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PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF GLUTATHIONE REDUCTASE ENZYME IN *Gammarus pulex*

İsmail POYRAZ, Mehtap KUTLU, Gözde AYDOĞAN

Anadolu University Faculty of Science, Department of Biology 26470 Eskişehir, Turkey

ÖZET

Uzun yıllardan beri tatlı su amfipodları, ekotoksikolojik çalışmalarda kullanılan en popüler canlı guruplarından birisi olmuştur. Dünyanın birçok bölgesinde yaygın olarak bulunması, elde edilebilme ve toplama kolaylığı, laboratuvar koşullarında yaşatılabilmesi konusunda büyük sorunlar çıkmayışı gibi avantajlarının yanı sıra bir çok toksik maddeye karşı da duyarlı bir organizmadır.

Glutatyon redüktaz enzimi hücrede okside glutatyonu redükte glutatyona dönüştürmeden sorumlu olan ve tüm dokularda tespit edilen flavoprotein yapısında bir enzimdir. Enzimin fonksiyonu hücrenin oksidatif strese karşı korunmasında ve deoksiribonükleotitlerin üretiminde rol almasıdır. Bu çalışmada sucul bir omurgasız olan ve balıkların besinini teşkil eden *Gammarus pulex'* deki glutatyon redüktaz enzimi 14 basamakta pürifiye edilmiş ve SDS-PAGE elektroforez yöntemi ile molekül ağırlığı tespit edilmiştir.

Anahtar Kelimeler: *Gammarus*, Glutathione reductase, Enzyme, Purification

GLUTATHIONE REDÜKTAZ ENZİMİNİN *Gammarus pulex*'TEN SAFLAŞTIRILMASI ve BİYOKİMYASAL YÖNDEN KARAKTERİZASYONU

ABSTRACT

Fresh water amphipods have been one of the most popular living organisms used in ecotoxicological studies for years. Besides its advantages like being wide spread throughout many regions in the world, being accessible and causing no problems in laboratory conditions, it is also a sensitive organism against many toxic substances.

Gutathione reductase is an enzyme that transforms oxidant glutathione into reduced glutathione in a cell and is in the structure of flavoprotein that is seen in all living tissues. The function of the enzyme is to take part in the protection of the cell against oxidative stress and to take part in the production of deoxiribonucleotides. In this current study, Glutathione reductase enzyme in *Gammarus pulex*, an aquatic invertebrate serving as aliment for the fish, has been purified after 14 stages and its molecular weight has been determined through SDS-PAGE electrophorese method

Keywords: *Gammarus*, Glutathione reductase, Enzyme, Purification

1. INTRODUCTION

In aquatic toxification tests, they are the amphipoda usually used as the most sensitive organisms to toxic substances. Transforming oxidant glutathione into reducted glutathione, Glutathione reductase has an important role in the protection of the cell [1]. Glutathione reductase can be observed in bacteria, ferment, peas, wheat, various plants, almost all animal tissues, erythrocytes, plates, human serum, and all other animal species.

Glutathione reductase (E.C.1.6.4.2) catalyzes the $GSSG + NADPH + H^{+} \rightarrow 2GSH + NADP^{+}$ reaction. The molecular weight is 120.000 daltons. It is an enzyme that has two subunits and is in the structure of flavoprotein [2].

The function of glutathione reductase enzyme is to keep the GSH (reduced glutathione) in a cell at the highest point. Moreover, it also provides adaptation against the stress in the cell division cycle and in the cell.

Glutathione reductase is important in the circle which is the defense center against the oxidative stress among cells in humans [3,4,5]. Reduced glutathione (GSH) has an important role in the protection of cells against the cytotoxic effects of various agents which include radiation and chemotherapotic medicine. The presence of GSH in liver even in milli-moles has a role in the cell detoxification.

GSH has also role in the inactivation of free radicals and in the repair of DNA damages caused by radiation [6,7,8].

2. MATERIALS AND METHODS

Gammarus pulex used in the experiments were collected from the Porsuk River (Eskişehir, around Regulator) and were taken to the laboratory. The

familiarities of the laboratory conditions were provided with the air flow and appropriate heat (adjusted to 10-12 °C) prepared by water and organic material taken from their natural environment. The living organisms, were fed with organic food added into the soil taken from their own environment. Organic food was given to the living organisms once in 20-25 days.

Living organisms in 5-8 mm body length were used in the experiments and animals were accustomed to the experimental conditions in 1 litter aquariums. No food was given to the living organisms before the experiment.

All buffers contained 1 mM EDTA. *Gammarus pulex* (5g) were homogenized in 0.25 M sucrose (6-7 ml/g) in ice. The homogenate was centrifuged for 60 minutes at 7.000 g. The supernatant was adjusted to pH 5.3 after being added 0.2 M acetic acid and was centrifuged for 40 minutes at 15.000 g. The pH is adjusted to 7.4 after adding 1 M Tris-base to the supernatant. A %20 (w/v) homogenate in 0.25 M sucrose was used as a starting material for the forthcoming purification stages. Neither PMSF (phenylymethanesulfonyl-fluoride) nor FAD was added.

After the supernatant fraction passed through the Sephadex G-25 (1.4 x 8 cm: 10 mM phosphate pH: 6,1), the same tampon was placed into CM-cellulose pillar. The elution in pH: 6.1 10 mM phosphate was exposed to NaCl linear concentration gradient (0-0.02M) .

The material procured in the second stage was concentrated with ultra-filtration in the way that it would be less than 30 ml and was placed into sephadex G-75 pillar (1.4 x 8 cm) which was balanced with 10 mM pH: 6.7 sodium phosphate.

This gel filtration parts glutathione reductase from other glutathione-bounded enzymes. In this stage, glutathione reductase can be frozen and kept up to several months.

The material procured in the third stage, before being put into the affinity pillar, was concentrated with the addition of solid ammonium sulfate (4gr / 10ml), was centrifuged and was dissolution in about 5 ml pH: 7.5 50mM potassium phosphate.

The dissolved precipitate was put into 2', 5' - ADP Sepharose (1.4 x 8 cm) which was balanced with pH 7.5 50 mM potassium phosphate (in 0,1 mM DTE (dithioerythritol). The column was washed and eluded.

The fraction procured in the fourth stage (after the concentration) was carried out in sephacryl S-200 pillar (1.4 x 8 cm) in pH: 7.5 50mM phosphate which includes 0,1 M KCl.

The material procured in the fifth stage was placed into a second 2', 5' - ADP Sepharose column (1.4 x 8 cm), balanced again, washed and eluded. In this stage the total gradient amount is 2 ml [9].

The protein procured in this stage was carried out in a percentage of 12 SDS-Polyacrylamide gel electrophorese and the molecular weight was calculated with the bands procured.

3. RESULTS AND DISCUSSION

Glutathione reductase is an enzyme whose geometry is known and is a flavoenzyme in the structure of homodymeric. Glutathione reductase enzyme was purified and defined from rat liver, pea shoots, *E. coli* and *Penicillium chrysogenum* This enzyme has mostly been studied in ferment and human erythrocytes [10,11]. In a study in which affinity finger print method was used, it was found out that ferment glutathione reductase-used as one of the objectives of model protein medicine and human glutathione reductase were homologues with a percentage of 48. XAN (6-hydroxy-3oxo-3H-3xsantine-9-propiyonic acid) ferment glutathione reductase inhibitor was tested on the human glutathione reductase and different types of inhibition were presented [4].

The subunits of the homodymeric enzyme consist of four parts and side groups join the active part. The detailed structural features of glutathione reductase in human erythrocytes and *E. coli* was also studied. Although these enzymes are similar with a percentage of 52, it was observed that the 16 N-terminal of erythrocyte glutathione reductase was lost in *E. coli* enzyme. The tertiary structure of the enzymes have little differencies [10].

As a result of the gel filtration in *Chlamydomonas reinhardtii* and SDS-PAGE, it was found out that the glutathione reductase enzyme is a monomer at a molecular weight of 54-56 kDa [12]. In our study, with SDS-PAGE analyze method, the molecular weight of *Gammarus pulex* glutathione reductase (GSSGr) enzyme, purified through gel filtration method, was also found as 56-58 kDa (Figure 1).

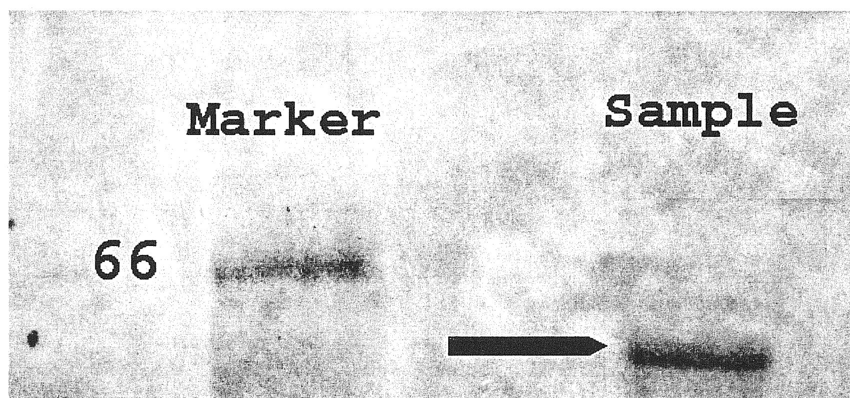

Figure 1. The SDS-Polyacrylamid gel electrophorese of *Gammarus pulex* glutathione reductase

In our study, the effect of EDTA on the *Gammarus pulex* glutathione reductase enzyme activity was observed. An inhibition of about 59.5% in an EDTA concentration of 100 µM, 66.2% in a concentration of 200 µM, and 73.9% in a concentration of 300 µM was observed. The results of the experiment were analyzed through t-test in MINITAB statistics program and ($p<0.05$) was found significant.

It was observed that the *Gammarus pulex* GSSGr enzyme activity which was tried to be purified by gel filtration method was about twice more active than the control group with a percentage of 159.6 .The enzyme procured after the gel filtration process was carried out in SDS-PAGE electrophorese [13,14] with a percentage of 12 and the molecular weight was found to be about 56-58 kDa.

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