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Properties of Barley Seed Chitinases and Release of Embryo-Associated Isoforms during Early Stages of Imbibition[1]

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ABSTRACT

Barley (*Hordeum vulgare* L.) seeds contain at least five proteins with chitinase (CH) activity. Two of these (CH1 and CH2) are found primarily in the aleurone and endosperm tissues, and the other three (CH3, CH4, and CH5) are enriched in the embryo. From the bran fraction, three of these CHs (CH1, CH2, and CH3) were purified to apparent homogeneity. These three CHs have apparent molecular masses of 27, 34, and 35 kilodaltons and isoelectric points of 9.3, 9.2, and 8.7, respectively. CH2 and CH3 have amino terminal sequences resembling a portion of the chitin-binding domain of lectins and other plant defense proteins. CH1 lacks this domain. All three CHs exhibit antifungal activity and inhibit the mycelial growth of some species of *Trichoderma* and *Fusarium in vitro*. During the early period of imbibition by seeds, two of the embryo-associated CHs are selectively released into the surrounding aqueous medium.

CHs[2] (EC 3.2.1.14) catalyze the hydrolysis of β(1,4) linkages between *N*-acetylglucosamine (2-acetamido-2-deoxyglucopyranoside) residues in the linear homopolymer, chitin. They are widely distributed enzymes and are found in microorganisms, plants, and animals. A role for the enzymes in plant defense against fungal attack is suggested by the absence of chitin in higher plants (1), its presence in fungal cell walls (2), and the finding that plant CHs inhibit spore germination and mycelial growth of certain fungi *in vitro* (5, 21, 24). When a CH was used in combination with a β(1,3)-glucanase (15) or a ribosome-inactivating protein (13), the mycelial growth of a wider range of fungal genera was inhibited.

CHs have been isolated from tissues of many higher plants (5) including wheat and barley (*Hordeum vulgare* L.) seeds (8, 12, 18) and from barley leaves infected with fungi (10). They are constitutively expressed at low levels in leaves and at high levels in roots (25) and seeds (13). Increased levels of gene expression or enzymic activity have been observed in leaves after inoculation with fungi (22), bacteria (6), or viruses (33). The enzymes also can be induced by wounding (7) or by exposure to ethylene (4), fungal cell wall preparations (7), or abiotic elicitors such as salicylic acid and mercuric chloride (19). Not only CHs, but also β(1,3)-glucanases and certain other proteins accumulate to high levels following these treatments or pathogen attacks. Collectively, all of these proteins are called b-proteins or pathogenesis-related proteins (31). The rather large number of factors that trigger the accumulation of these proteins and the lack of a high degree of pathogen specificity in their induction imply that they are part of a general response of plants to stress. Their induction has been correlated with greater resistance to subsequent pathogen attack (31). These proteins may be partially responsible for a systemically acquired resistance (32). The induction of CHs and other defense-related proteins during flowering and seed germination may protect plant tissues from microbial attack during abrupt developmental changes (20).

During a previous study of genes expressed in the aleurone layer cells of germinating barley seeds, we identified a cDNA clone encoding a CH (29). Results of western blot analysis indicated the presence of at least two CHs in barley seeds. We have now analyzed the distribution of CHs in barley seed tissues in greater detail and have purified and partially characterized three distinct enzymes. One of these purified CHs is located predominantly in the embryo and is released from the seed very early during imbibition. The other two enzymes are more abundant in the aleurone and endosperm tissues.

MATERIALS AND METHODS

Plant Material

Barley (*Hordeum vulgare* L. cv Himalaya) seeds were obtained from the Agronomy Department, Washington State University. Barley plants were maintained in a growth chamber at 22 to 26°C with a 15/9-h light/dark cycle. Developing spikes were harvested after anthesis at intervals of 5 d up to 45 DPA. Seed tissues were dissected, immediately frozen in liquid nitrogen, and stored at −70°C.

For imbibition studies, whole seeds were surface sterilized by mixing with 70% (v/v) ethanol for 4 min on a magnetic stirrer. They were rinsed four times with water, once quickly and three times for 3 min on the magnetic stirrer. One hundred seeds were placed crease side down in 15- × 100-mm Petri dishes containing 5 mL of water and kept at 25°C. Samples that imbibed longer than 12 h were supplemented with an additional 5 mL of water. At each sampling time, all

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[2] Abbreviations: CH, chitinase; SPB, sodium phosphate buffer; DPA, days postanthesis; ATCC, American Type Culture Collection; SSC, standard saline citrate; IEF, isoelectric focusing.

water was removed, and volumes were recorded. Samples for western blot analysis were prepared by TCA precipitation of 2% of the volume of recovered material, which was equivalent to the protein released from two seeds.

Fungal Material

Cultures of *Trichoderma harzianum* (ATCC 52443) and *Trichoderma viride* (ATCC 52438) were obtained from the ATCC. All other fungi were obtained from Dr. John Leslie, Kansas State University.

Mycelial growth inhibition assays were done on carrot juice agar plates at 25°C (21). An agar cube containing mycelia was placed at the center. When colonies were about 3 cm in diameter, sterile paper discs were placed 1 mm from the growing front. Test solutions were applied to the discs in 10-μL volumes, and the plates were incubated until discs spotted with water or buffer were overgrown.

CH Purification by Chitin Affinity Chromatography

Barley bran (250 g) was the starting material for protein purification. The bran abraded by pearling of whole seeds consisted of all of the pericarp and embryo, most of the aleurone, and some of the endosperm. The bran was mixed with 25 mM SPB, pH 7.0, at 5 mL/g. After insoluble material was removed by centrifugation at 13,000*g*, the supernatant was fractionated by ammonium sulfate precipitation. The 30 to 60% fraction was dialyzed against SPB and mixed in a beaker with SPB-equilibrated chitin. Colloidal crab shell chitin used for this step was prepared as described previously (21). After 4 h of mixing, the chitin was washed three times with 30 mL of SPB, followed by three more washes with 30 mL of 25 mM sodium citrate, pH 4.0, in a beaker. The chitin was then placed in a column (1 × 20 cm), and bound material was eluted with 20 mM acetic acid, pH 3.2. Two CHs (CH2 and CH3) were eluted in a single peak and were subsequently resolved in a Bio-Rad Rotofor (ampholytes 8–10; Serva) according to the manufacturer's directions. Pooled fractions were dialyzed against SPB.

To isolate CH1, which did not bind to chitin under these conditions, the first two batch supernatants from the procedure described above were precipitated with ammonium sulfate (60%) and dialyzed against 50 mM sodium citrate, pH 4.5. One- to 2-milliliter aliquots (60 mg protein/mL) were applied to the chitin column equilibrated with the same buffer. Elution with 1 M NaCl in 10 mM Tris, pH 7.4, resulted in substantial purification of CH1. Pooled fractions containing CH were dialyzed against 50 mM Tris, pH 7.5, and loaded onto a carboxymethyl-cellulose (Sigma) column (1.5 × 30 cm) equilibrated with the same buffer. A step gradient from 0 to 80 mM NaCl at 10 mM intervals was applied. The putative CH, CH4, was recovered in the flow-through fractions from the carboxymethyl-cellulose column. CH1 was eluted with 60 mM NaCl. Pooled fractions were dialyzed against SPB. Protein concentration was determined with the Bio-Rad protein assay reagent with immunoglobulin G as the standard.

Amino Acid Sequencing

Thirty micrograms of CH1, CH2, and CH3 were sequenced with an Applied Biosystems model 477A sequencer by Dr. William Morgan, University of Missouri-Kansas City. Amino acid derivatives were identified by HPLC.

PAGE

SDS-PAGE was performed as described previously (27) using 10% polyacrylamide minigels (Hoefer model SE250) for immunoblotting and 12% gels (BRL model V16) for mol wt estimation. Proteins were stained with Coomassie brilliant blue R-250. Mol wt markers were from Sigma. Discontinuous native PAGE at low pH was performed according to the method of Blackshear (3).

Activity Gels

Activity gels containing glycol chitin were prepared and processed as described by Trudel and Asselin (30).

Immunoblotting

A Polyblot apparatus (American Bionetics) was used according to the manufacturer's directions to transfer proteins to nitrocellulose (Schleicher and Schuell). Blots were incubated with antibodies raised in rabbits against CH1 or a bean leaf CH. The bean leaf CH antiserum was a gift from Dr. Richard Broglie, Du Pont Co. The antigen-antibody complex was detected with a goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Fisher) using the horseradish peroxidase color development reagent (Bio-Rad).

Enzyme Assays

Tritiated chitin (670,000 cpm/mg) was prepared as described by Molano *et al.* (17). Each 100 μL of standard assay mixture contained 0.5 mg of chitin, 5 μmol of indicated buffer, and enzyme solution. Reactions were stopped after 1 h at 25°C by the addition of 300 μL of 10% (w/v) TCA. After centrifugation for 5 min at 6,000*g*, the tritiated chitin oligosaccharides contained in 200 μL of supernatant were measured. Specific activities were determined from initial velocities at 30°C according to the method of Boller *et al.* (4) and expressed as nanokatals per milligram of protein. One nanokatal equals 1 nmol of 2-acetamido-2-deoxy-D-glucopyranoside equivalents released per second.

Lysozyme activity was measured as described by Mauch *et al.* (14). β-*N*-acetylglucosaminidase activity was measured at pH 4.5 as described by Roberts and Selitrennikoff (21). Chitosanase activity was tested by substituting glycol chitosan (Sigma) for glycol chitin in substrate overlay gels.

pH Optimum Determination

The effect of pH on enzymatic activity was determined with the following buffers at 50 mM concentrations: HCl-KCl at pH 2; sodium citrate at pH 3 to 6; sodium phosphate at pH 7 to 8; glycine at pH 9 to 10; and trisodium phosphate at pH 11.

Isoelectric Point Determination

Samples were subjected to electrophoresis in IEF grade agarose (FMC) slab gels according to the manufacturer's instructions. IEF standards were from Sigma.

RNA Analysis

Total RNA was recovered from tissues ground to a powder in liquid nitrogen and extracted with 4 mL of 100 mM sodium glycinate (pH 8.0), 10 mM EDTA, 100 mM NaCl, 1% (w/v) SDS, and 0.2% (w/v) proteinase K. After the mixture was centrifuged for 10 min at 6000*g*, the supernatant was brought to 8 mL by the addition of 70% guanidine isothiocyanate, mixed with 2 g of CsCl, placed over a 2-mL cushion of 5.7 M CsCl and 100 mM EDTA, and centrifuged for 20 h at 174,000*g*. Pellets were dissolved in 10 mM Tris, pH 8.0, containing 1 mM EDTA. Aliquots of 10 μg were subjected to electrophoresis in a 1.2% agarose-formaldehyde gel and blotted onto nitrocellulose with 20× SSC. (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate.) The blot was hybridized for 16 h with insert DNA from a barley CH cDNA clone (29) that was labeled using a random primer, [^{32}P]dCTP, and the Klenow fragment of DNA polymerase I. The blot was washed twice for 40 min at 50°C in 2× SSC and 0.1% SDS, once for 40 min at 65°C in 0.1× SSC and 0.1% SDS, and then exposed to X-ray film for 58 h. RNA size markers were from BRL.

RESULTS

Tissue Distribution

The distribution of CHs within barley seeds was examined with substrate overlay gels after electrophoresis of extracts from pericarp, aleurone, endosperm, and embryo (including the scutellum) tissues dissected from imbibed, mature seeds. Crude extracts were subjected to discontinuous native PAGE at low pH, and the resulting gel was assayed for CH activity with a glycol chitin-containing overlay gel (Fig. 1). Two CHs, CH1 and CH2, accounted for most of the activity in the aleurone and endosperm tissues (lanes 3 and 4). A third relatively abundant CH, CH3, was located primarily in the embryo (lane 5). Two additional proteins that appeared to be CHs (CH4 and CH5) also were found primarily in the embryo. Little CH activity was detected in the pericarp extract (data not shown).

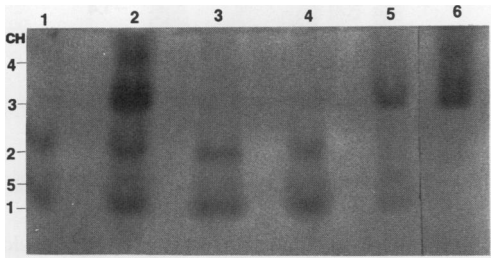

Figure 1. CH activity gel analysis of crude extracts of barley tissues. Lane 1, Flour (20 μg); lane 2, bran (60 μg); lane 3, aleurone (30 μg); lane 4, endosperm (30 μg); lane 5, embryo (90 μg); lane 6, protein released from one seed after 18 h of imbibition.

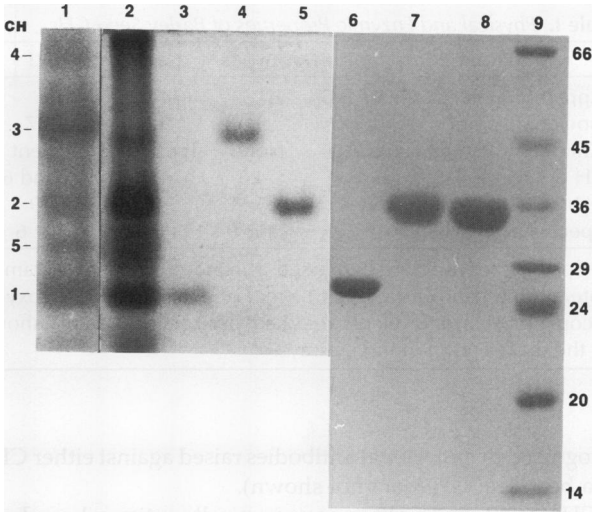

Figure 2. Native PAGE (lanes 1–5) and SDS-PAGE (lanes 6–9) of barley seed CHs. CHs are identified in the left margin. Lane 1, CH activity gel overlay of material in lane 2; lanes 2–9, stained with Coomassie blue; lane 2, 30 to 60% fraction of bran extract (450 μg); lane 3, CH1 (4 μg); lane 4, CH3 (4 μg); lane 5, CH2 (4 μg); lane 6, CH1 (10 μg); lane 7, CH3 (10 μg); lane 8, CH2 (10 μg); and lane 9, standard proteins with mol wts × 10^{-3} listed in right margin.

To determine the best milling fraction for use as a starting material for the purification of the CHs, mature barley seeds were pearled to remove the bran, which contained all of the embryo and pericarp, most of the aleurone layer, and some of the endosperm. The remaining polished seeds were milled into flour. Enzyme activity gel analysis of crude extracts of the flour and bran fractions showed that the former contained primarily CH1 and CH2 and that the latter had all five of the enzymes (Fig. 1, lanes 1 and 2). Therefore, the bran fraction instead of the whole seed was extracted as an initial enrichment step for purification of these enzymes.

CH Purification

A combination of purification methods, including chitin affinity chromatography and preparative scale IEF or cation exchange chromatography, was used to isolate CH1, CH2, and CH3 from bran extracts. The apparent homogeneity of these preparations was demonstrated by native PAGE and SDS-PAGE (Fig. 2). Yields of CH1, CH2, and CH3 were 90, 80, and 80 μg, respectively, per g of bran (equivalent to 20 mg of total extractable protein). A fourth putative CH, CH4, was partially purified but was not characterized further. CH5 was not purified. The apparent molecular masses of CH1, CH2, and CH3 were 27, 34, and 35 kD, respectively.

Physical and Enzymic Properties

Some of the properties of the three purified CHs are listed in Table I. Although CH2 and CH3 were similar in size, they differed in isoelectric point and in relative mobility under native PAGE conditions (Fig. 2, lanes 4 and 5). All of the enzymes were basic proteins with acidic pH optima and were

Table I. *Physical and Enzymic Properties of Barley Seed CHs*

	CH1	CH2	CH3
Apparent molecular mass (kD)	27	34	35
Isoelectric point	9.3	9.2	8.7
Chitin-binding domain	None	Present	Present
pH optimum	4.5	4	3.5 and 6
pH range for 50% activity	3.5–7	3–7	3–9
Specific activity[a]	94 ± 9	133 ± 12	70 ± 6

[a] Specific activities are expressed as nanokatals per milligram of protein. One nanokatal equals 1 nmol of 2-acetamido-2-deoxy-D-glucopyranoside equivalents released per second. Values shown are the means (±SD) of three assays.

recognized by polyclonal antibodies raised against either CH1 or a bean leaf CH (data not shown).

CH1, CH2, and CH3 were enzymically active when glycol chitin or tritiated chitin was used as a substrate, but they were inactive toward glycol chitosan (data not shown). A separate basic protein active toward glycol chitosan was detected in crude extracts of aleurone and endosperm tissues, but this chitosanase activity was not associated with any of the CHs and was not studied further. When a preparation of *Micrococcus lysodeikticus* cell walls was used as a substrate, all three of the CHs were found to exhibit a weak lysozyme-like activity. CH3 had the greatest lysozyme activity (5%) and CH1 the least (1%) on a per microgram basis, when compared to chicken egg white lysozyme (data not shown). These results indicated that the purified barley proteins are endochitinases.

The barley CHs also were tested for β-*N*-acetylglucosaminidase activity with the substrate analog *p*-nitrophenyl-β-D-*N,N'*-diacetylchitobiose (Sigma). Ten micrograms of each CH failed to release *p*-nitrophenol during 24-h incubations at 37°C. Molting fluid from *Manduca sexta* (tobacco hornworm), which contains a β-*N*-acetylglucosaminidase (9), was used as a positive control. Molting fluid samples containing 1.0 and 0.1 μg of total protein released *p*-nitrophenol within 1 and 24 h, respectively, under the same assay conditions. All of the results obtained with polysaccharide and oligosaccharide substrates indicated that the barley enzymes have a different mode of action from that of an exocleaving β-*N*-acetylglucosaminidase (21) and that they act primarily as endocleaving CHs with a greater specificity for a β(1–4) polymer of *N*-acetyl-D-glucosamine than for a β(1–4) copolymer of *N*-acetylglucosamine and *N*-acetylmuramic acid.

Antifungal Properties

Fungal growth assays on agar plates revealed that, at 1 μg/disc, all three CHs inhibited mycelial growth of *T. harzianum* (data not shown), a fungal saprophyte whose cell wall contains chitin (2). Greater inhibitions were seen with 10 μg. Nearly identical results were also obtained with *T. viride* and the pathogen, *Rhizoctonia solani*. Weak inhibition of the growth of *Fusarium culmorum* and *Fusarium graminearum* was also observed. None of these CHs slowed mycelial growth of *Pythium myriotylum*, an oomycete that lacks cell wall chitin (2).

CH mRNA Accumulation during Seed Development

A barley aleurone CH cDNA probe (29) was used for northern blot analysis of total RNA isolated from developing seed tissues (Fig. 3). Hybridizable mRNA was detected by 15 DPA in both the endosperm and aleurone and was present in both tissues through 40 DPA. The amount in whole seeds at 45 DPA was less than that observed at 40 DPA in either tissue alone, suggesting that a net loss of these messages occurred at the very end of seed development. CH mRNA was generally maintained at higher levels in the aleurone tissue than in the endosperm, except at 30 DPA, the time at which the mRNA level peaked in the latter tissue. Hybridizable mRNA was not detected in the developing embryo (data not shown).

CH Release during Imbibition

During imbibition by surface-sterilized barley seeds in deionized water, CH3 was released into the medium. CH3 was detected by immunoblotting as early as 2 h after the seeds were wetted (Fig. 4). In one experiment, 70 μg of CH3 was isolated by chitin affinity chromatography from the material released after 10 h of imbibition by 1000 seeds in

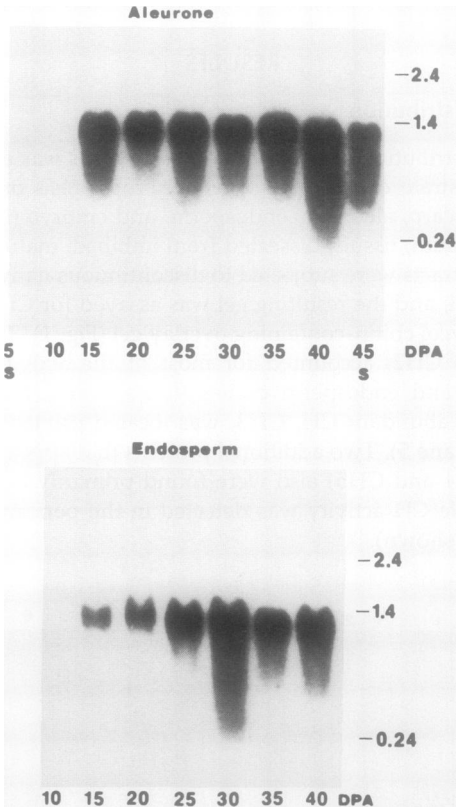

Figure 3. CH mRNA accumulation during seed development. Northern analysis of 10-μg aliquots of total RNA from developing aleurone (top) and endosperm (bottom) tissues. Developmental stages identified as DPA. Five- and 45-DPA samples were extracted from whole seeds (indicated by letter S). Mobilities of RNA markers and their sizes in kilobases are indicated in right margin.

water. CH1 appeared in the water by 24 h, by which time a large number of other proteins in addition to CH also had been released. Enzyme activity gel analysis of the leached proteins after 18 h of imbibition revealed the presence of CH3 as well as one of the other putative CHs (CH4) found primarily in the embryo (Fig. 1, lane 6). CH4 may be the additional immunoreactive band observed as early as 4 h after seed imbibition (Fig. 4).

Amino Acid Sequence Comparisons

Amino terminal sequences of CH1, CH2, and CH3 are compared with those of other plant CHs and lectins in Figure 5. CH1 and another barley CH, CHC (8), are identical for the first 10 residues at their amino termini. CH2 and CH3, on the other hand, are not similar to CH1 or CHC at their amino termini. Instead, they are identical with or similar to the amino termini of tobacco, barley, and bean CH, as well as wheat germ agglutinin, nettle lectin, and hevein (26, 28). A property common to these proteins is the ability to bind chitin.

CH 1	S V S S I V S R A Q
Chitinase C	S V S S I V S R A Q
CH 2	E Q X G S
CH 3	E Q X G S Q A G G A
Tobacco Ch	E Q C G S Q A G G A R C A S G L C C S K
Chitinase T	X * Q * * * * * * * T * P N X * * * * X
Bean Ch	* * * * R * * * * * L * P G * N * * * Q
WGA	K * * * * S * * K L * P N N * * * * Q

Figure 5. Amino terminal sequences of plant CHs and lectins. Barley CH C sequence is from Leah *et al.* (12). Barley CHs T and K sequences are from Kragh *et al.* (10, 11). Other sequences are from Stanford *et al.* (28). Stars indicate identity with tobacco CH sequence. WGA, Wheat germ agglutinin.

DISCUSSION

Two barley seed CHs, C and T, were described previously (8, 13). Both are basic proteins that are present in the aleurone and endosperm tissues and that act as endocleaving enzymes. A third CH, K, secreted by embryogenic cell suspensions of barley was described recently (11). In this report, we describe the isolation and partial characterization of three barley seed CHs, one of which (CH3) is located primarily in the embryo. Two other proteins that also have CH activity have been detected in the embryo, and one of these has been partially purified. All five of these proteins are present in the mature seed (Fig. 1).

CH1 and CH2 are localized primarily in aleurone and endosperm tissues. Those CHs appear to be similar if not identical in tissue localization, amino acid sequence, and physical properties to CHs C and T, respectively (11, 12).

CH3 is a CH relatively abundant in barley seeds that probably has not been reported previously. Although CH3 is like CH2 in size and amino terminal sequence, other properties distinguish it from CH2. CH3 is active over a broader pH range and has a lower isoelectric point of pH 8.7. CH3 has a lower specific activity than CH1 and CH2. Most important, CH3 is located predominantly in the embryo, whereas CH2 is found in the aleurone and endosperm. The properties of CH3 closely resemble those of the recently reported CH K from the medium of barley cell suspensions in culture (11). The barley embryo also contains two other putative CHs (CH4 and CH5) that have not been previously described.

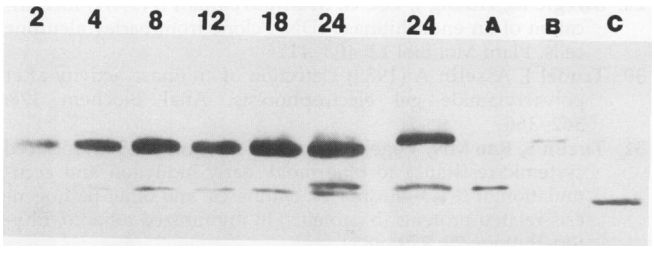

Figure 4. Immunoblot of proteins released during imbibition. Two seed equivalents per sample were treated with antibodies raised against CH1. Hours of imbibition are indicated at top. Lane A, CH1 (100 ng); lane B, CH3 (100 ng); lane C, sample containing partially purified CH4. The two 24-h samples are from two different experiments.

CH1, CH2, and CH3 digest tritiated chitin and glycol chitin, but they do not hydrolyze β-*N*-acetylglucosaminidase substrates. Like many plant CHs (5, 21, 24, 26), all three purified CHs inhibit the mycelial growth of several chitin-containing fungi *in vitro* and have basic isoelectric points. CH2 and CH3 have N-terminal lectin-like chitin-binding domain sequences and appear to represent class I CHs. CH1 and CHC lack this domain and are probably class II CHs (26).

Southern blot analyses indicate the presence of multiple CH genes in barley. Two reported barley CH cDNA clones, clone 10 (ref. 29) and cCHI26 (ref. 13), are derived from distinct genes, because the nucleotide sequences differ by 10% and their primary translation products differ in size. The translation product corresponding to clone 10 may be a precursor form for mature CH2 or CH3. Because the tissue distribution of transcripts detected by clone 10 (Fig. 3) is identical with that of CH2 in the mature seed (Fig. 1), it is likely that clone 10 encodes CH2 rather than CH3. Two segments of nine and 13 amino acids predicted from the clone 10 sequence match those determined directly from internal fragments of CH T, which is most closely related to CH2 (8, 11). Additional sequence information will be required to unequivocally identify the chitinase corresponding to clone 10.

Leah *et al.* (13) reported that the CHI26 gene is expressed only in aleurone cells of developing seeds. Our results showed that CH transcripts are present not only in aleurone cells but also in endosperm tissues during seed development (Fig. 3).

The CH released from seeds during imbibition, CH3, is localized primarily in the embryo from where it is likely,

released. The putative CH, CH4, also is released early during imbibition and is found primarily in the embryo (Fig. 1). This protein is detected in the material that is released as early as 4 h after imbibition (Fig. 4, *cf.* lanes 4–24 with lane C). The chitin-binding lectin, wheat germ agglutinin, is released from wheat embryos during the first hour of imbibition (16). CHs thus represent a second class of proteins that are released from embryos of grass seeds during the early stages of imbibition and interact with chitin.

Seed CHs presumably help protect seeds and seedlings from pathogenic fungi before and during germination. The significance of CH release during imbibition is not known. In nature, these enzymes would be released into the soil where they could contact soil-borne fungi, possibly causing direct inhibition of mycelial growth or the release of chitin fragments that may act as signals to elicit other plant defense responses (23). Further study is necessary to unequivocally define the actual physiological role of the seed CHs in plant development.

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