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**RESEARCH ARTICLE**

# Purification of glutathione *S*-transferase isoenzymes from tumour and nontumour human stomach and inhibitory effects of some heavy metals on enzymes activities

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**Abstract**

In this study, glutathione *S*-transferase (GST) enzyme was purified from nontumour and tumour human gastric tissue and *in vitro* effects of heavy metals on the enzyme were examined. GST was purified 3089 fold with a specific activity of 20 U/mg and a yield of 78% from gastric tumour tissue; and 1185 fold with a specific activity of 5.69 U/mg and a yield of 50% from nontumour tissue by using glutathione–agarose affinity column, respectively. Enzyme purity was verified by SDS-PAGE and subunit molecular mass was calculated around 26 kDa. The molecular weight of the enzyme was calculated as 52 kDa by using Sephadex G-75 gel filtration column. Then, inhibitory effects of metal ions on the enzymes were investigated. $Mg^{2+}$ and $Cd^{2+}$ had inhibitory effect on the enzymes activities. Other kinetic properties of the enzymes were also determined.

**Keywords:** Cancer, GST, enzyme, metal, inhibition



## Introduction

Gastric cancer is the second most common cause of cancer connected deaths in the world. In the developed countries, there are patients with advanced disease and die from metastases[1,2]. When we look from a global viewpoint, we can see that gastric cancer is the fourth most common malignant tumour and the second most common cause of cancer-associated death, accounting for a guessed 1.066.543 new cases and 800.230 deaths annually. Despite advances in surgery and chemotherapy, the prognosis of gastric cancer is poor, with an estimated relative 5-year survival proportion of 10–20% in USA and European countries[3,4].

Glutathione *S*-transferases are the member of detoxification enzyme family and play a vital role in drug resistance in various diseases[5,6]. Glutathione *S*-transferases (EC 2.5.1.18; GSTs), the family of multifunctional enzymes, are involved in the detoxification of both endogenous and xenobiotic compounds, in intracellular transport, biosynthesis of hormones, and protection against oxidative stress[7]. Mammalian GST is divided into seven classes. Five of this enzyme classes is cytosolic, two of them are membrane-bound. Cytosolic enzyme classes contain 13 subunits[8]. GSTs are found in mammals, insects, fish, birds, annelids, mollusca, and many microorganisms. GSTs are placed in the cytosols and membranes of various tissues primarily in the liver, small intestine, large intestine, kidney, lung, breast, muscle, spleen, testis and placenta[9]. In humans, GST enzymes can be separated into five main classes: Alpha (GSTA), Mu (GSTM), Pi (GSTP), Theta (GSTT), and Zeta (GSTZ). Each class made up of one or more isoenzymes (i.e., A1-A4, M1-M5, P1, T1- T2 and Z1), with a different but sometimes overlapping substrate specificity[10]. Mitochondria also includes GSTs which are believed to be similar to their cytosolic counterparts[11].

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Harris et al. were previously purified mitochondrial GSTs from rat liver[11,12].

Glutathione *S*-transferase (GST) enzyme families are formed in many cytosolic, mitochondrial, and microsomal (now designated as MAPEG) proteins. GSTs exist in eukaryotes and prokaryotes, where they catalyze several of reactions and admit endogenous and xenobiotic substrates[13]. The mammalian GST superfamily consists of cytosolic dimeric isoenzymes of 45–55 kDa size[14].

GSTs are important for the fight against the cancer because of their interactions with carcinogens and chemotherapeutic agents. They are the target of antiasthmatic and antitumour drugs[15]. Production of excessive amounts of GST enzyme in mammalian tumour cells leads to the formation of a resistance to some anticancer drugs and chemical carcinogens[16]. Erythrocyte GST activity of hemolytic anaemia was investigated in 513 patients and no significant correlation between the lack of GST and anaemia was detected. It is reported that decrease in GST levels, related with protecting cells from cellular damage in erythrocytes, causes a low stability of the cells and increased risk of anaemia[17].

Population growth and industrial activity lead an increase in environmental pollution continuously in many parts of the world. Industrial waste is frequently emptied to the surroundings without any prerefining. As a result, in some areas, the natural habitat of animals and balance of nature are threatened by pollution in freshwaters, seas and atmosphere[18]. In the environment, heavy metal concentration is increased by quick industrialization, poor emission control, disordered urbanization, and risen motor traffic. Plants and other organisms in the habitat are influenced by the contamination of soil, water, and atmosphere with heavy metals[19,20].

Therefore, in our study we wondered the effects of some heavy metals on the GSTs. For this purpose, we purified the enzyme from nontumour and tumour human stomach. Then we investigated inhibitory effects of $Pb^{2+}$, $Cu^{2+}$, $Fe^{2+}$, $Cr^{2+}$, $Al^{3+}$, $Ni^{2+}$, $Mn^{2+}$, $Cd^{2+}$, $Zn^{2+}$, and $Mg^{2+}$ on the enzyme.

## Materials and methods

The specimens of tumour and nontumour tissues of the human stomach were obtained from the Pathology Departments of Research Hospital of Ataturk University after operation and stored at −80°C until usage. Twenty grams of thawed samples of human stomach were separately cut into small pieces with a knife. The fragments were homogenized with liquid nitrogen. The homogenate was taken to 2 volumes of buffer solution (10 mM $KH_2PO_4$/150 mM NaCl, pH = 7.0) and 30 mL hexane was added into solution to solve the lipids. The homogenate was filtered through four layers of cheesecloth. Then, lipid fraction has been removed from the homogenate by using a separatory funnel. Next, it is centrifuged at 13.000 rpm for 1 h. The pellet was discarded. The homogenate was applied to the prepared Glutathione–Agarose affinity column equilibrated with 10 mM $KH_2PO_4$/150 mM NaCl, (pH = 7.0). The affinity gel was washed with 10 mM $KH_2PO_4$/0.1 M KCl, (pH = 8.0). The cancerous human tumour and non tumour stomach Glutathione *S*-Transferases enzymes were eluted with 50 mM Tris-HCl/1.25–10 mM GSH (pH = 9.5). All the purification steps were performed at 4°C.

### Determination of GST activity

Glutathione *S*-transferase activity was performed by using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate according to Habig et al. Among its many other substrates, the aromatic electrophile 1-chloro-2,4-dinitrobenzene is one that is the most often used substrate in GST enzyme activity determination. The reaction product, dinitrobenzene *S*-glutathione (DNB-SG), displays maximum absorbance at 340 nm. Activity measurements were thus carried out by measuring the absorbance increase at this wavelength[9].

### Protein determination

During each purification steps, protein determination was performed spectrophotometrically at 595 nm according to the Bradford method, by using bovine serum albumin as the standard[21].

### SDS polyacrylamide gel electrophoresis

After the purification steps, SDS polyacrylamide gel electrophoresis was performed to verify enzyme purity. It was carried out in 8% and 3% acrylamide for the running and the stacking gel, respectively, containing 0.1% SDS according to Laemmli procedure. A 20 µg sample was applied to the electrophoresis medium. Gels were stained for 1.5 h in 0.1% Coommassie Brilliant Blue R-250

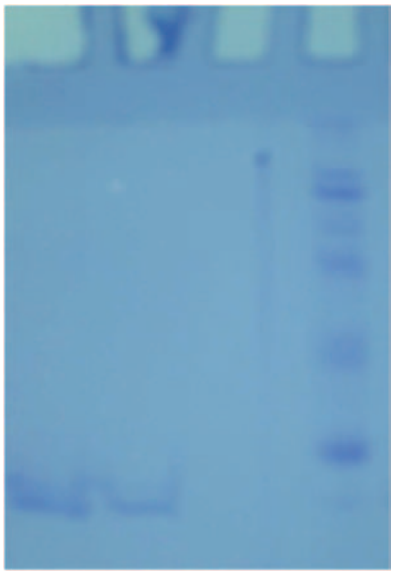

Figure 1. Lane (1) human tumour gastric GST. Lane (2) human nontumour gastric GST. Lane (3) standard proteins (*E. coli* β-galactosidase (116 kDa)), rabbit phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), chicken ovalbumin (45 kDa) and bovine carbonic anhydrase (29 kDa) SDS-PAGE analysis of purified.

in 50% methanol and 10% acetic acid, then destained with several changes of the same solvent without dye[22] (Figure 1).

### *In vitro* effects of compounds

Inhibitory effects of the metals were analysed at different inhibitor concentrations. Compounds showing inhibitory effects were tested in triplicate at each concentration used. We measured Glutathione *S*-transferase activity in the presence of metal concentrations. Control activity in the absence of inhibitor was taken as 100%. For each metal, activity (%) vs. inhibitor concentration graphs were drawn. In $K_i$ studies, we measured enzyme activities at three different inhibitor concentrations for five different substrate concentrations. Lineweaver–Burk curves were used to determine the $K_i$ values and inhibitor type of each inhibitor.

## Results and discussion

Gastric cancer is a disease in which malignant cells of the gastric tissue increases in number[23]. Although the incidence and the mortality rate of this disease have been decreasing significantly (approximately 5–6 fold) for last 30 years, it is the fourth most common cancer in the world. Prognosis of gastric cancer is evil and death ratio of this cancer ranked as second after lung cancer[24].

The aim of this study was to purify GST enzyme from tumour and nontumour human gastric tissue and investigate the effects of some heavy metals on enzyme activity. Cancerous human stomach and non cancerous tissues were chosen, because gastric cancer is the fourth highest cancer in the world. Tumour and nontumour GST enzymes were purified by using glutathione–agarose affinity column with high yields and purification folds (Tables 1 and 2). Enzyme purity was verified by SDS-PAGE and subunit molecular mass was calculated around 26 kDa. The molecular weight of the enzyme was calculated as 52 kDa by using Sephadex G-75 gel filtration column. (Figure 1). The apparent $K_m$ and $V_{max}$ values of tumour stomach were calculated as 3.46 μM, 43.7 (EU/mL·min) for GSH and 250 μM, 114.7 (EU/mL·min) for CDNB. These values for nontumour human stomach were calculated as 25.4 μM, 31 (EU/mL·min) for GSH and 79 μM, 25 (EU/mL·min) for CDNB, respectively. Inhibitory effects of metals were examined under *in vitro* conditions. $K_i$ values for each inhibitor were defined (Table 3).

All living organisms are subjected to frequent toxic chemicals. Metals come to the people's body with nutrition and bioaccumulation and they cannot be metabolized by the body because of their stability. The most well-known harmful metals are Al, Ar, Cd, Cu, Pb, Hg and Ni. Other heavy metals are not as harmful as these ones. Usually they do not have basic tasks in the body and they are very toxic. They are found in drinking water, food and thousands of man-made chemical substances and products. They are taken to the body by respiratory, digestion and skin absorption. If metals are taken to the body faster than detoxification, they are stored in the body and their toxic effects are formed. The harmful effects of toxic metals are formed by the generation of oxidative free radicals. Intense uptake of toxic substances and antioxidant deficiency triggers an uncontrolled free radical production. Uncontrolled free radicals in the body create tissue damage and lead to a number of degenerative diseases. The pH of the blood is decreased by toxic heavy metals. Body takes out $Ca^{2+}$ ions from the bones to balance the pH and an inflammatory condition occurs due to this $Ca^{2+}$ decrease in arteries and tissues[25].

In cell homeostasis, glutathione is an important modulator for the detoxification of oxiradical and carcinogens. Thus, in the absence of GSH, organism becomes sensitive to the pollutants. This situation shows the importance of GSH's ability of organisms fight against heavy metals which are known to inhibit antioxidant enzymes and increases the consumption of intracellular glutathione. When glutathione level increases, it stops increasing lipid peroxidation effects of Cd, Rb, Fe, Hg together with Zn or Se[26].

Wright et al.[27] suggested that Glutathione *S*-transferase enzyme is a tissue biomarker against Pb poisoning. Moreover, Glutathione plays an important role in arsenic toxicity. As the thiols are quite abundant in the cell, arsenic poisoning is known to be formed by thiol deficiency in the proteins. Lack of thiol causes toxicity and detoxification. Glutathione has been reported to increase the resistance of cells against As and Sb[28]. Cd, has basically non-essential task, is a toxic heavy metal even in

Table 1. Summary of purification procedure for tumour human gastric GST.

| Purification steps | Activity (EU/mL) | Total volume (mL) | Protein (mg/mL) | Total protein (mg) | Total activity | Specific activity (EU/mg) | Yield % | Purification fold |
|---|---|---|---|---|---|---|---|---|
| Homogenate | 0.094 | 23 | 14.43 | 331.89 | 2.162 | 0.0065 | 100.0 | 1.0 |
| Dialyzed enzyme after affinity chromatography | 0.241 | 7 | 0.012 | 0.084 | 1.687 | 20.08 | 78.03 | 3089.23 |

Table 2. Summary of purification procedure for nontumour human gastric GST.

| Purification steps | Activity (EU/mL) | Total volume (mL) | Protein (mg/mL) | Total protein (mg) | Total activity | Specific activity (EU/mg) | Yield % | Purification fold |
|---|---|---|---|---|---|---|---|---|
| Homogenate | 0.088 | 20 | 18.23 | 364.6 | 1.76 | 0.0048 | 100.0 | 1.0 |
| Dialyzed enzyme after affinity chromatography | 0.148 | 6 | 0.026 | 0.156 | 0.888 | 5.69 | 50.45 | 1185.4 |

Table 3. $K_i$ values and inhibition types for two metal ions of tumour and nontumour GST.

| Type of metals | For tumour tissue Average values of $K_i$ (mM) | Type of inhibition | For nontumour tissue average values of $K_i$ (mM) | Type of inhibition |
|---|---|---|---|---|
| $Zn^{2+}$ | Not Significant | – | Not Significant | – |
| $Cu^{2+}$ | Not Significant | – | Not Significant | – |
| $Fe^{2+}$ | Not Significant | – | Not Significant | – |
| $Cd^{2+}$ | 2.01 | Uncompetitive | 7.38 | Uncompetitive |
| $Pb^{2+}$ | Not Significant | – | Not Significant | – |
| $Cr^{2+}$ | Not Significant | – | Not Significant | – |
| $Al^{3+}$ | Not Significant - | – | Not Significant | – |
| $Ni^{2+}$ | Not Significant | – | Not Significant | – |
| $Mn^{2+}$ | Not Significant | – | Not Significant | – |
| $Mg^{2+}$ | 48.096 | Uncompetitive | 49.2 | Uncompetitive |

very small doses. Enzymatic activity in respiratory and urinary systems is impaired by Cadmium toxicity, and it causes kidney stones formation. Carcinogenic effects of this metal in epidemiological studies are also reported[29].

Fujian in China, Zn, Cu, Se levels in serum were investigated in people of 294 healthy and 109 gastric cancer. Quantities of these elements were found to be as 0.905 mg/L, 0.914 mg/L, 83.22 mg/L for healthy ones, while for the 109 gastric cancer individuals, the values were obtained as 0.843 mg/L, 1.045 mg/L and 73.58 mg/L, respectively[30].

Recent studies show that GST isoforms in cancer tissue were being overexpressed compared to normal ones. Tsuchida and his friends demonstrated that GST-Pi content in cancer colon mucosa was 6-fold higher than the level found in normal mucosa by using an enzyme-linked immunosorbent assay (ELISA) (in-house method)[31]. In addition, concentration of GSTs was found to be higher in the lavage fluid taken from the area of tumour lung when compared with the normal area of tissue[32]. Besides, in 19 lung, 27 colon and 9 gastric cancer patients, it was determined by chromatography that GST-Pi concentration was significantly higher in tumour tissue according to normal tissue samples[33]. Similar studies in various malignancies such as stomach, colon, breast and ovarian cancers, cancer GST levels were detected to be higher than in normal tissues[33,34].

In this study, we studied the effects of some metals on GST enzymes of tumour and nontumour tissues. Inhibitory effects of $Mg^{2+}$ and $Cd^{2+}$ metals were observed in both tumour and nontumour tissue. $K_i$ values were calculated for both metals. $K_i$ values of tumour tissue for $Mg^{2+}$ and $Cd^{2+}$ metals were calculated as 48.096 mM and 2.01 mM, respectively. $K_i$ values of $Mg^{2+}$ and $Cd^{2+}$ metals from nontumour tissue were calculated as 49.2 mM and 7.38 mM, respectively. According to the data we obtained, metal ions had an inhibition at a lower concentration on GST enzyme purified from tumour tissue than that of nontumour tissues. We demonstrated that metals had toxic effects at lower concentrations in cancerous tissues.

It is known that GST removes reactive oxygen species in metabolism. We observed in our study that metals inhibit enzymes of tumour tissues at lower concentrations. $K_i$ value of the tumour GST enzyme was lower than nontumour one. Nontumour and tumour tissues exposed to metals like Cd and Mg, it is expected nontumour tissues have more reactive oxygen species. Thus reactive oxygen species may have more negative effects on tumour tissues. As a result metals, Cd and Mg were found to have inhibitory effects on both tumour and nontumour GST enzyme. They may increase the spread of tumour tissues. Hence, exposure to metals of humans should be limited because of their harmful effects in the metabolism.

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Research ethics committee approval needed for the cancerous tissue used in this study was taken from Ataturk University, Faculty of Medicine.

## Declaration of interest

The authors report no conflicts of interest.

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