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Thiocalsin: A thioredoxin-linked, substrate-specific protease dependent on calcium

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ABSTRACT **We describe a protease, named "thiocalsin," that is activated by calcium but only after reductive activation by thioredoxin, a small protein with a redox-active disulfide group that functions widely in regulation. Thiocalsin appeared to be a 14-kDa serine protease that functions independently of calmodulin. The enzyme, purified from germinating wheat grain, specifically cleaved the major indigenous storage proteins, gliadins and glutenins, after they too had been reduced, preferentially by thioredoxin. The disulfide groups of the enzyme, as well as its protein substrates, were reduced by thioredoxin via NADPH and the associated enzyme, NADP-thioredoxin reductase. The results broaden the roles of thioredoxin and calcium and suggest a joint function in activating thiocalsin, thereby providing amino acids for germination and seedling development.**

Thioredoxin, a small protein with a catalytically active disulfide group, regulates a spectrum of cellular processes, ranging from photosynthesis to the proliferation of virus-infected cells to DNA replication (1–3). Recent studies in our laboratory indicate that thioredoxin, reduced with NADPH via the enzyme, NADP-thioredoxin reductase (NTR), functions in germinating wheat by reducing specific disulfide groups of gliadins and glutenins, storage proteins of the endosperm, thereby increasing their susceptibility to proteolysis and availability as a nitrogen source (4). This process can be described by the following three chemical equations.

$$\underset{\text{(-S—S-)}}{\text{NADPH} + \text{H}^+ + \text{thioredoxin}_{ox}} \xrightarrow{\text{NTR}} \text{NADP}^+ + \underset{\text{(-SH HS-)}}{\text{thioredoxin}_{red}} \quad [1]$$

$$\underset{\text{(-SH HS-)}}{\text{thioredoxin}_{red}} + \underset{\substack{\text{(-S—S-)}\\ \text{insoluble,}\\ \text{protease-resistant}}}{\text{gliadins}_{ox}\ \text{glutenins}_{ox}} \longrightarrow \underset{\text{(-S—S-)}}{\text{thioredoxin}_{ox}} + \underset{\substack{\text{(-SH HS-)}\\ \text{soluble,}\\ \text{protease-sensitive}}}{\text{gliadins}_{red}\ \text{glutenins}_{red}} \quad [2]$$

$$\underset{\text{(-SH HS-)}}{\text{gliadins}_{red}\ \text{glutenins}_{red}} \xrightarrow{\text{proteases}} \text{aminoacids} \quad [3]$$

Thioredoxin has also been found to reduce and thereby inactivate low molecular weight disulfide proteins that inhibit specific enzymes (5, 6) as follows.

$$\underset{\text{(-SH HS-)}}{\text{thioredoxin}_{red}} + \underset{\substack{\text{(-S—S-)}\\ \text{active}}}{\text{enzyme inhibitor}} \longrightarrow \underset{\text{(-S—S-)}}{\text{thioredoxin}_{ox}} + \underset{\substack{\text{(-SH HS-)}\\ \text{inactive}}}{\text{enzyme inhibitor}} \quad [4]$$

There is evidence that thioredoxin acts in the reduction of disulfide groups of proteins that specifically inhibit amylolytic enzymes, α-amylase and pullulanase (limit dextrinase), thus leading to the deinhibition of these target enzymes and the mobilization of carbon in the endosperm (7, 8) as follows.

$$\text{starch} \xrightarrow{\text{active enzymes}} \text{glucose} \quad [5]$$

In extending these studies, we have identified a type of protease that, following reduction and limited activation by thioredoxin, is modulated by calcium. The enzyme, named "thiocalsin," is specific for gliadins and glutenins, preferably after reduction by thioredoxin. The evidence suggests that thioredoxin functions jointly with calcium in regulating the hydrolysis of storage proteins in germinating wheat.

MATERIALS AND METHODS

Plant Materials. Durum wheat (*Triticum durum*, Desf. cv. Monroe) was a kind gift from K. Khan (North Dakota State University, Fargo).

Chemicals and Enzymes. Reagents for SDS/PAGE were obtained from Bio-Rad. Dithiothreitol (DTT) and the protease inhibitors were from Boehringer Mannheim. 2,4,6-Trinitrobenzenesulfonic acid (TNBS, also called picrylsulfonic acid) and biochemicals were from Sigma. Pure thioredoxin and NTR were isolated and purified from *Escherichia coli* cells transformed to overexpress each protein as described (5). Thioredoxin *h* was isolated from wheat germ by using the procedure of Florencio *et al.* (9) as modified (5).

Growth of Seedlings. Typically, 10,000 wheat grains were surface sterilized in 1% sodium hypochlorite for 30 min and washed 8–10 times with sterile deionized water. Grains were then set to germinate in lots of 30 in clean Petri dishes lined with three layers of Whatman #1 filter paper. Plates were wrapped with aluminum foil and left for 2 days at 20°C. Following harvest, the endosperm plus pericarp were severed from the embryonic axis, including roots and shoots, with a scalpel. The endosperm component was stored at −20°C until use.

Abbreviations: DTT, dithiothreitol; IEF, isoelectricfocusing; GR, glutathione reductase; mBBr, monobromobimane; NTR, NADP-thioredoxin reductase; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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Preparation of the Protein Substrates. Following removal of albumins and globulins by saline extraction, storage protein substrates were obtained from 1 g of flour by sequential extraction for 2 h at 20°C with 5 ml each of 70% ethanol (gliadin fraction) and 0.1 M acetic acid (glutenin fraction). Samples were extracted on an electrical rotator and, in addition, were periodically agitated with a vortex mixer. After extraction with each solvent, the fractions were centrifuged (25,000 × *g* for 30 min), and the supernatant solutions were saved for analysis. Between the ethanol and acetic acid extractions, pellets were washed once with 5 ml of water and collected by centrifugation as before. The supernatant wash solution was discarded.

The gliadin and glutenin substrates were reduced as described by Kobrehel *et al.* (4). NTR (0.7 μg) and thioredoxin (0.8 μg) (both from *E. coli*) were added to 70 μl of 30 mM Tris·HCl buffer, pH 7.9, containing 1 mM NADPH and 20 μg of target protein substrate (final volume, 100 μl). After incubation for 20 min at room temperature, sodium iodoacetate was added to 10 mM, and the reaction was continued for another 1 h. The samples were then taken to dryness by a 2 h treatment in a SpeedVac concentrator (Savant). The dried, reduced proteins were dissolved in 30 mM Tris·HCl buffer, pH 7.9. Reduction with glutathione was performed under the same conditions by replacing thioredoxin with 1 mM reduced glutathione and NTR with 1 μg of glutathione reductase (GR) (purified from spinach leaves). Reduction by DTT was accomplished by adding DTT to a final concentration of 0.5 mM in the absence of thioredoxin and NTR. Electrophoretic analysis revealed that both low and high molecular weight species were present in the glutenin fraction.

Protease Assay. Protease activity was determined in 96-well ELISA plates by the trinitrophenylation method, which is based on the reaction of protein amino groups with TNBS (10). The reaction product has a yellow color with absorbance measured at 405 nm. Gliadins and glutenins that had been reduced by the NADP–thioredoxin system and derivatized in 50 mM Tris·HCl buffer, pH 7.9, were used as substrates to follow protease activity during purification. A 20-μg aliquot of the indicated substrate, dissolved in 50 mM Tris·HCl buffer, pH 7.9, was supplemented with a suitable amount of the enzyme solution in 50 mM sodium acetate buffer, pH 5.0, to give a final volume of 100 μl. As indicated, thiocalsin was activated by reduced thioredoxin and Ca^{2+}. The indicated amount of $CaCl_2$ and 0.1 μg of thioredoxin (from *E. coli*), together with 0.1 μg of NTR (from *E. coli*) and 0.5 mM NADPH, were incubated with thiocalsin in 50 mM Tris·HCl buffer, pH 7.9, for 1 h at room temperature. In certain cases, NTR was replaced by 0.1 μg of GR (spinach leaves), and thioredoxin was replaced by 1 mM glutathione. When DTT (0.5 mM) was used, NTR and NADPH were omitted and 0.1 μg of thioredoxin (from *E. coli*) or wheat germ (*h* type) was supplied. Substrate was added, and the reaction mixture containing the activated enzyme in 50 mM sodium acetate buffer, pH 5.0, was incubated for 4 h at 37°C. Assays were conducted in quintuplicate. Values consistently agreed within ±5% variation. The amount of the fractions assayed was chosen so that activity was proportional to added enzyme (up to an A_{405} of 1.5). Assays using pure thiocalsin were routinely conducted with 10 ng of protein. Assays with protease inhibitors were carried out in 50 mM sodium acetate buffer, pH 5.0. For varying pH in the ranges 3.0–6.0 and 5.0–9.0, reaction mixtures were buffered with 50 mM sodium acetate and 50 mM Tris-maleate, respectively. To stop the reaction, 400 μl of 1% SDS solution was added, and the samples were incubated at 75°C for 30 min. A 20-μl aliquot of the reaction mixture was applied to a well of a 96-well microtiter plate containing 90 μl of 0.21 M phosphate buffer, pH 8.2, and 90 μl of 0.1% TNBS (final assay volume was 200 μl). Following incubation for 1 h (in the dark) at 37°C, the plate was read at 405 nm with a Biolog model Emax microplate reader. One unit of enzyme activity corresponds to the formation of 1 nmole of amino group per min from the reduced gliadin or glutenin substrate under the described reaction conditions, using glycine as a standard. The assay was specific for amino groups (11).

Purification of Thiocalsin. Unless indicated otherwise, all steps were carried out at 4°C, and protein was monitored during purification at 280 nm.

Extraction. A total of 400 g of deembryonated kernels were ground with a mortar and pestle and extracted overnight in 100 mM sodium acetate buffer, pH 4.5 at a ratio of 1:4 (wt/vol). The extract was centrifuged for 60 min at 30,000 × *g*, the pellet was discarded, and the supernatant fraction was dialyzed overnight against 50 mM sodium acetate buffer, pH 4.5.

Ammonium sulfate precipitation. The clarified extract was brought to 80% saturation with solid ammonium sulfate. The supernatant fraction was removed by centrifugation at 30,000 × *g* for 30 min, and the precipitate was dissolved in 50 mM sodium acetate buffer, pH 4.5. The solution was dialyzed overnight against the same buffer and then clarified by centrifugation for 20 min at 30,000 × *g*.

Affinity chromatography. The dialyzed ammonium sulfate fraction was applied to a hemoglobin-agarose column (2 × 15 cm) prepared according to the manufacturer's instructions (Sigma). Following a wash with three column volumes of 50 mM sodium acetate buffer, pH 4.5, the sample was eluted with 50 mM sodium acetate buffer, pH 3.8. The flow rate was approximately 2 ml/min; 4.0-ml fractions were collected. Fractions having protease activity were combined, concentrated by ultrafiltration in an Amicon cell fitted with a YM-3 membrane (3-kDa cutoff; Amicon), and then dialyzed against 50 mM sodium formate buffer, pH 3.0.

Cation-exchange chromatography. The concentrated protein solution was filtered through a 0.22-μm pore size Millex-GV filter (Millipore) and chromatographed at room temperature on a 5 mm × 5 cm Pharmacia Mono-S cation-exchange column, equilibrated with 50 mM sodium formate buffer, pH 3.0. The column was eluted with a linear gradient of NaCl (0–1 M NaCl in 50 mM sodium formate buffer, pH 3.0) for 60 min (flow rate of 0.5 ml/min); 1-ml fractions were collected. Fractions containing protease activity were pooled and concentrated by using an Amicon Centricon-3 microconcentrator.

Reverse-phase chromatography. High-resolution purification was achieved by reverse-phase FPLC using a ProRPC HR 5/10 column (5 μm; C_1/C_8; 100 × 5 mm; Pharmacia). Trifluoroacetic acid (TFA) was added to 0.1% to the concentrated enzyme fraction, which was applied to a column preequilibrated with 0.1% TFA as above and eluted with a linear gradient of 0–60% (vol/vol) acetonitrile in 0.1% TFA (flow rate of 0.5 ml/min). Fractions of 1 ml were collected, pooled according to protein peak, and freeze dried. The dry protein fractions were dissolved in 50 mM sodium acetate buffer, pH 4.5. Individual peaks showing protease activity with reduced gliadins and/or glutenins were analyzed by SDS/PAGE and isoelectric focusing (IEF).

Analytical Methods. Samples from the different purification steps were analyzed by SDS/PAGE using a 10–20% acrylamide gradient resolving slab gel (12). Proteins were identified by silver staining by using the Bio-Rad kit. The following protein standards were used for molecular mass determination: rabbit skeletal muscle myosin (M_r 213,000), *E. coli* β-galactosidase (M_r 123,000), bovine serum albumin (M_r 85,000), hen egg white ovalbumin (M_r 50,300), bovine carbonic anhydrase (M_r 33,300), soybean trypsin inhibitor (M_r 28,500), hen egg white lysozyme (M_r 18,900), and bovine pancreas aprotinin (M_r 7,800).

Protein concentration of the fractions analyzed was determined by the Bio-Rad microassay using bovine γ-globulins as a standard (13).

Molecular weight of the native thiocalsin was determined on an electrospray mass spectrometer (model 5989A, Hewlett Packard) by D. King (University of California, Berkeley).

Analyses of cysteine were performed on performic acid-oxidized thiocalsin samples by the Protein Structure Laboratory, University of California, Davis. One milliliter of a 30% (vol/vol) solution of hydrogen peroxide and 9 ml of 88% (vol/vol) formic acid were mixed and allowed to stand for 1 h. One milliliter of this solution was added to a lyophilized thiocalsin sample and left at 4°C for 24 h. Amino acids were analyzed on an Applied Biosystems model 477 automated amino acid analyzer.

IEF. The enzyme purified through the reverse-phase chromatography step was mixed with 1% Bio-Lytes, pH range 3–10, (Bio-Rad) in distilled water (total volume 18 ml) and separated by a semipreparative IEF on a Rotofor Cell (Bio-Rad) as described in the manufacturer's instruction manual. Electrophoresis was carried out for 4 h at 12 W at 4°C. Protease activity and pH were measured on each fraction (1.2 ml). Analytical IEF was carried out at 25 W for 1 h in Isogel agarose IEF plates (FMC) containing isogel ampholytes in a pH range of 3–10. Samples (10 μg) were applied to pieces of filter paper on the gel surface. Protein bands were detected by Coomassie blue R-250 staining.

RESULTS

The present study was designed to determine whether thioredoxin functions in germination as established for photosynthesis—i.e., by reducing regulatory disulfide bonds, thereby activating a target enzyme. Such a role seemed feasible in view of results demonstrating that thioredoxin of wheat endosperm is converted from the oxidized to the reduced form during germination in a manner reminiscent of the redox changes observed with illuminated chloroplasts (36). To this end, we examined the endosperm of germinating wheat for a protease whose activity increased in the presence of reduced thioredoxin. Thioredoxin-reduced forms of the native storage proteins gliadins and glutenins were used as substrate. Both types of protein undergo extensive reduction *in vivo* in the first several days of germination in reactions that appear to be linked to thioredoxin (4). We selected an incubation time of 2 days, reasoning that a protease of the type sought would be formed at the onset of reduction activity. Exploratory studies yielded evidence for the occurrence of a thioredoxin-linked protease specific for the native storage proteins. We proceeded, therefore, to purify and characterize the enzyme from germinating grain. Activity of thiocalsin was not detected in dry (ungerminated) seeds.

Purification of Thiocalsin. The purification of the protease (thiocalsin) from wheat grain germinated for 2 days is summarized in Table 1. The final enzyme fraction contained about 2% of the total original proteolytic activity measured with either reduced gliadins or glutenins and 0.003% of the original protein, resulting in a 550-fold increase in specific activity. Activity was proportional to pure enzyme added from 0.1 to 20 ng of protein.

Properties of Thiocalsin. The purified fraction showed a single protein component with a molecular weight of 12,800 and a molecular mass of 14,000 Da, as determined by mass spectrometry (data not shown) and SDS/PAGE (Fig. 1*A*), respectively. To confirm that the electrophoretic band corresponded to the new protease, the fraction was subjected to isoelectric focusing using a horizontal agarose gel in a pH 3–10 gradient. This procedure resolved three protein components with isoelectric points of 5.55, 5.65, and 6.00 (Fig. 2), each of which showed similar activity in specifically hydrolyzing the reduced forms of glutenins and gliadins. Moreover, the mobility of each component in SDS/polyacrylamide gel was similar to that of the parent fraction (cf. Fig. 1*A*).

Further evidence that the 14-kDa protein band in Fig. 1*A* corresponded to thiocalsin was provided by employing a combination of nondenaturing and SDS/PAGE. First, the active component was isolated and identified on nondenaturing electrophoresis gels with substrate (carboxymethylated gliadin fraction) incorporated into the gel matrix (14, 15); the catalytically active component—i.e., that hydrolyzing the gliadins—was observed as a clear band after staining with Coomassie blue (I.B., J.H.W., and B.B.B., unpublished results). Second, following elution, the excised active component was again subjected to electrophoresis, using nondenaturing gels and SDS gels in parallel. After development, each gel was stained for protein with silver stain. In both cases, the catalytically active band corresponded to the major protein band. Furthermore, in the SDS gel, the component showed the same molecular mass as that in Fig. 1*A*—i.e., 14 kDa. It was concluded, therefore, that the band seen in Fig. 1*A* was thiocalsin and that the enzyme existed in three isoforms which,

Table 1. Purification of thiocalsin from wheat grain after 2 days of germination

Purification steps	Total protein, mg	Total activity, units	Specific activity, units/mg	Yield, %	Purification, Fold
1. Crude extract clarified	4800				
Glutenins		7248	1.5	100	1.0
Gliadins		7488	1.6	100	1.0
2. Ammonium sulfate fraction	1846				
Glutenins		9285	5.0	128	3.3
Gliadins		9655	5.2	129	3.4
3. Hemoglobin-agarose fraction	38				
Glutenins		1720	45.3	24	30.0
Gliadins		1490	39.2	20	25.1
4. Mono-S fraction	0.67				
Glutenins		43	63.5	0.6	42.1
Gliadins		42	62.5	0.6	40.1
5. Reverse-phase fraction	0.16				
Glutenins		140	865	1.9	573
Gliadins		140	865	1.9	555

The endosperm components from 2-day germinated wheat seeds were collected, and protease activity was followed during purification using as substrates 20 μg of glutenins or 20 μg gliadins, both of which were reduced by DTT/thioredoxin and alkylated by iodoacetate at pH 5.0 and 37°C. One unit of protease activity corresponds to the formation of 1 nmol of amino groups per ml and per min under the incubation conditions, using glycine as a standard.

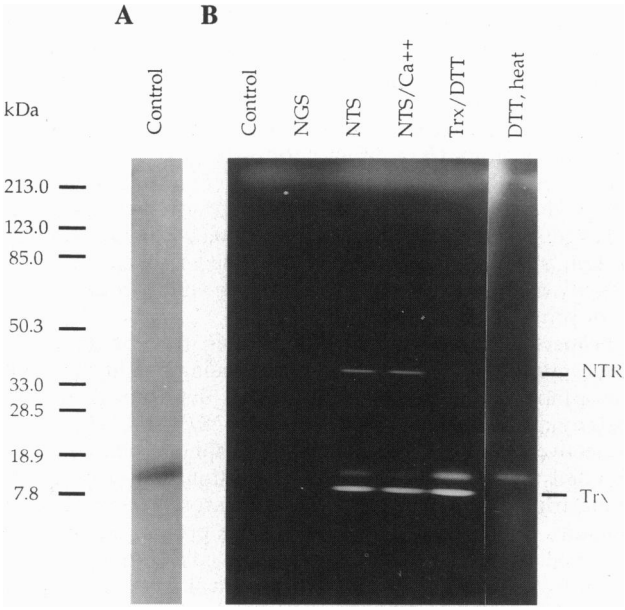

FIG. 1. Thiocalsin and its reduction by the NADP/thioredoxin system determined by an SDS/polyacrylamide gel/mBBr procedure. (*A*) Silver stain for protein. (*B*) Fluorescence showing newly formed sulfhydryl groups. Following incubation with the indicated additions, the thiocalsin samples were derivatized with mBBr and subjected to SDS/PAGE. Two micrograms of thiocalsin in 30 mM Tris·HCl, pH 7.9, was applied to all lanes. Control: no addition; NGS: 2 mM glutathione, 1 μg of GR (from spinach leaves), and 2 mM NADPH; NTS: 1 μg of thioredoxin, 1 μg of NTR (both from *E. coli*), and 2 mM NADPH; NTS/Ca^{++}: same as NTS, except incubation was with Ca^{2+} (final concentration 20 μM); Trx/DTT: 1 μg of thioredoxin (from *E. coli*) and 0.5 mM DTT; DTT, heat: the sample was heated 3 min in boiling water after reduction by 0.5 mM DTT. Trx, thioredoxin.

while having different charges, showed similar activity and molecular weight, as well as temperature and pH optima (30°C; pH 5.0). Proteolytic activity was not observed with any of the forms at temperatures higher than 55°C or at pH values below 3.0 or above 8.0 (data not shown).

Purified thiocalsin showed no discernible reaction with mBBr, a probe that becomes fluorescent after covalently binding free sulfhydryl groups (16) (Fig. 1*B*). Fluorescence indicative of sulfhydryl groups was observed, however, following reduction with *E. coli* thioredoxin that itself had been reduced either enzymatically with NADPH (and NTR) or chemically with DTT. Similar results were obtained with native wheat thioredoxin *h* (data not shown). Amino acid composition analyses indicated that thiocalsin contained 10 half-cysteine residues per molecule, which were concluded to be present in the disulfide form because of the absence of reactivity with mBBr. Densitometric scanning revealed that, in the presence of NADPH and NTR, thioredoxin reduced two of the five disulfide bonds based on the assumption that all disulfide groups were reduced in samples heated in DTT and SDS under the conditions of Fig. 1 (treatment 6, far right, Fig. 1*B*). Reduction of the enzyme was not observed with the reduced form of glutathione, a cellular monothiol (treatment 2: glutathione, GR, and NADPH; Fig. 1*B*). The thiocalsin disulfide groups appeared, therefore, to be specific for thioredoxin or its nonphysiological substitute, DTT.

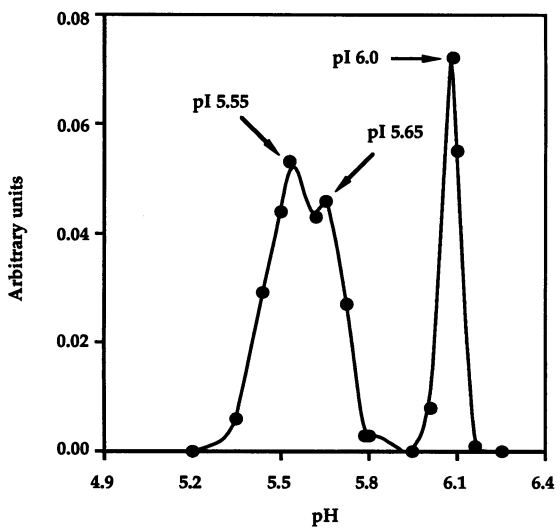

FIG. 2. Densitometric scan of IEF gel showing isozymic forms of thiocalsin.

Requirements for Proteolysis. As indicated in the initial exploratory experiments, reduced thioredoxin activated thiocalsin. As shown in Table 2, thioredoxin (reduced enzymatically with NADPH and NTR) effected a 2-fold increase in activity of the pure enzyme with the reduced forms of either glutenins or gliadins as substrate (treatment 1 vs. 5). Following activation by thioredoxin, the enzyme was activated an additional 2- to 3-fold by a divalent metal, of which calcium was most effective. That activation was due to calcium was confirmed by the finding that an equimolar concentration of chloride (added as NaCl) did not alter activity. Typical of other calcium-linked enzymes (17), the divalent metal requirement could be met by 1.5-fold higher concentration of Ba^{2+} and Sr^{2+} (data not shown). Calcium had no significant effect on the activity of the oxidized enzyme—i.e., in the absence of reduced thioredoxin (treatment 1 vs. 2). Reduced glutathione was also without effect in either the absence (treatment 3) or presence of Ca^{2+} (treatment 4), in accord with its above noted inability to reduce the enzyme. Thiocalsin appeared to act independently of calmodulin. Activity was not affected by the addition of bovine brain calmodulin or known calmodulin inhibitors added at 100 μM [trifluoroperazine dihydrochloride (TFP) and *N*-(6-aminohexyl)-5-chloronaphtalene sulfonamide (W7)].

While equally active as substrates, gliadins and glutenins required different calcium concentrations for hydrolysis. Thus, when gliadins were used as substrate, 5 μM Ca^{2+} supported maximal activity, whereas with glutenins, 20 μM Ca^{2+} was required (Fig. 3). In addition, higher than optimal calcium

Table 2. Requirements of thiocalsin for proteolysis

Treatment	Specific activity, units per mg of enzyme	
	Glutenins	Gliadins
1. Control	865	865
2. Ca^{2+}	1007	1213
3. NGS	886	870
4. NGS/Ca^{2+}	933	1034
5. NTS	1691	1848
6. NTS/Ca^{2+}	4392*	5249*

Protease activity was measured by using 20 μg of glutenins or 20 μg of gliadins as substrates, both reduced by DTT/thioredoxin and alkylated by iodoacetate at pH 5.0 and 37°C. All mixtures were preincubated for 60 min at 37°C prior to addition of the substrate. One unit of protease activity corresponds to the formation of 1 nmol of amino groups per ml and per min under the incubation conditions, using glycine as a standard. NTS: NADPH, NTR, and thioredoxin (both proteins from *E. coli*). NGS: NADPH, GR (from spinach), and glutathione. The final concentration of Ca^{2+} is 20 μM.

*Value was corrected for the apparent activity of 687 units/mg observed with NTS and 20 μM Ca^{2+} in the absence of substrate. Addition of NADPH, NTR, and thioredoxin singly gave 846, 833, and 835 units/mg, respectively, in the presence of substrate.

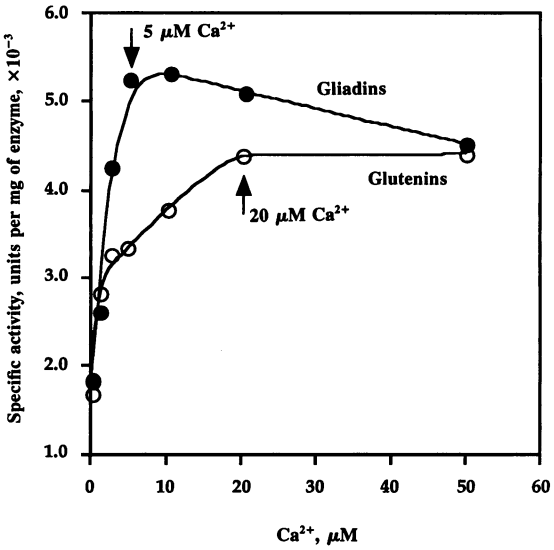

FIG. 3. Effect of Ca^{2+} on thiocalsin activity with glutenin and gliadin as substrates. Protease activity was measured by using 20 μg of gliadins or glutenins that had been reduced by DTT/thioredoxin and alkylated by iodoacetate. The enzyme was activated by the NADP/thioredoxin system and Ca^{2+} as indicated, prior to addition of substrate.

concentrations were slightly inhibitory with gliadins but not with glutenins.

Effect of Substrate Concentration. The question arises as to the mechanism by which thioredoxin and Ca^{2+} increase the activity of thiocalsin—i.e., whether activation is due to an increase in reaction rate or substrate affinity. To test this point, we varied the concentration of the protein substrates in experiments in which the enzyme was first activated (preincubated with reduced thioredoxin and calcium) and then assayed (added to the reaction mixture). The results (Fig. 4) illustrate that, while having relatively little effect on maximal velocity (15,000 units per mg of enzyme), activation by thioredoxin and calcium increased the affinity of thiocalsin for both glutenins and gliadins ≈4-fold. Following activation, thiocalsin showed an $S_{0.5}$ of 100 μg (glutenins) and 50 μg (gliadins) vs. control values of 400 μg and 200 μg, respectively. At a substrate concentration of 600 μg or greater, the enzyme was largely independent of thioredoxin and calcium. Because of the limited availability of the soluble substrates, it seems likely that calcium would contribute to the *in vivo* function of thiocalsin, at least in germination and early seedling development.

Substrate Specificity. The specificity of thiocalsin was tested with seed storage proteins, as well as with substrates conventionally used in protease purification and characterization. As seen in Fig. 5, thiocalsin showed activity only with the seed proteins tested and then only after reduction (conversion from disulfide to sulfhydryl form). Activity was greatest with the storage proteins native to wheat (gliadins and glutenins) but, while low, was detectable with a soluble counterpart from hemp seed (edestin). Based on mBBr/SDS/PAGE analyses, thiocalsin most actively cleaved the reduced forms of high molecular weight glutenins and highly hydrophobic gliadins that had been isolated by a reverse-phase chromatography procedure to be described elsewhere. Consistent with earlier results (4), thioredoxin was more effective than glutathione in reducing the gliadin and glutenin substrates for thiocalsin. There was little or no activity with substrates routinely used to monitor protease activity—i.e., gelatin, casein and hemoglobin.

Effect of Protease Inhibitors. Proteases are classified according to their response to selected inhibitors (18). When thiocalsin was tested with classical inhibitors, we observed consistent inhibition with compounds effective for serine proteases (Table 3). Thus, thiocalsin was inhibited 80% by 0.1 mM 3,4-dichloroisocoumarin (3,4-DCI) and 90% by 4 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), both specific serine protease inhibitors (The 3,4-DCI had to be present during activation as well as during catalysis.) Phenylmethylsulfonyl fluoride (PMSF), an inhibitor of serine as well as cysteine proteases was also effective (50–60% inhibition at 1 mM). Other types of inhibitors, iodoacetic acid and E-64 (cysteine proteases), pepstatin (aspartic proteases), and 1,10-phenanthroline (metalloproteases) were without appreciable effect. In short, the experiments with inhibitors indicated that thiocalsin is a serine protease.

DISCUSSION

The present results extend our knowledge of proteolytic enzymes. The enzyme described above, thiocalsin, appears to be representative of a new type of protease that depends on two well-known cellular agents, thioredoxin and calcium.

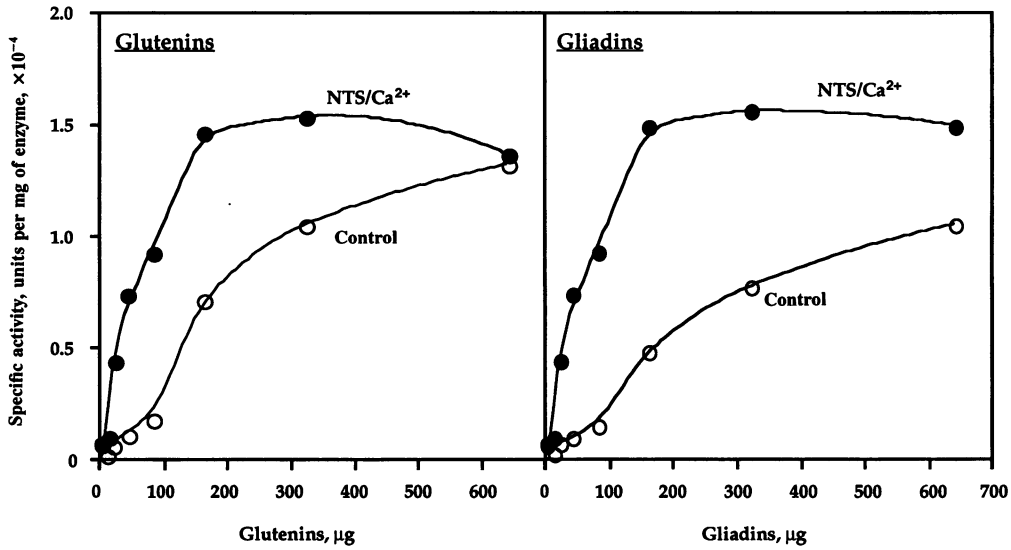

FIG. 4. Effect of glutenin and gliadin substrate concentration on the activity of thiocalsin before (control) and after activation by thioredoxin and calcium. Activity was measured by using a gliadin fraction or a glutenin fraction, both reduced by DTT/thioredoxin and alkylated by iodoacetate. As indicated, thiocalsin was untreated (control) or activated by preincubation with the NADP/thioredoxin system and 20 μM $CaCl_2$ (NTS/Ca^{2+}).

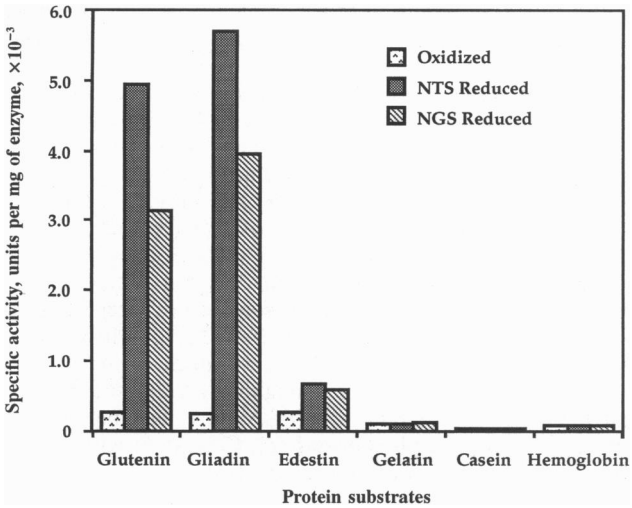

FIG. 5. Substrate specificity of thiocalsin. Activity was measured with enzyme activated by the NADP/thioredoxin system and 20 μM $CaCl_2$. Substrates were untreated (oxidized) or reduced, as indicated, with the NADP/thioredoxin system (NTS) or the NADP/glutathione system (NGS).

Certain proteases—e.g., calpain—are known to be activated by calcium, but none require thioredoxin. Similarly, while the activity of yet other proteases can be enhanced by thiols under certain conditions, none appear to be specific for thioredoxin. A specific dependency on thioredoxin thus distinguishes thiocalsin from cysteine-type proteases for which a variety of thiols (19), including thioredoxin (20), are able to restore activity following oxidative inactivation. A similar lack of thiol specificity differentiates thiocalsin from the kallikrein serine proteases (21). The joint requirement for thioredoxin and calcium thus seems to distinguish thiocalsin from proteases described so far.

The discovery of thiocalsin attests to the importance of using physiological substrates in studying protease activity. There are examples of proteases that degrade native storage proteins (22–24), but these enzymes are also active with heterologous protein substrates or modified oligopeptides. To our knowledge, none of the enzymes isolated so far is specific for indigenous proteins. In view of the current data, the use of heterologous substrates appears to be of limited value in relating regulatory events to proteolysis, events that may be critical to germination and seedling development. Its substrate specificity is consistent with the view that thiocalsin functions

Table 3. Effect of inhibitors on thiocalsin activity

Treatment	Concentration, mM	Relative activity, %	
		Glutenins	Gliadins
1. None		100	100
2. PMSF	1	41	48
3. 3,4-DCI	0.1	16	20
4. AEBSF	4	9	6
5. Iodoacetic acid	0.1	122	100
6. E-64	0.01	96	89
7. Pepstatin	0.001	111	93
8. 1,10-Phenanthroline	0.01	102	93

The protease activity is expressed as the percent of control (100%), when is reduced by NTS in presence of 20 μM Ca^{2+}, in absence of inhibitors. Activity of 100% corresponds to 4392 units per mg of enzyme when using 20 μg of glutenins and 5249 units per mg of enzyme when using 20 μg of gliadins, both substrates reduced by DTT/thioredoxin and alkylated by iodoacetate at pH 5.0 and 37°C. All mixtures were preincubated for 30 min at 37°C prior to addition of the substrate. The values were calculated from five different experiments. PMSF, phenylmethylsulfonyl fluoride; 3,4-DCI, 3,4-dichloroisocoumarin; and AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride.

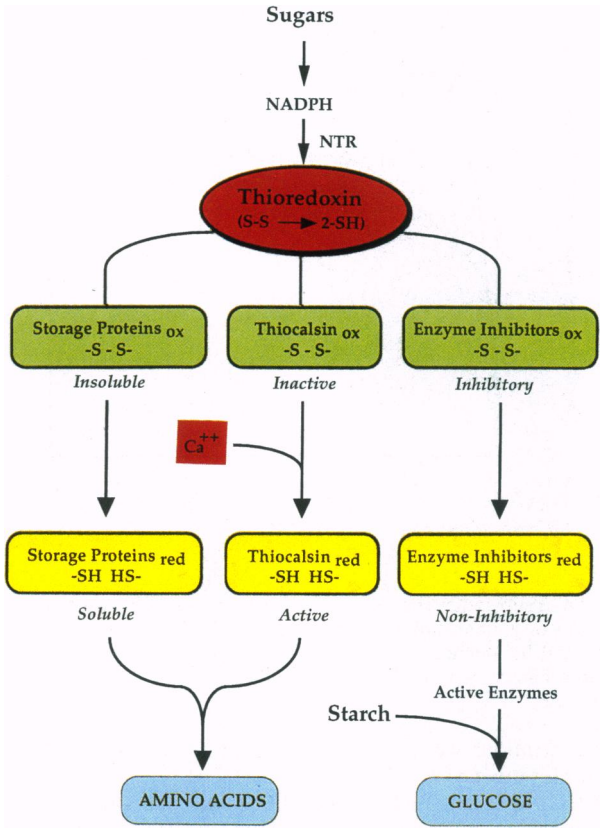

FIG. 6. Role of thioredoxin in the germination of wheat.

early in germination and seedling development for the hydrolysis of specific proteins (25), in a manner reminiscent to the barley aleurone cysteine proteases differentially regulated by giberellic acid (26), perhaps before large increases in the level of general (nonspecific) proteolytic activity take place (27, 28). The relative importance of thiocalsin to the other proteases functional at this time is a question for the future.

The present findings also add a new dimension to our understanding of the role of thioredoxin in germination. The results demonstrate that thioredoxin functions to enhance proteolysis through the activation of thiocalsin, in addition to its earlier identified function in reducing (solubilizing) storage proteins (4) and neutralizing enzyme inhibitor proteins (5–7) (Fig. 6). In the activation of thiocalsin, thioredoxin acts jointly with calcium, a signal known to promote the release of hydrolytic enzymes from the aleurone to the endosperm (29) and to serve as a cofactor for one of them, α-amylase (30).

In this newly found role, thioredoxin appears to function in a manner reminiscent of its mode of action in photosynthesis—i.e., in the reduction of a regulatory disulfide bond(s) on a target enzyme. Further, as with thiocalsin, thioredoxin-linked reductive activation is also enhanced by calcium with two enzymes of photosynthesis (chloroplast fructose bisphosphatase and sedoheptulose bisphosphatase) (31–33). It will be of interest to see whether the joint regulatory function of thioredoxin and calcium applies to other enzymes and proteins that bind calcium, including those containing a thioredoxin-like site (34, 35).

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