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Characteristics of Thiol:Protein Disulfide Oxidoreductase from Wheat (*Triticum aestivum* L.) Grain

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Abstract—Biochemical properties of a homogenous preparation of thiol:protein disulfide oxidoreductase (TPDO, EC 1.8.4.2) isolated for the first time from mature wheat (*Triticum aestivum* L.) grain were studied. According to polyacrylamide gel electrophoresis data, the molecular weight of TPDO is around 167 kD, the enzyme consisting of two subunits of 77 and 73 kD, which differentiates TPDO from known enzymes of SH/SS-metabolism of wheat caryopses. In substrate specificity and enzymatic characteristics (pH and temperature optima) TPDO is similar to analogous enzymes of animal tissues. Inhibition of disulfide reductase activity by alkylating agents and heavy metal ions suggests the participation of active center SH-groups in the catalytic act and classes the enzyme as a member of the thioredoxin superfamily. The SS-reductase reduces aggregating capacity of acetic acid-soluble fraction of wheat storage proteins. The proposed physiological role of TPDO is participation in creation and regulation of SH/SS-status of wheat endosperm proteins and formation of the rheological properties of gluten.

Key words: thiol:protein disulfide oxidoreductase, molecular mass, substrate specificity, inhibition, enzymatic properties, wheat storage proteins, aggregation

Wheat storage proteins are known to form on hydration a viscoelastic matrix, gluten, which determines the rheological properties of dough and the quality of wheat flour in general. The traditional classification divided gluten proteins into two groups—gliadins and glutenins. Gliadins are alcohol-soluble proteins consisting of a number of individual polypeptides with molecular mass of about 30-60 kD, most having intramolecular disulfide bonds. Wheat glutenin is a macromolecular complex of many heterogeneous polypeptides associated with each other via noncovalent interactions and intermolecular disulfide bonds. The molecular masses of glutenins range between 20-80 kD and higher [1, 2]. Glutenin subunits are essential to high baking quality of wheat flour [3]. They contain a large number of free SH-groups capable of forming intermolecular disulfide bonds stabilizing gluten protein complex [4]. The balance of SH/SS-bonds in wheat glutenins to a significant extent determines the rheological properties of gluten [5].

Recent investigations have shown that coagulation and association of wheat storage proteins occur with the participation of molecular chaperons and enzymes [6, 7], with an important role assigned to enzymes of SH/SS-metabolism. Thus, in *in vitro* tests protein disulfide isomerase significantly accelerated formation of disulfide bonds in γ-gliadins [8]. Shimoni et al. extracted protein disulfide isomerase (PDI) from endosperm of developing wheat caryopsis [9]. Increase in PDI expression during maximal synthesis of gluten proteins, between 14 and 28 days after blooming, may point to the participation of this enzyme in formation of storage protein structure [9, 10]. It has been shown that under physiological conditions the system NADPH/thioredoxin h/thioredoxin reductase catalyzes *in vitro* dissociation of disulfide bonds in wheat gliadins [11]. The balance between the activities of enzymes catalyzing reactions of thiol-disulfide exchange may affect SH/SS status of wheat storage proteins [1, 7].

We were the first to isolate a GSH-dependent enzyme thiol:protein disulfide oxidoreductase (TPDO, EC 1.8.4.2) from spring wheat *Triticum aestivum* L. caryopses. It is similar to insulin transhydrogenase of animal tissues catalyzing dissociation of protein disulfides [12]. Study of this enzyme is of interest from the point of view of its possible participation in the control of SH/SS status

Abbreviations: TPDO) disulfide reductase, thiol:protein disulfide oxidoreductase; DTNB) Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid); PDI) protein disulfide isomerase; NEM) N-ethylmaleimide; PCMB) *p*-chloromercuribenzoate.

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of storage proteins and their association in the protein complex of gluten [13].

MATERIALS AND METHODS

Enzyme extraction. TPDO was extracted and purified from coarsely ground flour of Siberian selection Tulunskaya 12 according to the previously described method [12]. Protein fractions with TPDO activity not containing activities of other enzymes of SH-metabolism (glutathione reductase, lipoxygenase) were lyophilized in an Inei apparatus (Biopribor, Russia) and used for further studies.

Determination of activity. TPDO activity was determined on the basis of the rate of SH-group formation in reaction mixture aliquots withdrawn at selected time intervals at 37°C. In the typical case in 0.1 M Tris-HCl buffer + 1 mM EDTA, pH 7.5, we introduced insulin (4 mg/ml) (Endocrine Preparations Plant, Kaunas, Lithuania) or BSA (19 mg/ml) (ICN, USA) as substrates, purified enzyme (20-30 μg), and the cofactor GSH (1 mM) (Reanal, Hungary).

Proteins of the acetic acid-soluble fraction of Tulunskaya 12 variety spring wheat extracted following standard methods [14] were used as substrate. Lyophilized gluten proteins (50 mg) were dissolved in 5 ml of 0.1 M Tris-HCl buffer, pH 7.5, containing 1 mM of EDTA and 10% sodium salicylate for 30 min. Soluble proteins were separated by centrifuging at 8000*g* for 10 min and added to the incubation medium at the concentration 5 mg/ml. To precipitate protein, aliquots were put in acidulous acetone and then centrifuged at 8000*g* for 20 min. The precipitates were twice washed with acidulous acetone and dissolved in 3 ml 0.1 M Tris-HCl buffer, pH 8.0, containing 1% SDS and 0.27% 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) (Sigma, USA). After 40 min, the optical density was measured at 412 nm [15]. Increase in SH-group concentration per minute (μmol/min) in the incubation medium was taken as an activity unit. Specific activity was expresses in activity units (U) per mg enzyme protein, where $U = \Delta SH/t$. Protein concentration was determined by the Lowry method [16].

Determination of molecular mass of TPDO. To determine molecular mass of the native enzyme, we used non-denaturing electrophoresis in the presence of SDS [17]. TPDO proteins reduced by 5% β-mercaptoethanol (5 min in boiling water bath) were analyzed by electrophoresis according to Laemmli [18]. In case of low protein concentration in the samples, gels were stained with silver nitrate (ICN) according to a modified method [19]. For this purpose, the gel (earlier stained with Coomassie R-250 and washed with 7% acetic acid) was washed with 10% ethanol for 30 min, then with distilled water (2 min) and placed in a solution of 0.1% $AgNO_3$ with incubation for 30 min. After incubation, the gel was washed with distilled water for 10-15 sec and placed in developing solution containing 3% Na_2CO_3 and 0.02% formaldehyde. Staining of protein bands was monitored visually, and the reaction was stopped in 1% acetic acid. Reagents produced by Sigma were used for electrophoresis. Protein markers (Sigma) were used to calculate molecular mass.

Enzymatic characteristics. Michaelis constant was determined using the Lineweaver–Burk method [20] for two substrates—insulin and BSA; pH and temperature optima of TPDO were determined with BSA (40 mg/ml) as the substrate.

Effect of inhibitors. N-Ethylmaleimide (NEM, 0.5 and 1.0 mM, Sigma), 0.5 and 1.0 mM *p*-chloromercuribenzoate (PCMB) (Sigma), and 0.5 and 1.0 mM solutions of $ZnSO_4$ and $CuSO_4$ (Reakhim, Russia) were tested as inhibitors. The enzyme was subjected to preliminary incubation with the inhibitors for 30 min at 30°C. Then we determined remaining TPDO activity with BSA (40 mg/ml) as the substrate according to the standard method. TPDO activity in the control was 0.1 μmol SH/min per mg protein.

Effect of TPDO on the aggregation of gluten proteins. The acetic acid-soluble gluten fraction was extracted from Tulunskaya 12 variety wheat following the standard method [14]. Gluten from defatted flour was first washed with 0.1 M solution of sodium pyrophosphate, pH 7.0, then with water. Raw gluten was dissolved in 0.1 M acetic acid and lyophilized. Aggregation parameters were determined according to published methods [21, 22]. In the control, we mixed equal volumes of gluten proteins solution in 0.1 M acetic acid (final protein concentration 0.01%) and 0.2 M sodium phosphate buffer, pH 5.6, containing 0.5 M sodium chloride and 1 mM GSH. In the experimental sample, we added TPDO (3-5 U).

Optical density due to aggregation versus time was monitored by spectrophotometry. Aggregation index τ_{10}/C (solution turbidity per protein concentration unit in 10 min) was calculated using the formula $\tau_{10} = 2A/C$, where A is optical density at $\lambda = 350$ nm and C is protein concentration, %.

The results were subjected to statistical analysis using the Microsoft Excel program and average values ± standard error are given.

RESULTS AND DISCUSSION

Molecular mass of TPDO. Analysis of the fractions with disulfide-reductase activity by non-denaturing SDS-PAGE in 7% gels showed electrophoretic homogeneity of native protein with molecular mass about 167 kD (Fig. 1). Treatment of native disulfide reductase with 5% β-mercaptoethanol in the presence of 2% SDS its dissociated into two subunits with molecular masses of about 73 and 77 kD (Fig. 2). Molecular masses of earlier described

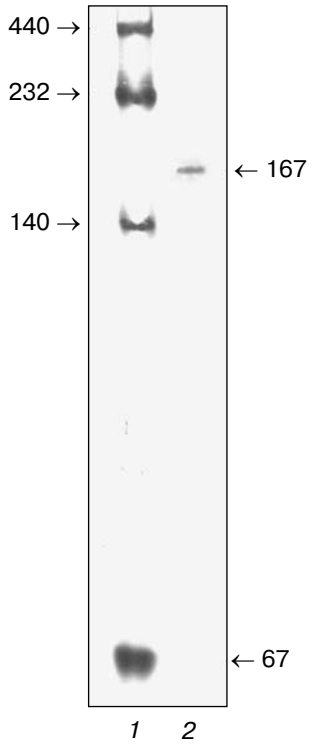

Fig. 1. Non-denaturing SDS-PAGE of wheat TPDO in 7% gel: *1*) markers (ferritin, 440 kD; catalase, 223 kD; lactate dehydrogenase, 140 kD; BSA, 67 kD); *2*) native TPDO.

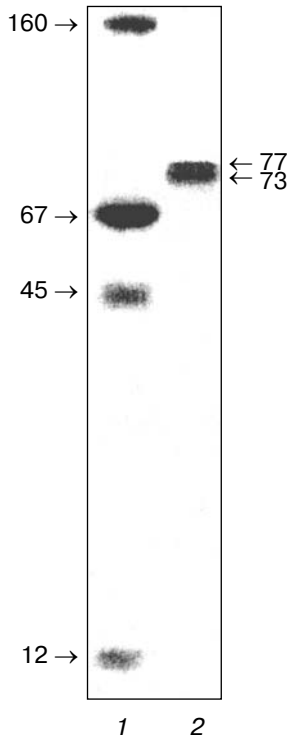

Fig. 2. Laemmli SDS-PAGE of wheat TPDO in 10% gel: *1*) markers (γ-globulin, 160 kD; BSA, 67 kD; ovalbumin, 45 kD; cytochrome *c*, 12 kD); *2*) TPDO reduced by β-mercaptoethanol.

GSH-dependent protein disulfide reductases from animals and microorganisms vary from 50 to 120 kD [23-25]. Enzymes with high molecular mass are usually dimers. For example, 60 kD TPDO from bovine liver dimerized under long-term freezing or heating to 60°C due to formation of intermolecular S–S-bonds [23]. TPDO extracted from wheat caryopses had higher molecular mass of both native protein and individual subunits as compared to the previously described GSH-dependent protein disulfide reductases of animals. However, TPDO proved similar to them in terms of the ability of the subunits to dimerized due to formation of intermolecular disulfide bonds.

The enzyme extracted by us differed from the known wheat caryopsis enzymatic systems of SH/SS metabolism. The thioredoxin system consists of NADPH, the flavoprotein thioredoxin reductase (EC 1.6.4.2) (the molecular weight of which in wheat caryopses according to different authors is 35 and 65 kD [11, 26]), and low molecular weight protein thioredoxin h: 13.5 kD in soft wheat (*T. aestivum* L.) and 13.8 kD in hard wheat (*T. durum* L.) [27]. The glutaredoxin system consists of NADPH, flavoprotein glutathione reductase (EC 1.6.4.2), oxidized glutathione, and glutaredoxin (EC 2.5.1.18) with molecular weight of about 50 kD (*T. aestivum* L.) [28]. Glutathione reductase [29] and NADPH-dependent protein disulfide isomerase (EC 5.3.4.1) purified by Shimoni et al. [9] from maturing wheat caryopses had molecular mass of 60 kD.

Substrate specificity. Substrate specificity was determined for standard substrates insulin having three S–S bonds and BSA having 17 S–S bonds [30], and also for gluten proteins dissolved in 0.1 M Tris-HCl, pH 7.5, containing 1 mM EDTA and 10% sodium salicylate.

Figure 3 presents the rate of S–S bond formation as a function of substrate concentration. With 1 mM GSH in the incubation medium, the K_m for insulin was 2.18 ± 0.2 μM, which is 5-10 times less than K_m values for insulin of the analogous enzymes of animal tissues [31]. Enzyme activity is maximal for insulin concentration 3.1 μM (Fig. 3a). For BSA under the same conditions the K_m was 570 ± 15.7 μM. Maximal TPDO activity was found at 700 μM BSA (Fig. 3b).

The rather high affinity to insulin to wheat TPDO is similar to that of the protein disulfide reductases from animals [23-25]. In incubation medium consisting of gluten proteins, TPDO, and GSH, the content of SH-groups during 10 min of incubation increased from 60.1 ± 2.0 to 73.9 ± 1.5 μmol. At the same time, the content of SH-groups in the control remained practically unchanged, 29.1 ± 1.8 μmol. The reaction rate was 1.65 μmol SH per min, and the specific activity was

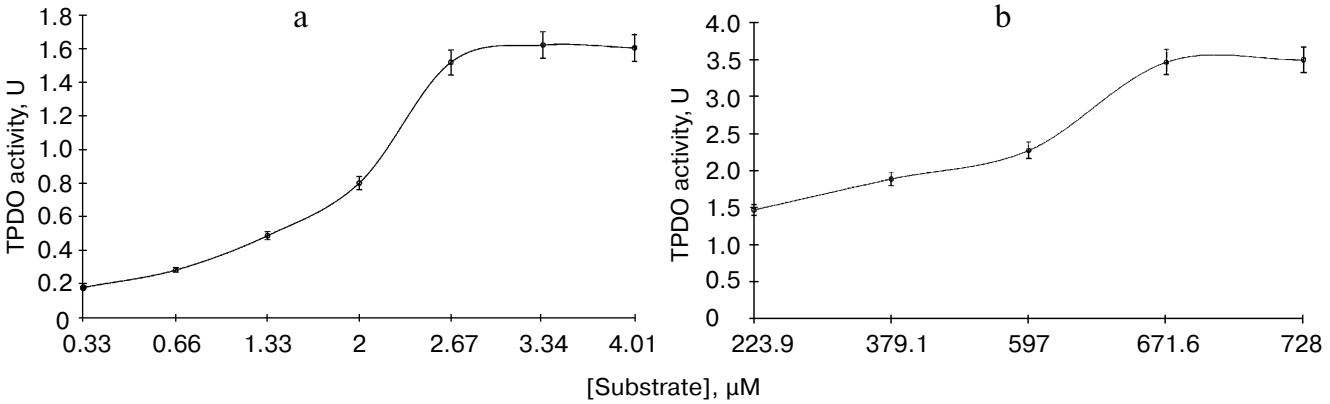

Fig. 3. Wheat TPDO activity ($\Delta SH/t$) as a function of substrate concentration: a) insulin; b) BSA. Average values of three independent experiments are presented.

46.7 μmol/min per mg protein. This is consistent with our data on the action of partially purified TPDO extract on acetic acid-soluble gluten proteins [12].

pH and temperature dependences of disulfide reductase activity. The dependence of TPDO activity on temperature is shown in Fig. 4a. The temperature optimum for activity is around 33-38°C, with maximal activity at 36°C. NADP-dependent wheat thioredoxin reductase has temperature optimum of 25°C [26], and the enzymes extracted from animal tissues had similar temperature optimum, 37°C [33, 34].

The dependence of disulfide reductase activity on pH was determined in 0.1 M Tris-HCl buffer + 1 mM EDTA, with pH values in the interval pH 5-8 at 36°C. GSH (1 mM) was used as the cofactor. Figure 4b shows that wheat TPDO has pH optimum in the interval 6.5-7.5 with maximal activity at pH 7.0. At the extreme pH values studied (5.0 and 8.0), about 20% of maximal TPDO activity was retained.

The pH optimum values for wheat enzyme in general are similar to the values for disulfide reductases of animal tissues [23, 34]; only some researchers report more alkaline values of pH optimum, 7.5-8.5 [24, 32]. Thus, with respect to pH and temperature optima of activity, wheat disulfide reductase is similar to the analogous enzymes from animal tissues.

Effect of inhibitors. For inhibition analysis, we used specific reagents for SH-groups. Bivalent copper and zinc ions linking SH-groups with mercaptide formation caused 100% inhibition of SS-reductase activity. Alkylating agents, NEM and PCMB, also inhibit SS-reductase activity, with NEM suppressing its activity to a higher extent, which may be accounted for by different accessibility of relevant SH-groups of this enzyme for these compounds (table).

The inhibition analysis suggests that wheat caryopsis TPDO as well as PDI is a representative of the thioredoxin superfamily, which has two cysteine residues in a

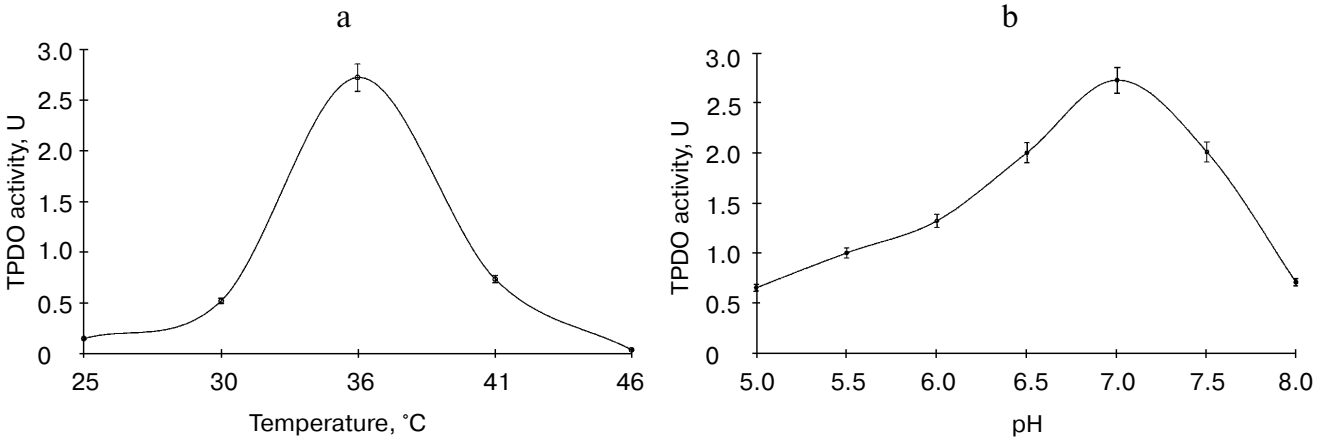

Fig. 4. Wheat TPDO activity as a function of temperature (a) and pH (b). Average values of three independent experiments are presented.

Effect of inhibitors on the activity of thiol:protein disulfide oxidoreductase from wheat caryopses (average values of three independent experiments are presented; the standard deviation is less than 10%)

Inhibitor	Concentration, mM	Inhibition, %
$CuSO_4$	0.5	100
	1.0	100
$ZnSO_4$	0.5	100
	1.0	100
NEM	0.5	70
	1.0	100
PCMB	0.5	20
	1.0	53

thioredoxin-like domain, -Cys-Gly-His-Cys-, in the active center [35].

Effect of wheat TPDO on gluten protein aggregation. The ability of storage proteins to aggregate is one of the important parameters characterizing rheological properties of dough and gluten quality [21, 22]. To study the effect of TPDO on wheat storage protein aggregation, we used the acetic acid-soluble fraction of these proteins consisting largely of gliadin and partially of glutenin subunits with admixture of polypeptides of albumin-globulin nature [1].

As stated above, TPDO is a glutathione-dependent enzyme, so in the course of experiments 1 mM GSH was introduced into both experimental and control samples. Figure 5a presents the results on aggregation tests conducted at pH 5.6 according to standard methods [21]. Protein aggregating ability in the presence of TPDO is reduced compared to the control. The difference in the aggregation value τ_{10}/C between control and experiment on average was from 15 to 22%. Thus, aggregation parameter τ_{10}/C (for 10 min) in control was 134.1 ± 4.6 and in the experimental sample was 114.6 ± 1.2. In the course of further experiments (before termination of the aggregation) during 25 min, aggregation index was 143.6 ± 5.1 in the control and 119.4 ± 1.2 in the experimental sample. In an experiment conducted at pH 7.5, optimal for TPDO activity (Fig. 5b), aggregation parameter τ_{10}/C was reduced even more considerably with enzyme addition, by 25-30%. Reduction of aggregation parameters with TPDO addition is apparently due to dissociation of proteins S–S bonds and consequent weakening of gluten matrix rigidity.

Unlike PDI, which catalyzes isomerization of proteins S–S bonds, TPDO catalyzes reduction of S–S bonds. The system NADPH/thioredoxin h/thioredoxin reductase also reduces S–S bonds of storage proteins, though the functional role of thioredoxin h in wheat caryopses is largely attributed to mobilization of storage proteins in the course of germination [11].

Comparing the aggregating capacities of acetic acid-soluble gluten proteins from various wheat varieties, it was found that physical properties of gluten correlate with the intensity and the nature of aggregation, the aggregation parameters of storage proteins from strong wheat varieties being significantly higher than those from weak varieties [22]. The direct effects of TPDO on gluten proteins presented in this paper as well as the coincidence in maturing wheat caryopsis (at the phase of late milky ripeness) of disulfide reductase activity maximum with period of intense formation of protein structures characteristic for gluten earlier established by us suggests that *in vivo* the enzyme is involved in the regulation of SS/SH-balance in the endosperm protein in the post-synthetic period [36]. The balance of PDI and TPDO activities apparently has

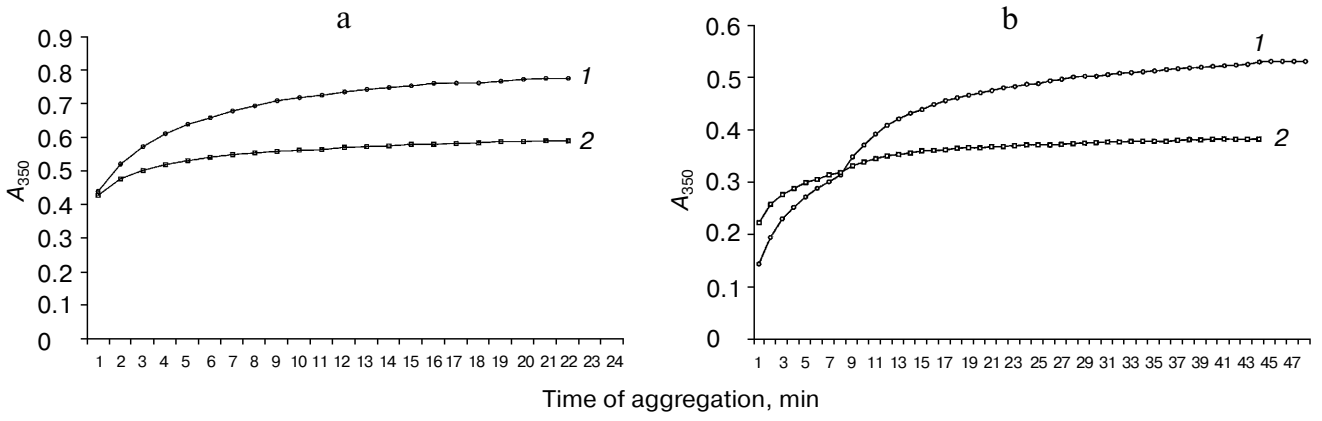

Fig. 5. Effect of wheat TPDO on aggregation of acetic acid-soluble gluten proteins: a) pH 5.6; b) pH 7.5. *1*) Control (without TPDO); *2*) experiment (+ TPDO). Average values of three independent experiments are presented; the error of experiments did not exceed 5%.

a direct impact on the formation of gluten structural matrix.

Sucrose density gradient centrifugation of homogenate from wheat caryopses of milk and wax-milk ripeness demonstrated that TPDO, unlike PDI, is localized not only in microsomal and proteins body fractions, but also in the soluble cytoplasmic protein and vacuole fractions [37]; hence, TPDO has wider distribution in the cell than PDI [9]. Wheat TPDO, along with PDI, thioredoxin h, and thioredoxin reductase, may participate in the regulation of cell oxidation-reduction potential and activation of other enzymes via maintenance of the required proportion of S–S bonds and SH-groups.

The results presented in this paper characterize differences of TPDO from known plant enzymes of SH/SS-metabolism in terms of molecular mass and functional features. The enzyme is similar to GSH-dependent protein disulfide oxidoreductases from animals in the nature of the catalyzed reaction and some enzymatic characteristics (pH and temperature optima) [23-25, 33, 34]. It will become possible to arrive at more exact conclusions after determination of the primary structure of this enzyme.

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