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Some Kinetic Properties and Inhibition of Glutathione S-Transferase from a Hybridized Wheat (*Triticum aestivum L.*)

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Abstract: Glutathione S-transferase enzymes (GSTs) play central roles in phase II detoxification of both xenobiotics and endogenous compounds in almost all living organisms. The enzyme was extracted and partially purified from wheat leaves through a procedure including ammonium sulfate fractionation followed by dialysis and gel filtration chromatography. These procedures yielded a 7.14-fold purification with 71% recovery. Optimum activity conditions-pH, temperature and ionic strength-of the enzyme were determined. Its some kinetic properties such as Vmax, KM, and kcat were calculated for GSH and CDNB substrates. The kcat/KM values of the enzyme were 603.5 for GSH and 385.3 for CDNB. The native molecular weight of the enzyme was estimated to be 52 kDa based on its mobility in gel filtration column.

Keywords: Wheat; Glutathione S-transferase; Purification; Kinetic.

1. Introduction

Glutathione *S*-transferases (GSTs, EC 2.5.1.18) are a family of multifunctional enzymes involved in the detoxification processes through several different mechanisms. These proteins detoxify electrophilic xenobiotics and endogenous compounds by catalysing their conjugation with the tripeptide glutathione to the electrophilic centre of lipophilic compounds, thereby increasing their solubility and removing toxic compounds from circulation through covalent and non-covalent binding [1-3]. The enzymes are also involved in several additional functions including the binding, transport, storage of hydrophobic ligands, [4] the isomerization of maleylacetoacetate [5] and the regulation of stress kinases and apoptosis.[6] Furthermore, these proteins are known to protect cellular integrity from endogenous oxidative stress by expressing selenium–independent glutathione peroxidase activity with organic hydroperoxides. [6] Plant GSTs are a multigene family of enzymes that constitute about 1% of the soluble protein in photosynthetic plant cells. [7] These enzymes are known to function in herbicide detoxification, tyrosine metabolism, hormone homeostasis, vacuolar sequestration of anthocyanin, hydroxyperoxide detoxification, regulation of apoptosis and in plant responses to biotic and abiotic stresses [8].

Glutathione S-transferase and its izozymes have been extensively purified from different sources such as plant, [9, 10] mammalian [11] and microbial sources [12, 13]. In order to purify the enzymes, various chromatographic techniques such as, affinity, ion-exchange, reversed phase, hydrophobic, and size-exclusion columns were used in different reports [14, 15].

In the current study, we partially purified glutathione S-transferase from a hybridized wheat leaves and searched for its some kinetic and characteristic properties. We identified its different properties from the other GST enzymes purified and characterized so far from the other sources.

2. Experimental

2.1. Materials

Wheat (*Triticum aestivum* L.), used in this study was harvested from a field in Erzurum, Turkey. Sephadex G-100 used as gel filtration column, substrates, and other chemicals were purchased from Sigma Chem. Co. All chemicals used in this study were the best grade available. Substrates and other solutions were prepared freshly in distilled water.

2.2. Enzyme Extraction and Purification

15 grams of wheat samples were cleaned and prepared for the extraction. The samples were immersed in liquid nitrogen, in a Dewar flask to disrupt cell membranes, and were homogenized in 75 ml of 0.1 M potassium phosphate buffer pH 6.0. The crude extract samples were centrifuged at 14,000g for 30 min. The process was conducted at 4 ^{o}C. The supernatant was precipitated at 0-20%, 20-40%, 40-60% and 60-80% neutral salt concentrations to find

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proper saturation point with solid $(NH_4)_2SO_4$. The proper saturation of GST was determined to be in 20-60% $(NH_4)_2SO_4$ fraction. The precipitate was dissolved in a small amount of 0.1 M phosphate buffer (pH 6.0) and the proteins were dialyzed in 10 mM potassium phosphate buffer (pH 6) containing 0.01% (w/v) β-mercaptoethanol and 1 mM EDTA overnight to completely remove low molecular inhibitors. In order to conduct further purification, the dialysate was applied to a column (2.5-70 cm) filled with Sephadex G-100 gel and equilibrated with 50 mM phosphate buffer pH 6.0 containing 50 mM KCl. The enzyme was eluted with the equilibration buffer by means of a peristaltic pump. The flow rate was adjusted to 20 mL/h [15, 16].

The eluates were collected in test tubes with volumes of 2 mL and elution continued until zero absorbance was obtained at 280 nm. Each eluate fraction that showed absorbance at 280 nm was assayed for GST activity. A_{280} and the enzyme activity were plotted versus the tube number. The fractions having GST activity were collected and degree of purification was determined by measuring specific activity before and after purification. Specific activity was determined from GST activity and quantitative protein data obtained.

2.3. Activity Determination

Glutathione S-transferase activity was determined as described by Habig and Jakoby [17]. The reaction medium contained 0.1 M citrate buffer pH 4.5, 1.0 mM GSH, 1.0 mM CDNB, and 1% absolute ethanol with a total volume of 1.0 mL. The reaction was monitored by an increase in A_{340} for 3 min. The reactions were initiated by the addition of the enzyme solution. All enzymatic activities were determined spectrophotometrically at 25 ^oC, with a Beckman Spectrophotometer (DU 730). One unit of activity was defined as the formation of 1.0 μmol product min^{-1} (extinction coefficient at 340 nm = 9.6 mM^{-1} cm^{-1} for GSDNB). Finally, the protein concentrations were calculated from measurements of absorbance at 595 nm according to the method of Bradford, with bovine serum albumin as a standard [18].

2.4. Optimum pH Determination

GST activities determined in appropriate buffers (citrate for pH 3.0-5.0, phosphate for pH 5.0-7.5, and Tris-HCl for pH 7.5-8.5) were used for the determination of optimum pH of the enzyme. The optimum pH values obtained from this assay were used in all subsequent experiments.

2.5. Stable pH Determination

In order to determine pH stability of the enzyme, equal volumes of purified enzyme sample and the buffers (citrate at pH of 3.5, 4.0, 4.5, and 5.0, phosphate at pH of 5.5, 6.0, 6.5, 7.0, and 7.5, and Tris-HCl at pH of 7.5, 8.0, and 8.5) were mixed and kept in refrigerator (+4 ^oC). The enzyme activity was assayed at the beginning and at each three-day interval for twelve days.

2.6. Optimum Temperature Determination

For the determination of the optimum temperature, enzyme activity was assayed in 0.1 M citrate buffer at different temperatures ranging from 5 ^oC to 70 ^oC. The desired temperature was provided by using Polyscience bath (model 9105).

2.7. Optimum Ionic Strength Determination

Effect of ionic strength on the enzyme was measured in a range from 0.02 M to 0.2 M citrate buffer concentrations at the assay conditions.

2.8. Determination of the Native Molecular Weight

The native molecular weight of wheat GST was found out on the gel filtration column according to the method of Andrews [19]. Ten grams of dried Sephadex G-100 was used for about 180 mL column size. The gel was incubated in distilled water at 90 ^oC throughout a night. After removing the air in the gel, it was loaded onto the column (2.5x70 cm). Flow rate was adjusted to 8 mL/h by means of peristaltic pump. The column was equilibrated with 50 mM Tris-HCl, 50 mM KCl buffer, pH 7.0 until the final absorbance difference became zero at 280 nm, and pH value was the same with that of equilibration buffer. As a first step, to establish the void volume, Blue Dextran (2000 kDa) was passed through the column; then, horse heart cytocrome-c (12.4 kDa), bovine erythrocyte carbonic anhidrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), and sweet potato β-amylase (200 kDa) were used as standards (MW-GF-200; Sigma). The enzyme sample from gel filtration chromatography was mixed with glycerol to form a mixture including 50 mM glycerol and applied to the column. K_{av} values were calculated for each standard protein and the GST, and a standard K_{av}-Log MW graph was drawn as seen in Figure 5.

2.9. Effect of Substrate Concentrations

GST activities were measured with GSH and CDNB at varying concentrations to determine the optimum substrate concentration for the best activity of the enzyme under optimum conditions of pH, temperature, and ionic strength. Michaelis constant (K_M) and maximum velocity (V_{max}) values of the enzyme for GSH and CDNB substrates were calculated from a plot of 1/V against 1/[S] by the method of Lineweaver and Burk [20]. The k_{cat} values were obtained by the division of the V_{max} and the total enzyme concentration, and the values of k_{cat}/K_M to both GSH and

CDNB were also calculated [21]. The higher k_{cat}/K_M value represents more efficient substrate specificity of the enzyme.

3. Results and Discussion

3.1. Extraction and Purification of GST

Glutathione S-transferase enzyme was extracted and partially purified from wheat leaves through a procedure that included ammonium sulfate fractionation followed by dialysis and gel filtration chromatography on Sephadex G-100 column. These procedures yielded a 7.14-fold purification with 71% recovery of the total activity having a specific activity of 4.0 EU/mg proteins (Table 1).

3.2. Effect of pH

The enzyme activity was measured in buffers having different pH values. The optimum pH for enzymic activity, after compensation for non-enzymic conjugation of GSH with CDNB, exhibited one peak at pH 4.5 in citrate buffer (Fig. 1). Different optimum pHs for the enzyme obtained from various sources have been reported in the literature. Since non-enzymic conjugation of GSH with CDNB increases with increased pH, the measurements in high pH values are not useful. Thus, although Habig and Jakoby [17] have found a sharp peak at pH 7.5, they have performed their assays at pH 6.5. In general, vegetables and fruits show maximum activity at or near neutral pH values, but these values may vary with the source of enzyme and substrate within a relatively wide range of pH. Although, in most cases, pH optima have been reported between 4.0 and 8.5, it should be noted that the optimum pH can also be affected by the type of buffer and the purity of enzyme [16].

Stability of wheat GST activity was studied at 11 different pHs ranging from 3.5 to 8.5 over a period of 12 days. The enzyme showed a maximum activity of 19.0% at the end of 12 days in K-phosphate buffer (pH 6.0). Consequently, stable pH of the enzyme was regarded as 6.0 in K-phosphate buffer (Figure 2).

3.3. Effect of Temperature

As shown in Fig. 3, maximum enzyme activity was obtained at 40 ^{o}C. While the enzyme was able to show 30% of the total activity at 10 ^{o}C, it retained 68% of its enzymatic activity at high temperatures such as 70 ^{o}C. Therefore, the optimum temperature of the enzyme could be regarded as 40 ^{o}C (Figure 3).

3.4. Effect of Ionic Strength

To determine the affect of ionic strength on the enzyme activity, measurements were performed between 0.04 and 0.40 M citrate concentrations. The ionic strength profile analysis of the enzyme indicated that the optimum ionic strength for wheat GST activity was 0.1 M citrate concentration (Figure 4).

3.5. Molecular Weight Determination

The native molecular weight of wheat GST enzyme was estimated to be approximately 52 kDa by gel filtration chromatography on Sephadex G-100 column (Figure 5).

3.6. Enzyme Kinetics and Substrate Specificity

The kinetic parameters, V_{max} and K_M, were determined using CDNB and GSH as co-substrates. V_{max} values were calculated as 0.227 U/mg proteins and 0.715 U/mg proteins, K_M constants were also estimated as 0.118 mM and 0.237 mM for CDNB and GSH, respectively, as seen in Table 2. k_{cat} values were also determined as 45.46 min^{-1} for CDNB and 143.04 min^{-1} for GSH. Since k_{cat} and k_{cat}/K_M values obtained for GSH are higher than that for CDNB, it may be said that GSH is a better substrate for the enzyme.

4. Conclusions

Glutathione S-transferases (GSTs) play central roles in phase II detoxification of both xenobiotics (drugs, insecticides, and herbicides) and endogenous compounds in plants [22]. Lots of GSTs from plants have been purified, and characterized, and their genes have been cloned. Among them, the enzymes from arabidopsis, soybean, sorghum and maize have been extensively studied for their structure, function, and physiological significance in detail [23, 24]. To our knowledge, there was no adequate research on the hybridized wheat (*Triticum aestivum* L.) GST enzyme in the literature. Therefore, we trayed to identify its different properties from the GST enzymes purified and characterized from the other plant sources. For this reason, we have partially purified and characterized the GST from hybridized wheat leaves for the first time in the current work. The enzyme was extracted and partially purified from wheat by a procedure that included ammonium sulfate fractionation followed by dialysis and gel filtration chromatography. Our procedure led to 7.14-fold purification with 71% recovery having a specific activity of 4.0 EU/mg proteins (Table 1). Maize GST has been purified approximately 200 fold with 3.6% recovery by ammonium sulfate precipitation and two chromatographic methods. Sorghum GST isozymes-A1/A2 and B1/B2-have also been isolated 190 and 107 fold with 0.7% and 0.8% recovery by four chromatographic methods.

The wheat GST showed a maximum activity in 0.1 M citrate buffer (pH 4.5) at 40 ^{o}C and its stability was determined to be pH 6.0 at the end of 12 days in citrate buffer. Optimum pH of maize GST was determined to be between 7.5 and 8.0.[9]

$V_{max,}$ $K_{M,}$ and k_{cat} values were calculated as 0.715 U/mgp, 0.237 mM, and 143.04 min^{-1} for GSH and 0.227 U/mgp, 0.118 mM, and 45.46 min^{-1}for CDNB substrates, respectively. The k_{cat}/K_M values of the enzyme were 603.15 for GSH and 385.3 for CDNB. K_M values have been determined to be 0,292 mM on the maize enzyme [9] and 0.915 mM on a sorghum isozyme [22] for GSH substrate. For another sorghum isozyme, the K_M values for GSH and CDNB have also been reported to be 0.118 mM and 1.913 mM, respectively. In previous reports of kinetic parameters for purified plant GSTs, initial velocity data have been analyzed the standard Michaelis equation for a unireaction.[9] However kinetic models describing bireactant mechanizms are better suited for the determination of kinetic constant for GSTs, which utilize two substrates [10]. For wheat GST, the random, rapid equilibrium Bi-Bi model best described the kinetics with GSH and CDNB as substrates. Although the data, further kinetic analyses would be required to confirm the random, rapid equilibrium Bi-Bi model.

The native molecular weight of the enzyme was estimated to be 51 kDa based on its mobility in gel filtration column. But, the quarternary structure of the enzyme could not be determined. The native molecular weight of sorghum GST A1/A2 has been determined to be 26 kDa with homodimeric structure, but its another isozyme's molecular weight has been calculated 26 and 28 kDa with hetero dimeric structure [10]. The native molecular weight of bovine erythrocytes GST have been found to be 53 kDa by gel filtration column and 27 kDa on SDS-PAGE [11]. The molecular weight of the wheat native enzyme is similar to those of that calculated from mentioned reports.

5. Funding

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Table-1. Partial purification of glutathione *S*-transferase from wheat leaves

Purification steps	Total volume (mL)	Activity (U/mL)	Total activity (U)	Protein (mg/mL)	Total protein (mg)	Specific Activity (U/mg)	Yield (%)	Purifi-cation n-fold
Crude extract	20	0.014	0.28	0.025	0.500	0.56	100	1.00
$(NH_4)_2SO_4$ precipitation and dialysis	6	0.038	0.23	0.044	0.264	0.86	82	1.54
Gel filtration chromatography	10	0.020	0.20	0.005	0.050	4.00	71	7.14

Table-2. V_{max}, K_M, and k_{cat} values of wheat GST

Substrate	V_{max} (U/mg proteins)	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (mM/min)
1-Chloro-2,4-dinitrobenzene	0.227	0.118	45.46	385.3
Glutathione	0.715	0.237	143.04	603.5

Figure-1. Effect of pH on activity of wheat GST. The activity of the enzyme was determined in the assay conditions by using three different buffers.

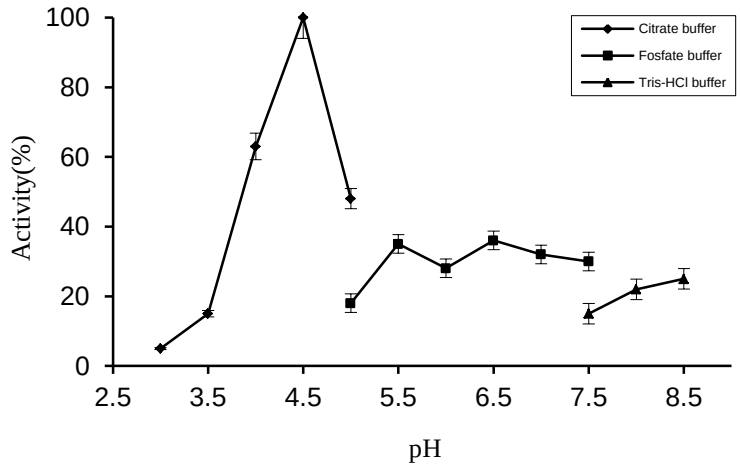

Figure-2. pH stability of wheat GST in citrate buffer.

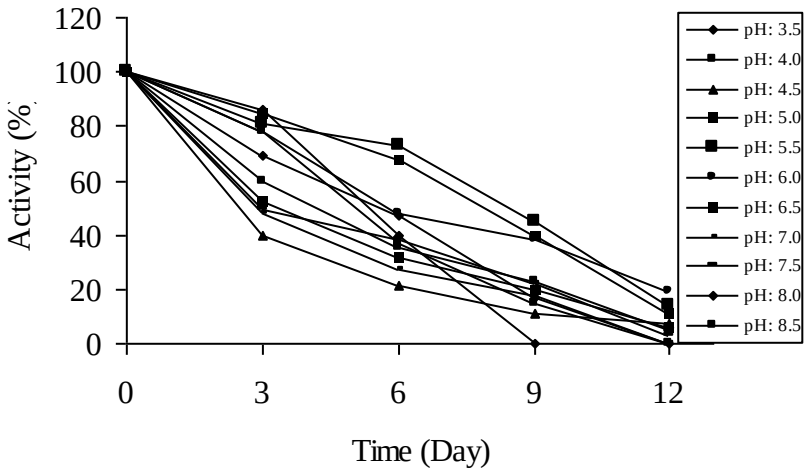

Figure-3. Effect of temperature on activity of wheat GST. The measurements were performed in 0.1 M citrate buffer (pH 4.5).

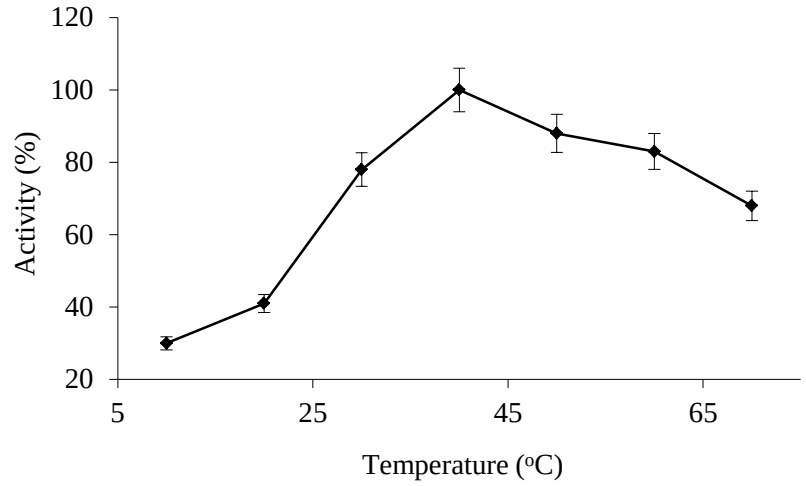

Figure-4. Effect of ionic strength on activity of wheat GST. The activity was assayed in different concentrations of citrate buffer at 25 °C.

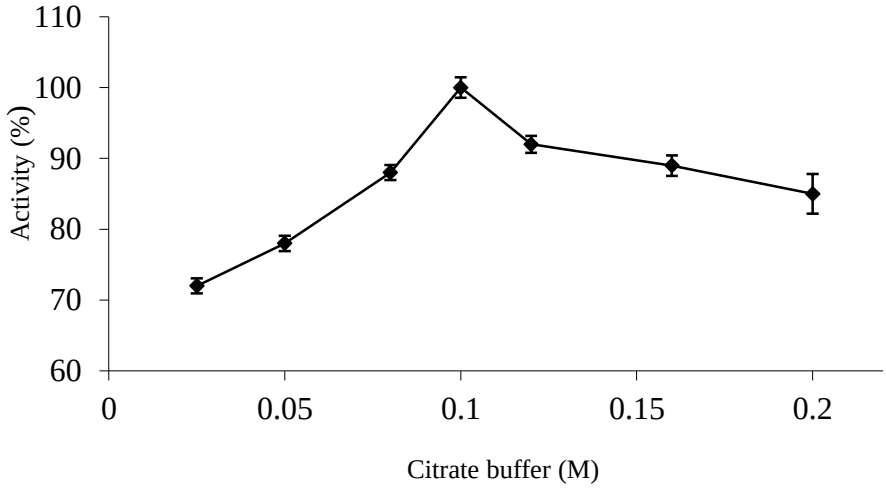

Figure-5. Standard K_{av}-LogMW graph of wheat GST using gel filtration. Standard proteins: horse heart cytocrome c (12.4 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), and sweet potato β-amylase (200 kDa).