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PURIFICATION AND CHARACTERIZATION OF GLUTATHIONE REDUCTASE ENZYME FROM *Arum Maculatum* LEAF

Gürkan BİLİR[1], Mücella SARIAHMET[1], Deniz EKİNCİ[1]*

[1]*Ondokuz Mayıs University, Faculty of Agriculture, Department of Agricultural Biotechnology, 55139, Samsun, Türkiye*

Abstract: *Arum* species grow in temperate and Mediterranean climates and have been used for hundreds of years for food and medicinal purposes, although they are highly toxic if not cooked using proper techniques. Glutathione reductase (GR) is a member of the pyridine nucleotide disulfide oxidoreductase family of flavoenzymes that catalyzes the reduction of glutathione disulfide (GSSG) to reduced GSH using NADPH or NADH. In this study, GR enzyme was characterized by partial purification processes including homogenate preparation, ammonium sulfate precipitation and dialysis from the leaf of *Arum maculatum* plant. The highest enzyme activity was found at 40-60% saturation range. Optimum ionic strength, pH and substrate concentration were investigated for GR enzyme from *A. maculatum* leaf. As a result of the study, these values were found to be 150 mM potassium phosphate buffer, pH 7.00, and 0.18 mM, respectively. The GR enzyme was partially purified from the leaf of the *A. maculatum* with a specific activity of 1.640 EU mg^{-1} in 34.9% yield, 1.108-fold. This study is the first study in terms of purification and characterization of GR enzyme from *A. maculatum* leaf.

Keywords: Glutathione reductase, *Arum maculatum*, Purification, Enzyme, Characterization

***Corresponding author:** Ondokuz Mayıs University, Faculty of Agriculture, Department of Agricultural Biotechnology, 55139, Samsun, Türkiye
E mail: deniz.ekinci@omu.edu.tr (D. EKİNCİ)
Gürkan BİLİR https://orcid.org/0000-0003-3526-6610
Mücella SARIAHMET https://orcid.org/0000-0001-7743-3670
Deniz EKİNCİ https://orcid.org/0000-0001-7849-4117

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1. Introduction

Glutathione reductase [NADPH:oxidized-glutathione oxidoreductase, EC 1.8.1.7: GR] is a member of the pyridine-nucleotide disulfide oxidoreductase family of flavoenzymes (Meister and Anderson, 1983). This enzyme catalyzes the reduction of glutathione disulfide (GSSG) to reduced glutathione (γ-L-glutamyl-Lcysteinyl glycine; GSH) using NADPH or NADH as a reducing agent. Thus, GR maintains the reduced glutathione/oxidized glutathione (GSH/GSSG) ratio in the cell environment (Şentürk et al., 2009). GSH, which is present in both prokaryotic and eukaryotic cells, is a crucial thiol that protects the cell against the harmful effects of oxidized molecules, thanks to the -SH groups in its tripeptide structure, and it constitutes a large part of the intracellular free sulfhydryl groups (Alscher, 1989). It is also involved in DNA and protein synthesis, detoxification of some metabolic end products and drugs, transport of amino acids, and breaking the disulfide structures of some proteins, such as insulin, which contain disulfide bonds (Couto et al., 2016; Çakmak et al., 2011; Townsend et al., 2003). Moreover, GSH is an abundant metabolite in plants that can protect enzyme thiol groups and is also known to be involved in signal transduction and protect plant cells from oxidative damage caused by reactive oxygen species (ROS) (Foyer and Noctor, 2005; Liu et al., 2020). Due to these critical tasks, metabolic disorders occur as a result of the decrease in the concentration of GSH in the cell. GR activity has been associated with reactive oxygen species generated by abiotic stresses such as salinity, drought, UV radiation, high light intensity, heavy metals and herbicides (Kaur et al., 2022; Romero-Puertas et al., 2006). GR is a very critical enzyme in cellular redox balance, as it can maintain the GSH/GSSG ratio to protect plant cells from oxidative damage by reactive oxygen species (Gill et al., 2013; Liu et al., 2020; Şentürk and Şentürk, 2020).

Arum maculatum is a perennial herb with glossy green leaves that look like arrowheads at the ends of long stems and whose roots form tubers (Gibernau et al., 2004). *Arum* species, which grow in temperate and Mediterranean climates and are known by common local names such as nivik, tirsik, snakeshead, adder's root, lords and ladies, devils and angels, cuckoo-pint, have been used for food and medicinal purposes for hundreds of years, although they are highly poisonous if not cooked with appropriate techniques (Ceylan and Sahingoz, 2022; Dayisoylu, 2010; Raju et al., 2018; Yurt et al., 2019). Compounds such as gum, mucilage, starch, glycoside saponin and an alkaloid (conicine) have been reported in its fresh leaves and tubers (Baytop, 1999). In addition, a high rate of protein 56.93% was identified in *A. maculatum* leaves (Ali, 2008). Alcoholic macerate of *A.*

maculatum leaves is used as antirheumatic and antineuralgic in Lebanon (Marc et al., 2008), also it is reported that the infusion in the form of compresses is used in the treatment of rheumatism and gout, anti-inflammatory, digestive, gastrointestinal and respiratory tract, joint pain, hemorrhoids, liver diseases, lung diseases (Tetik et al., 2013). Previous studies have shown that plant extracts of *A. maculatum* prepared with chemicals such as petroleum ether and methanol result in antimicrobial activity (Çolak et al., 2009; Farahmandfar et al., 2019; Uzun et al., 2004).

To date, GRs from various plants have been purified and characterized, including *Zea mays* (Mahan and Burke, 1987), *Oryza sativa* (Wu et al., 2013), *Pisum sativum* (Madamanchi et al., 1992) and Scots pine needles (Wingsle, 1989). To the best of our knowledge, no previous study has been reported in the literature on the GR enzyme from the *A. maculatum* plant. Therefore, in this study, it was aimed to partially purify GR enzyme from *A. maculatum* leaves and to determine some of its characteristic properties.

2. Materials and Methods

2.1. Materials

Ethylenediaminetetraacetic acid (EDTA), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), ammonium sulfate ($(NH4)_2SO_4$), potassium phosphate (KH_2PO_4), dithiothreitol (DTT), oxidized glutathione (GSSG), β- nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH), sodium hydroxide, coomassie brilliant blue G-250, polyvinylpyrrolidone (PVP) and bovine serum albumin (BSA) were analytically graded and obtained from Sigma Chemical Co., MO, USA.

2.2. Preparation of Homogenate

In the study, fresh leaf tissues of *A. maculatum* plant were used for partial purification of the GR enzyme. In order to determine the enzyme activity, 3 g of tissue samples were ground in a porcelain mortar with the help of liquid nitrogen, and then homogenized in 30 ml of 50 mM Tris-HCl / 2 mM EDTA (pH 7.8) buffer containing 5% (w/v) PVP and 1mM DTT. After a short vortexing process, it was centrifuged (Hermle Z 326 K, Hermle Labortechnink, Wehingen, Germany) at 15,000 x*g* for 30 min at 4 °C. The supernatant was separated from the precipitate using filter paper and used in further analysis.

2.3. Ammonium sulfate precipitation and dialysis

In order to the purification of GR enzyme from the homogenate prepared from *A. maculatum* leaves, ammonium sulfate precipitation processes were carried out separately at intervals of 0-40% and 40-60%. For the precipitation of ammonium sulfate, solid $(NH_4)_2SO_4$ was added very slowly to the homogenate at 4°C on the cooled magnetic stirrer. In each precipitation process, the homogenates were centrifuged at 5,000 x*g* for 15 minutes. The supernatant was taken into a separate beaker and the precipitates were dissolved with a sufficient amount of 150 mM Tris (pH 7.8) buffer and enzyme activities were measured both in the supernatant and in the precipitate. By repeating the procedures at all ammonium sulfate precipitation intervals, the activity measurement was carried out, and the range in which the enzyme was active was determined. Dialysis was used to desalinate the protein solution. For this purpose, the mixture obtained as a result of ammonium sulfate precipitation was placed in a dialysis bag and dialyzed for 2 hours by changing it against 10 mM Tris (pH: 7.8) buffer twice (Smith et al., 1988). Dialysis was carried out on a magnetic stirrer at 4 °C. The product obtained at the end of dialysis was stored at -20 °C to be used in the following analysis.

2.4. Activity Assay of GR Enzyme

Enzyme activity was measured spectrophotometrically using the modified method of Carlberg and Mannervik (1975) at 25 °C with a Shimadzu UV-1800 spectrometer (Shimadzu Corporation, Shimadzu, Japan). In this method, the activity was determined by the decreasing amount of NADPH due to the oxidation of NADPH in the presence of GSSG. The enzyme activity assay was carried out in a one ml reaction mixture consisting of assay buffer (50 mM Tris-HCl, pH 7.0), 2 mM GSSG, 2 mM NADPH and 25 µl sample. Spectrophotometric measurements were made in the kinetics rate program at 340 nm for 3 minutes. In the calculation of the enzyme unit, the molar extinction coefficient (ε) of NADPH at 340 nm was used as 6.22 mM^{-1} cm^{-1}. Enzyme activity was expressed as specific activity (EU mg $protein^{-1}$).

2.5. Protein Determination

Protein contents of all samples were determined spectrophotometrically at 595 nm according to Bradford (1976) method using BSA containing 1 mg protein ml^{-1} as a standard.

2.6. Characterization Studies

2.6.1. Optimum ionic strength determination

In order to determine the optimum ionic strength, the partially purified *A. maculatum* GR enzyme activity was measured using different concentrations of Tris-HCl (pH 7.0) and K-phosphate (pH 7.0) buffers, in the range from 5 to 250 mM (Tekman et al., 2008).

2.6.2. Optimum pH determination

For the optimum pH characterization, buffers were prepared in the range of pH 5.0 - 10.0 using the buffer concentration showing the highest activity and enzyme activity measurements were carried out (Tekman et al., 2008).

2.6.3. Optimum substrate concentration determination

The optimum substrate concentration was determined by using the buffer and pH with the highest activity in the previous steps. Activity measurements were performed using NADPH substrate in the 0.02 - 0.4 mM cuvette concentration range (Tekman et al., 2008).

3. Results and Discussion

Within the scope of this study, *A. maculatum* leaves GR enzyme was first partially purified and characterized. The purification procedure was carried out by

preparation of the homogenate, ammonium sulfate precipitation and dialysis. In the homogenate prepared for the partial purification of the GR enzyme from the leaf tissue of *A. maculatum*, the highest activity was obtained at 40-60% ammonium sulfate saturation, as a result of the activity measurements performed after the ammonium sulfate precipitations at the previously mentioned intervals. In previous studies, the ammonium sulfate precipitation range of GR enzyme purified from soybean seed (Bilir, 2017), human and bovine erythrocytes (Erat et al., 2003; Senturk et al., 2008, 2009), sheep brain (Kocaoğlu et al., 2019) and sheep liver (Ulusu et al., 2005) was found to be 40-60%, 30-70%, 35-55% and 0-60%, respectively. In our study, ammonium sulfate precipitation range for GR, which was partially purified for the first time from *A. maculatum*, was found to be compatible with the literature. Afterward, dialysis was applied and the activity measurement was repeated. GR was purified from leaf tissues of *A. maculatum* in 34.9% yield and 1.108 purification coefficient with a specific activity of 1.64 EU mg^{-1} (Table 1.).

Table 1. The summary of purification steps for *A. maculatum* GR

Purification step	Activity (EU/mL)	Total volume (mL)	Protein (µg/mL)	Total activity (EU)	Total protein (µg)	Specific activity (EU/mg)	Yeild (%)	Purification factor
Homogenate	0.280	25	0.194	7.00	4.852	1.479	100	1
ASPD	0.407	6	0.248	2.44	1.490	1.640	34.9	1.108

ASPD= ammonium sulfate precipitation and dialysis

In order to determine the most suitable ionic strength for GR enzyme activity, activity measurements were performed in Phosphate and Tris buffers at different concentrations and the obtained values are given in Figure 1. According to the results obtained, it was determined that the most suitable ionic strength for the *A. maculatum* leaf tissue GR enzyme was 150 mM KH_2PO_4 buffer. In order to determine the optimum pH, the selected 150 mM KH_2PO_4 buffer was prepared at different pH levels and GR activities were determined spectrophotometrically. The data obtained are shown in Figure 2. The optimum pH value for the GR enzyme of *A. maculatum* leaf tissue was determined as pH 7.0 in 150 mM KH_2PO_4 buffer. In a study by Lascano et al. (2001), it was reported that the optimum activity of chloroplastic GR of the wheat plant they purified was at pH 8.0 and an ionic strength between 60 and 100 mM. GR obtained from the plant *Larix kaempferi* has been reported to have an optimum pH ranging from 7.0 to 9.0 (Wang Xin, 2013). GRs purified from *Pisum sativum* have been reported to have optimum activity at pH 7.8 (Madamanchi et al., 1992). In addition, the optimum pH of the GR purified from the seed of the soybean plant was determined as pH 8.5 (Bilir, 2017). Moreover, it has been observed that GR optimum pH values purified from different organisms such as fish, sheep, chicken, humans and turtles are in similar ranges such as 6.5 - 8.5 (Acan and Tezcan, 1989; Erat et al., 2005; Ogus and Ozer, 1998; Tekman et al., 2008; Willmore and Storey, 2007). The optimum concentration of substrate for the GR enzyme activity obtained from the leaf of *A. maculatum* was determined by the activity measurements using the optimum buffer solution at the optimum pH value containing different concentrations of NADPH (Figure 3). According to the results obtained, it was observed that the most suitable substrate concentration for the *A. maculatum* GR enzyme was 0.18 mM NADPH in 150 mM phosphate buffer (pH 7.0).

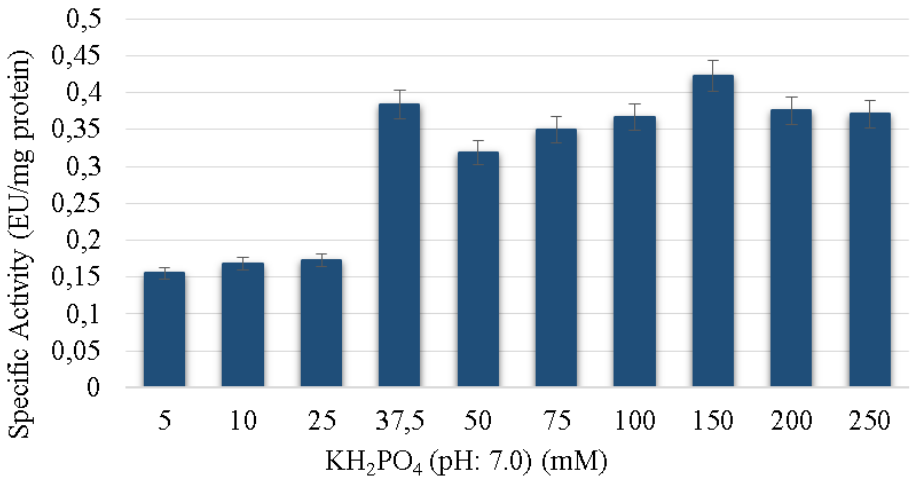

Figure 1. Specific activity – [ionic strength] graph for *A. maculatum* GR enzyme using K-phosphate buffer solutions at different concentrations.

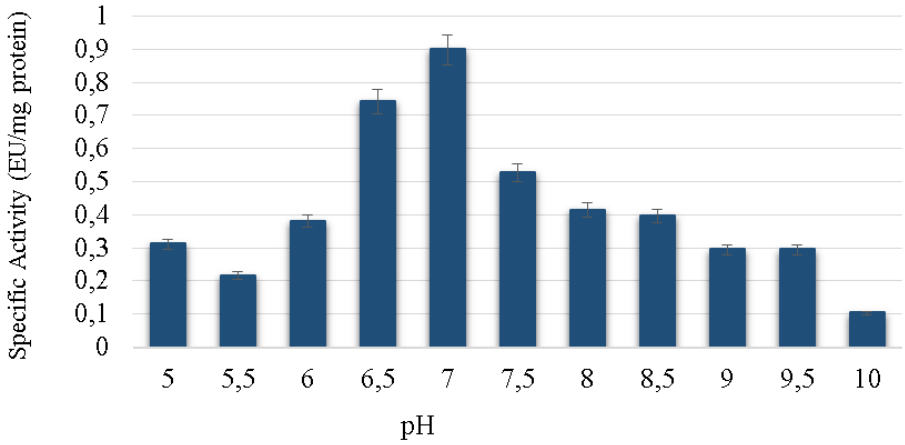

Figure 2. Specific activity – [pH] graph for determination of optimum pH for GR enzyme from *A. maculatum*.

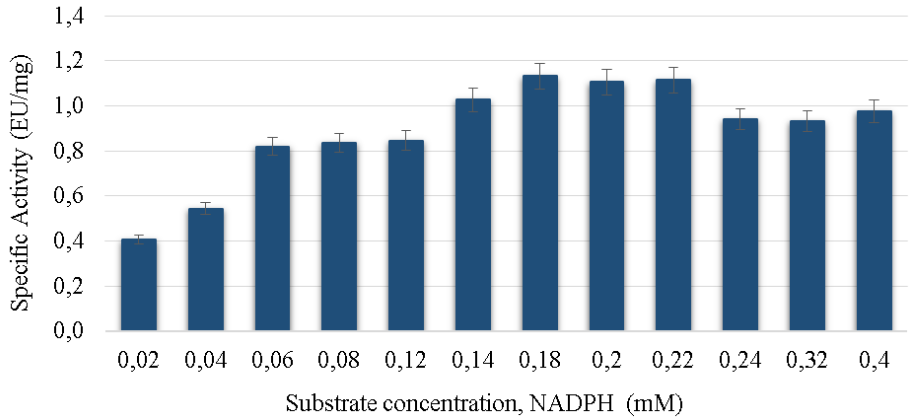

Figure 3. Specific activity – [Substrate concentration] graph for *A. maculatum* GR enzyme using NADPH solutions at different concentrations.

4. Conclusion

This study is the first research on the partial purification and characterization of the GR enzyme from the leaf of *A. maculatum*, which has been reported to have positive effects on health. To the best of our knowledge, no previous studies have reported on the purification of GR from A. maculatum. As a result of the study carried out using the leaf of the *A. maculatum* plant, the GR enzyme was obtained at a saturation of 40-60% ammonium sulfate. With the studies carried out for the characterization of the partially purified enzyme, it was determined that the optimum ionic strength was 150 mM potassium phosphate buffer, the optimum pH value was 7.0 and the optimum substrate concentration was 0.18 mM. It is thought that the results obtained will be a guide for future studies on the GR enzyme, which is of great metabolic importance.

Author Contributions

The percentage of the author(s) contributions is presented below. All authors reviewed and approved the final version of the manuscript.

	G.B.	M.S.	D.E.
C	30	20	50
D			100
S			100
DCP	30	20	50
DAI	30	20	50
L	30	20	50
W	40	10	50
CR			100
SR			100
PM	40		60
FA			100

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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