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Purification and Identification of Barley (*Hordeum vulgare* L.) Proteins That Inhibit the Alkaline Serine Proteinases of *Fusarium culmorum*

ANJA I. PEKKARINEN*,†,‡ AND BERNE L. JONES†,§

Department of Agronomy, University of Wisconsin, Madison, Wisconsin 53706, VTT Biotechnology, P.O. Box 1500, FIN-02044 VTT, Finland, and Cereal Crops Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Madison, Wisconsin 53726

It has been proposed that microbial proteinase inhibitors, which are present in abundance in cereal grains, protect the seed against plant pathogens. So far, however, very little is known about the interactions of those inhibitors with the proteinases of phytopathogenic microbes. The increased alkaline proteinase activities of Fusarium head blight (FHB) diseased wheat and barley grain imply that the *Fusarium* fungi synthesize those enzymes during the colonization of the kernel. To study which barley proteins can inhibit *Fusarium* proteinases, and hence, possibly protect the seed from FHB, the proteins of a grain extract have been separated and tested for their abilities to inhibit two alkaline serine proteinases that we previously isolated from *F. culmorum*. The proteins were separated by size exclusion, ion exchange, and reversed-phase-HPLC chromatographies. The purified inhibitors were identified by their molecular masses and N-terminal amino acid sequences. The proteins that inhibited the subtilisin-like *Fusarium* proteinase were the chymotrypsin/subtilisin (CI) inhibitors 1A, 1B, and 2A and the barley α-amylase/subtilisin inhibitor (BASI). Only one of the purified proteins inhibited the trypsin-like proteinase, the barley Bowman−Birk inhibitor (BBBI). No novel inhibitors were detected.

KEYWORDS: ***Fusarium*; phytopathogen; proteinase; inhibitor; barley (*Hordeum vulgare* L.)**

INTRODUCTION

Cereal seeds contain serine proteinase inhibitors that may defend them against pathogens and pests (*1* and the references therein). These proteins have generally been detected and characterized by their abilities to affect bovine trypsin or α-chymotrypsin, bacterial subtilisin, or the *Aspergillus oryzae* proteinase (oryzin). However, their interactions with the enzymes of plant pathogenic fungi have scarcely been studied (*2*−*4*). To establish whether the proteinase inhibitors play any role in seed protection, it is important to identify the proteins that inhibit the plant pathogen enzymes that occur in infected grain and to then characterize their interactions in vitro.

The proteinase inhibitors that may protect the cereal seeds against proteolysis by pathogenic microbes are the Bowman−Birk type trypsin inhibitors (*5*), the α-amylase/subtilisin inhibitors (*6*, *7*), and the chymotrypsin/subtilisin inhibitors (*8*, *9*), all of which have been shown to inhibit microbial proteinases. In addition, a trypsin/α-amylase inhibitor from barley had antifungal properties (*10*), but it did not inhibit certain microbial proteinases (*11*). The Bowman−Birk inhibitor from barley embryos (BBBI) strongly inhibited the bovine and microbial (Pronase) trypsins and, to a lesser extent, bovine chymotrypsin (*5*). Proteins that are closely related to BBBI have been isolated from wheat, rice, and several other grasses and legumes (*1*, *12*), and an immunologically cross-reacting protein has been detected in rye (*5*). The bifunctional α-amylase/subtilisin inhibitor strongly inhibited bacterial subtilisin and weakly some fungal (*Aspergillus* spp.) proteinases (*6*). It did not inhibit bovine chymotrypsin or trypsin (*7*), even though it belongs to the Kunitz trypsin inhibitor family (*13*). Similar inhibitors occur in several other cereals (*1*, *14*−*16*). These proteins inhibit endogenous seed α-amylases, but their inhibition of microbial amylases has not been detected. CI-1 and CI-2, which belong to the potato inhibitor I family (*17*, *18*), inhibit the activities of chymotrypsin, elastase, bacterial subtilisin and certain other microbial proteinases (*8*, *9*, *19*). An inhibitor that is very similar to CI-2A is present in wheat grain endosperm (*9*, *20*).

Fusarium head blight (FHB, scab) of wheat and barley is a worldwide problem in agriculture. This disease has caused drastic yield and quality losses in both of these cereals, especially during the past decade in North America (*21* and the references therein). *Fusarium graminearum* and *F. culmorum*, two fungal pathogens that cause the disease FHB, both produced alkaline proteinases in infected kernels (*22*, *23*). It is likely that these proteinases function in the grain to provide nutrients for

* Corresponding author. Phone: +358-9-456-4461. Fax: +358-9-455-2103. E-mail: anja.pekkarinen@vtt.fi.
† University of Wisconsin.
‡ VTT Biotechnology.
§ U.S. Department of Agriculture.

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the growing mycelium, so it is possible that inhibitors that affect these proteinases could restrict the fungal growth in the kernel. Previously, we have purified and characterized the two alkaline serine proteinases that are synthesized by *F. culmorum* in the presence of grain proteins and in infected grain (*23*−*25*). The aim of this study was to determine which barley proteins, if any, can inhibit these *F. culmorum* serine proteinases.

MATERIALS AND METHODS

Purification of the Inhibitors. *Extraction and Size-exclusion chromatography.* Barley (*Hordeum vulgare* L. cv. Morex) grain was ground with a Retsch ZM1 (Brinkmann, Haan, Germany) mill to pass a 0.5 mm screen. An extract was prepared by mixing 30 g of the ground grain with 90 mL of 50 mM NH_4 acetate, pH 5.0, buffer for 30 min at 22 °C. The mixture was then centrifuged at 12,000 ***g*** for 10 min at 5 °C, and the supernatant was filtered through six layers of cheesecloth, divided into 20 mL aliquots, and stored at −20 °C until used.

An aliquot of the barley extract was thawed, centrifuged as above, and 15 mL of the supernatant was applied to a 2.5 × 64 cm Bio-Gel P-30 (Bio-Rad, Hercules, CA) size exclusion column that had been equilibrated with pH 5.0, 50 mM, NH_4 acetate buffer. The column was eluted with the same buffer, and the absorbances of the collected 5.2-mL fractions were measured at 280 nm. The fractions were tested for their abilities to inhibit the previously described (*24*, *25*) *F. culmorum* subtilisin-like (SL) and trypsin-like (TL) proteinases using the assay described below. The fractions that inhibited were combined into two pools, F18−23 and F24−31. The corresponding fractions from three identical separations were combined and diluted 2.5-fold for cation exchange chromatography.

Ion Exchange Chromatography. The inhibitors from each of the size exclusion column pools were applied to a 1 × 8 cm carboxymethyl cellulose (CMC, CM52, Whatman, Maidstone, U.K.) column that was equilibrated with 20 mM NH_4 acetate, pH 5.0. The same buffer was used to wash any unbound proteins from the column. For both pools, the proteins were first separated with a linear gradient that ran from 20 to 150 mM, pH 5.0, NH_4 acetate (130 mL of each buffer). When the proteins of the F24−31 pool were being purified, a second linear gradient that ran from 150 to 500 mM buffer (100 mL of each) was applied to the column after the 20−150 mM one had finished.

The majority of the inhibitors in the F18−23 pool did not bind to the CMC column, and this unbound protein was collected and subjected to anion exchange chromatography. The pH of the sample was adjusted to 8.3 with NH_4OH, and it was applied to a 1 × 7.5 cm quaternaryammonium cellulose (QAC, QA52, Whatman) column that had been equilibrated with 20 mM NH_4HCO_3, pH 8.5. The inhibitors were eluted with a linear, pH 8.5, NH_4HCO_3 buffer gradient that ran from 20 to 200 mM (150 mL of each). During all of the ion exchange steps, 4.2-mL fractions were collected and their inhibitory activities were tested as described below. The fractions that contained inhibitors were pooled and stored at −20 °C.

Reversed-Phase-HPLC Separation. The inhibitors were further purified by reversed-phase (RP)-HPLC on a Zorbax SB−C18 4.6 mm × 7.5 cm column (MAC-MOD Analytical, Inc., Chadds Ford, PA) that was eluted with gradients made with water and acetonitrile, each of which contained 0.1% TFA. The thawed samples were filtered through PTFE 0.45 μm filters and 2−10-mL aliquots were applied to the column, which was washed with 2 mL of the equilibration buffer at a flowrate of 1 mL/min. The linear water-acetonitrile gradients differed slightly and were between 25% and 50% as shown in **Figure 3**. The 280 nm-absorbing fractions that eluted were collected, freeze-dried twice, redissolved in 300 or 500 μL of water, and their inhibitory activities were tested. When necessary, the RP-HPLC step was repeated.

Inhibitor Assay. The enzymes that were used to detect the inhibitors during the chromatograpy steps were purified from a *F. culmorum* culture medium preparation by CMC, as described previously (*24*). The enzyme preparation was diluted with pH 5.0, 50 mM, NH_4 acetate buffer to make solutions that contained approximately 270 nM SL or 180 nM TL proteinase. These concentrations yielded activities of about 5 nkat/mL of sample under the assay conditions. When testing fractions that inhibited the TL proteinase, 63 μg/mL of bovine serum albumin (BSA, Pierce, Rockford, IL) was added to the enzyme preparation to stabilize its activity. A 10 μL sample of each fraction or of 50 mM NH_4 acetate buffer (control) was mixed with 40 μL of each enzyme solution and incubated on ice for 30 min. The SL or TL activities of these mixtures were then measured at 28 °C with 3 min assays, as described previously (*24*). The substrate solutions used were 5.0 mM *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide for the SL and 0.5 mM *N*-benzoyl-Val-Gly-Arg *p*NA for the TL. The enzymes and inhibitors were diluted 10-fold when mixed with the substrate solution. The substrates were dissolved in 175 mM Na citrate, pH 6.0, that contained 4% dimethyl sulfoxide (DMSO). The chromatography fractions were diluted, when necessary, with the NH_4 acetate buffer.

SDS−PAGE. Samples of the RP-HPLC separated inhibitor preparations were separated with High-Density PhastGels (Amersham Pharmacia Biotech, Uppsala, Sweden) under denaturing and reducing conditions according to manufacturer's instructions. A mixture of trypsinogen (24.0 kDa), lysozyme (14.3 kDa), cythochrome c (12.4 kDa) and aprotinin (6.5 kDa), all purchased from Sigma, was used for calibrating the gels. The gels were stained with 0.1% Coomassie Brilliant Blue R-250 (Sigma) dissolved in 40% methanol and 1% acetic acid and destained in the same solution without CBB.

Protein Identification. The molecular masses of the purified proteins were determined by MALDI-TOF mass spectrometry on a Bruker Biflex III (Bruker Daltonics, Billerica, MA) instrument at the University of Wisconsin Biotechnology Center, Madison, WI. The N-terminal amino acid sequences of the proteins were analyzed using the Edman degradation method with an ABI 420/H Amino Acid Analysis System at the Protein Chemistry Laboratory of the University of Texas Medical Branch Cancer Center, Galveston, TX.

A Comparative Measurement of the Inhibition Activities. The purified inhibitor preparations were filtered through 0.45 μM PTFE filters, and their absorbances were measured at 280 nm. The extinction coefficients (ϵ_{280}) of the inhibitors were estimated from their tyrosine (Tyr) and tryptophan (Trp) compositions (ϵ, $mM^{-1}cm^{-1} = 1.4 \times nTyr + 5.6 \times nTrp$) (26). To compare the abilities of the various inhibitors to inactivate the *F. culmorum* proteinases, the inhibitor samples were diluted with water to concentrations between 0.2 and 6.6 μM. To relate the inhibition due to the purified barley inhibitors with that of commercial class-specific inhibitors, 0.2−1.4 μM of chymostatin (Sigma) or 1−10 μM of soybean Bowman−Birk inhibitor (Sigma) solutions were prepared in water (the chymostatin solutions contained less than 0.01% DMSO). The *F. culmorum* proteinases were purified by cation exchange-HPLC (*24*). The enzyme concentrations were calculated from their approximate k_{cat} values (33 s^{-1} for the SL and 45 s^{-1} for the TL enzyme) after their maximal velocities were measured (*24*, *25*). Twenty-microliter samples of each inhibitor solution were incubated on ice with either 200 nM SL or 90 nM TL proteinase in a total volume of 50 μL. All of the incubation mixtures contained 50 μg/mL BSA to stabilize the enzymes. The activities were measured as above, after 30 and 40 min of incubation.

RESULTS

Separation of the *Fusarium* Proteinase Inhibitors. A schematic diagram of the purification procedure is presented in **Figure 1**. A Bio-Gel P-30 size exclusion column separation resolved the SL proteinase inhibitors into two fractions that were denominated F18−23 and F24−31 (**Figure 2**). The TL proteinase inhibitor(s) eluted together with the second SL-inhibitor fraction. The majority of the inhibitors in F18−23 did not bind to a cation exchange column at pH 5.0 (chromatogram not shown), but four inhibitory fractions were separated from F18−23 by anion exchange chromatography at pH 8.5 (fractions A−D of **Figure 3A**). Application of the 20 mM buffer wash lowered the elution pH to ∼6.2, which eluted the fraction A inhibitors. The other inhibitors eluted at buffer (pH 8.5 NH_4HCO_3) concentrations of 85, 105, and 130 mM. The RP-HPLC separations of the ion exchange fractions B and C are shown in **Figure 4A**,**B**. The proteins that eluted in the peaks indicated

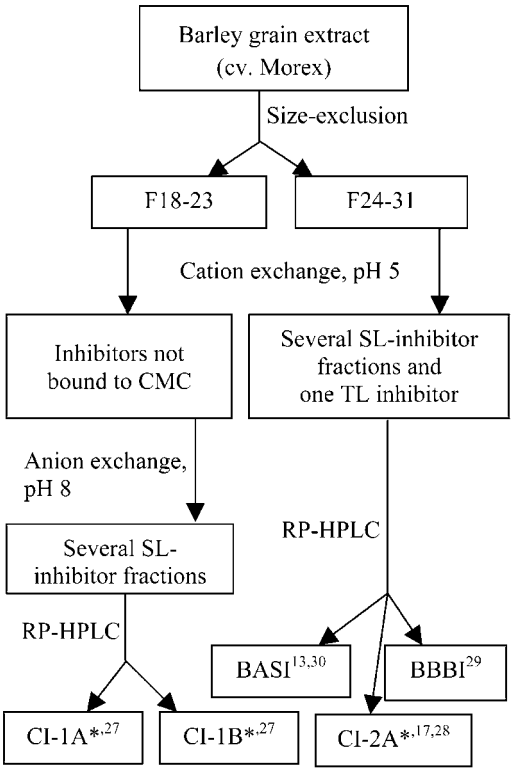

Figure 1. A schematic diagram of the inhibitor purification procedures. The chromatography methods are indicated beside the arrows at each step. The inhibitors that occurred in multiple size-forms are indicated with an asterisk (*), and the superscript numbers refer to the original articles in the list of references.

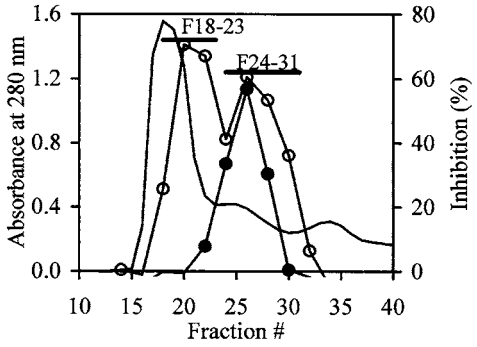

Figure 2. A Bio-Gel P-30 size exclusion separation of barley extract proteinase inhibitors. A 15 mL sample of barley extract was applied to a 2.5 × 64-cm column, and the eluted proteins were collected as 5.2-mL fractions. (—) Protein, absorbance at 280 nm, (○) inhibition of the SL proteinase activity (fractions 14–36 were diluted 2-fold before the analysis), and (●) inhibition of the TL proteinase.

by the heavy lines all inhibited the SL enzyme and were purified further by repeating the RP-HPLC step (not shown). When the components of the pool F24−31 fraction were separated with cation exchange chromatography, the SL inhibitors eluted at buffer concentrations of 50, 75, 110, and 370 mM (fractions E−H, **Figure 3B**), and the single TL proteinase inhibitor eluted at 400 mM buffer (I). The RP-HPLC separations of the proteins in fractions E, F, and I are shown in **Figure 4C−E**, respectively. The RP-HPLC separated fractions were called barley inhibitor number *n* (BI_n) and were numbered in the order of elution, as indicated on each chromatogram.

Identifying the Inhibitors. The masses of all of the RP-HPLC fraction components (BI_n) that inhibited either SL or TL

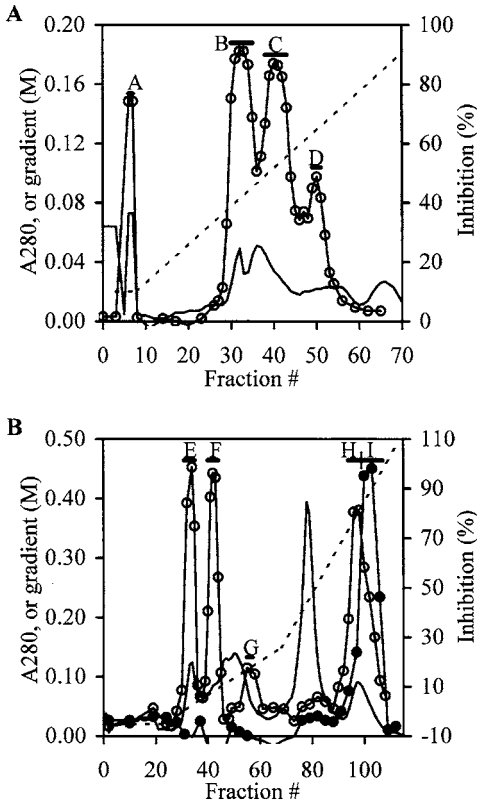

Figure 3. Ion exchange separations of barley proteins that inhibit *Fusarium* proteinases. (—) Protein, absorbance at 280 nm; (---) molarity of the NH_4HCO_3 gradient; (○) inhibition of the SL proteinase activity; and (●) inhibition of the TL proteinase. (A) Anion exchange chromatography with QAC at pH 8.5. Fractions 1–5 and 6–7 contained the nonbound sample and a 20mM NH_4HCO_3 wash, respectively. (B) Cation exchange chromatography at pH 5.0. Fractions 1–22 contained the nonbound sample and a 20mM NH_4 acetate wash. Fractions, 4.2 mL each, were collected, pooled as indicated by the bars, and designated as A–I.

proteinases were measured by MALDI-TOF mass spectrometry (**Table 1**), and the N-terminal amino acid sequences of selected inhibitor proteins were analyzed and compared with those stored in the SWISS-PROT database (**Table 2**).

The QAC-Separated Inhibitors. Fraction B contained several inhibitor forms that separated with RP-HPLC (**Figure 4A**). The molecular mass and N-terminal amino acid sequence of BI_1 indicated that it was a fragment of CI-1A whose N-terminus started with residue Lys_{10} (*27*). The mass of BI_2 was identical to that of the complete molecule of CI-1A (*27*). However, no amino acid sequence data was obtained from this fraction, because its N-terminus was apparently blocked. The mass spectrometric data indicated that the BI_2 preparation was homogeneous, but it still gave two bands, of 5.7 and 7.3 kDa, on an SDS−PAGE gel (**Figure 5**, lane 2). The two major proteins in BI_3 were CI-1B protein fragments whose N-termini started with the residues Lys_{10} and Gly_6 (**Tables 1** and **2**) (*27*). Small amounts of other proteins whose masses corresponded to various CI-1B fragments were also present. BI_4 contained a major protein with a mass of 8657 Da and traces of other proteins. Because preliminary studies indicated that the 8657 Da protein might be N-terminally blocked, it was digested with trypsin and the amino acid sequences of two of the resulting peptides were analyzed. Both of these peptides had sequences that were identical with portions of the CI-1B molecule. On the basis of its molecular mass, this protein was apparently a CI-1B fragment that started with Ser_3.

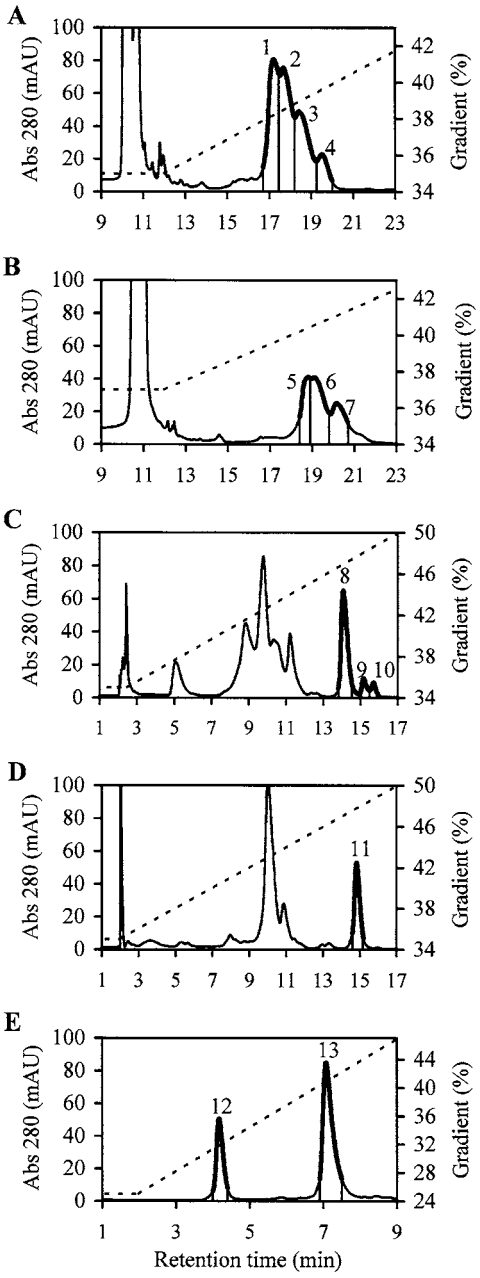

Figure 4. Reversed-phase-HPLC separations of the *Fusarium* proteinase inhibitors. (A and B) QAC pools B and C; (C–E) CMC pools E, F, and I, respectively. (—) Absorbance at 280 nm; (---) % acetonitrile in gradient. The inhibitor fractions are indicated by the heavier lines. These were individually collected and designated BI_n.

Inhibitors from the QAC-fraction C were separated into three peaks with the RP-HPLC (**Figure 4B**). BI_5 and BI_6 both contained fragments of CI-1A, the most prevalent of which started with residues Lys_{10} and Tyr_{11} (**Tables 1** and **2**). The masses of the proteins that were present in BI_7 corresponded to fragments of CI-1B that started with Glu_5 and Lys_{10}. The RP-HPLC separations of QAC-pools A and D (not shown) were very similar to those of B and C. The molecular masses of the major proteins from QAC A were 7957, 7984, and 8325 Da, indicating that they were various forms of CI-1A and -1B that also occurred in BI_1 and BI_3. The molecular mass (8456 Da) and N-terminal amino acid sequence of the inhibitor in QAC fraction D indicated that it was CI-1B that started with Glu_5 (not shown).

Table 1. The Molecular Masses of the RP-HPLC-Separated Inhibitors (BI_n), as Measured by MALDI-TOF Mass Spectrometry (M_d)[a]

BI_n	M_d (Da)	M_c (Da)	$inhibitor_{aa}$
1	7963	7960	$CI\text{-}1A_{10}$
2	8795	8882[b]	$CI\text{-}1A_1$
3	7990	7988	$CI\text{-}1B_{10}$
	8331	8329	$CI\text{-}1B_6$
	8186	8185	$CI\text{-}1B_8$
	8273	8272	$CI\text{-}1B_7$
4	8657	8676	$CI\text{-}1B_3$
5	7961	7960	$CI\text{-}1A_{10}$
	8447	8446	$CI\text{-}1A_5$
6	7831	7832	$CI\text{-}1A_{11}$
	8793	8882[b]	$CI\text{-}1A_1$
7	8458	8458	$CI\text{-}1B_5$
	7989	7988	$CI\text{-}1B_{10}$
	8328	8329	$CI\text{-}1B_6$
8	9291	9250[c]	$CI\text{-}2A_1$
9	7399	7400	$CI\text{-}2A_{19}$
	7285	7286	$CI\text{-}2A_{20}$
	9290	9250[c]	$CI\text{-}2A_1$
10	7397	7400	$CI\text{-}2A_{19}$
11	7865	7865	$CI\text{-}2A_{15}$
12	13851	13823	$BBBI_1$
13	19886	19879	$BASI_{23}$

[a] The calculated masses (M_c) were acquired from the SwissProt database. The number listed after the inhibitor name indicates the amino acid (aa) located at the N-terminus of each protein. [b] Reported by Williamson et al., 1988, as 8790 Da. [c] The mass of the protein with N-terminus Ser_1. The calculated mass of the protein that begins at the Met residue is 9380 Da (Williamson et al., 1987).

Table 2. The N-Terminal Amino Acid Sequences of Selected Purified Inhibitors (BI_n)[a]

BI_n	amino acid sequence	inhibitor
1	(10)KYPEPTEGSIGASSAKTSWPEVVGMSAEK	CI-1A
3	(10)KYPEPTEGSIGASGAKRSWPEVVGM	CI-1B
	(6)GSVPKYPEPTEG	
4	(27)SWPEVVGMSAEK	CI-1B
	(46)DKPDAQIEVIPVDAMVPLDF	
6	(11)YPEPTXGSIXAXSAKT	CI-1A
9	(20)LKTEXPELVGK	CI-2A
	(19)NLKTEXPELVGK	
10	(19)NLKTEWPELVGKSVEEAKKVILQDKPEAQIIVLPVGTIVT	CI-2A
11	(15)GDRHNLKTEWPELVGKSVEEAKKVILQDKPEAQIIVLP	CI-2A
12	(1)AGKKRPWKXXDQAVXTRSIPPIXT	BBBI
13	(23)ADPPPVHDTDGHELRADANY	BASI

[a] Two sequences were sometimes observed that started from different locations within a single protein. The residue number of the N-terminal amino acid, as listed in SwissProt, is indicated in brackets.

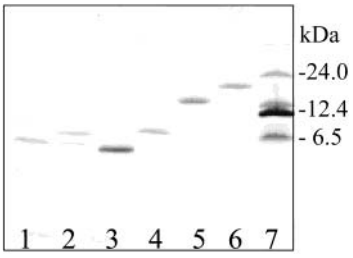

Figure 5. SDS–PAGE separations of selected purified inhibitors. Lane 1, BI_1, $CI\text{-}1A_{10-83}$ (0.6 μg); lane 2, BI_2, $CI\text{-}1A_{1-83}$ (1 μg); lane 3, BI_{11}, $CI\text{-}2A_{15-83}$ (0.8 μg); lane 4, BI_8, $CI\text{-}2A_{1-83}$ (0.3 μg); lane 5, BI_{12}, $BBBI_{1-124}$ (0.7 μg); lane 6, BI_{13}, $BASI_{23-203}$ (0.8 μg); and lane 7, molecular weight standard.

CMC-Separated Inhibitors. As shown in **Figure 4C**,**D**, the major inhibitors in fractions E and F eluted from the RP-HPLC column at essentially the same positions. BI_8 and BI_{11} each

contained single proteins, whose molecular masses corresponded to a complete molecule (BI_8) and a fragment (BI_{11}) of CI-2A (**Table 1**). As shown in **Table 2**, its N-terminal amino acid sequence analysis confirmed that BI_{11} was identical to CI-2A, starting from residue Gly_{15} (*17*, *28*). As found previously with BI_2, the Edman N-terminal sequence analysis of BI_8 was not successful, indicating that its N-terminus was blocked (see ref. *17*). The Ser_1 in the N-terminus of CI-2A may be acetylated, because the observed molecular mass of BI_8 was about 40 kDa higher than the calculated mass of CI-2A (**Table 1**). The molecular masses of BI_{11} and BI_8 on SDS−PAGE analysis were 5.2 and 7.3 kDa, respectively (**Figure 5**, lanes 3 and 4). The minor fractions, BI_9 and BI_{10}, contained CI-2A fragments that started with residues Leu_{20} and Asn_{19}, respectively, (**Tables 1** and **2**). The proteins of the CMC fraction G were also separated with RP-HPLC (not shown), and the purified preparations contained small amounts of proteins with molecular masses of 8849 and 7693 kDa, which corresponded to CI-2A fragments that began with residues Lys_5 and Arg_{17}, respectively.

Because the last two fractions, H and I, overlapped (**Figure 3B**) and both contained the same inhibitors, although in different proportions, only the I RP-HPLC separation is shown (**Figure 4E**). The molecular masses and amino acid sequence data for BI_{12} and BI_{13} showed that they were comprised of BBBI (*29*) and BASI (*13*, *30*), respectively. Their molecular sizes, when analyzed with SDS−PAGE, were 14.2 and 19.5 kDa respectively (**Figure 5**, lanes 5 and 6).

Inhibitor Yields. After the compositions of each of the inhibitor fractions were determined, their yields were calculated, using the Lambert−Beer Law. The absorbance of each sample was measured and its concentration was calculated from its molar extinction coefficient (which in turn was computed from its Tyr + Trp composition, as determined from its published amino acid sequence). When the amounts of all of the CI-1 fractions (homogeneous samples or mixtures) were totaled, their yield was ~8 μg per g of ground grain. Nearly equal amounts of CI-1A and -1B were recovered, as would be expected on the basis of their respective mRNA levels in the developing endosperm (*27*). CI-1B occurred as several different fragments while the majority of the CI-1A was present in two forms that started with the residues Met_1 or Lys_{10}. BASI, BBBI, and the major forms of CI-2A yielded 35, 13, and 18 μg per g of ground grain, respectively.

Comparison of the Inhibitory Activities. *Inhibitors of the SL Proteinase.* To determine which proteins were the most potent inhibitors of the *Fusarium* proteinases, enzyme assays were carried out in the presence of varying concentrations of the purified inhibitors. The inhibition of ~20 nM SL proteinase was carried out with chymostatin, BASI, and the different CI-1A and CI-2A forms at concentrations that varied from 4 to ~250 nM (**Figure 6A**). BASI and CI-2A were equally strong inhibitors and inactivated the enzyme almost totally at concentrations of 40−60 nM, while over 200 nM solutions of CI-1A were required to reach 90% inhibition. The effect of CI-1A was similar to that of chymostatin, which is a class specific inhibitor of chymotrypsin and subtilisin. The abilities of the CI-1A and 2A proteins to inhibit the SL proteinase differed only little, whether they were complete proteins or fragments whose structures started at residues Lys_{10} or Gly_{15}.

The TL Proteinase Inhibitors. The abilities of 3−200 nM concentrations of purified BBBI and 40−200 nM soybean Bowman−Birk inhibitor to inhibit 9 nM solutions of the TL proteinase were measured. As shown in **Figure 6B**, an approximately 3-fold ratio of BBBI to proteinase inhibited 90% of the TL activity, while the effect of the 8-kDa soybean inhibitor was much weaker. The soybean inhibitor never inhibited more than ~80%, even when its concentration was increased to 400 nM (not shown).

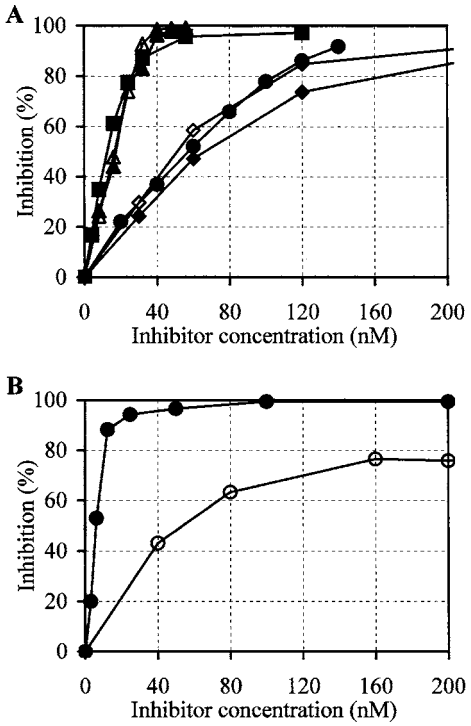

Figure 6. The inhibition of the *Fusarium* proteinases by the various inhibitors. (A) SL enzyme (20 nM in the reaction mixture) inhibited with CI-$1A_{1-83}$ (◆), CI-$1A_{10-83}$ (◇), CI-$2A_{1-83}$ (▲), CI-$2A_{15-83}$ (△); BASI (■, and chymostatin (●). (B) TL enzyme (9 nM) inhibited with BBBI (●) or soybean Bowman–Birk inhibitor (○).

DISCUSSION

Two *F. culmorum* alkaline serine proteinase activities were used to monitor the HPLC and open column chromatographic separations of a barley protein extract in order to identify the various inhibitors of these enzymes that it contained. The SL proteinase activity was inhibited by the proteins CI-1, CI-2, and BASI. All of these proteins have been previously shown to inhibit proteinases from various nonpathogenic microbes (*2*, *6*, *9*, *19*, *31*). Mikola and Suolinna (*2*) showed that an alkaline proteinase inhibitor, which was later called CI-1 (*8*), inhibited a proteinase from the phytopathogen *Alternaria tenuissima*. To our knowledge, the interaction of either CI-2A or BASI with a plant pathogen proteinase has never been demonstrated previously. Only a single protein, BBBI, inhibited the TL proteinase. This inhibition may be related to that in which a trypsin inhibitor from kidney bean (*Phaseolus vulgaris*) inactivated the trypsin-like activity of *Fusarium solani* (*32*). None of the endosperm trypsin/α-amylase inhibitors, also called CM-proteins (*33*), were detected, implying that these proteins are not strong inhibitors of the *Fusarium* proteinases. Although these are called chloroform-methanol soluble proteins, they have also been purified from acidified aqueous solutions (*11*), so it seems likely that they would have been detected if they inhibited the *Fusarium* enzymes.

Various methods for purifying all of these inhibitors have been described previously (*2*, *5*−*8*, *11*, *18*, *29*). Using the *Fusarium* proteinases to monitor the purification procedures allowed us to screen and collect all of the possible inhibitors

from the entire buffer-soluble barley protein fraction. No completely novel inhibitors were detected during this study, indicating that it is unlikely that there are other inhibitor classes that play a part in controlling the activities of these *Fusarium* proteinases. It also allowed us to purify several inhibitors at the same time. Pure protein preparations were obtained for all of the inhibitors except CI-1B, whose various fragments were particularly difficult to separate. However, even when the fragments were not separated, only very small amounts of other proteins were present in those fractions. A third homologous CI-1 protein, CI-1C, has been co-purified with the other two CI inhibitors by others (*18*), but it was neither recovered or detected during this study. Either the method does not work for this protein or there was little or no CI-1C protein in Morex barley. The fragmentation of CI-1 and CI-2 has been reported previously (*17*, *18*), but the physiological functions of these modifications, if any, is not known. At least part of the cleavages occurred during the extraction and purification steps because the various forms were obtained in different proportions when the entire extraction procedure was repeated (not shown).

CI-2A, BASI, and BBBI eluted concomitantly from the small scale size exclusion column, but these proteins were totally separated when the purification scale was enlarged by using a 5×77 cm column (not shown). However, in that case the BASI and BBBI eluted later than CI-2A. The CI-proteins reportedly tend to form di-, tri-, and tetramers (*8*), which may have affected their elution rates. However, the observed inhibitor elution patterns seem to indicate that BASI and BBBI probably interacted with the column.

Williamson et al. showed that despite the fact that CI-2 has a slightly larger calculated molecular mass (9250 Da, starting with Ser_1) than CI-1 (8790 Da), it migrated faster in urea/SDS−PAGE than CI-1 (*27*). In our SDS−PAGE analysis (30% ethylene glycol, no urea), all of the different inhibitor forms migrated slightly faster than expected on the basis of their calculated molecular masses, but the previously reported anomalous separation of CI-1 and CI-2 did not occur.

CI-2A and BASI were more potent inhibitors of the SL proteinase than CI-1A. Both CI-1 proteins also inhibited bovine chymotrypsin and bacterial subtilisin more weakly than CI-2A (*19*). BBBI was a stronger inhibitor than its soybean relative. The soybean inhibitor has only a single binding site for trypsin (*34*), whereas the double domain BBBI can bind two trypsin molecules simultaneously (*5*, *35*). However, the difference between the inhibitory activities of the soybean and barley inhibitors was not proportional to their numbers of binding sites, implying that the *F. culmorum* TL proteinase binds more tightly to the barley inhibitor. As seen with CI-1 and -2, which inhibit microbial proteinases more strongly than bovine chymotrypsin (*19*, *31*), BBBI inhibited a microbial proteinase (Pronase) more effectively than it did bovine trypsin (*5*). Previous studies with bovine chymotrypsin and bacterial subtilisin have shown that CI-1 and CI-2A are slow-binding inhibitors (*19*, *31*). Our preliminary inhibition studies (not shown) have indicated that Michaelis−Menten kinetic analysis was not applicable to any of the purified inhibitors, so more detailed studies are being carried out to define the kinetic properties of these inhibitors with the *Fusarium* proteinases.

CI-1, CI-2, and BASI all are seed-specific proteins (*27*, *28*, *30*, *36*). Inhibitors of chymotrypsin and *Aspergillus oryzae* alkaline proteinase(s) have been detected in young barley leaves, rootlets and embryos, but they were different from those that were present in the endosperm (*37*). The presence of these inhibitors is supported by the finding of a mRNA in barley shoots and leaves that is related to, but not identical with, that of CI-2A (*28*). The synthesis of the inhibitors (or their mRNAs) has been detected 2 weeks after anthesis in the endosperm tissue (*27*, *30*, *38*−*40*). CI-1 and CI-2 were synthesized in both the aleurone layer and starchy endosperm (*38*, *41*, *42*), but they have not been detected in embryos (*38*). In several studies, BASI mRNA has been detected in the starchy endosperm of seeds and in cultured aleurone cells, but not in the developing aleurone (*30*, *42*, *43*). However, in other studies the BASI protein has been detected in the aleurone layers of various cultivars (*40*, *44*). These variant findings may be due to differences in the detection techniques that were used. Alternatively, the synthesis of BASI in aleurone layers may vary in different cultivars, eg. BASI mRNA has been extracted from both the starchy endosperm and aleurone layer of Bomi, but was not present in the aleurone layer of its high-lysine mutant, Risø 1508 (*41*). The BASI protein was also present in the embryos of several cultivars (*40*, *44*).

Bifunctional Bowman−Birk inhibitors, which inhibit both bovine trypsin and chymotrypsin, have been detected in cereals and legumes (*1*, *12*). A Bowman−Birk-type trypsin inhibitor has been purified from barley embryos (*5*) and rootlets (*29*) and there are indications of its presence in the aleurone layer (*5*). Two BBBI isoforms have been isolated from Risø 1508 barley (*5*), but only one form was detected in Morex. BBBI contains two homologous domains and can bind two bovine (*5*, *29*, *35*) or microbial (Pronase) (*5*) trypsin molecules simultaneously. A similar inhibitor has been purified from wheat embryos, together with a homologous protein that had a molecular mass of about 7 kDa (*45*).

The only previously known physiological functions of CI-1 and CI-2 were to serve as storage proteins, although it has been presumed that they may confer resistance to grains against microbial pathogens and insects (*46*). A seed-protective role has also been proposed previously for BBBI (*5*). This was supported by studies in which two Bowman−Birk-type inhibitor forms from barley and one from wheat endosperm apparently had antifungal properties in vitro (*4*, *10*). A trypsin inhibitor from corn, which differs from BBBI, has been shown to inhibit the sporulation and growth of plant pathogenic fungi (*47*). However, this effect was not necessarily due to the proteinase inhibition properties of this protein, but could be due to its effect on the fungal α-amylase (*48*). It has been proposed that BASI may regulate the release of sugars from starch, prevent precocious germination and protect the seed from invasion by pathogens because it inhibits both endogenous α-amylase 2 and the microbial proteinase subtilisin (*1*, *7*, *30*, *49*, *50*).

In summary, the inhibition of microbial proteinases by CI-1 and -2, BASI and BBBI, and their abundances in the grain have encouraged researchers to presume that they protect the seed from pathogen and pest attacks. Our results strongly support this hypothesis, as we have shown that these proteins inhibit the two alkaline serine proteinases of a potent phytopathogenic fungus in vitro. We have found that these proteinases were synthesized in heavily infected developing barley kernels (*23*). However, the inhibitors apparently cannot fully prevent the fungal invasion, because it has been shown that *F. culmorum* and *F. graminearum* can grow into the endosperm tissues where most of these inhibitors are located (*22* and the references therein). Our future research will focus on localizing the fungal proteinases in infected seeds with immunohistochemical methods, to establish when and where they are synthesized. This should give a good indication of whether the proteinases may interact with the inhibitors inside the seed.

Proteins. SwissProt (http://www.expasy.ch) entry: BASI, P07596; BBBI, P12940; CI-1A, P16062; CI-1B, P16063; CI-1C, P01054; CI-2A, P01053. Enzymes: chymotrypsin (E.C. 3.4.21.1), trypsin (E.C. 3.4.21.4), subtilisin (E.C. 3.4.21.62), oryzin (E.C. 3.4.21.63).

Abbreviations. BASI, barley α-amylase/subtilisin inhibitor; BBBI, barley Bowman−Birk inhibitor; CI, chymotrypsin/subtilisin inhibitor; CMC, carboxymethyl cellulose; DMSO, dimethyl sulfoxide; FHB, Fusarium head blight; QAC, quaternaryammonium cellulose; SL, subtilisin-like; TL, trypsin-like.

LITERATURE CITED

(1) Shewry, P. R. Enzyme inhibitors of seeds: types and properties. In *Seed Proteins*; Shewry, P. R., Casey, R., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1999; pp 587−615.

(2) Mikola, J.; Suolinna, E.-M. Purification and properties of an inhibitor of microbial alkaline proteinases from barley. *Arch. Biochem. Biophys.* **1971**, *144*, 566−575.

(3) Dunaevskii, Ya. E.; Pavlikova, E. B.; Belyakova, G. A.; Belozerskii, M. A. Physiological role of protease inhibitors in plants: two groups of active inhibitors in buckwheat seed. *Mol. Biol.* **1995**, *29*, 747−750.

(4) Chilosi, G.; Caruso, C.; Caporale, C.; Leonardi, L.; Bertini, L.; Buzi, A.; Nobile, M.; Magro, P.; Buonocore, V. Antifungal activity of a Bowman−Birk-type trypsin inhibitor from wheat kernel. *J. Phytopathol.* **2000**, *148*, 477−481.

(5) Boisen, S.; Djurtoft, R. Protease inhibitor from barley embryo inhibiting trypsin and trypsin-like microbial proteases. Purification and characterisation of two isoforms. *J. Sci. Food Agric.* **1982**, *33*, 431−440.

(6) Yoshikawa, M.; Iwasaki, T.; Fujii, M.; Oogaki, M. Isolation and some properties of a subtilisin inhibitor from barley. *J. Biochem.* **1976**, *79*, 765−773.

(7) Mundy, J.; Svendsen, I.; Hejgaard, J. Barley α-amylase/subtilisin inhibitor. I. Isolation and characterization. *Carlsberg Res. Comm.* **1983**, *48*, 81−90.

(8) Boisen, S.; Yding Andersen, C.; Hejgaard, J. Inhibitors of chymotrypsin and microbial serine proteases in barley grains. *Physiol. Plant* **1981**, *52*, 167−176.

(9) Hejgaard, J. Isoelectric focusing of subtilisin inhibitors: detection and partial characterization of cereal inhibitors of chymotrypsin and microbial proteases. *Anal. Biochem.* **1981**, *116*, 444−449.

(10) Terras, F. R. G.; Schoofs, H. M. E.; Thevissen, K.; Osborn, R. W.; Vanderleyden, J.; Cammue, B. P. A.; Broekaert, W. F. Synergistic enhancement of the antifungal activity of wheat and barley thionins by radish and oilseed rape 2S albumins and by barley trypsin inhibitors. *Plant Physiol.* **1993**, *103*, 1311−1319.

(11) Mikola, J.; Suolinna, E.-M. Purification and properties of a trypsin inhibitor from barley. *Eur. J. Biochem.* **1969**, *9*, 555−560.

(12) Prakash, B.; Selvaraj, S.; Murthy, M. R. N.; Sreerama, Y. N.; Rajagopal Rao, D.; Gowda, L. R. Analysis of the amino acid sequences of plant Bowman−Birk inhibitors. *J. Mol. Evol.* **1996**, *42*, 560−569.

(13) Svendsen, I.; Hejgaard, J.; Mundy, J. Complete amino acid sequence of the α-amylase/subtilisin inhibitor from barley. *Carlsberg Res. Commun.* **1986**, *51*, 43−50.

(14) Zawistowska, U.; Langstaff, J.; Friesen, A. D. Purification and characterization of two double-headed triticale isoinhibitors of endogenous alpha-amylase and subtitlisin. *J. Food Biochem.* **1989**, *13*, 215−239.

(15) Mosolov, V. V.; Shul'gin, M. N. Protein inhibitors of microbial proteinases from wheat, rye and triticale. *Planta* **1986**, *167*, 595−600.

(16) Yamagata, H.; Kunimatsu, K.; Kamasaka, H.; Kuramoto, T.; Iwasaki, T. Rice bifunctional α-amylase/subtilisin inhibitor: characterization, localization, and changes in developing and germinating seeds. *Biosci., Biotechnol., Biochem.* **1998**, *62*, 978−985.

(17) Svendsen, I.; Jonassen, I.; Hejgaard, J.; Boisen, S. Amino acid sequence homology between a serine protease inhibitor from barley and potato inhibitor I. *Carlsberg Res. Comm.* **1980**, *45*, 389−395.

(18) Svendsen, I.; Boisen, S.; Hejgaard, J. Amino acid sequence of serine protease inhibitor CI-1 from barley. Homology with barley inhibitor CI-2, potato inhibitor I, and leech eglin. *Carlsberg Res. Comm.* **1982**, *47*, 45−53.

(19) Greagg, M. A.; Brauer, A. B. E.; Leatherbarrow, R. J. Expression and kinetic characterization of barley chymotrypsin inhibitors 1a and 1b. *Biochim. Biophys. Acta* **1994**, *1222*, 179−186.

(20) Khludnev, D. V.; Pivnenko, N. V.; Mosolov, V. V. Chymotrypsin/subtilisin inhibitor from wheat grain endosperm. *Biochemistry (Moscow)* **1992**, *57*, 1303−1309.

(21) Gilbert, J.; Tekauz, A. Review: Recent developments in research on fusarium head blight of wheat in Canada. *Can. J. Plant Pathol.* **2000**, *22*, 1−8.

(22) Nightingale, M. J.; Marchylo, B. A.; Clear, R. M.; Dexter, J. E.; Preston, K. R. Fusarium head blight: effect of fungal proteases on wheat storage proteins. *Cereal Chem.* **1999**, *76*, 150−158.

(23) Pekkarinen, A. I.; Kleemola, T. H.; Laitila, A. T.; Haikara, A. I.; Jones, B. L. *Fusarium* species synthesize alkaline proteinases in infested barley. *J. Cereal Sci.* **2003**, *37*, 349−356.

(24) Pekkarinen, A. I.; Jones, B. L.; Niku-Paavola, M.-L. Purification and properties of an alkaline proteinase of *Fusarium culmorum*. *Eur. J. Biochem.* **2002**, *269*, 798−807.

(25) Pekkarinen, A. I.; Jones, B. L. Trypsin-like proteinase produced by *Fusarium culmorum* grown on grain proteins. *J Agric. Food Chem.* **2002**, *50*, 3849−3855.

(26) Copeland, R. A. *Methods for Protein Analysis. A Practical Guide to Laboratory Protocols*; Chapman & Hall International Thomson Publishing: New York, 1994.

(27) Williamson, M. S.; Forde, J.; Kreis, M. Molecular cloning of two isoinhibitor forms of chymotrypsin inhibitor 1 (CI-1) from barley endosperm and their expression in normal and mutant barleys. *Plant Mol. Biol.* **1988**, *10*, 521−535.

(28) Williamson, M. S.; Forde, J.; Buxton, B.; Kreis, M. Nucleotide sequence of barley chymotrypsin inhibitor-2 (CI-2) and its expression in normal and high-lysine barley. *Eur. J. Biochem.* **1987**, *165*, 99−106.

(29) Nagasue, A.; Fukamachi, H.; Ikenaga, H.; Funatsu, G. The amino acid sequence of barley rootlet trypsin inhibitor. *Agric. Biol. Chem.* **1988**, *52*, 1505−1514.

(30) Leah, R.; Mundy, J. The bifunctional α-amylase/subtilisin inhibitor of barley: nucleotide sequence and patterns of seed-specific expression. *Plant Mol. Biol.* **1989**, *12*, 673−682.

(31) Longstaff, C.; Campbell, A. F.; Fersht, A. R. Recombinant chymotrypsin inhibitor 2: expression, kinetic analysis of inhibition with α-chymotrypsin and wild-type and mutant subtilisin BPN′, and protein engineering to investigate inhibitory specificity and mechanism. *Biochemistry* **1990**, *29*, 7339−7347.

(32) Mosolov, V. V.; Loginova, M. D.; Fedurkina, N. V.; Benken I. I. The biological significance of proteinase inhibitors in plants. *Plant Sci. Lett.* **1976**, *7*, 77−80.

(33) Garcia-Olmedo, F.; Salcedo, G.; Sanchez-Monge, R.; Gomez, L.; Royo, J.; Carbonero, P. Plant proteinaceous inhibitors of proteinases and α-amylases. *Oxf. Surv. Plant Mol. Cell Biol.* **1987**, *4*, 275−334.

(34) Odani, S.; Ikenaka, T. Studies on soybean trypsin inhibitors IV. Complete amino acid sequence and the anti-proteinase sites of Bowman−Birk soybean proteinase inhibitor. *J. Biochem.* **1972**, *71*, 839−848.

(35) Song, H. K.; Kim, Y. S.; Yang, J. K.; Moon, J.; Lee, J. Y.; Suh, S. W. Crystal structure of a 16 kDa double-headed Bowman−Birk trypsin inhibitor from barley seeds at 1.9 Å resolution. *J. Mol. Biol.* **1999**, *293*, 1133−1144.

(36) Robertson, M.; Hill, R. D. Accumulation of an endogenous alpha-amylase inhibitor in barley during grain development. *J. Cereal Sci.* **1989**, *9*, 237−246.

(37) Kirsi, M.; Mikola, J. Occurrence and heterogeneity of chymotrypsin inhibitors in vegetative tissues of barley. *Physiol. Plant.* **1977**, *39*, 110−114.

(38) Rasmussen, U. Immunological screening for specific protein content in barley seeds. *Carlsberg Res. Commun.* **1985**, *50*, 83−93.

(39) Rasmussen, U.; Williamson, M. S.; Mundy, J.; Kreis, M. Differential effects of the Hiproly *lys 1* gene on the developmental synthesis of (lysine-rich) proteins from barley endosperm. *Plant Sci.* **1988**, *55*, 255−266.

(40) Hill, R. D.; Gubbels, S. M.; Boros, L.; Sumner, M. J.; MacGregor, A. W. Location of α-amylase/subtilisin inhibitor during kernel development and germination. *Can. J. Bot.* **1995**, *73*, 982−990.

(41) Jakobsen, K. S.; Kalvenes, C.; Olsen O.-A. mRNA levels in the developing aleurone and starchy endosperm in wild type and a high lysine (*lys 3a*) mutant of barley. *Physiol. Plant.* **1991**, *83*, 201−208.

(42) Mundy, J.; Hejgaard, J.; Hansen, A.; Hallgren, L.; Jorgensen, K. G.; Munck, L. Differential synthesis *in vitro* of barley aleurone and starchy endosperm proteins. *Plant Physiol.* **1986**, *81*, 630−636.

(43) Mundy, J.; Rogers, J. C. Selective expression of a probable amylase/protease inhibitor in barley aleurone cells: comparison to the barley amylase/subtilisin inhibitor. *Planta* **1986**, *169*, 51−63.

(44) Jensen, L. G. Developmental patterns of enzymes and proteins during mobilization of endosperm stores in germinating barley grains. *Hereditas* **1994**, *121*, 53−72.

(45) Odani, S.; Koide, T.; Ono, T. Wheat germ trypsin inhibitors. Isolation and structural characterization of single-headed and double-headed inhibitors of the Bowman−Birk type. *J. Biochem.* **1986**, *100*, 975−983.

(46) Shewry, P. R.; Tatham, A. S.; Halford, N. G.; Barker, J. H. A.; Hannappel, U.; Gallois, P.; Thomas, M.; Kreis, M. Opportunities for manipulating the seed protein composition of wheat and barley in order to improve quality. *Transgenic Res.* **1994**, *3*, 3−12.

(47) Chen, Z.-Y.; Brown, R. L.; Lax, A. R.; Cleveland, T. E.; Russin, J. S. Inhibition of plant pathogenic fungi by a corn trypsin inhibitor overexpressed in *Escherichia coli*. *Appl. Environ. Microbiol.* **1999**, *65*, 1320−1324.

(48) Chen, Z.-Y., Brown, R. L.; Russin, J. S.; Lax, A. R.; Cleveland, T. E. A corn trypsin inhibitor with antifungal activity inhibits *Aspergillus flavus* α-amylase. *Phytopathol.* **1999**, *89*, 902−907.

(49) Weselake, R. J.; MacGregor, A. W.; Hill, R. D. An endogenous α-amylase inhibitor in barley kernels. *Plant Physiol.* **1983**, *72*, 809−812.

(50) Mundy, J. Hormonal regulation of α-amylase inhibitor synthesis in germinating barley. *Carlsberg Res. Commun.* **1984**, *49*, 439−444.

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