Wheat (*Triticum aestivum*) storage proteins fold and assemble into complexes that are linked by intra- and intermolecular disulfide bonds, but it is not yet clear whether these processes are spontaneous or require the assistance of endoplasmic reticulum (ER)-resident enzymes and molecular chaperones. Aiming to unravel these processes, we have purified and characterized the enzyme protein disulfide isomerase (PDI) from wheat endosperm, as well as studied its developmental expression and intracellular localization. This ER-resident enzyme was previously shown to be involved in the formation of disulfide bonds in secretory proteins. Wheat PDI appears as a 60-kD glycoprotein and is among the most abundant proteins within the ER of developing grains. PDI is notably up-regulated in developing endosperm in comparison to embryos, leaves, and roots. In addition, the increase in PDI expression in grains appears at relatively early stages of development, preceding the onset of storage protein accumulation by several days. Subcellular localization analysis and immunogold labeling of electron micrographs showed that PDI is not only present in the lumen of the ER but is also co-localized with the storage proteins in the dense protein bodies. These observations are consistent with the hypothesis that PDI is involved in the assembly of wheat storage proteins within the ER.

It is now well documented that the initial folding of secretory proteins within the ER is not spontaneous but is assisted by a variety of molecular chaperones as well as enzymes that catalyze these processes (Gething and Sambrook, 1992). One of these ER-localized enzymes, PDI, was first described about 30 years ago (Freedman et al., 1994). PDI was initially suggested to be involved in the formation and isomerization of disulfide bonds in nascent secretory proteins (Freedman et al., 1994). Later, this enzyme was shown to be a multifunctional protein, assisting a variety of protein maturation process within the ER (Freedman et al., 1994). In addition to its primary role in disulfide bond formation, PDI is also (a) the β-subunit of prolly 4-hydroxylase that adds hydroxyl groups to some secretory proteins (Pihlajaniemi et al., 1987) and (b) a component of the triglyceride transfer complex that catalyzes the incorporation of lipids into nascent core lipoproteins (Wetterau et al., 1990, 1991). Several additional functions were also attributed to PDI, but these are still questionable (Freedman et al., 1994).

Genes and cDNAs encoding PDI have been cloned from a large number of animal species (Freedman et al., 1994, and refs. therein) and recently also from several plant species (Shorrough and Dixon, 1991; Shimonri et al., 1995; GenBank accession Nos. L32520 and D23769). Analysis of these clones showed that PDI contains a C-terminal tetra amino acid sequence, K/HDEL, that apparently functions in its retention within the ER. In addition, both animal and plant PDI sequences were shown to contain two highly conserved, thioredoxin-like, active sites (Freedman et al., 1994).

PDI has been extensively studied both structurally and functionally in a variety of animal species (Freedman et al., 1994). Still, very little is known about the characteristics of plant PDIs, the regulation of their expression during plant development, and their involvement in the folding of plant secretory proteins. Plants contain a variety of vacuole-resident proteins that contain disulfide bonds whose folding is apparently assisted by PDI. These include storage proteins, hydrolases, proteases, and α-amylase inhibitors, as well as enzymes involved in plant defense (Herman, 1993).

The role of PDI in folding of plant proteins has so far been studied in wheat (*Triticum aestivum*) sulfur-rich gliadins as a model system (Bulleid and Freedman, 1988). These storage proteins contain six to eight Cys residues that are located in the C-terminal region and are apparently linked by three to four intramolecular disulfide bonds (Shewry and Tatham, 1990). Bulleid and Freedman (1988) suggested that formation of intrachain disulfide bonds within the gliadins is assisted by PDI. In that study, a wheat γ-type gliadin, translocated in vitro into canine microsomes that were previously depleted of luminal soluble proteins, showed very inefficient acquisition of intramolecular disulfide bonds. Supplementation of the depleted microsomes with purified PDI significantly increased the efficiency of disulfide bond formation in this gliadin (Bulleid and Freedman, 1988).

Detailed analyses of the role of PDI in the assembly of the wheat gliadins as well as other plant vacuolar proteins

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requires a detailed characterization of PDI as well as the development of in vitro systems in which purified PDI can be mixed with purified target proteins. In the present report we describe the purification of PDI from wheat endosperm to homogeneity, as well as the characterization, developmental expression, and intracellular localization of this protein.

MATERIALS AND METHODS

Tissue Homogenization and Preparation of Enriched Rough Microsomes

Enriched wheat rough microsomes were prepared essentially as described previously (Walter and Blobel, 1983). Developing grains (about 50 g) at 18 DAA or young wheat leaves were frozen in liquid N₂ and placed into a chilled mortar on ice and ground with a pestle in 200 mL of 0.25 M Suc, 100 mM Tris-HCl, pH 8.0, 100 mM KCl, 50 mM magnesium acetate, 1 mM EDTA, 1 mM PMSF (buffer A). The resulting homogenate was filtered through four layers of cheesecloth. The homogenate was then centrifuged for 10 min at 1,000g and the supernatant was recentrifuged for 10 min at 10,000g. Enriched rough microsomes were collected by ultracentrifugation of the 10,000g supernatant for 2.5 h at 140,000g (Beckman Ti60 rotor at 45,000 rpm) through a cushion of 1.3 M Suc in buffer A as previously described (Walter and Blobel, 1983). The microsomal membrane pellets were either used immediately or stored at -80°C.

For extraction of Triton X-100-soluble proteins, tissue was homogenized in 10 mM Hepes, pH 7.5, 0.2 M Suc, 2 mM EGTA, 5 mM MgCl₂ supplemented with 1 mM PMSF, 1 μg/mL leupeptin, and Triton X-100 to 0.5% (v/v). Following centrifugation, protein concentrations in the Triton X-100 supernatant were determined by the Bradford method (Bradford, 1976), with the Bio-Rad protein assay dye reagent according to the manufacturer's instructions, using BSA as a standard.

Con A Affinity Chromatography

The chromatography on a Con A column (Pharmacia) was performed according to the instructions of the manufacturer. Enriched rough microsomes from wheat grains were resuspended in Con A buffer (20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM MnCl₂) supplemented with 1% Nonidet P-40 and applied on a Con A column with a bed volume of approximately 0.5 mL. The column was then washed with 10 column volumes of the above Con A buffer. Elution was performed with the same buffer containing 0.2% methyl-a-D-mannopyranoside. Fractions of 0.5 mL were collected from both wash solution and eluate.

PNGase F Treatment

PDI purified by Con A chromatography or the enriched microsomes were enzymatically deglycosylated essentially as described by Tarentino et al. (1985) with the following modifications. The protein sample was boiled for 3 min in the presence of 1% SDS and cooled. The sample was adjusted to 100 mM sodium phosphate, pH 7.2, 25 mM EDTA, 1% Triton X-100, 1% β-mercaptoethanol, 0.2% SDS with or without 0.2 units/mL of peptide PNGase F. The samples were incubated at 37°C for 6 h, after which they were examined by SDS-PAGE.

Production and Use of Antibodies

New Zealand White rabbits were injected subcutaneously three times at 2-week intervals with gel slices containing about 100 μg of wheat PDI that was Con A purified and then fractionated in SDS gels. Serum was obtained from several bleedings beginning 10 d after the second injection. Antibodies raised against alfalfa PDI (Shorros et al., 1993) were kindly provided by Dr. R.A. Dixon. Antibodies raised against yeast BiP (Rose et al., 1989) were kindly provided by Dr. J.P. Vogel. The monoclonal antibodies 1D3, raised against the C-terminal KDDDQKAVKDEL sequence of rat PDI (Vaux et al., 1990), were kindly provided by Dr. S. Fuller. Anti-γ-gliadin serum (Rubin et al., 1992) was kindly provided by R. Rubin.

SDS-PAGE and Western Blot Analysis

SDS-PAGE was on 7.5% or 10% polyacrylamide gels as described by Laemmli (1970). After electrophoresis, gels were stained with either Coomassie blue or silver. For western blot analysis, proteins were transferred to 0.2-μm nitrocellulose filters and immunoblotted with the desired antiserum. Protein bands were detected using the ECL kit (Amersham) according to the instructions of the manufacturer. Anti-wheat PDI, anti-yeast BiP, anti-γ-gliadin, and anti-alfalfa PDI were used for western blots at dilutions of 1:1000, 1:1000, and 1:20,000, respectively. Monoclonal 1D3 supernatant was used without further dilution. Densitometer analysis of western blot films was performed by a Molecular Dynamics (Sunnyvale, CA) 300A computing densitometer, using Image Quat III software.

45Ca Overlay

Samples were subjected to SDS-PAGE (10% polyacrylamide resolving gel). Proteins were then transferred onto nitrocellulose membranes. After the proteins were transferred, a 45Ca overlay assay was performed essentially as described by Maruyama et al. (1984).

Proteolytic Cleavage and N-Terminal Amino Acid Sequencing

Digestion of the Con A-purified PDI with endoprotease Lys-C was carried out in 100 mM NH₄HCO₃, pH 7.8, containing 1.5% SDS for 2 h at 35°C at a substrate:enzyme ratio of about 1:0.2 (w/w). Following digestion, samples were boiled for 5 min to inactivate the protease. "In gel" digestion with endoproteinase Glu-C (V8) was carried out during electrophoresis on a gel slice containing PDI as previously described (Cleveland et al., 1977). When the dye was about halfway down the stacking gel, the power was switched off for 30 min to allow digestion to occur. Electrophoresis was then continued as normal. Following elec-
Figure 1. Identification of calcium-binding proteins in wheat microsomes. Equal amounts of proteins from enriched rough microsomes from developing wheat grains 18 DAA (lanes 1) or from leaves (lanes 2) were separated by 10% SDS-PAGE. The gels were either Coomassie blue stained (A) or transferred onto a nitrocellulose membrane, probed with $^{45}$Ca, and autoradiographed (B). The positions of marker proteins are indicated on the left and the positions of P60 are indicated by arrows.
Figure 2. Isolation of P60 by Con A affinity chromatography. Enriched rough microsomes from developing grains 18 DAA were solubilized in Con A buffer and separated on a Con A Sepharose column. Unbound substances were washed away with Con A buffer. Bound substances were eluted with methyl-α-D-mannopyranoside. Fractions from the flow-through wash (lanes 1–5) and the methyl-α-D-mannopyranoside eluate (lanes 6–9) were fractionated by 10% SDS-PAGE and either stained with Coomassie blue (A) or transferred onto a nitrocellulose membrane, followed by the 45Ca overlay assay and autoradiography (B). The positions of marker proteins are indicated on the left and the positions of P60 are indicated by arrows.

deduced sequence of a wheat PDI cDNA (Shimoni et al., 1995) showed 100% identity for the N-terminal and V8-generated peptide and a significant, although incomplete, identity with the Lys-C peptide. This confirmed that P60 is indeed PDI. The incomplete homology of the Lys-C peptide sequence to that of the deduced amino acid sequence of the cloned wheat PDI cDNA may be due either to a sequencing error or to the presence of multiple PDI genes in wheat.

Reactivity of P60 with a Polyclonal Anti-Alfalfa PDI Serum and a Monoclonal Anti-KDEL Antibody

To confirm further that P60 is the wheat PDI homolog, this protein was reacted in a western blot with polyclonal serum raised against alfalfa PDI (Shorrosh et al., 1993). As shown in Figure 5, A and B, P60 reacted positively with this serum. In addition, we also reacted P60 in a western blot with 1D3, a monoclonal antibody that was raised against a synthetic peptide comprising the last 12 amino acids of rat PDI, and was shown to specifically recognize its ER retention signal, KDEL (Vaux et al., 1990). As shown in Figure 5C, lanes 1 and 2, the monoclonal antibody reacted positively with P60, showing that P60 indeed contained this signal. When the monoclonal antibody was reacted against enriched ER proteins from pig kidney, it cross-reacted with a single protein band of about 57 kD. This apparently represents the mammalian PDI, which migrates slightly faster than wheat PDI (Lambert and Freedman, 1983). The fact that the antibody against the KDEL-containing synthetic peptide of rat PDI preferentially recognized PDI from either the pig or the wheat microsomes in our study is in agreement with a previous study in which this antibody preferentially recognized PDI in mammalian microsomes (Vaux et al., 1990). Nevertheless, after a longer exposure, additional faint bands were detected, which probably represent other KDEL-harboring ER-resident proteins (data not shown). In contrast to the monoclonal antibodies that recognized PDI from both animal and plant tissues, the anti-wheat PDI polyclonal serum did not recognize the mammalian PDI homolog (Fig. 5D).

Developmental Expression of PDI

To study the regulation of PDI expression, we analyzed the level of this protein during plant development in comparison with that of another ER-resident protein, BiP. In the first experiments root tips and shoots of young wheat plants as well as developing endosperm or embryos were homogenized in a Triton X-100-containing buffer and the soluble proteins were reacted in western blots with anti-PDI and anti-BiP sera. PDI was the most abundant in developing endosperm and the least abundant in leaves (Fig. 6, A and B). The level of BiP was quite variable between experiments, but in general it was higher in root tips and developing endosperm than in leaves and developing embryos (Fig. 6, A and C). Yet, the relative levels of PDI and BiP varied between the different tissues, suggesting that the expression of their genes is not subject to a coordinated regulation. It is impossible to compare the actual abundance of PDI and BiP in each tissue because the different antisera might possess different affinities to their antigens. However, the relative level of PDI in endosperms,

Figure 3. PNGase F treatment of the Con A-purified P60 and grain-enriched rough microsomes. A, Con A-purified P60; B, enriched rough microsomes. Proteins were treated with (lane 1) and without (lane 2) PNGase F, separated on 7.5% SDS-PAGE, and either stained with silver (A) or subjected to a western blot analysis with anti-wheat P60 serum (B). The migration of mol wts of protein standards (MW) is indicated on the left.
Purification and Analysis of Wheat Protein Disulfide Isomerase

Figure 4. Amino acid sequence alignment of partial proteolytic digestion products at the N-terminal region of P60 with that of wheat PDI. A comparison of the amino acid sequences derived from the purified wheat P60 protein (by direct peptide sequencing) to the wheat PDI amino acid sequence deduced from its cDNA-coding sequence (Shimoni et al., 1995) is shown. Amino acid residues that are identical are connected by vertical lines. The sequence resembling the thioredoxin/PDI-like catalytic sites is boxed, with its two redox-active Cys residues shown in bold type. Also indicated are the proteolytic enzymes from which the peptide sequences were derived, as well as the signal peptide of PDI.

compared to its own level in other tissues, was higher than the relative level of BiP in endosperm compared to BiP level in other tissues (Fig. 6).

We also tested the level of PDI and BiP during wheat grain development in comparison with the onset of storage protein accumulation. The levels of both PDI and BiP increased notably between 5 and 9 DAA, remained relatively high until 17 to 21 DAA, and then declined toward seed maturation and desiccation (Fig. 7, B and C). It is interesting that PDI and BiP were already up-regulated several days before storage proteins are detected in the western blots (Fig. 7, cf. parts B, C, and D).

Figure 5. Reactivity of P60 with various anti-PDI sera. Con A-purified P60 (lane 1), enriched wheat grain rough microsomes (lane 2), and enriched pig kidney microsomes (lane 3 in C and D) were separated on 10% SDS-PAGE and either stained with Coomassie blue (A) or subjected to a western blot analysis with anti-alfalfa PDI (B), anti-rat PDI KDEL-tail 1D3 monoclonal serum (C), or anti-P60 sera (D). Mol wt markers (MW) were also included with sizes indicated on the left.

Figure 6. Analysis of PDI and BiP levels in various wheat tissues. Equal amounts of Triton X-100-protein extracts from root tips of wheat seedlings (lane 1) and young leaves (lane 2), as well as 18-DAA developing grain endosperm (lane 3) and embryos (lane 4) were separated on 10% SDS-PAGE. A, Coomassie blue-stained gel including marker proteins (MW) with sizes indicated on the left. B and C, Gels identical with that shown in A were subjected to western blot analysis with anti-alfalfa PDI or anti-yeast BiP sera, respectively. The relative abundance of either PDI or BiP in the various tissues tested, as measured by densitometry quantitation, is plotted below the blots. Protein levels in endosperm (B) and in root tips (C) were compared to its own level in other tissues, was higher than the relative level of BiP in endosperm compared to BiP level in other tissues (Fig. 6).
Subcellular Localization of P60

We have previously shown that the ER-resident chaperone BiP is co-localized with the storage proteins in dense PB within the vacuoles (Levanony et al., 1992). We also suggested that this may have resulted from trapping BiP during the process of assembly of the storage proteins within the ER. Based on this observation, it was tempting to test whether PDI also co-localized with the storage proteins in the dense PB. To address this, developing grains were homogenized in a Suc-containing buffer and fractionated on a 10 to 50% metrizamide density gradient. As shown in Figure 8, PDI localized in two peaks, one that co-sedimented with the ER marker enzyme NADH Cyt c reductase (Fig. 8, fractions 4-6) and a second peak that localized at the bottom of the gradient (Fig. 8A, fractions 11-13). This second peak also co-sedimented with the storage proteins present in dense PB (data not shown; Rubin et al., 1992).

The intracellular localization of PDI in developing endosperm was also studied by immunogold labeling of electron micrographs with the anti-PDI serum and a preimmune serum as a control. The efficiency of detection of PDI in the electron micrographs was much lower than that obtained in western blots, and the anti-alfalfa PDI was proven to be much more efficient than the anti-wheat PDI. For this reason, we used the anti-alfalfa PDI at a dilution of 1:100 for immunogold labeling. At this dilution, a faint band in the size of PDI was also detected in western blots treated with the preimmune serum in overexposed films. This band was, however, much more intense with the immune serum at an identical dilution (data not shown).

Upon immunogold labeling of the electron micrographs with the anti-PDI serum, gold particles were detected both in the lumen of the ER and in the dense PB (Fig. 9, a and c). There was also some labeling inside the ER and the PB upon treatment with the preimmune serum, but the numbers of gold particles per unit area in both organelles were significantly higher upon treatment with the immune serum compared to the preimmune serum (Fig. 9, cf. a and b as well as c and d; see also Table I). This supported the results of the density gradient analysis showing the presence of PDI both within the ER and in the PB.

DISCUSSION

Purification and Characterization of Wheat PDI

PDI is an ER-resident protein that is ubiquitous to animal and plant cells and catalyzes the formation and isomerization of disulfide bonds in nascent proteins within the ER (Bulleid and Freedman, 1988; Shorrosh and Dixon, 1991; Shorrosh et al., 1993; Freedman et al., 1994). To study in more detail the role of PDI in the maturation of plant proteins, we have attempted to purify PDI from wheat endosperm to homogeneity and study its developmental expression and intracellular localization. PDIs from animals and plants are calcium-binding proteins that gener-
Figure 9. Immunogold labeling of PDI in developing wheat grains. Thin sections of wheat endosperm cells were immunogold labeled with anti-alfalfa PDI at a dilution of 1:100 (a and c) or with preimmune serum (b and d). I, Inclusion body; V, vacuole. Bars: a and b, 0.25 μm; c, 0.5 μm; d, 1 μm.
ally range in size between 55 and 60 kD. Moreover, previous studies suggested that PDI may be extremely abundant in developing wheat grains (Roden et al., 1982). Thus, among the ER proteins of developing wheat grains, P60 appeared to qualify best for these requirements. Indeed, several lines of evidence clearly showed that P60 is the wheat PDI homolog. These included amino acid sequence analyses of the N-terminal and two additional internal peptides of P60 as well as the demonstration that P60 cross-reacts with anti-alfalfa PDI serum and a monoclonal antibody that is specific for the KDEL sequence of mammalian PDI (Vaux et al., 1990; Shorosh et al., 1993).

Our study showed that wheat PDI is a glycoprotein. This possibility was anticipated, since previous studies showed that PDI from yeast and perhaps also from several mammalian species appear to be glycoproteins. Previous analysis of the deduced amino acid sequence of a wheat PDI cDNA demonstrated the presence of a single N-glycosylation site (Shimoni et al., 1995). Thus, our study implies that this site is indeed utilized. It seems that in wheat PDI appears mainly in a glycosylated form because only a single band cross-reacted with the anti-alfalfa PDI serum and a monoclonal antibody that is specific for the KDEL sequence of mammalian PDI (Vaux et al., 1990; Shorosh et al., 1993).

The observation that PDI binds to Con A Sepharose was used in this study as a mean for a simple and rapid purification of PDI. Following passage of ER-enriched fractions from developing grains on a Con A Sepharose column, PDI appeared as the only band detected by Coomassie blue staining, suggesting that this process yielded a nearly homogeneous preparation of PDI. Moreover, based on the abundance of PDI in wheat grains, our calculations show that this simple purification process may yield more than 100 µg of PDI from about 50 g of seeds. Such quantities appear to be sufficient to perform in vitro studies and thus may overcome the need to produce recombinant PDI in heterologous systems.

Developmental Expression of PDI and BiP

Both PDI and BiP are parts of the machinery that assists in the folding, assembly, and sorting of secretory proteins via the ER. Thus, our observation that both proteins were more abundant in developing endosperm and root tips than in leaves is consistent with the notion that the quality control system is expected to be up-regulated in cells that are more active in the synthesis of secretory proteins. However, the relative proportions of PDI and BiP varied in the different tissues, with PDI being the most abundant in developing endosperm and BiP being the most abundant in root tips (Fig. 6). This suggests that expression of these two proteins is not entirely coordinated during plant development. Interestingly, the expression of both of these proteins in developing endosperm was up-regulated several days prior to the detection of storage proteins in western blots (Fig. 7). The control of induction of PDI and BiP expression during grain development and whether it is related to the onset of storage protein synthesis has still to be established. The levels of PDI and BiP (based on equal amount of Triton X-100-extracted proteins) declined after 17 DAA. This may represent an underestimation of their actual levels, because from 17 DAA on, storage proteins accumulate, contributing significantly to the total Triton X-100-extractable proteins (Fig. 7D).

We also analyzed the levels of PDI and BiP during development of grains from two isogenic lines of tetraploid Triticum durum, one of which lacked the entire set of the high mol wt glutelins. No significant difference was evident between these isogenic lines in the pattern of appearance of both proteins (data not shown), suggesting that expression of PDI and BiP may also not be related to the synthesis of high mol wt glutelins.

Intracellular Localization of PDI

The demonstration that PDI is co-localized with the storage proteins in the PB extends our previous works showing a similar co-localization of BiP (Levanony et al., 1992; Rubin et al., 1992) and suggests that the PB possibly contain the entire set of ER-resident proteins. Several lines of evidence have recently shown that a considerable amount of the storage proteins assemble into PB within the ER (Levanony et al., 1992; Altschuler and Galili, 1993; Altschuler et al., 1993; Galili et al., 1993; Rosenberg et al., 1993). Thus, the presence of ER-resident proteins inside the PB may be due to their trapping inside the storage protein complexes during PB formation within the ER. Whether PDI is also involved in the folding and assembly of the storage proteins still remains to be established, although indirect evidence supports this possibility (Galili et al., 1993, and this work). The availability of large amounts of purified wheat PDI will enable researchers to address this issue further.

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