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Investigation of the effects of some water-soluble vitamins on glutathione reductase enzyme purified from bovine liver

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Abstract: Glutathione reductase (GR, EC 1.8.1.7) is an antioxidant enzyme and is involved in the reducing reaction of oxidized glutathione. Glutathione and reduced glutathione are very crucial for micro- and macroorganisms because of their barrier function against radicals. Therefore, the aim of this article was to purify GR and evaluate the relationship between purified GR and vitamins. GR was purified from bovine liver using 2',5'-ADP Sepharose 4B column materials. To check the purity of each subunit of the enzyme, SDS-PAGE electrophoresis was performed and each one was 55 kDa. Afterwards, specific activity and purified ratio for the enzyme were calculated as 54 EU/mg × protein and 2700 times, respectively. Vitamins have a regulatory role in organisms and also some, like vitamin B, are coenzymes due to having cofactor effects. In this project, variable concentrations of some water-soluble vitamins, riboflavin, ascorbic acid, nicotinamide, folic acid, and thiamine, were tested. As a result of these kinetic findings, nicotinamide and folic acid increased the activity of glutathione reductase, while thiamine decreased it. Riboflavin and ascorbic acid did not show a stable effect on enzyme activity. Ki and IC_{50} constants were found as 50.16 ± 5.63 mM and 21.04 mM, respectively, when Lineweaver–Burk and activity % vs. inhibitor concentration graphs were drawn for thiamine. The results of this article illustrate that the behaviors of vitamins could be complicated and vary from one living organism to other; in other words, they are unpredictable.

Key words: Glutathione reductase, thiamine, folic acid, riboflavin, nicotinamide, ascorbic acid

1. Introduction

In organisms, an enzyme is a biological catalyst that increases velocity and provides 100% yield without byproducts in chemical reactions [1]. In 1951, glutathione reductase enzyme (GR, E.C.1.8.1.7) was first identified. It catalyzes the reduction of oxidized glutathione (GSSG), which occurs due to the reduction reaction of hydrogen peroxide and organic peroxide by glutathione peroxidase (GPX). Thereby, a small amount of glutathione (GSH) in the organism is ready for reuse. However, reduced nicotinamide adenine dinucleotide phosphate (NADPH) is needed to complete this reaction; for this aim, sufficient amounts of NADPH are obtained from the pentose phosphate pathway. Due to NADPH, the relationship between GR and glucose-6-phosphate dehydrogenase (G6PD) is observed. If G6PD does not react, it leads to a decrease in the amount of NADPH and this results in a small amount of GSH based on the low activity of GR [2–4]. GR is localized to the cytosol and is a structural dimer enzyme. Flavin adenine dinucleotide (FAD) is present in each subunit of GR. NADPH reduces to FAD and then the electrons of FAD are transferred to glutathione disulfide. The activity of GR is

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spectrophotometrically measured by the reduction of GSSG to GSH with the consumption of NADPH. This reaction is as follows: GSSG + NADPH + $H^+ \rightarrow$ 2 GSH + $NADP^+$. When GR intracellularly increases the GSH/GSSG ratio, it protects erythrocytes against hemolysis [2,4–8]. As seen in the reaction, GSH is produced by glutathione reductase and it is consumed by many enzymes like GPX and glutathione S-transferase (GST). This has inspired scientific studies such as those about growing rats [9].

Vitamins are essential nutrients, which are macro or organic molecules. Organisms require sufficient amounts of these to ensure that their metabolism operates optimally [10]. Sufficient amounts of essential nutrients may not be present or synthesized by the organism and therefore these should be ingested from either animal or herbal foods. Vitamin C is synthesized by some organisms while not by others. Moreover, vitamin C is a required vitamin for some organisms, while it is not for others. The term "vitamin" does not contain the three other main nutritional molecule groups, which are minerals, essential amino acids, and essential fatty acids [10].

Vitamin B1, also called thiamine, is known as an antineuritic and beriberi factor and is also called aneurine in European countries. Thiamine has a white crystalline structure and has double rings of pyrimidine, and thiazole. Moreover, thiamine pyrophosphate (TPP) or diphosphothiamine (DPP) forms when it is linked to two phosphoric acids. This compound is involved as a coenzyme for carboxylase enzymes. TPP is part of the decarboxylation of oxidative, nonoxidative, and transketolase reactions [11].

Nicotinamide is also called niacinamide, niacin, or a type of vitamin B3 [12]. Lack of nicotinamide causes pellagra, with symptoms such as inflamed skin, diarrhea, dementia, and sores in the mouth and it is frequently seen in the south of Europe and the Americas [11].

Riboflavin, also called vitamin B2, is involved in biological oxidation and the electron transport chain by complexing with proteins as flavoprotein. In addition, it is present in the structures of D- and L-amino acid, xanthine oxidase, cytochrome c reductase, and some dehydrogenases. Flavin also exists in FAD and flavin mononucleotide (FMN) as a prosthetic group. Flavoproteins exist in different forms and are enzymes that perform oxidation and reduction reactions in intracellular structures [11].

Folic acid is known as folacin or pteroyl glutamic acid and consists of three compounds, pteridine, p-amino benzoic acid, and L-glutamic acid. The main biochemical function of folic acid is to transmit the one-carbon units by means of its 5,6,7,8-tetrahydro derivative of tetrahydrofolic acid as a coenzyme [11].

The aim of this study was to investigate the effects of some water-soluble vitamins on GR purified from bovine liver in vitro. As is known, the truest findings for enzyme kinetics are obtained from the purified enzyme. Therefore, GR has been purified many times and from many different sources in unicellular and multicellular organisms, such as liver and erythrocytes of Lake Van pearl mullet, *Streptococcus pneumoniae*, and bovine liver [13–15]. This revealed the value of GR. Moreover, although there are many studies about the effects of vitamins on GR, such as the effects in vivo of folate deficiency on GR, GPX, and GST [16] and mice fed with niacin and nicotinamide [17], the effects of some soluble-water vitamins on GR in vitro were researched for the first time in this article. With this purpose, the effects of some water-soluble vitamins like ascorbic acid, nicotinamide, riboflavin, folic acid, and thiamine were investigated by using their diluted and concentrated homogeneous solutions with bovine liver GR.

2. Material and methods

2.1. Materials

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. or Merck (Turkey); only 2',5'-ADP Sepharose-4B was obtained from Amersham Biosciences.

2.2. Preparation of bovine liver homogenate

This preparation was obtained by using the reported protocol [15]. In brief, bovine liver was obtained from a local slaughterhouse and brought to the laboratory in an ice bath. Frozen bovine liver was cut into small pieces with a bistoury and about 15 g of minced liver was obtained. After this procedure, the minced liver was added to the homogenate buffer (0.05 M Na-phosphate, 0.24 M sucrose, pH 7.4) and the mixture was homogenized by a WIGGEN HAUSER ultrahomogenizer at 8000–30000 rpm in an ice-water bed for approximately 15 min. The homogenate was first centrifuged three times at 4 °C, 1500 rpm, and 20 min and then at 4 °C, 11000 rpm, and 60 min to remove cellular residues. Then 75 mL of bovine liver homogenate was used to perform purification of GR and the homogenate activity was measured from this homogenate after it was obtained.

2.3. Affinity chromatography

The affinity column was prepared by using the reported protocol [15]. In brief, 2.5 g of dried 2',5'-ADP Sepharose-4B was used for a column with bed volume of 10 mL. The gel was washed with 400 mL of bidistilled water to remove foreign bodies and air, suspended in balancing buffer (50 mM K-phosphate/1 mM EDTA, 1 mM DTT, pH 7.3), and packed in the column (1 × 10 cm). After being filled with the gel, the column was equilibrated with balancing buffer by means of a peristaltic pump over 3 days. The flow rates for washing and equilibrium were adjusted to 20 mL/h. The homogenate prepared previously was loaded into the 2',5'-ADP Sepharose-4B affinity column and the column was washed with balancing buffer and washing buffer I (0.1 M K-acetate/0.1 M K-phosphate, pH 7.85) and then washing was continued with washing buffer II (0.1 M K-phosphate/0.1 M KCl, pH 7.85) until the final absorbance difference became 0.05 at 280 nm. The enzyme was eluted with the elution buffer [1 mM GSH/0.5 mM NADPH in 50 mM K-phosphate/1 mM EDTA (pH 7.0)] and collected with volumes of 3 mL in each tube. Active fractions were detected by measuring the enzyme activity as mentioned below. All of the procedures were performed at 4 °C.

2.4. Assay of GR activity and its kinetic behavior

This activity was evaluated by using the assay [15] reported according to Carlberg and Mannervik [18] with minor configurations and a Shimadzu UV-1280 spectrophotometer at room temperature. In brief, in the study the assay protocol was 50 μL of 50 mM Tris-HCl/1 mM EDTA buffer (pH 8.0), 30 μL of 20 mM GSSG, 50 μL of 2 mM NADPH, 30 μL of purified/hemolysated GR, and double-distilled water to complete the total reaction volume of 1 mL. The enzyme activity protocol added 30 μL of purified/hemolysated GR to this protocol mixture with or without the presence of various concentrations of water-soluble vitamins after 3 min of incubation. One enzyme unit is defined as the oxidation of 1 μmol NADPH per minute under assay conditions [15].

2.5. Protein determination

To determine the protein content in all eluents from the affinity column, a quantitative protein assay was carried out spectrophotometrically at 595 nm with respect to Bradford's method [19] using bovine serum albumin as

the standard. Figure 1 represents the qualitative and quantitative assay for purified GR.

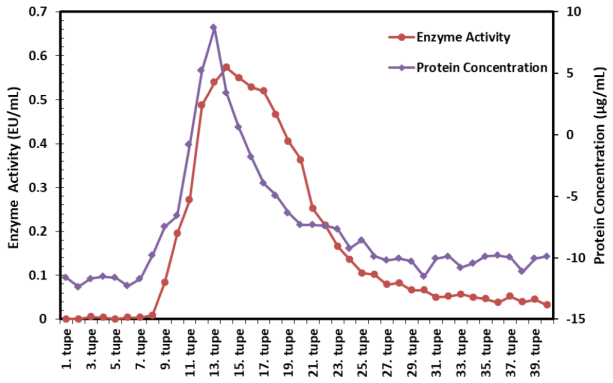

Figure 1. Qualitative and quantitative assays of the purified GR.

2.6. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

This method was carried out by using the published protocol [15]. In brief, the acrylamide concentrations of the stacking and separating gels were 3% and 8%, respectively, and 1% SDS was used for denaturing secondary, tertiary, and quaternary structures. The Fermentas protein ladder SM0671 was used as a standard protein mixture for SDS-PAGE electrophoresis. The gel was added to stabilization solution containing 50% propanol, 10% TCA, and 40% double distilled water for about 30 min. After that, the gel was placed in staining solution including 0.1% Coomassie Brilliant Blue R-250, 50% methanol, 10% acetic acid, and 39.9% double-distilled water for about 3 h. Lastly, the gel was washed with the same staining solvent without dye until the protein bands were clearly seen. Electrophoresis bands are shown in Figure 2.

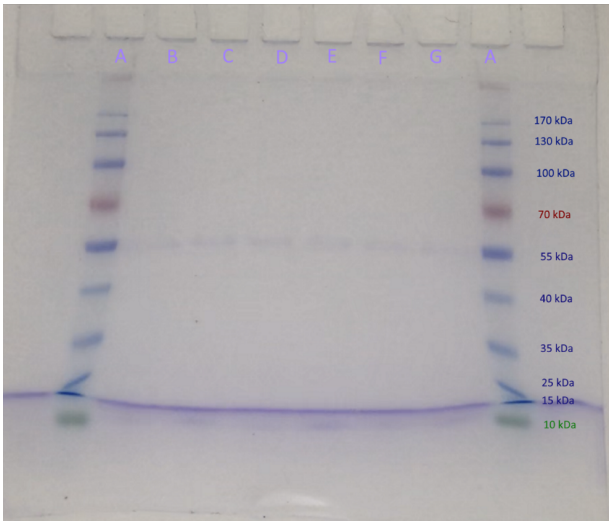

Figure 2. SDS-PAGE electrophoresis gel for bovine liver GR. A column: Fermentas SM 00671 marker; B–G columns: GR purified from affinity column chromatography.

2.7. In vitro kinetic studies

The effects of various concentrations of the water-soluble vitamins on bovine GR enzyme activity were researched. In brief, the assay protocol of the in vitro kinetic studies was 50 μL of 50 mM Tris-HCl/1 mM EDTA

buffer (pH 8.0), 30 μL of 20 mM GSSG, five different concentrations from 30 μL to 70 μL of 2 mM NADPH, various concentrations (μL) of vitamin solutions, 30 μL of purified GR, and double-distilled water to complete the total reaction volume of 1 mL. The purified GR was added to the above protocol after 3 min of incubation. Afterwards, reactions between the purified GR and vitamin were spectrophotometrically recorded at 340 nm for 3 min [15]. Spectrophotometric data were converted to activity (EU/mL) by drawing the Lineweaver–Burk, activity % vs. inhibitor concentration, and activity % vs. activator concentration graphs. The kinetic results, IC_{50}, and Ki values for vitamins on GR activity are given in the Table. Lineweaver–Burk graphs, which are used to determine inhibition type, were drawn by using 1/[S] vs. 1/[V] at different fixed inhibitor concentrations, where [S] is substrate (NADPH) concentration and [V] is activity value of the inhibitor (vitamin) on GR. The IC_{50} values were calculated from activity % vs. inhibitor concentration plots.

Table 1. The interactivity of some vitamins, soluble in water, with GR activity.

Vitamins	**Solubility**	**Concentrations**	**IC_{50}^{a}**	**Ki[b]**	**TKB***
Ascorbic acid	+	2.05, 2.24, 4.1, 8.2, 10.25, 41.0, and 82.0 μM	-	-	NE
Nicotinamide	+	20.0, 41.0, 62.0, 82.0, and 103.0 mM	-	-	ACT
Folic acid	+	2.27, 4.53, 6.80, 9.06, and 11.33 mM	-	-	ACT
Thiamine	+	3.0, 6.0, 9.0, 12.0, and 15.0 mM	21.04 mM	50.16 mM	NCI
Riboflavin	+	1.3, 2.6, 3.9, 5.2, 6.5, 7.8, 10.4, and 13.0 μM	-	-	NE

* Kinetic behaviors.
+ Vitamin soluble in water.
[a] Half maximal inhibitory concentration.
[b] Coefficient of inhibition.
NE: No effect; ACT: activator; NCI: noncompetitive inhibition.

3. Results and discussion

In the present study, GR was purified from bovine liver using 2',5'-ADP Sepharose-4B affinity column chromatography. The specific activity, yield %, and purity ratio were calculated as 54 EU/mg protein, 43.2%. and 2700-fold, respectively. Moreover, the molecular weight of each subunit of the purified enzyme was found as 55 kDa using the SDS-PAGE electrophoresis method. This result has very good fit compared to purified GR enzymes such as from liver and erythrocytes of Lake Van pearl mullet [13], bovine liver [15], bovine filarial worms *Setaria cervi* [20], and rainbow trout (*Oncorhynchus mykiss*) liver [21].

GR, as a flavo- and homodimeric protein, catalyzes the reduction of GSSG to GSH using NADPH. To ensure high amounts of GSH/GSSG, GR affects several critical events such as reactive oxygen species (ROS), the defensive response against free radicals, and protein and DNA biosynthesis [22]. Oxidative stress causes oxidants including ROS, which produce GSSG by the oxidation of GSH [23]. The GSH/GSSG ratio is decreased when oxidative stress occurs. This situation means that GSH and GSSG form a cellular redox buffer [24]. Accordingly, from past to present, GR was purified from a variety of sources a number of times including the

liver and erythrocytes of Lake Van pearl mullet [13], bovine filarial worms *Setaria cervi* [20], human erythrocytes [25], bovine erythrocytes [26], sheep brain [27], and rodent malaria parasite *Plasmodium berghei* [28]. Activation energy is the specific energy required at the beginning of each reaction. The principal role of enzymes is to ease the reactions by decreasing the activation energy. This occurs when the enzymes bind to reactants and use energy more effectively. Reactant chemicals, known as substrates, especially bind to active sites located on enzymes, which are very complicated three-dimensional structures. Coenzymes consist of vitamins, or are synthesized directly from vitamins, and these help enzymes bind to their substrate [11].

The % activator vs. concentration graphs were drawn for nicotinamide and folic acid and these graphs are given in Figures 3 and 4. Increased concentrations of nicotinamide and folic acid in the test tube improved the GR activity, while thiamine concentrations inhibited the activity. There is a larger relationship between coenzymes and vitamins. Structures of many coenzymes consist of vitamins. In some conditions, a lack of coenzyme occurs due to deficiency of the vitamin, and in this case, the enzymatic reaction cannot be completed. Besides, many different concentrations of riboflavin and ascorbic acid were used with GR, but no effect was observed. This probably results from the structures of both vitamins not fitting the active sites on the enzyme. All concentrations of vitamins used in this study are given in the Table.

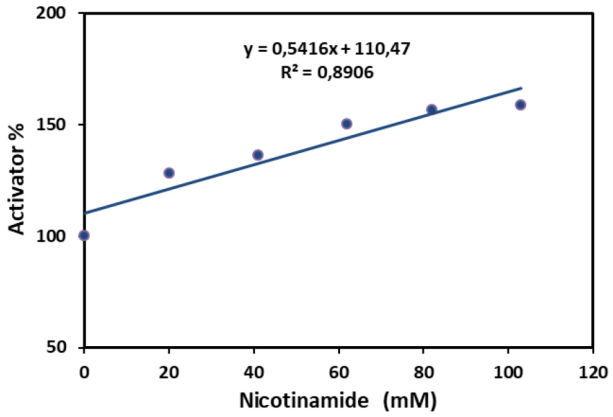

Figure 3. Activator % vs. concentration for nicotinamide.

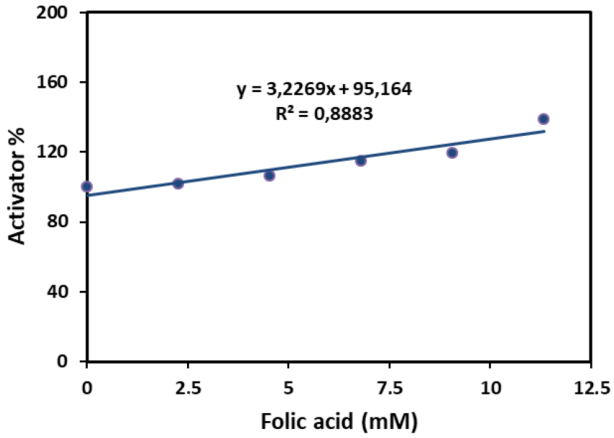

Figure 4. Activator % vs. concentration for folic acid.

The Lineweaver–Burk inhibition graph was drawn for thiamine and the inhibition type was understood to be noncompetitive inhibition from this graph (Figure 5). Using this graph plot, the Ki value of thiamine was 50.16 ± 5.63 mM. Also, the activity % against inhibitor concentration graph was drawn, and the IC_{50} value for thiamine from these plot equations was 21.04 mM (Figure 6). The Ki value of thiamine was larger than the K_i values of $NADP^+$ and GSH obtained from previous purification of GR such as from bovine liver [29], sheep liver [30], and turkey liver [31]. Therefore, thiamine is considered to be an inhibitor or coenzyme for GR with weaker binding from GSH and $NADP^+$ to GR.

One of our results is that folic acid and nicotinamide stimulated the activity of GR. Both nicotinamide and folic acid increased the efficiency of GR, and when the amount of GSH increases, the oxidative stress may decrease. Similar results were seen in other reports that showed that nicotinamide was an antioxidant and also regulated oxidative stress [17]. In another study, oxidative stress features developed in rats due to deficiency of folate. Moreover, this situation caused an increase in the activity of enzymes involved in liver metabolism such as GR, GPX, and GST but did not affect catalase and superoxide dismutase [16]. Contrary to this, in two

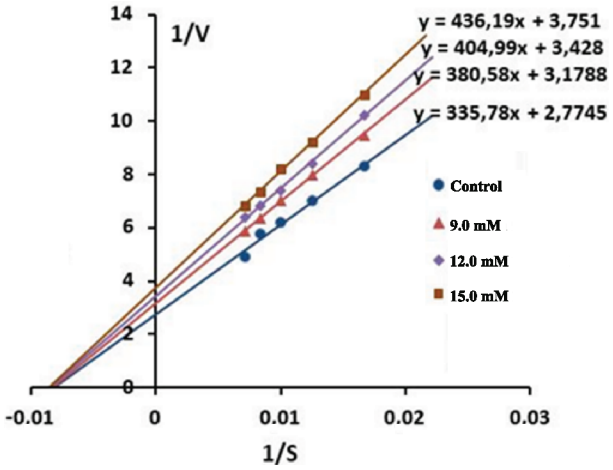

Figure 5. Lineweaver-Burk graph for thiamine.

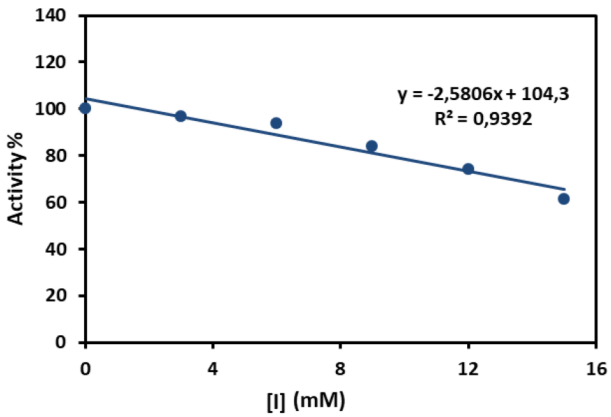

Figure 6. Inhibitor concentration vs. GR activity % of thiamine.

different in vivo studies, Ctenopharyngodon idella was fed with niacin or nicotinamide and the results of these studies revealed that niacin decreased GR activity [32,33].

In addition, different concentrations of riboflavin did not show remarkable efficiency on GR activity or indirectly on the amount of GSH. This argument is similar to some in vivo reports about erythrocyte antioxidant enzyme activities of rats with deficiency of riboflavin [34], the levels of antioxidant GSH/antioxidant enzymes of rats with riboflavin deficiency [35], and also erythrocytes of pigs with riboflavin deficiency.

Of the findings in this article, ascorbic acid did not regularly stimulate the activity of bovine liver GR. This result is supported by Zhao et al., who found that the effect of ascorbic acid and vitamin E was not consistent on the antioxidant enzymes in bull plasma [36].

In some studies similar to ours, the effects of vitamins E, A, and C on rats with diazinon were investigated and the levels of AST and ALT decreased after these compounds were administered. According to these results, the vitamins protected against liver effects of diazinon and also may have radical cleansing and antioxidant properties [37]. In another study, the effect of vitamin E (600 mg) on sick and normal groups was investigated for 6 days. As a result of this study, vitamin E promoted the activity of antioxidant enzymes including superoxide dismutase, GR, and catalase in both groups [38]. Jeya Sheela et al. researched the effect of different supplements of folic acid, vitamin B (especially biotin, riboflavin, nicotinic acid, thiamin hydrochloride, and pyridoxine), vitamin C, and mixed minerals on Channa striatus, which is a freshwater fish. According to them, these supplements stimulated the activity of some enzymes such as amylase, protease, and lipase [39].

4. Conclusions

Although the GR enzyme was not purified from bovine liver for the first time here, the reaction and reaction products of GR and also the mechanism in which it is involved are very important. Furthermore, the enzyme may vary structurally from one tissue to another and from one living organism to the next, as mentioned above. However, the reaction is unique. Therefore, in this study, we investigated the effect of some water-soluble vitamins on this enzyme. This is the first study to investigate this aspect and we believe that it contributes to the literature.

Acknowledgment

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