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Biochemical Changes of Glutathione S-Transferase Activity During the Germination of *Trifolium alexandrinum*

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Abstract: The key objective of this study was to investigate the effect of germination on biochemical and enzymatic antioxidant activities of *Trifolium alexandrinum* seeds. The *T. alexandrinum* was chosen for GST purification and characterization due to its highest GST activity and antioxidant capacity, beside its economical importance. Seeds of *T. alexandrinum* were germinated for 6 days. Changes in glutathione S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx) in germinating and dormant seeds of *T. alexandrinum* were evaluated. Also the changes in the total phenolic and flavonoid contents as well as the antioxidant capacities were monitored for 6 days of germination using spectrophotometeric methods. Simple reproducible procedures for the purification of *T. alexandrinum* GST from both dormant and 6 days germinated seeds were established using DEAE-Sepharose and Sephadex G-100 columns chromatography. The results showed the presence of three isoenzymes for both dormant and germinated seeds designated as GST1, GST2 and GST3. The major GST of the dormant seed was GST2 while for the germinated one was GST3. Dormant GST2 was expressed as a heterodimer with molecular weight of 27.5 and 28.5 KDa while germinated GST3 was expressed as a homodimer with molecular weight of 27 KDa. Characterization of both dormant GST1 & GST2 and germinated GST2 and GST3 including optimum pH, kinetic parameters [k_m^{GSH} of 1.17±0.39, 1.22±0.13, 0.84±0.19, 0.96±0.24mM, respectively and k_m^{CDNB} of 0.65±0.095, 0.57±0.11, 0.59±0.14, 1.02±0.18 mM, respectively]. The substrate selectivity using different electrophilic compounds and inhibitor effects were carried out. In opposite to other isoenzymes, germinated GST3 exhibited enzymatic activity towards ethacrynic acid with specific activity of 0.073±0.005 µmol/min/mg protein while cibacron blue was the most potent inhibitor for these isoenzymes.

Keywords: Antioxidant Enzymes, Inhibition, Kinetic Parameters, Seed Germination, *Trifolium alexandrinum*

1. Introduction

Plants, similar to animals, face several problems such as a need for regulation of metabolic processes, reproduction and efficient defense against enemies. They respond to a variety of environmental stresses (e.g. change in temperatures, drought, salinity, UV or ozone stress and pathogen infections) through the induction of antioxidant defense enzymes that provide protection against further damage [1].

Glutathione S-transferases (GSTs; EC 2.5.1.18) are a family of enzymes involved in detoxification of different xenobiotics. The main function of GST is to catalyze the conjugation of glutathione (GSH) to an electrophilic site of a broad range of potentially toxic and carcinogenic compounds, thereby making such compounds less biologically active and enabling their excretion [2]. In plants, all the GSTs described to date are dimmers composed of 25-30 kDa subunits. On the basis of sequence similarity and gene organization, plant GSTs appear to have evolved from a common ancestral GST into four distinct cytosolic classes, namely the Phi, Tau, Zeta, and Theta GSTs. The two largest classes are the plant-specific Phi and Tau GSTs. Both classes have major roles in herbicides detoxification [3]. In addition, plant GSTs have less well characterized roles in endogenous metabolism including functioning as glutathione peroxidases counteracting oxidative stress and also act as flavonoid-

binding proteins, stress signaling proteins and regulators of apoptosis [4]. Plant GST has been concerned in the agricultural chemistry and biochemistry because of its major factors involved in the resistance of a variety of herbicides [5].

The plant, *Trifolium alexandrinum* (common name: Egyptian clover, berseem clover) represents the main forage crops for livestock and have produced milk and /or meat in Egypt [6] and its seeds are used as an antidiabetic treatment [7]. It is traditionally as human ailments, including renal dysfunctions [8]. The leaves, seeds and sprouts of *T. alexandrinum* also have medicinal use in many metabolic deficiencies, are phytonutrient-rich, provide significant amounts of antioxidants [9], delay the aging processes, help to strengthen the immune system, especially protect against infection, prevent heart disease and coronary heart disease (through decreasing plasma cholesterol) [10]. The *T. alexandrinum* contains two to three percent saponin glycosides and phenolic compounds [11]. Phytochemical investigations of *T. alexandrinum* have revealed the presence of terpenoind saponin, flavonoids, isoflavonoids, and fatty acids in different parts of the same plants.

Germination is one of the most common and effective processes for improving the quality of legumes [12]. Exposure to free radicals from a variety of sources has led organisms to develop a series of defense mechanisms [13]. Defense mechanisms against free radical-induced oxidative stress involve: (i) preventative mechanisms, (ii) repair mechanisms, (iii) physical defenses, and (iv) antioxidant defenses. Enzymatic antioxidant defenses include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and GST. Non-enzymatic antioxidants are represented by ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids, and other antioxidants [14].

Thus the key objective of this study was to investigate the effect of germination on biochemical and enzymatic antioxidant activities of *T. alexandrinum* seeds. The changes in the total phenolic and flavonoid contents as well as the antioxidant capacities were also monitored for 6 days of germination.

2. Materials and Methods

2.1. Materials

The dormant seeds of *T. alexandrinum, Leguminosae* family were collected from Agricultural Research Centre, El-Dokki, Giza. The plants were identified and authenticated by the Department of Herbachium Botany at the National Research Center. The healthy seeds were selected and stored in sealed polyethylene bags with silica gel included as a desiccant for one week. The samples were kept in a refrigerator at 4^{o}C until ready for extraction. 1-chloro-2, 4-dinitrobenzene (CDNB), Folin-Ciocalteu's (FC) reagent, 1,1 diphenyl-2-picryl-hydrazyl (DPPH), nicotinamide adenine dinucleotide phosphate reduced form (NADPH), sulfobromophthaliene are products of Sigma-Aldrich Company. DEAE-Sepharose was obtained from GE Healthcare Co. The reduced GSH was obtained from Bio-Basic INC. Glutathione disulphide (GSSG) was purchased from Fluka Company.

2.2. Determination of Total Phenolics

The *T. alexandrinum* seeds (0.5 g) were gently powdered, homogenized and extracted with 70% ethanol at room temperature (25^{o}C). The total phenolics were determined by Folin-Ciocalteu's reagent method with some modifications of the method proposed by Singleton and Rossi [15]. The total phenolic content was determined as gallic acid equivalents (mg gallic acid / g dry seed).

2.3. Determination of Total Flavonoids

Total flavonoids were determined using the procedure reported by Woisky and Salatino [16]. Total flavonoids of the sample were expressed as catechin equivalents (mg catechin / g dry seed).

2.4. Determination of Antioxidant Capacity

The free radical scavenging activities were determined by DPPH method with some modifications of the method proposed by Blois [17]. DPPH free radical scavenging ability of the extract was expressed as µg ascorbic acid equivalents per gram dry seed.

2.5. Seed Germination

Sterile *T. alexandrinum* seeds were germinated in a growth chamber with a photoperiod of 16 h day/ 8 h night at 23±1ºC. Germinated seeds were firstly collected and then homogenized using mortar in 25 mM Tris-HCl buffer, pH 7.5. The homogenate was centrifuged at 11,000 × g for 30 min and the clear supernatant was designated as crude homogenate and saved at –20°C for further analyses.

2.6. Purification of GST from T. Alexandrinum Seeds

Crude homogenate of the dormant and germinated seeds was incubated with DEAE-Sepharose for 30 min, filtered, washed with 25 mM Tris-HCl, pH 7.5 containing 10 mM $CaCl_2$ and 2 mM DTT (buffer A) and then packed to the column (8.5 x 2.6 cm i.d.). The bound proteins were eluted with buffer A using step gradient of NaCl (0.05, 0.1 and 0.2 M). Fractions in 3 ml volume were collected at a flow rate of 60 ml/h. The GST containing fractions under each peak were pooled separately and designated as GST1, GST2 and GST3 for dormant seeds and germinated GST1, GST2 and GST3 for germinated seeds according to their elution order and saved at -20^{o}C for further analyses. The purified DEAE-Sepharose enzyme was applied to a Sephadex G-100 column (85 × 1.6 cm i.d.) equilibrated with buffer A to estimate molecular weight [18]. Protein concentration was determined by the method of Bradford [19] using bovine serum albumin as a standard.

Native- PAGE (12%) was carried out using a horizontal electrophoresis system (Amersham Pharmacia). The gel was stained for GST activity and for protein with 0.1% (w/v) Coomassie Brilliant Blue R250 (CBB R250) dissolved in 30%methanol and 10% glacial acetic acid. The subunit molecular weight of the pure enzyme was determined by SDS-PAGE (12%) as described by Laemmli [20].

2.7. Enzyme Assays

2.7.1. Glutathione S-Transferase

The specific activities of the GST were measured towards CDNB, 4-nitrophenthyl bromide (NPB), 1,2-epoxy-3-[4-nitrophenoxy]-propane (EPNP), ethacrynic acid (EA), and 1, 2 - dichloro-4-nitrobenzene (DCNB) according to the method described by Habig *et al*. [21]. The activity was measured with cumene hydroperoxide (CuOOH) by coupling the glutathione reductase-dependent NADPH oxidation [22]. The activity was also measured with styrene oxide (SO) [23]. One unit of activity is defined as the formation of 1 µmol product min^{-1} at 30°C. One unit of GST activity is defined as the amount of enzyme which catalyzes the formation of 1.0 µmole of thioether per min.

2.7.2. Glutathione Reductase (GR)

The GR activity was determined spectrophotometrically at 25°C following the decrease in absorbance at 340 nm according to the method described by Zanetti [24].

2.7.3. Glutathione Peroxidase (GPx)

GPx was measured spectrophotometrically at 25°C according to the method described by Weinhold *et al.* [25]. One unit of GPx and GR activity is defined as the amount of enzyme, which oxidize 1 µmole of NADPH (ε_{340} = 6.22 $mM^{-1}\,cm^{-1}$) per min.

2.7.4. Catalase (CAT)

The CAT activity was assayed according to the method described by Aebi [26]. The method is based on monitoring the rate of decomposition of H_2O_2 at 25°C. One unit of activity was defined as the calculated consumption of 1 µmol of H_2O_2 (ε340 = 43.6 $mM^{-1}\,cm^{-1}$) per min.

2.7.5. Superoxide Dismutase (SOD)

The SOD activity was determined using a commercial kit purchased from Biodiagnostic (Cat. No. SD 25 21) based on the method described by Nishikimi *et al*. [27]. One unit of enzyme activity is defined as the amount of enzyme that gave 50% inhibition of nitroblue tetrazolium reduction in one minute. Specific activity of all antioxidant enzymes is expressed as unit/mg protein.

2.8. Effect of pH

Activity of purified GST fractions were assayed at different pH ranges using 0.1 M citrate phosphate buffer for pH ranged from 4.5 to 6, 0.1 M potassium phosphate buffer for pH ranged from 6 to 7.5, and 0.1 M Tris-HCl buffer for pH ranged from 7.5 to 10.

2.9. Kinetic Parameters

Kinetic measurements were performed at 25ºC in 0.1M potassium phosphate buffer, pH 6.5. The concentration of CDNB was varied between 0.1-2 mM at constant concentration of GSH of 5.0 mM. The GSH concentration was varied between 0.1 and 2.0 mM at constant concentration of CDNB of 2.0 mM. The K_m and V_{max} values were calculated by using the Michaelis-Menten equation in GraphPad Prism 5 soft ware (GraphPad Prism Soft ware Inc., USA).

2.10. Inhibition Studies

The IC_{50} value of each inhibitor (hematin, cibacron blue, sulfobromophthaliene and EA) for *T. alexandrinum* dry and germinated seeds GST was determined according to Yalcin *et al.* [28].

2.11. Statistical Analysis

The Student's t-test was performed to examine the difference between means. Experimental results were mentioned as mean ± SD of three parallel measurements. *P* values < 0.05 were regarded as significant, *p* values < 0.01 as highly significant and *p* values > 0.05 as insignificant.

3. Results

3.1. Changes in the Total Phenolic, Flavonoid Contents and Antioxidant Capacity during T. Alexandrinum Seed Germination

Total phenolics, total flavonoids and antioxidant capacity in 70% ethanolic extract of dormant and germinated seeds were determined (Table 1). The highest phenolic and flavonoid content was found in *T. alexandrinum* dormant seeds. This was followed by significant decrease ($P < 0.001$) by day 4 and 6. The highest antioxidant capacity was observed in dormant seeds and significantly increased by 2 days of germination ($P > 0.001$).

Table 1. *Total phenolic, total flavonoid contents (mg/g dry seeds) and antioxidant capacity (mg dry weight/g dry seeds) of ethanolic extract of dormant and germinated T. alexandrinum seeds.*

T. alexandrinum	Phenolics	Flavonoids	IC_{50}
Dormant seeds	64±7.5	19.2±1.0	1.27±0.06
Germinated seeds			
2 days	50.3±2.5	4.70±0.3**	2.70±0.30**
4 days	41±1.0**	6.60±0.6**	3.30±0.30**
6 days	41.7±1.1**	7.80±0.6**	3.30±0.15**

** Highly significant $P < 0.001$

3.2. Effect of Germination on Seed Antioxidant Enzyme Activities

The final germination percentage of the *T. alexandrinum* dormant seeds after 6 days was over 95%. The crude homogenate of *T. alexandrinum* dormant and 2, 4 and 6 days

old germinated seeds was prepared as previously described in the materials and methods section. The total protein content was decreased during seed germination stages. The total protein content of dormant seeds (46 mg/g tissue) was decreased to 6.95 mg/g tissue in six days old germinating seeds. The catalytic activity of GST, GR, GPx, CAT and SOD were assayed in the crude homogenates of dormant as well as 2, 4 and 6 days old germinated seeds (Table 2). In opposite to other enzymes, GST was significantly decreased ($P < 0.001$) by germination while the other examined antioxidant enzymes were significantly increased by germination.

Table 2. *Antioxidant Enzyme Activities of T. Alexandrinum Seeds during Germination.*

T. alexandrinum	Protein (mg/g tissue)	Specific activity (Unit /mg protein)				
		GST	CAT	SOD	GPx	GR
Dormant seeds	46.4±4.80	0.35±0.02	31.2±2.8	5±0.50	ND	0.01±0.001
Germinated seeds						
2 days	20.1±2.9	0.15±0.01*	122±21*	28±3*	0.05±0.006*	0.01±0.001
4 days	11.7±0.50	0.11±0.007*	90±7.2*	27±2.5*	0.05±0.004*	0.02±0.002*
6 days	6.95±0.87	0.14±0.02*	111±2.9*	50±4*	0.07±0.007*	0.03±0.004*

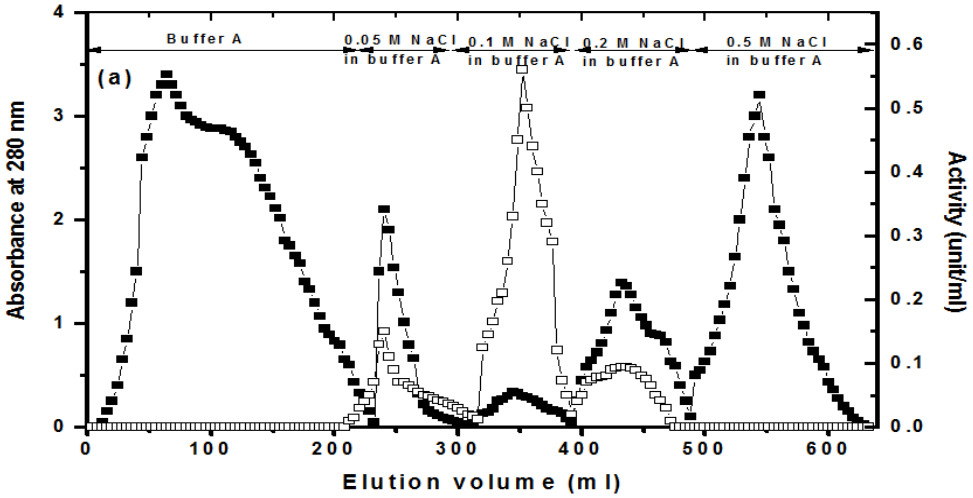
* Highly significant $P < 0.001$.
ND: not detected under the experimental conditions

3.3. GST Purification and Molecular Weight Determination

Simple reproducible procedures for the purification of GST from dormant (46 units & 365 mg protein) and germinated (16.2 units & 164 mg protein) *T. alexandrinum* seeds are summarized in Tables 3 & 4. The procedure included ion exchange chromatography on DEAE-Sepharose followed by gel filtration on Sephadex G-100. The specific activity of dormant GST2 and germinated GST3 increased to 3.3 and 0.85 unit/mg protein after gel filtration on Sephadex G-100, respectively. Typical elution profiles for the chromatography of *T. alexandrinum* dormant and germinated crude homogenates on DEAE-Sepharose are shown in Fig. 1 a, b. The bound proteins were eluted with buffer A containing stepwise gradients of NaCl (0.05, 0.1 and 0.2 M) at a flow rate of 60 ml/h. Three protein peaks with GST activity were eluted with 0.05, 0.1 and 0.2 M NaCl. The GST containing fractions under each peak were pooled separately and designated GST1, GST2 and GST3 for dormant seeds and germinated GST1, GST2 and GST3 for germinated seeds according to their elution order, respectively. The specific activity of the major GST peaks of dormant GST2 and germinated GST3 of *T. alexandrinum* seeds was changed to 0.85 & 0.09 µmol/min/mg protein with an overall recovery of 88% & 41.7%, respectively (Table 3 & 4). The molecular weight of dormant GST2 and germinated GST3 was calculated from Sephadex G-100 calibrated curve to be 52.5 and 47.8 KDa, respectively (Fig. 2).

T. alexandrinum crude homogenate and DEAE-Sepharose purified fractions (GST1, GST2 and GST3) were applied to native-PAGE and stained for activity (Fig. 3). PAGE of *T. alexandrinum* crude homogenate indicated the presence of three main GST activity spots, GST1, GST2 and GST3 exhibited low, medium and high relative mobility, respectively.

Subunit molecular weights of *T. alexandrinum* dormant seeds GST2 and germinated seeds GST3 were estimated using 12% SDS-PAGE and the molecular weight was calculated from previously established standard curve for known molecular weight proteins. *T. alexandrinum* GST2 dormant seed was found to be a heterodimer with subunit molecular weight of 27.5 and 28.5 KDa while GST3 germinated seeds was a homodimer with molecular weight of 27.0 KDa (Fig. 4).



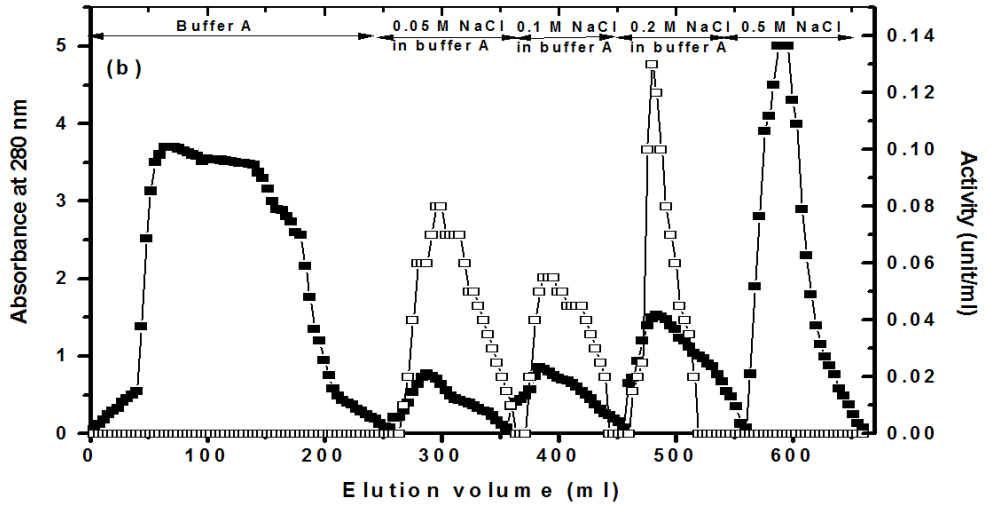


Fig. 1. *Typical elution profiles of T. alexandrinum dormant (a) and germinated (b) seeds on DEAE-Sepharose column. Absorbance at 280 nm (▪) and GST activity at 340 nm (□).*

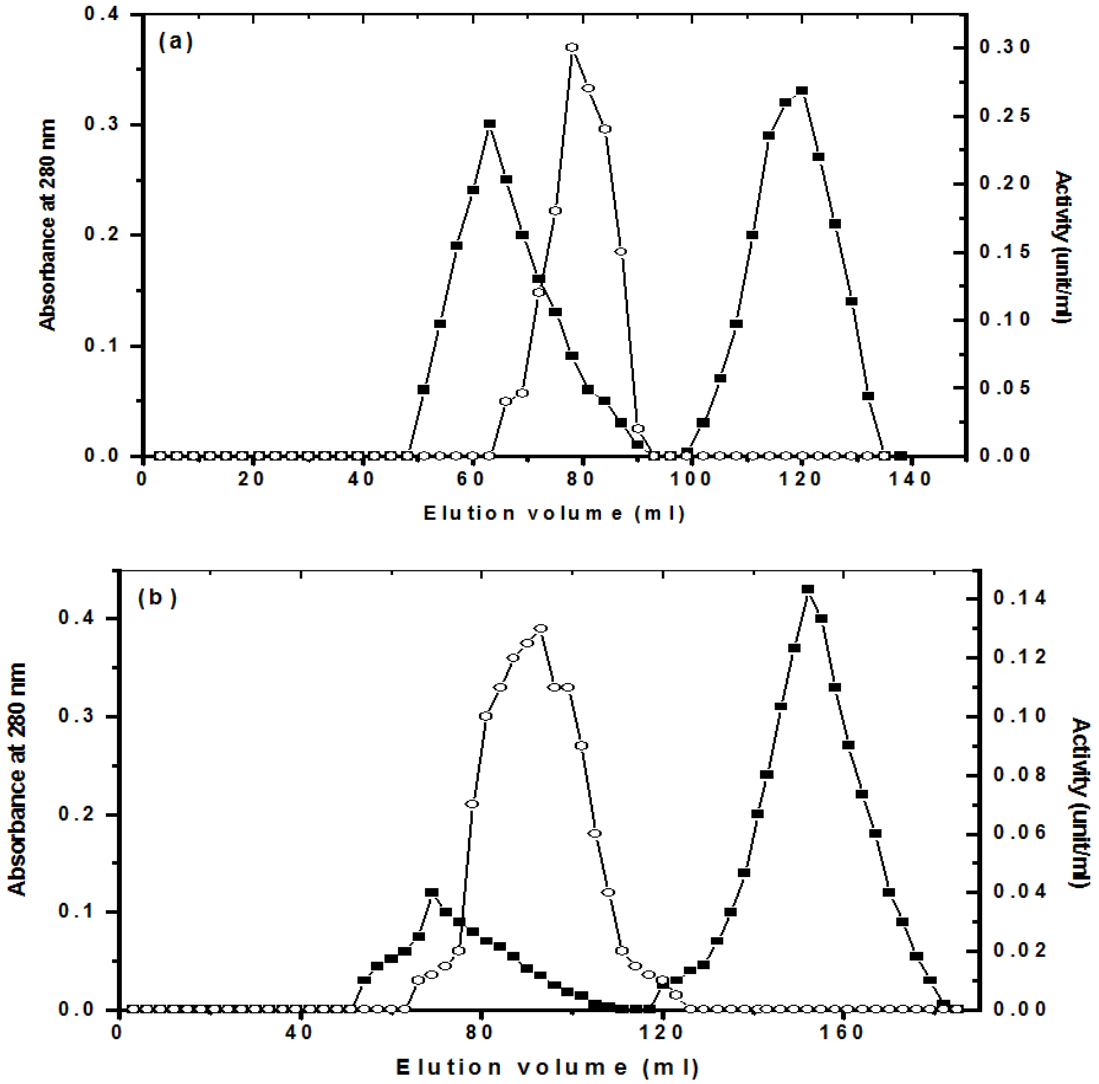


Fig. 2. *Typical elution profiles for the chromatography of T. alexandrinum of dormant GST2 (a) and germinated GST3 (b) seeds on Sephadex G-100 column at a flow rate of 20 ml/h and 3 ml fractions volume. Absorbance at 280 nm (▪), GST activity at 340 nm (o).*

Fig. 3. *Native PAGE of DEAE-Sepharose column fractions stained for GST activity using CDNB as substrate. Crude homogenate (1) and DEAE-Sepharose column fractions: GST1 (2), GST2 (3), and GST3 (4).*

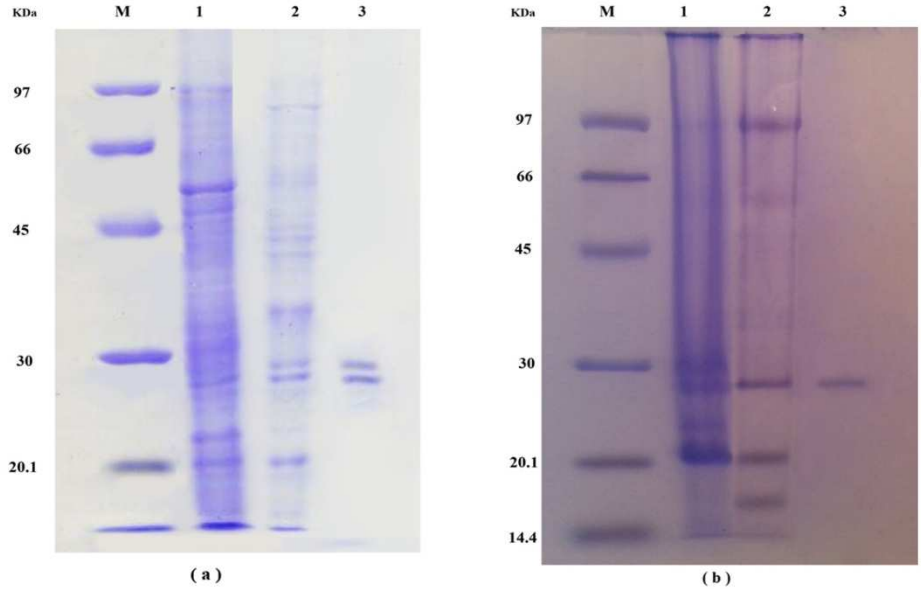


Fig. 4. *SDS-PAGE (12%) of dormant GST2 (a) and germinated GST3 (b) stained for protein by Coomassie brilliant blue R-250. Standard low molecular weight markers (M), crude homogenate (1), DEAE-Sepharose fraction (2), gel filtration fraction (3).*

Table 3. *Purification scheme of GST of dormant T. alexandrinum seeds.*

T. alexandrinum	**Activity (units)**	**Protein (mg)**	**Specific activity***	**Recovery (%)**	**Fold purification**
Dormant seeds	46	365	0.12	100	1
DEAE-Sepharose					
GST1	4.1	12.1	0.33	9	2.75
GST2	36	42	0.85	78	7.08
GST3	0.6	55	0.01	1	0.09
Sucrose concentration					
GST2	17	20	0.85	37	7.08
Gel filtration					
GST2	12	3.6	3.3	26	27.5

* Specific activity was expressed in μmole/min/ mg protein.

Table 4. *Purification scheme of GST from 6 days old germinated T. alexandrinum seeds*

T. alexandrinum	Activity (units)	Protein (mg)	Specific activity*	Recovery (%)	Fold purification
Germinated seeds	16.2	164	0.09	100	1
DEAE-Sepharose					
GST1	2.16	4.2	0.51	13.3	5.7
GST2	1.93	2.9	0.67	12	7. 4
GST3	2.63	29	0.09	16.2	1.0
Sucrose concentration					
GST3	2.5	10	0.25	15.6	2.9
Gel filtration					
GST3	0.85	1	0.85	5.32	10

* Specific activity was expressed in μmole/min/ mg protein.

3.4. Characterization of Purified Dormant and Germinated GST Isoenzymes

3.4.1. Effect of pH

The effect of pH on the activity of GST1 and GST2 isoenzymes of dormant seed as well as GST2 and GST3 of germinated seed of *T. alexandrinum* was examined. Dormant GST1 exhibited a sharp optimum pH at 7.0 while dormant GST2 exhibited a bell shape optimum pH between pH 7.0-7.5 (Fig. 5 a, b). On the other hand, the germinated GST2 and GST3 exhibited a sharp optimum pH at pH 8.0 (Fig. 5 c, d).

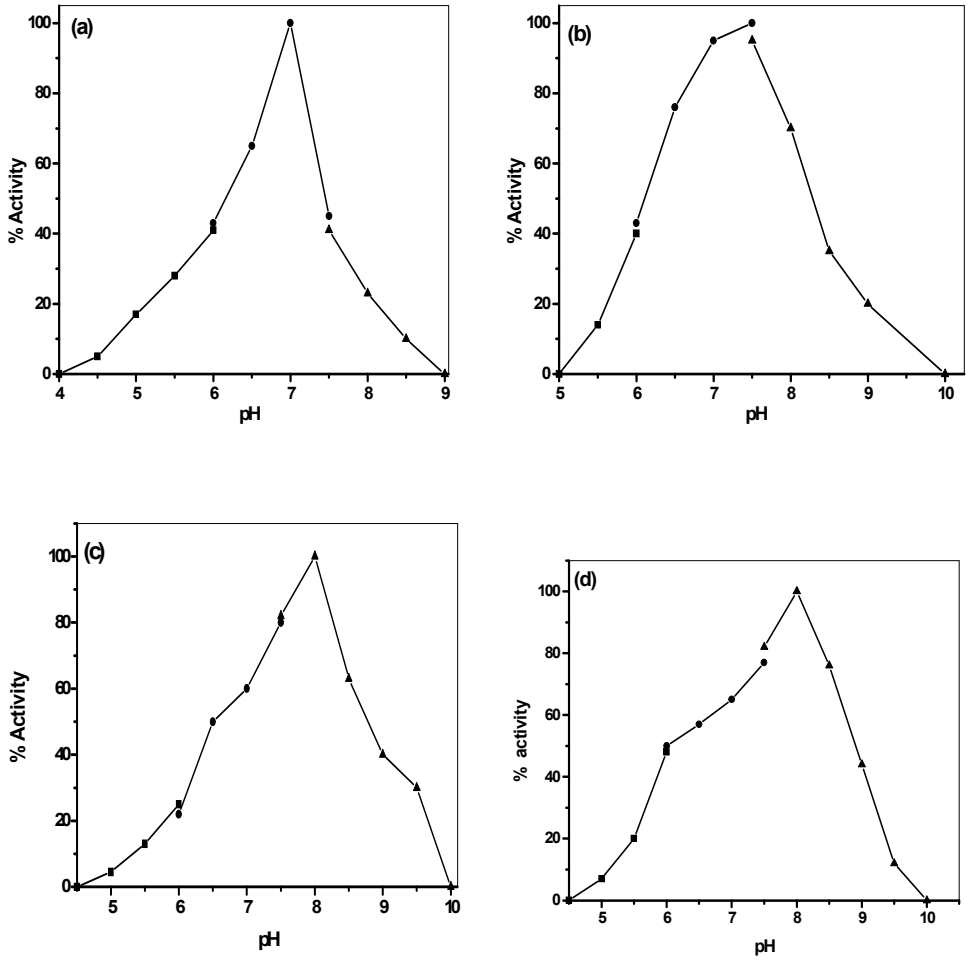


Fig. 5. *Effect of pH on T. alexandrinum dry seeds GST1 (a), GST2 (b) and germinated seeds GST2 (c), GST3 (d). The maximum activity was expressed as 100%. 0.1 M citrate phosphate buffer (▪), 0.1 M sodium phosphate buffer (•), and 0.1 M Tris-HCl buffer (▲).*

3.4.2. Substrate Selectivity

Germinated and dormant GST isoenzymes did not show any detectable activity toward NPB, EPNP, SO and DCNB with the exception of germinated GST3, GST activity could be detected on EA. Germinated GST3 exhibited high activity on EA (73%). The four isoenzymes exhibited peroxidatic activity toward CuOOH. While dormant GST2 and germinated GST3 exhibited high peroxidatic activity (74 and 76%) of the activity on CDNB, dormant GST1 and germinated GST2 exhibited 33 and 28% (Table 5).

Table 5. *GST activity towards different electrophilic substrates.*

Seeds	CDNB		CuOOH		EA	
	Specific activity*	%Relative activity	Specific activity*	%Relative activity	Specific activity*	%Relative activity
Dormant						
GST1	0.33±0.05	100	0.11±0.007	33	ND	ND
GST2	0.47±0.08	100	0.35±0.02	74	ND	ND
Germinated						
GST2	0.75±0.08	100	0.21±0.03	28	ND	ND
GST3	0.10±0.01	100	0.08±0.01	76	0.07±0.005	73

*Specific activity was expressed in µmol/min/mg protein
ND: not detected under the experimental conditions

3.4.3. Kinetic Parameters

The K_m values and V_{max} for dormant GST1 and GST2 and germinated GST2 and GST3 are summarized in Table 6. The K_m of CDNB for GST2 is almost the same for both germinated and dormant seeds, while K_m of CDNB for GST3 is almost double that of GST2 either germinated or dormant seeds (significant increase $p < 0.001$). K_m values for GSTs of dormant and germinated for GSH are comparable; the changes in the values are statistically insignificant ($P< 0.1$).

3.4.4. Inhibition Studies

Cibacron blue, sulfobromophthaliene, EA and hematin were tested for their ability to inhibit CDNB-conjugating activity of the purified *T. alexandrinum* dormant GST1, GST2, germinated GST2 and GST3 isoenzymes. IC_{50} values (Table 7) were determined by measuring the activity of the isoenzyme in the presence of varying concentrations of the inhibitor. The most potent inhibitor for dormant and germinated GSTs is cibacron blue followed by hematin. Dormant GST1 is the most sensitive one for cibacrone blue and hematin with IC_{50} of 0.23 and 0.65 µM, respectively while EA is the most potent inhibitor for germinated GST3 but had almost no effect on dormant seed GST1.

Table 6. *Kinetic parameters of T. alexandrinum dormant GST1, GST2, germinated GST2 and GST3.*

T. alexandrinum	GSH		CDNB	
	K_m (mM)	V_{max}*	K_m (mM)	V_{max}*
Dormant seeds				
GST1	1.17±0.39	0.06±0.009	0.65±0.095	0.05±0.003
GST2	1.22±0.13	0.23±0.009	0.57±0.11	0.32±0.026
Germinated seeds				
GST2	0.84±0.19	0.04±0.004	0.59±0.14	0.04±0.003
GST3	0.96±0.24	0.16±0.016	1.02±0.18	0.13±0.01

* V_{max} was expressed in µmol/min/mg protein.

Table 7. *Effect of inhibitors (IC_{50} values) on purified T. alexandrinum dormant (GST1, GST2) and germinated (GST2, GST3) seeds.*

Inhibitor	IC_{50} (µM)			
	Dormant seed		Germinated seed	
	GST1	GST2	GST2	GST3
Hematin	0.65	30	20	12
Cibacron blue	0.23	7	1	9
Sulfobromophthaliene	200	50	400	110
Ethacrynic acid	NI	100	74	0.60

NI: No inhibition under the experimental conditions.

4. Discussion

The germination process changed the total phenolic and flavonoid contents of legume seeds and their antioxidant capacity were also altered. Our results indicated that *T. alexandrinum* dormant seeds had phenolic contents of 64±7.5 mg/g dry seed and flavonoid content of 19.2±1.12 mg/g dry seed. The ratio of flavonoids to phenolics for the plants used in our study is less than 10% except for *T. alexandrinum* which has a higher ratio (30%). Among six *Trifolium* species examined by Koldziejczyk-Czepas *et al*. [29], it was found that the aerial parts of *T. alexandrinum* had the highest contents of phenolic (52.55 mg/g dry matter) and flavonoid (22.30 mg/g dry matter). Seeds of 57 species of the genus *Trifolium* have been studied for the occurrence and concentration of flavonoids [30]. All but three tested species contained flavonoids. flavonoid contents in *Trifolium* species ranged from 0.04 to 8.16 mg/g dry matter. The majority of species contained quercetin as a sole flavonoid or in the mixture with a number of unidentified flavonoid components. The concentration of quercetin was in the range 0.05-3 mg/g dry matter in the *Trifolium* species [31].

Trifolium incarnatum extract was particularly rich in flavonoids (41.54 mg/g dry matter), but *T. alexandrinum*

contained isoflavones (18.97 mg/g dry matter) and significant amounts (9.63 mg/g dry matter) of clovamides. The interaction between phenolic compounds and DPPH depends on their structural conformation. Phenolics with a high number of hydroxyl groups can react very rapidly and reduce a large number of DPPH molecules [32]. The observed antioxidant effect of *Trifolium* extracts may be attributed to scavenging of both peroxynitrite and radicals derived from its decomposition [29].

It was found that the total phenolic and flavonoid contents of mature legume seeds significantly decreased after short-term germination. However, their antioxidant capacity increased after long-term germination [33]. In the present study, total phenolic and flavonoid contents were detected by germination period. The concentration of flavonoids was significantly changed after germination and influenced by the varieties and stages of germination. The contents and compositions of bioactive compounds varied greatly between types of legumes and their varieties, due to genetic characteristics. Also the compositions of flavonoids differed in each germination stage of *T. alexandrinum* and the degree of seed germination. This observation is analogous with the results obtained by Lin and Lai [33] but in difference with data obtained by Cevallos-Casals and Cisneros-Zevallos [34] for edible seed species.

Regarding the antioxidant capacity, the germination process modifies the antioxidant activity, measured by its free radical scavenging capacity of *T. alexandrinum* seeds. After a germination of 2, 4, 6 days, seeds show higher antioxidant capacity than dormant seeds. Time may affect the antioxidant activity, as they reach the highest values of antioxidant activity after 6 days of germination. This observation was found to be analogous to peas, beans and lentils [12].

The main detoxifying systems involve enzymes, mainly SOD, CAT and enzymes of the ascorbate glutathione cycle, the antioxidant compounds such as reduced glutathione and ascorbate [35]. However, the efficiency of active oxygen species scavenging through antioxidant enzyme activities, mainly CAT, has been increased during seed development [36]. On the other hand, ascorbate peroxidase (APX) and GR activities decreased during desiccation of *Triticum durum* seeds [37].

In our study, the dormant seeds exhibited the highest GST activity as compared to the germination stages and this may be due to the biochemical metabolism changes of the seeds during germination, which might produce some secondary plant metabolites such as anthocyanins and flavonoids or release aglycones from conjugated glycosides from seed coats and cotyledons due to the enzymatic activation [38]. These metabolites may inhibit GST activity during germination stages. On the other hand, GR, CAT, SOD and GPx increase during germination stages as compared to dormant seeds.

The DEAE-Sepharose profile for GST activity changed by germination. Although the isoenzyme pattern is almost comparable, where three isoenzyme peaks were observed, they changed in amounts. The GST2 is the major in dormant seeds (78% of the total activity) while the germinated GST3 was increased from 1 to 16% of the total activity by germination. Five GSTs isoenzymes were separated and designated GST1, GST2, GST3, GST4 and GST5 according to their elution order from onion bulb [39].

Our results indicated that germinated GST3 was expressed as homodimer (M.wt. 27 KDa) while the presence of two subunits with different molecular weight may suggest that dormant GST2 is either a heterodimer (M.wt. 27.5 and 28.5 KDa) or the presence of more than one protein having slightly different molecular weights. These results are in agreement with the molecular weights reported by Gronwald and Plaisance [40] for sorghum GST in which GST isoenzymes A1/A1 and B1/B2 were purified from sorghum shoots. GST A1/A1, a constitutively expressed as homodimer, had a molecular mass of 26 KDa, GST B1/B2 was a heterodimer with subunit molecular masses of 26 KDa for B1 and 28 KDa for B2. Also four GSTs were identified in *Festuca arundinacea* in which GST1 was purified until homogeneity was determined to be a heterodimer consisting of two subunits of 28.0 and 27.2 KDa [41].

In the present investigation, the kinetic studies of the purified *T. alexandrinum* GSTs exhibited typical Michaelis-Menten equation with respect to GSH as substrate. The K_m^{GSH} values are ranged from 0.84±0.19 to 1.22±0.13 mM which was in general agreement with published K_m^{GSH} values of approximately 0.34-0.84 mM for the *Oryza sativa* GSTF5 [42]. In contrast, The K_m^{CDNB} values of *T. alexandrinum* GST are ranged from 0.57±0.11 to 1.02±0.18 mM. These values are similar to K_m^{CDNB} values reported for the GST of *Oryza sativa* which calculated to be 0.55 mM [42], *Lactuca sativa* which calculated to be 1.42 mM [43]. It can be concluded from our results that K_m^{CDNB} for GST2 is almost the same for both germinated and dormant seeds; while K_m^{CDNB} for GST3 is almost double of GST2 either germinated or dormant seeds ($p< 0.0001$). This indicates that the affinity of GST2 to CDNB of either germinated or dormant is higher than germinated GST3.

The activity of *T. alexandrinum* GSTs toward CDNB was significantly lower than the enzymes from corn and *Lactuca sativa* which has a value of 4.03±0.23 μmol/min/mg protein [43]. Also they showed selenium-independent GPx activity toward CuOOH as an alternative substrate with specific activity ranged from 0.076±0.01 to 0.35±0.02 μmol/min/mg protein. The theta-class GSTs purified from *Arabidopsis thaliana* also exhibited selenium-independent GPx activity of 1.32±0.01 μmol/min/mg protein [43]. In the present investigation, GST activity in dormant seeds is significantly higher than that of the 6 days GST, while no GSH peroxidatic activity could be detected. This may suggest that GST2 of dormant seeds is mainly responsible for the GSH peroxidatic activity. However GST activity significantly dropped by germination and GST3 activity increased having high peroxidatic activity beside that GSH peroxidase significantly increased. This role of GST is represented by its function as

glutathione-dependent peroxidase catalyzing the reduction of oxidative stress products, such as organic hydroperoxides [3]. Beside that the presence of GSTs having peroxidatic activity may have a role in the tolerance to herbicides by effectively scavenging toxic byproducts of herbicide activity and to the protection of the cell from lipid peroxidation [44, 45]. In opposite to other isoenzymes under this investigation, inated seed GST3 exhibited enzymatic activity towards EA with specific activity 0.073±0.005 µmol/min/mg protein. However, all *T. alexandrinum* GST isoenzymes are not significantly active towards DCNB, NPB, SO and EPNP.

In this study, the most potent inhibitor for dormant and germinated GSTs is cibacron blue followed by hematin while EA is the most potent inhibitor for germinated GST3 with IC_{50} of 0.60 µM but had almost no effect on dormant seed GST1. The present data is analogous to the results reported by Gyamfi *et al*. [46] for *Thonningia sanguinea* in which the effect of EA is very strong with IC_{50} of 0.83±0.04 µM and differs from that reported by Jo *et al*. [47] for *Oryza sativa* in which the effect of EA is week with IC_{50} of 268±19 µM. The lower IC_{50} value for EA indicates a higher affinity of *T. alexandrinum* germinated GST3 for electrophilic substrate which is similar to IC_{50} of 0.28±0.04 µM of *Lactuca sativa* reported by Park *et al*. [43]. On the other hand, sulphobromophthaliene was less potent inhibitor among the substances tested with IC_{50} ranged from 50 to 400 µM for both dormant and germinated GSTs.

5. Conclusion

The *T. alexandrinum* was chosen for GST purification and characterization due to its highest GST activity and antioxidant capacity, beside its economical importance. This study is the first report on the purification and characterization of GSTs from *T. alexandrinum* seeds. The increase in GST3 with different properties from GST2 may consider with the active metabolism that increased by germination and to compensate the increase of ROS and the decrease in phenolics and flavonoids. GR, CAT, SOD and GPx increase during germination stages as compared to dormant seeds. It was shown that GST2 is the major enzyme of the dormant seeds while GST3 was increased in 6 days germinated seeds, which were kinetically different.

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