Purification of Glutathione S-Transferase From Bonito (*Sarda Sarda*) Liver And Investigation of Metal Ions Effects on Enzyme Activity

Glutatyon S-Transferaz Enziminin Palamut (Sarda sarda) Karaciğerinden Saflaştırılması ve Bazı Metal İyonların Enzim Aktivitesi Üzerine Etkilerinin İncelenmesi

Research Article

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ABSTRACT

The Glutathione S-transferase (GST, EC.2.5.1.18) catalyzes the conjugation of hydrophobic compounds including electrophilic centre to the glutathione (GSH). In this study, purification of GST from bonito liver for the first time and examination of some metal ions' effects on enzyme activity were aimed. Purification procedure was performed in two steps as preparation of homogenate and glutathione-agarose affinity chromatography. The purity of enzyme was controlled by SDS-PAGE, and it exhibited two bands. It was found as heterodimer structure. Additionally, effects of some metal ions were examined on the enzyme activity. For metal ions showing inhibitory effects, IC_{50} values were calculated by Activity% [metal ion] graphs and K_i constants and inhibition types were determined via Lineweaver-Burk graphs. Pb^{2+}, Cr^{2+} and Fe^{3+} ions had not any effects on the enzyme activity. Yet, while Co^{2+} activated the enzyme, Ag^+, Cu^{2+}, Cd^{2+} and Zn^{2+} showed inhibitory effect. Inhibitory order was found as Cu^{2+}>Ag^+>Cd^{2+}>Zn^{2+} with the K_i values of 0.166 ± 0.046 µM; 0.0146±0.0047 mM; 0.0883±0.0335 mM and 1.39±0.44 mM respectively and enzyme was inhibited noncompetitively by these metals.

Key Words
glutathione S-transferase, purification, bonito, metal ions

ÖZET

Glutatyon S-transferaz (GST, EC. 2.5.1.18) elektrofilik merkeze sahip hidrofobik bileşiklerin glutatyonla (GSH) konjugasyonunu katalizler. Sunulan bu çalışmada GST enziminin palamut karaciğerinden ilk kez saflaştırılması ve bazı metal iyonların enzim aktivitesi üzerine etkilerinin araştırılması amaçlanmıştır. Saflaştırma işlemi homojenatın hazırlanması ve glutatyon-agaroz afinite kromatografisi olmak üzere iki basamakta gerçekleştirildi. Enzimin saflığı SDS-PAGE ile kontrol edildi ve elektroforezde çift bant görüldü. Enzimin heterodimer yapıya sahip olduğu bulundu. Ayrıca bazı metal iyonların enzim aktivitesi üzerine etkileri incelendi. İnhibisyon etkisi gösteren metal iyonlar için IC_{50} değerleri % aktivite-[metal iyon] grafiğinden hesaplandı ve K_i sabitleri ve inhibisyon türleri Lineweaver-Burk grafiği kullanılarak belirlendi. Pb^{+2}, Cr^{+2} ve Fe^{+3} iyonları enzim aktivitesini etkilemediği, Co^{+2} iyonunun enzimi aktive ettiği, Ag^+, Cu^{+2}, Cd^{+2} ve Zn^{+2} inhibisyon etkisi gösterdiği bulundu. İnhibisyon sırası 0,166±0,046 µM; 14,6± 4,7 µM; 88,3±33,5 µM ve 1390±440 µM K_i sabitleriyle, Cu^{+2}>Ag^+>Cd^{+2}>Zn^{+2} olarak bulundu. Bu metallerin hepsinin enzimi yarışmasız olarak inhibe ettiği tespit edildi.

Anahtar Kelimeler
Glutatyon S-transferaz, saflaştırma, palamut, metal iyonları

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INTRODUCTION

Throughout their lives, human beings are exposed to xenobiotics via nutrients and medicines. But in order to cope up with these noxious matters, our bodies have detoxification systems in which most of enzymes take part. Mostly these systems are enough to minimize the damage originated from xenobiotics. Yet, many studies were reported that damaged detoxification systems are closely related to some diseases such as Parkinson's diseases, fibromyolgie, immune dysfunction syndrome and cancer [1,2]. When non-reactive compounds are taken in to body, they are removed by converting through two phases. Phase I is functionalization reactions which use oxygen to perform reactive region and Phase II is conjugation reactions which add water-soluble groups to the previously comprising reactive region.

Glutathione S-transferases (GSTs) are an important part of detoxification enzymes. They catalyze conjugation of reduced glutathione (GSH) to the some compounds including electrophilic and/or hydrophilic group [3]. GSTs are composed identical or similar subunits and generate a large group of Phase II enzymes relation with detoxification of xenobiotics having electrophilic centre [4,5]. It is thought that these enzymes protect cells against foreign chemicals such as pesticides, drugs and carcinogens [6]. GSTs have important physiological role on taking hepatic organic ion because of having function in conjugation, binding and transport of organic anions. They are present in all organisms from bacterium to vertebrates and all cells of eukaryotes. GSTs are divided into seven classes as μ, π, α, θ, δ, K and ε according to substrate specificity, immunological activities and protein sequences [7-10].

Up to now, enzyme was purified and characterized from some mammalians such as human, mouse, bovine, and rat. Additionally, hepatic GSTs was purified and partially characterized from different aquatic species [11-19].

In this study toxic effects of some metal ions such as Ag^+, Cu^{2+}, Cd^{2+}, Fe^{3+}, Pb^{2+}, Cr^{2+}, Co^{2+} and Zn^{2+} on GST from bonito liver were investigated.

Concurrently with developments in the industry, releasing of the chemicals that are injurious for ecosystem and living organisms, increased. Heavy metals which were produced by pulp, paper, iron and steel industry, petrochemical, refining, fertilizer and automobile industry, poison biological environment [20]. Unfortunately waste substances from all these sources contaminate sea and fresh water.

Metals are the oldest known pollutants and toxic for humans. For instance, some acute and chronic poisonings originated from cadmium and mercury are known [21, 22]. Furthermore cadmium and lead are carcinogens for human and may increase the probability of neurodegenerative diseases [23- 25].

It is thought that metal ions such as Cd^{2+}, Hg^{2+}, Pb^{2+}, Cr^{2+}, Ni^{2+} and Pt^{2+} show their toxic effects by interacting with proteins or DNA. There are two type models about how metals affect protein. One of them is, metal ions bind to free thiol groups or other functional groups of native proteins. The other is, metal ions may change place with Zinc or another metal ions in metal-binding proteins [26-29]. So we aimed to enlighten what extend metal ions effect GST being such an important enzyme in detoxification reactions.

MATERIALS AND METHODS

Materials
Glutathione-agarose was purchased from Sigma-Aldrich. GSH, 1-chloro 2,4-dinitro benzene (CDNB), the protein assay reagent and the chemicals for electrophoresis were purchased from Sigma Chem. com. All other chemicals used were analytical grade and purchased from either Sigma or Fluka.

Preparation of the homogenates
The frozen bonito liver was cut into small pieces and cell membranes were rended with liquid nitrogen, and the powder was suspended in 50 mM Tris/HCl (pH 7.5) containing 1 mM DTT, 1 mM EDTA, and 1 mM PMSF. The homogenate was centrifuged at 13,000 × g for 1 h, and then the precipitate was removed.

Glutathione-agarose affinity chromatography
Freshly prepared homogenates were loaded on glutathione-agarose affinity column which was

equilibrated with 10 mM K-phosphate buffer, pH 7.4 containing 150 mM NaCl. The column was washed with the same buffer until final absorbance difference of buffer and eluate became 0.05 at 280 nm. Then the enzyme was eluted with a gradient of 5 to 10 mM GSH in 50 mM Tris/HCl (pH 9.5), and active elutions were combined.

Activity determination

Enzymatic activity was determined spectrophotometrically with a Shimadzu spectrophotometer (UV-1208) at 280 nm and 25°C according to the method described by Habig *et al*. 1974. The assay system contained 100 mM K-phosphate buffer, pH 6.5, including 1 mM EDTA, 20 mM GSH, and 25 mM CDNB.

Protein determination

Quantitative protein determination was performed spectrophotometrically at 595 nm according to Bradford's method, using bovine serum albumin as a standard (30).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

To control the purity of enzyme, SDS-PAGE was performed according to Laemmli's method (31). The acrylamide concentration of the stacking and separating gels were 3% and 15%, respectively, and 1% SDS was also added to the gel solution. β-galactosidase (120 kDa), albumin (85 kDa), ovalbumin (50 kDa), carbonic anhydrase (35 kDa), β-lactoglobulin (25 kDa) and lysozyme (20 kDa) were used as standards (peqGOLD Protein Marker III).

In vitro effects of metal ions

To determine the effects of metal ions on bonito liver GST activity, different concentrations of Ag^+, Cu^{2+}, Cd^{2+}, Fe^{3+}, Pb^{2+}, Cr^{2+}, Co^{2+} and Zn^{2+} were added into the activity medium. Activity of the enzyme

was measured, and the absence of metal ions was used as control (100% activity). IC_{50} values were calculated from activity (%)-metal ion concentration graph. In order to determine K_i constants, at five different fixed concentrations of substrate (GSH), three different inhibitor concentrations added to the reaction medium. Lineweaver-Burk graphs were drawn by using $1/V$ vs. $1/[GSH]$ values, and K_i constants and inhibition types were obtained from these graphs.

RESULTS

In this study bonito liver GST was purified at two steps as preparation of homogenate and glutathione-agarose affinity chromatography. Pure enzyme was gained 1690 fold with 25.375 EU/mg specific activity and a yield of 53.2% as shown in Table 1. Purity of enzyme was controlled by SDS-PAGE (Figure 1), and it exhibited two bands. It was found that bonito liver GST has heterodimer structure.

According to inhibition studies IC_{50} values and K_i constants were determined by graphs shown in Figures 2-9. Pb^{2+}, Cr^{2+} and Fe^{3+} ions had not any effects on the enzyme activity. While Co^{2+} activated the enzyme, Ag^+, Cu^{2+}, Cd^{2+} and Zn^{2+} showed inhibitory effect. Inhibitory order was found as $Cu^{2+}>Ag^+>Cd^{2+}>Zn^{2+}$ with the IC_{50} values of 64 μM, 216 μM, 355 μM, 182.8 μM and K_i constants of 0.166±0.046 μM; 14.6±4.7 μM; 88.3±33.5 μM; 1390±440 μM and respectively. And they inhibited enzyme noncompetitively (Table 2).

DISCUSSIONS

GSTs prohibit combination of biomacromolecules with products formed by oxidation or foreign toxic matters externally taken. And they provide

Table 1. Purification steps of GST from bonito liver.

Purification Step	Total Volume (mL)	Activity (EU/mL)	Protein (mg/mL)	Total protein (mg)	Total Activity (EU)	Spesific Activity (EU/mg)	Purification Coefficient	Yield (%)
Homogenate	18	0.064	4.280	77.04	1.145	0.0149	1	100
Glutathione-agarose affinity chromatography	3	0.203	0.008	0.024	0.609	25.375	1690	53.2

Table 2. K_i constants and IC_{50} values obtained from regression analysis graphs for GST from bonito liver in the presence of different ions.

Metal ion	IC_{50}	K_i	Inhibition type
Cu^{2+}	64 μM	0.166±0.046 μM	noncompetitively
Ag^+	216 μM	14.6±4.7 μM	noncompetitively
Cd^{2+}	355 μM	88.3±33.5	noncompetitively
Zn^{2+}	1828 μM	1390±440 μM	noncompetitively
Co^{2+}	activated		
Pb^{2+}	ineffective		
Cr^{2+}	ineffective		
Fe^{3+}	ineffective		

removal of matter at issue without damage the cell components. Because of this GSTs are important member of protection enzyme group [32-34].

Most harmful substances are firstly metabolized by phase I detoxification systems. At this step toxic reactive molecule which is more dangerous than starting molecule may be produced. If these molecules are not metabolized by phase II including conjugation enzymes, cell proteins, RNA and DNA can be damaged [35]. Most study have proved that induced phase I and/or decreased phase II activities are closely related with some diseases such as; cancer, systemiclupus erythematosus and Parkinson's diseases [36-41]. Yet, it is desired to inhibit GSTs activity in damaged or cancer tissues in contrast to
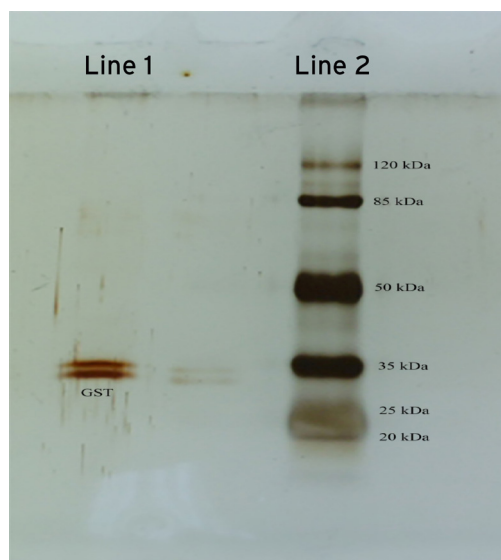
the healthy tissues. In this respect GST enzymes are therapeutic targets for cancer drugs. Thus inhibition studies on GSTs are extremely important researches. In this paper we aim to examine effects of some metal ions on GST activity from bonito liver. Wide range of chemicals having extremely important toxic effects for ecosystems are produced and these dangerous wastes are finally thrown to the aquatic environment.

Chemicals indicate their toxic effects by inhibiting enzymes. Metals reduce enzymes activity via binding thiol groups of proteins and by this way mercaptans are performed. For instance it is reported
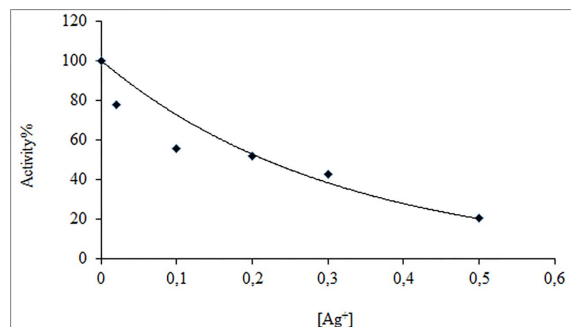
Figure 1. SDS-PAGE photograph: lane 1, purified enzyme from Glutathione agarose affinity chromatography; lane 2, standard proteins: β-galactosidase (120 kDa), albumin (85 kDa), ovalbumin (50 kDa), carbonic anhydrase (35 kDa), β-lactoglobulin (25 kDa) and lysozyme (20 kDa) were used as standards (peqGOLD Protein Marker III).

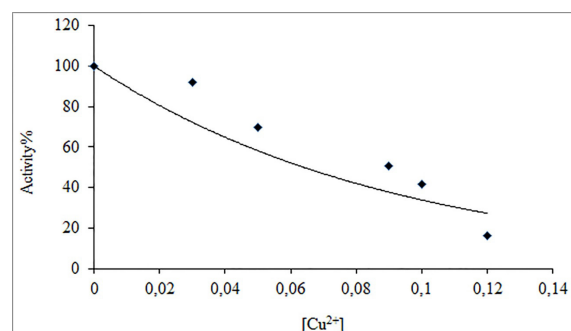**Figure 2.** Activity%-[Ag^+] graph for determining IC_{50} value of Ag^+ ion.

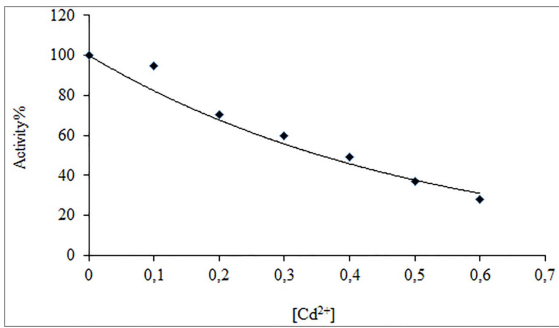**Figure 3.** Activity%-[Cu^{2+}] graph for determining IC_{50} value of Cu^{2+} ion.

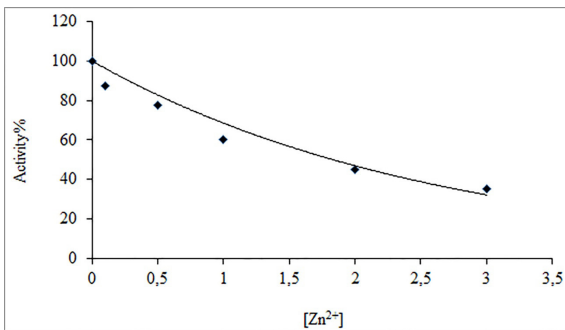**Figure 4.** Activity%-[Cd^{2+}] graph for determining IC_{50} value of Cd^{2+} ion.

Figure 5. Activity%-[Zn^{2+}] graph for determining IC_{50} value of Zn^{2+} ion.

that Ag^+ inhibits Na^+/K^+ ATPase activity by binding to the sulfhydryl group of enzyme. Besides, metals may bind to the sulfhydryl group of glutathione and this case causes damages at antioxidant systems and increases the reactive oxygen species (ROS) in cells [42].

Nowadays, the level of metals in environment reached very dangerous for the health of aquatic and terrestrial life. Although trace amounts of some metals are required for enzymatic activities as a cofactor, it is known that metal ion concentrations at mg/L are toxic because heavy metals may cause irreversible inhibition effects on some enzymes activities [43].

Henczova et al. 2008 examined the effects of Cu^{2+} and Pb^{2+} ions on 7-ethoxyresorufin-o-deethylase (EROD), 7-ethoxycaumarin-o-deethylase (ECOD) and aminopyrene-N-demethylase (APND) from silver carp, carp and wells liver. According to their *in vivo* studies, Cu^{2+} (1 mg/L) activated enzymes from silver carp liver. When 2 mg/kg Pb^{2+} was given to fishes, it was seen that EROD was inhibited at the end of a day. *In vitro* studies showed that Cu^{2+} and Pb^{2+} caused a decrease at the CYP450 content in the carp liver

microsomes [44].

Effect of some metal ions such as Cd^{2+}, Hg^{2+}, and Pb^{2+} on refolding of chemically denatured luciferase, lactate dehydrogenase, malate dehydrogenase and glucose-6-phosphete dehydrogenase were studied. It was demonstrated that all three metals inhibited the refolding of in question proteins and inhibition order was $Hg^{2+} > Cd^{2+} > Pb^{2+}$ [45].

Soyut *et al.,* 2012 investigated the effects of Co^{2+}, Cu^{2+}, Zn^{2+}, and Ag^+ on expression of Hsp 70 gene and *in vitro* carbonic anhydrase (CA) enzyme activity from rainbow trout muscle. They reported that Cu^{2+}, Zn^{2+} and Ag^+ were non-competitive; Co^{2+} was competitive inhibitor of rainbow trout muscle CA [46]. According to a similar study Ag^+, Co^{2+}, Cu^{2+} and Zn^{2+} exhibit inhibitory effect on CA from rainbow trout liver and brain as well [47, 48]. When the effects of Zn^{2+}, Cd^{2+} and Cu^{2+} metals on glucose-6-phosphode dehydrogenase (G6PD) from amphibian *X laevis* and *P.waltl* were examined, it was seen that in
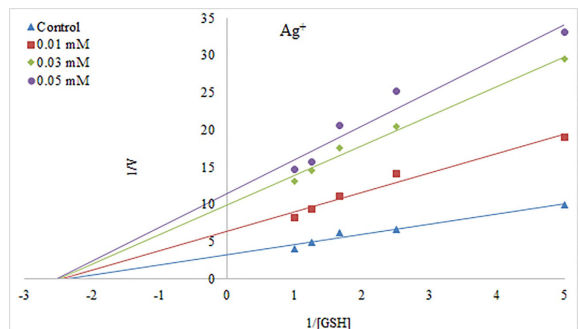

Figure 6. Lineweaver-Burk graph with 5 different substrate (GSH) concentrations and 3 different Ag^+ concentrations for determination of K_i.
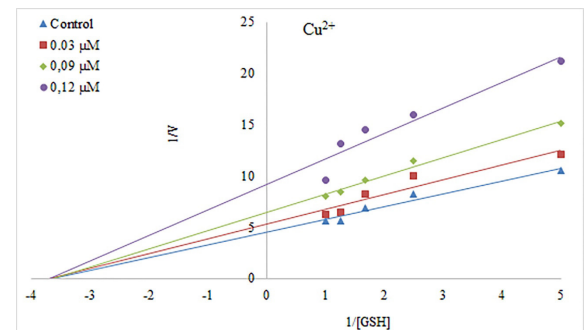

Figure 7. Lineweaver-Burk graph with 5 different substrate (GSH) concentrations and 3 different Cu^{2+} concentrations for determination of K_i.

Figure 8. Lineweaver-Burk graph with 5 different substrate (GSH) concentrations and 3 different Cd^{2+} concentrations for determination of K_i.
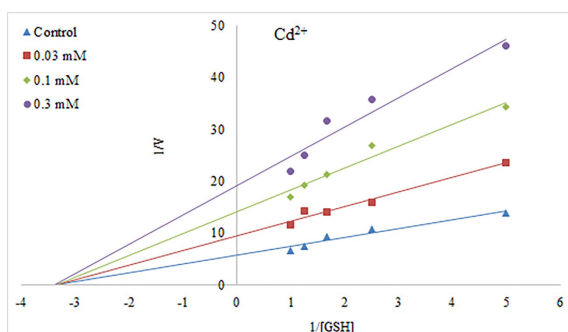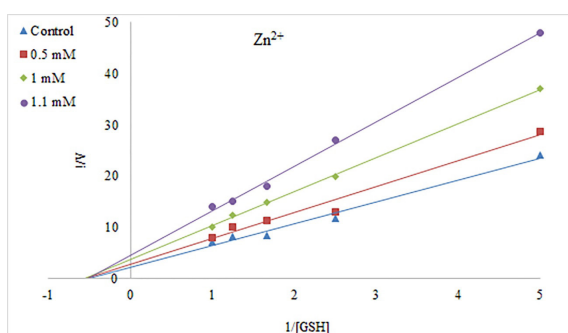

Figure 9. Lineweaver-Burk graph with 5 different substrate (GSH) concentrations and 3 different Zn^{2+} concentrations for determination of K_i.

both species enzyme can tolerate all concentrations of Zn^{2+} and Cd^{2+} but not Cu^{2+} so Cu^{2+} caused so much decreases in G6PD activity [49].

For determining the inhibition effects of Pb^{2+}, Cr^{2+},Fe^{3+},Co^{2+}, Ag^+, Cu^{2+}, Cd^{2+} and Zn^{2+}, firstly we isolated GST from bonito liver in two steps as preparation of homogenate and glutathione-agarose affinity chromatography in this study. GST having a specific activity of 25.375 EU/mg proteins was purified 1690 fold with a yield of 53.2% (Table 1). The purity of enzyme was controlled by SDS-PAGE (Figure 1) it exhibited two bands because of it heterodimer structure like as turkey liver GST and Van lake fish liver GST [50, 51].

Inhibitory effects of metal ions (Cu^{2+}, Hg^{2+}, Fe^{2+}, Zn^{2+}, Ag^+, Mg^{2+}, Ni^{2+}, and Mn^{2+}) on turkey liver GST were examined *in vitro* and it was demonstrated that Cu^{2+}, Hg^{2+}, Ag^+ were inhibited enzyme non-competitively (50). According to *Özaslan* 2014's study Ag^+, Cu^{2+}, Cd^{2+}, Co^{2+} ions inhibited GST from Van lake fish liver. While Cu^{2+}, Cd^{2+}, Co^{2+} exhibited competitive inhibition,

Ag^+ showed non-competitive inhibitory effect on the enzyme [51].

In our research, we determined that while Pb^{2+}, Cr^{2+} and Fe^{2+} did not affect GST from bonito liver, Co^{2+} exhibited activation affect and Ag^+, Cu^{2+}, Cd^{2+} and Zn^{2+} inhibited the enzyme activity. Similar to GST from Van lake fish liver IC_{50} values for metal ions, inhibiting enzyme, were calculated by drawing Activity% [metal ion] graphs given at Figure 2-5. K_i constants and inhibition type of metal ions are determined from Lineweaver-Burk graphs as given at Figure 6-9. According to our study in question metal ions inhibited the enzyme non-competitively. And the most effective inhibitor was Cu^{2+} with the IC_{50} value of 64 μM and K_i constants of 0.165±0.046 μM. The inhibition order of metal was Cu^{2+}>Ag^+>Cd^{2+}>Zn^{2+} and IC_{50} values and K_i constants were given at Table 2.

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