of the kinetic studies, gentamicin, cefuroxime, and amikacin were found to display inhibitory effects and Activity %-[I] graphs were drawn for these medications, whose IC50 values were calculated as 0.568, 1.104, and 7.637 mM, respectively (Figures 2-3; Table 2). For gentamicin, cefuroxime, and amikacin inhibitors, Lineweaver-Burk plots were drawn (Figures 4-6) and their Ki constants were determined as 1.506 ± 0.176 (non-competitive), 0.915 ± 0.490 (competitive), and 6.43 ± 3.627 (non-competitive) (Table 2). Similar studies can be found in the literature. For instance, the researchers in a study examined the in vitro interaction between GST and medications with wide clinical use (analgesic, antipyretic, antimicrobial

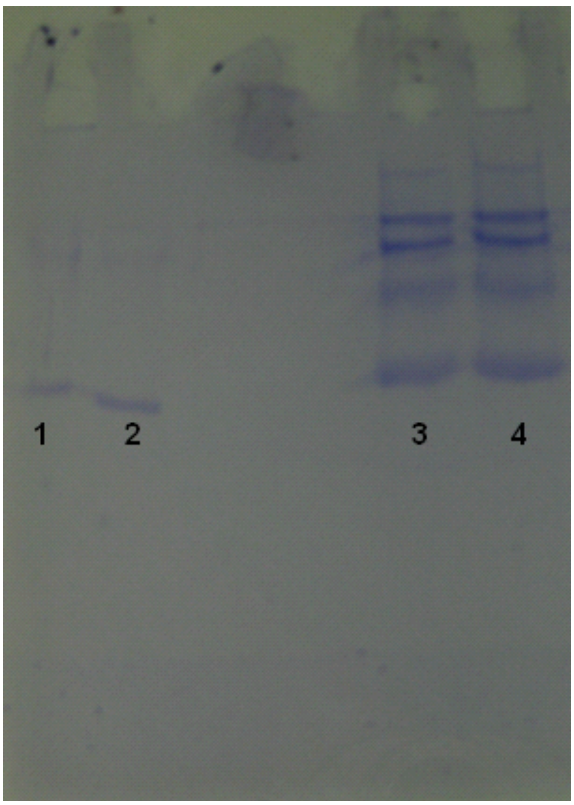

Figure 1. Purification control of GST from affinity column by SDS-PAGE (line 1, 2: GST from affinity column. Line 3, 4: Standard proteins (Rabbit muscle phosphorilase b: 97 kDa; BSA: 66 kDA; Ovalbumin: 45 kDa; Bovine erythrocytes CA: 29 kDa).

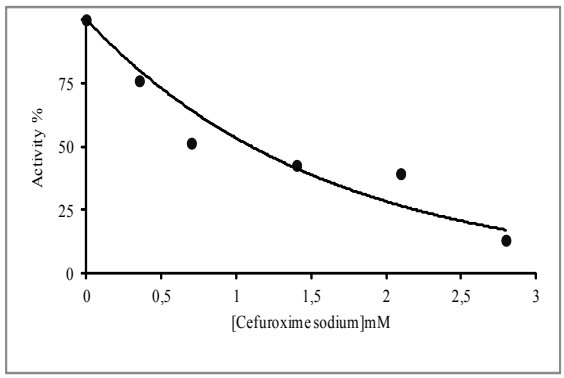

Figure 2. Activity % vs [Cefuroxime sodium] regression analysis graphs for GST in the presence of 5 different Cefuroxime sodium concentrations.

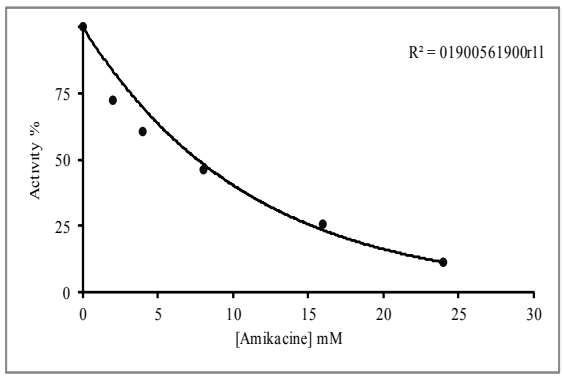

Figure 3. Activity % vs [Amikacine] regression analysis graphs for GST in the presence of 5 different Amikacine concentrations.

Table 2. K_i and IC50 values obtained from regression analysis graphs for GST in the presence of different drugs.

Inhibitor	IC_{50}(mM)	K_i constant	Inhibition type
Gentamicin sulfate	0.568	1.506 ± 0.176	Non-competitive
Sefuroksim sodium	1.104	0.915 ± 0.490	Competitive
Amikacine	7.637	6.430 ± 3.627	Non-competitive

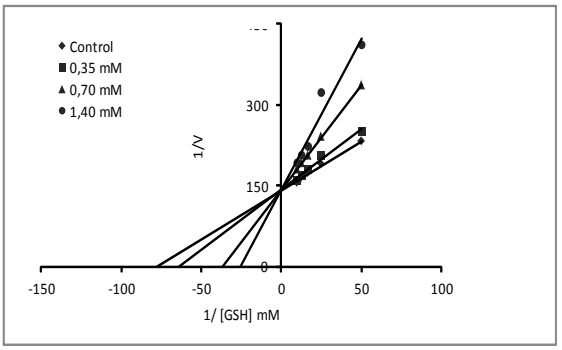

Figure 4. Lineweaver-Burk graph for 5 different substrate concentrations and 3 different Cefuroxime sodium concentrations for determination of K_i.

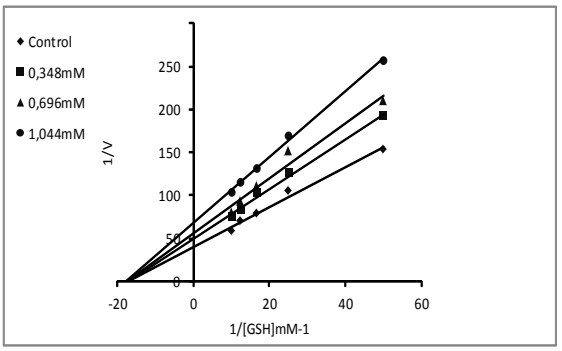

Figure 5. Lineweaver-Burk graph for 5 different substrate concentrations and 3 different gentamicin sulfate concentrations for determination of K_i.

etc.) in full-term human placenta and reported that the ampicillin antibiotic among the medications used reduced GST enzyme activity by 45%, while Novalgine, an analgesic, decreased it by 70% [18]. On the other hand, the present study found that ampicillin had no in vitro inhibitory effect on rainbow trout erythrocyte GST enzyme activity.

Other studies in the literature have examined and reported on the inhibitory effects of the antibiotics whose effects on GST we have studied in this research upon the enzyme activity of glucose 6-phosphate dehydrogenase and glutathione reductase, which are involved in the antioxidant defense system [22. 12,23,24]. For instance, one study examined the in vitro effects of gentamicin sulfate and ampicillin on the enzyme activity of glutathione reductase

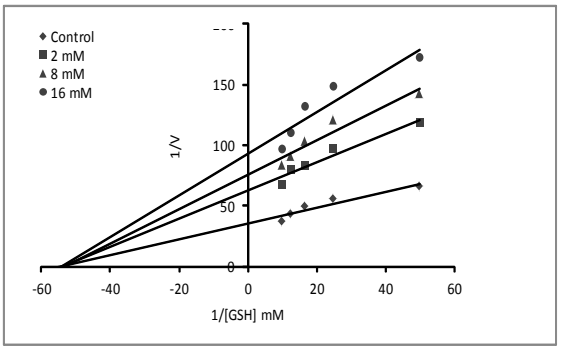

Figure 6. Lineweaver-Burk graph for 5 different substrate concentrations and 3 different amikacine concentrations for determination of K_i.

purified from human erythrocytes, reporting that these antibiotics exhibited no inhibitory effect [22]. However, in the present study, we found that gentamicin caused inhibition on rainbow trout erythrocyte GST enzyme. Another study examined the in vitro effects of six antibiotics, one of which was gentamicin sulfate, upon the enzyme activity of glutathione reductase purified from sheep liver and gentamicin was observed to have no inhibitory effect [23]. In our study, on the other hand, gentamicin was found to cause inhibition on rainbow trout erythrocyte GST enzyme. Another study in the literature examined the in vitro effects of a series of antibiotics including cefuroxime and amikacin on the enzyme activity of glutathione reductase (GR) purified from human erythrocytes and reported that cefuroxime had inhibitory effects, while amikacin did not [12]. In our research, however, we found that both antibiotics caused inhibition on rainbow trout erythrocyte GST enzyme. Still another study examined the effects of some antibiotics including thiamphenicol, amikacin, gentamicin, and netilmicin upon the enzyme activity of glucose 6-phosphate dehydrogenase (G6PD) purified from rainbow trout (oncorhynchus mykiss) erythrocytes, reporting on their inhibitory effects [24].

The most efficient parameters showing the inhibitory effect of any inhibitor are Ki constant and IC_{50} values [25]. An examination of the Ki values of the medications with inhibitory effect on the

activity of rainbow trout erythrocyte GST enzyme (Table 2) demonstrates that all of them are strong inhibitors of GST, but cefuroxime sodium is the medication displaying the highest inhibitory effect. Both the results of our study and those of others in the literature we have mentioned have revealed that the antibiotics used in the present research are potential inhibitors for metabolic enzymes. Such medications inhibit not only GST enzyme, but also other enzymes that produce glutathione as a GST substrate (glutathione reductase), which will result in a serious failure of detoxification reactions. Therefore, in determining the doses of these antibiotics used for treatment, it will be particularly important for metabolic defense systems to take into consideration the IC_{50} values obtained as a result of kinetic studies.

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