

## Purification and Characterization of a New Class of Insect $\alpha$ -Amylase Inhibitors from Barley

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### ABSTRACT

Cereal Chem. 74(2):119–122

Barley seeds contain proteins that apparently protect them against attack by microorganisms and insects. Studies of these barley defensive proteins may lead to the development of barleys with improved natural resistance to pests. We have purified two low molecular weight proteins, designated BI $\alpha$ 1 and BI $\alpha$ 2, from barley grain, using ion-exchange chromatography and reversed-phase and gel-permeation high-performance liquid chromatography (HPLC). Both BI $\alpha$ 1 and BI $\alpha$ 2 inhibited insect (yellow meal worm, *Tenebrio molitor*)  $\alpha$ -amylase activities. For the *T. molitor*  $\alpha$ -amylase, the IC<sub>50</sub> values of BI $\alpha$ 1 and BI $\alpha$ 2 were 80  $\mu$ g/mL (12.5  $\mu$ M) and 34  $\mu$ g/mL

(6.8  $\mu$ M), respectively. Neither protein inhibited either human salivary  $\alpha$ -amylase, barley  $\alpha$ -amylase, or trypsin activities. N-terminal amino acid sequences of the inhibitors were highly homologous with those of the plant proteins called *defensins*. The first 20 N-terminal amino acids of BI $\alpha$ 2 were identical to those of  $\gamma$ -hordothionin, but neither BI $\alpha$ 1 nor BI $\alpha$ 2 protein showed any homology with the chloroform-methanol (CM) soluble protein amino acid consensus sequence. The two inhibitors therefore apparently comprise another group of low molecular weight barley proteins that inhibit the  $\alpha$ -amylase activities of some insects that attack cereal grains.

Resistance of barley grain to insect infestation during storage is important to barley producers and consumers, and it is of special importance to the malting and brewing industries, where the use of insecticides on malting barley and malt is discouraged. Increasing the intrinsic resistance of barley grain to insect attack would reduce storage losses, lower costs, and ultimately increase industry profits.

Over 10% of the proteins of the barley seed starchy endosperm are reportedly toxic or inhibitory to microorganisms, insects, and human cells (García-Olmedo et al 1982, 1983, 1989). Two of the major barley toxic-inhibitory protein groups are the  $\alpha$ -amylase-trypsin inhibitors and the hordothionins. The  $\alpha$ -amylase-trypsin inhibitors, also known as the chloroform-methanol (CM) proteins, have molecular weights ranging from 9,000 to 16,000 and function as inhibitors of trypsin or insect  $\alpha$ -amylases (Shewry 1993). The properties of these proteins and the potential for conferring pest resistance to cereals have been reviewed recently (Buonocore et al 1977, Garcia-Olmedo et al 1992, Shewry 1993).

The hordothionins are also low molecular weight (LMW) barley proteins. There are three known endosperm thionin forms in barley:  $\alpha$ -,  $\beta$ -, and  $\gamma$ -hordothionins. These proteins contain high levels of the basic amino acids and cysteine, and have molecular weights of  $\approx$ 5,000. The thionins are toxic to some microbes (Stuart and Harris 1942, Fernandez de Caleyá et al 1972, Bowles 1990) and to cultured mammalian cells (García-Olmedo et al 1982). They cause changes in cell membrane permeabilities (Carrasco et al 1981) and inhibit *in vitro* protein synthesis in both wheat germ and rabbit reticulocyte cell free systems (García-Olmedo et al 1983).

Although the protein called  $\gamma$ -hordothionin shares some properties with the  $\alpha$ - and  $\beta$ -hordothionins, it differs from them in important ways. Its amino acid sequence is unlike those of the  $\alpha$ - and  $\beta$ -hordothionins but similar to those of a group of plant pro-

teins called *defensins*. The defensins also have antifungal or insecticidal properties (Terras et al 1995).

This article reports the purification and partial characterization of two LMW barley proteins, BI $\alpha$ 1 and BI $\alpha$ 2, which inhibit the  $\alpha$ -amylase activity of the cereal grain insect pest *Tenebrio molitor*, but do not affect the activities of the barley  $\alpha$ -amylases. These proteins differ greatly from the barley CM-proteins, which also inhibit the insect  $\alpha$ -amylase activities, but both BI $\alpha$ 1 and BI $\alpha$ 2 were highly homologous with the plant defensin proteins (Terras et al 1995). They apparently comprise a previously unstudied group of proteins that inhibit insect  $\alpha$ -amylase activities and possibly defend barley seeds from insect attack.

### MATERIALS AND METHODS

#### Extraction of Barley Proteins

Barley (*Hordeum vulgare* L. cv. Morex) flour (200 g) was suspended in 500 mL of 50 mM H<sub>2</sub>SO<sub>4</sub> and stirred at room temperature for 1 hr. The extracts were centrifuged at 10,000  $\times$  g (10 min), after which the supernatant was adjusted to pH 7.4 with NH<sub>4</sub>OH and held at room temperature. After 30 min, the sample was centrifuged at 10,000  $\times$  g for 10 min, and the supernatant was adjusted to pH 5.2 with glacial acetic acid. Any precipitate that formed was removed by centrifugation and the supernatant was termed crude extract.

#### Ion-Exchange Chromatography

The crude extract was loaded onto a 2-  $\times$  14-cm carboxymethyl cellulose (CMC) column equilibrated with 50 mM, pH 5.2, ammonium acetate (NH<sub>4</sub>Ac) buffer. The  $\alpha$ -amylase inhibiting proteins were eluted with a 600 mL, linear, 0.3–1.2M NH<sub>4</sub>Ac gradient.

#### Reversed-Phase HPLC

The  $\alpha$ -amylase inhibiting fractions from CMC chromatography were pooled, freeze-dried, redissolved in 5 mL of H<sub>2</sub>O and loaded onto a Zorbax C-18 HPLC column (4.6 mm  $\times$  7.5 cm) equilibrated with a 20% acetonitrile solution containing 0.1% trifluoroacetic acid (TFA). The adsorbed proteins were eluted with a linear 20–40% (v/v) acetonitrile gradient in 15 min, at a 1 mL/min flow rate. The various separated protein fractions were freeze-dried and tested for inhibitory activity.

#### Size-Exclusion HPLC

After the RP-HPLC separation, the  $\alpha$ -amylase inhibiting fractions were further separated by HPLC chromatography on a 7.8 mm  $\times$  30 cm SynChropak GPC peptide gel permeation column in 100 mM NH<sub>4</sub>Ac, at a flow rate of 0.5 mL/min.

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### $\alpha$ -Amylase Activity Assay

Enzyme extracts were prepared from whole *T. molitor* larvae using the method of Buonocore et al (1975). The  $\alpha$ -amylase activity of the preparation was assayed by the method of Bernfeld (1955) using a 1% starch substrate dissolved in 1 mM  $\text{CaCl}_2$  and 100 mM NaCl. Purified inhibitor protein samples varying in size from 5 to 200  $\mu\text{g}$ , or 50- $\mu\text{L}$  fractions collected during purification steps, were preincubated with 2  $\mu\text{L}$  of the  $\alpha$ -amylase extract for 10 min, after which the reaction was started by adding 250  $\mu\text{L}$  of 1% soluble potato starch substrate (Sigma Chemical Co., St. Louis, MO). Maltose (50–500  $\mu\text{g}$ ) was used as standard in this colorimetric assay. One unit of  $\alpha$ -amylase activity released 1 mg of maltose from starch in 10 min, at 30°C, and one unit of inhibitory activity reduced the  $\alpha$ -amylase activity by one unit.

### Protein Assay

Proteins were measured by a modification of the Bradford (1976) method, using the Bio-Rad (Hercules, CA) protein assay dye solution.

### Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SDS-PAGE analyses were conducted with the Pharmacia PhastSystem, using 20% acrylamide PhastGels and Pharmacia SDS buffer strips. The purified BI $\alpha$ 1 and BI $\alpha$ 2 proteins (1  $\mu\text{g}$  each) were applied to the gel and separated by maintaining a constant current (10 mA/gel) for 40 min. The gels were silver-stained using the method described in the PhastSystem methods manual.

### N-Terminal Amino Acid Sequencing

Sequencing was performed with a pulsed liquid phase amino acid sequencer (model 477A, Applied Biosystems) equipped with a model 120A on-line phenylthiohydantoin amino acid analyzer. The purified BI $\alpha$ 1 and BI $\alpha$ 2 samples (1–4 nmol) were dissolved in 0.2N acetic acid and applied to a preconditioned filter. Sequencer data were analyzed by both the instrument's data analysis program and the operator.

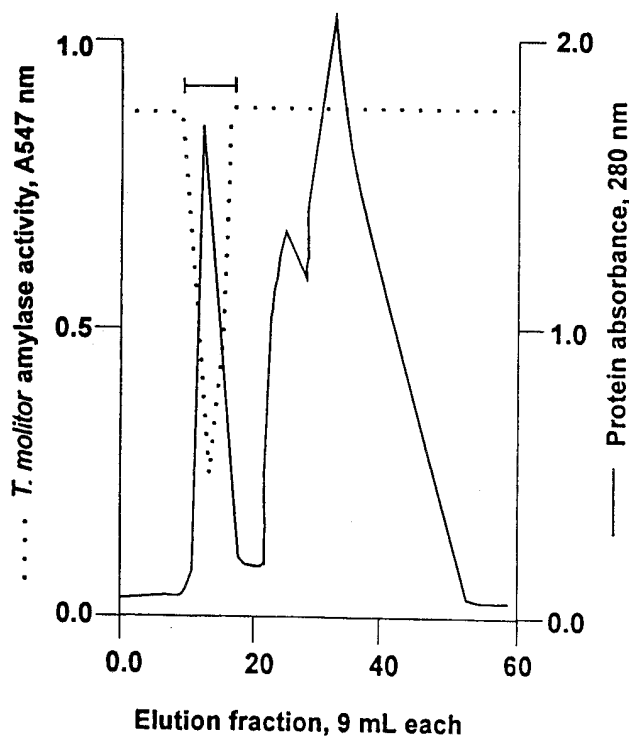


Fig. 1. Carboxymethyl cellulose (CMC) ion-exchange separation of the compounds in a crude barley extract that inhibit *Tenebrio molitor*  $\alpha$ -amylase activities. Extract was applied to the column and eluted with a 0.3–1.2M  $\text{NH}_4\text{Ac}$  gradient. Bar = pooled inhibiting fractions.

### Amino Acid Composition Analyses

Purified BI $\alpha$ 1 and BI $\alpha$ 2 were hydrolyzed with sequanal grade 6N HCl (Pierce Chemical Co., Rockford, IL) using the method of Lookhart et al (1982). The resulting amino acids were derivatized with phenylisothiocyanate, separated by RP-HPLC, and quantified as reported by Jones and Poulle (1990).

## RESULTS

### Purification of BI $\alpha$ 1 and BI $\alpha$ 2

All of the  $\alpha$ -amylase inhibitory activity of the crude extract eluted from a CMC column in the presence of 0.6–0.7M, pH 5.2,  $\text{NH}_4\text{Ac}$  buffer (fractions 11–18 of Fig. 1). Further fractionation of the CMC separated inhibitory material by RP-HPLC and SE-HPLC yielded two inhibitor protein preparations (Fig. 2), each homogeneous on SDS-PAGE analysis (Fig. 3, lanes 2 and 3). The estimated molecular weights of BI $\alpha$ 1 (lane 2) and BI $\alpha$ 2 (lane 3) were 6,400 and 5,000, respectively, as determined by SDS-PAGE analysis (Fig. 3). The pI values of BI $\alpha$ 1 and BI $\alpha$ 2, determined by isoelectric focusing, were 9.3 and 9.6 (gel not shown), respectively. BI $\alpha$ 1 and BI $\alpha$ 2 comprised 0.29 and 0.37%, respectively, of the crude extract protein, and they were purified by factors of 70- and 154-fold (Table I). Of the original inhibitory activity, 79% was accounted for by the two purified preparations.

### Characterization of BI $\alpha$ 1 and BI $\alpha$ 2

Both BI $\alpha$ 1 and BI $\alpha$ 2 inhibited *T. molitor*  $\alpha$ -amylase activity at concentrations as low as 5  $\mu\text{g}/\text{mL}$  of assay solution (Fig. 4), but neither had any effect on human salivary  $\alpha$ -amylase, barley  $\alpha$ -amylase, or bovine trypsin activities (data not shown). The IC<sub>50</sub> (concentrations causing 50% inhibition of  $\alpha$ -amylase activity) values of BI $\alpha$ 1 and BI $\alpha$ 2 for inhibiting the *T. molitor*  $\alpha$ -amylase were 80  $\mu\text{g}/\text{mL}$  (12.5  $\mu\text{M}$ ) and 34  $\mu\text{g}/\text{mL}$  (6.8  $\mu\text{M}$ ) (calculated from Fig. 4 using the Lineweaver-Burk equation), respectively.

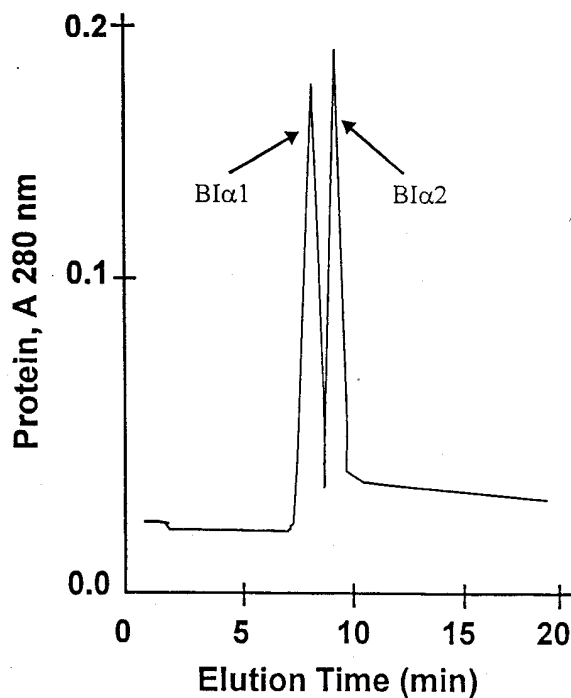
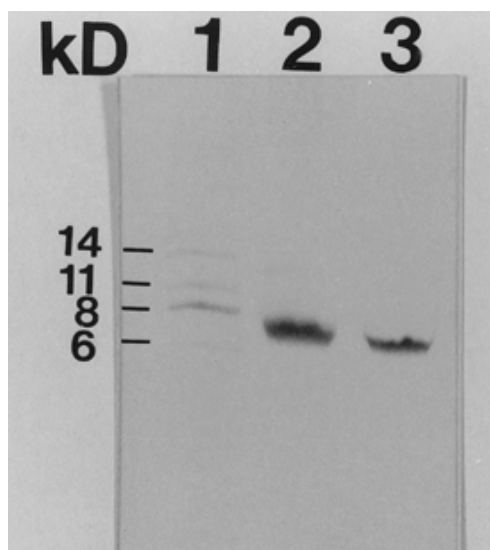


Fig. 2. Separation of BI $\alpha$ 1 and BI $\alpha$ 2 on a high-performance liquid chromatography (HPLC) gel-permeation column. Pooled fractions from Fig. 1 were applied to a reverse-phase HPLC column. *Tenebrio molitor*  $\alpha$ -amylase inhibitory fractions from the RP-HPLC step were concentrated, applied to a peptide gel-permeation column, and eluted with 100 mM  $\text{NH}_4\text{Ac}$ .

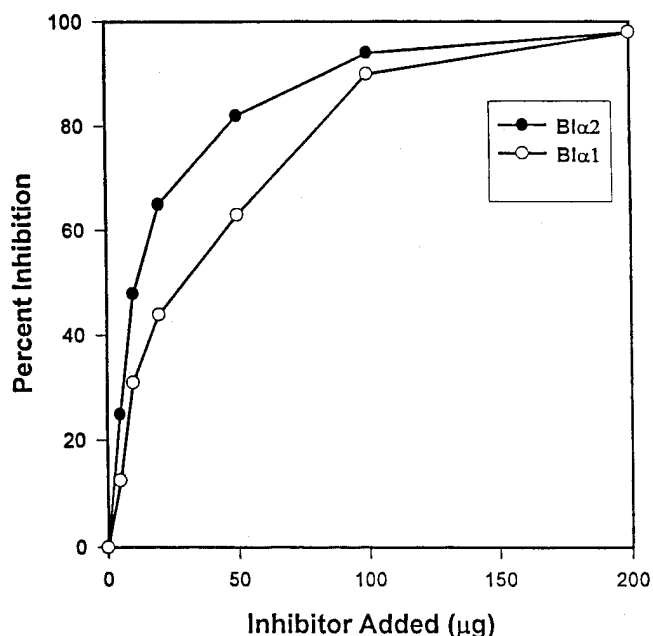
## DISCUSSION

The N-terminal amino acid sequence of BI $\alpha$ 1 was highly homologous (14 of 20 amino acids sequenced were identical) with that of the previously reported sorghum insect  $\alpha$ -amylase inhibitor SI $\alpha$ 1 (Fig. 5) and showed less homology with BI $\alpha$ 2 (9 of 20 amino acids were identical). The sequence of the first 20 N-terminal amino acids of BI $\alpha$ 2 was identical with that of the protein that has been called  $\gamma$ -hordothionin (Mendez et al 1990), and was also highly homologous with the sequences of the sorghum insect  $\alpha$ -amylase inhibitors SI $\alpha$ 2 and SI $\alpha$ 3 (Bloch and Richardson 1991).

Like those of the barley and wheat thionins, the amino acid compositions of both BI $\alpha$ 1 and BI $\alpha$ 2 are rich in the amino acids cysteine and arginine (Table II). Not surprisingly, BI $\alpha$ 2 had an amino acid composition nearly identical to that of  $\gamma$ -hordothionin (Table II). The BI $\alpha$ 1 and BI $\alpha$ 2 amino acid compositions were quite different from those of the CM-proteins, which are rich in proline, glutamic acid, and aspartic acid (Barber et al 1986).



**Fig. 3.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of BI $\alpha$ 1 and BI $\alpha$ 2. Proteins were silver stained. Lane 1, protein standards, molecular weights indicated on left; lane 2, purified BI $\alpha$ 1; lane 3, purified BI $\alpha$ 2.



**Fig. 4.** Inhibition of *Tenebrio molitor*  $\alpha$ -amylase activity by BI $\alpha$ 1 and BI $\alpha$ 2. Control (no added inhibitor)  $\alpha$ -amylase activity = 0.5 unit.

Barley CM-proteins and hordothionins are two groups of LMW endosperm proteins that are directly or indirectly involved in the plant defense system (Bowles 1990). Three thionin forms ( $\alpha$ -,  $\beta$ -, and  $\gamma$ -hordothionins) have been purified from barley seed endosperm (Bohlmann and Apel 1991, Bohlmann 1994). These were all toxic to certain microbial and mammalian cells (Stuart and Harris 1942, Fernandez de Caleyra et al 1972, Bowles 1990). The  $\alpha$ - and  $\beta$ -hordothionins are very similar to each other and have highly homologous amino acid sequences. The  $\gamma$ -hordothionin, like  $\alpha$ - and  $\beta$ -hordothionins, is rich in basic amino acids and cysteine and has molecular weight close to 5,000, but its amino acid sequence is quite different from those of the  $\alpha$ - and  $\beta$ -thionins. However, there is a newly recognized group of plant proteins with amino acid

	1	5	10	15	20
BI $\alpha$ 1:	NH <sub>2</sub>	R-I-C-T-G-K-S-Q-H-H-H-F-P-C-F-S-D-K-S-C-			
BI $\alpha$ 2:	NH <sub>2</sub>	R-I-C-R-R-R-S-A-G-F-K-G-P-C-V-S-N-K-N-C-			
SI $\alpha$ 1:	NH <sub>2</sub>	R-V-C-M-G-K-S-Q-H-H-S-F-P-C-I-S-D-R-L-C-			
SI $\alpha$ 2:	NH <sub>2</sub>	R-V-C-M-G-K-S-A-G-F-K-G-L-C-M-R-D-Q-N-C-			

**Fig. 5.** Comparison of the amino acid sequences of the first 20 residues of BI $\alpha$ 1, BI $\alpha$ 2, SI $\alpha$ 1, and SI $\alpha$ 2 (SI $\alpha$ 1 and SI $\alpha$ 2 sequences from Bloch and Richardson 1991).

**TABLE I**  
Protein Yield and Inhibitory Activity of Each Purification Step

Purification Steps	Total Protein (mg)	Protein Yield (%)	Total Inhibitory Activity <sup>a</sup>	Specific Activity <sup>b</sup>	Purification Factor
Crude extract	1,023	100	202	0.20	
CMC <sup>c</sup> separation	308	30	173	0.56	
C-18 HPLC <sup>d</sup> separation	10	1	169	16.9	
Size-exclusion HPLC					
BI $\alpha$ 1	3.0	0.29	42	14.0	70-fold
BI $\alpha$ 2	3.8	0.37	117	30.8	154-fold

<sup>a</sup> Activity units (1 inhibitory activity unit reduced the  $\alpha$ -amylase activity by 1 unit).

<sup>b</sup> Activity unit/mg of protein.

<sup>c</sup> Carboxymethyl cellulose.

<sup>d</sup> High-performance liquid chromatography.

**TABLE II**  
Amino Acid Compositions of BI $\alpha$ 1, BI $\alpha$ 2, and  $\gamma$ -Hordothionin

Amino Acid	BI $\alpha$ 1		BI $\alpha$ 2		$\gamma$ -Hordothionin <sup>a</sup>	
	Residues/6,400 Da	Mole %	Residues/5,000 Da	Mole %	Residues/Mole	Mole %
Asx <sup>b</sup>	2.4 (2) <sup>c</sup>	3.5	2.2 (2)	4.4	4	8.5
Glx <sup>d</sup>	5.4 (5)	8.8	2.7 (3)	6.7	3	6.4
Ser	4.1 (4)	7.0	1.9 (2)	4.4	2	4.3
Gly	3.7 (4)	7.0	7.0 (7)	15.6	7	14.9
His	7.2 (7)	12.3	0.1 (0)	0.0	0	0.0
Thr	3.3 (3)	5.3	0.4 (0)	0.0	0	0.0
Ala	4.4 (4)	7.0	2.1 (2)	4.4	2	4.3
Arg	7.2 (7)	12.3	8.4 (8)	17.8	8	17.0
Pro	1.4 (1)	1.8	2.2 (2)	4.4	2	4.3
Tyr	1.5 (2)	3.5	0.0 (0)	0.0	0	0.0
Val	1.8 (2)	3.5	2.0 (2)	4.4	2	4.3
Met	0.0 (0)	0.0	2.1 (2)	4.4	2	4.3
Cys	7.5 (8) <sup>e</sup>	14.0	7.5 <sup>e</sup> (8)	17.8	8	17.0
Ile	1.3 (1)	1.8	1.0 (1)	2.2	1	2.1
Leu	1.4 (1)	1.8	1.1 (1)	2.2	1	2.1
Phe	2.6 (3)	5.3	1.1 (1)	2.2	1	2.1
Lys	2.7 (3)	5.3	2.7 (3)	6.7	3	6.4
Trp	nd <sup>f</sup>	nd	nd	nd	1	2.1

<sup>a</sup> Mendez et al (1990).

<sup>b</sup> Asp + Asn.

<sup>c</sup> Nearest whole residue.

<sup>d</sup> Glu + Gln.

<sup>e</sup> Determined as pyridylethyl cysteine.

<sup>f</sup> Not determined.

sequences that are highly homologous with that of  $\gamma$ -hordothionin. These proteins have been called plant defensins (Terras et al 1995) because of their antifungal (Terras et al 1992) or antipest (Bloch and Richardson 1991) activities. The BI $\alpha$ 1 and BI $\alpha$ 2 proteins reported in this article show considerable amino acid sequence homology with the members of the plant defensin protein group.

The sequence of the first 20 amino acids of BI $\alpha$ 2 was identical to that of  $\gamma$ -hordothionin (Fig. 5). Analysis of the amino acid composition of BI $\alpha$ 2 (Table II) indicated that BI $\alpha$ 2 and  $\gamma$ -hordothionin are probably the same protein. The amino acid sequence of BI $\alpha$ 1 shared homology with that of  $\gamma$ -hordothionin (BI $\alpha$ 2), but BI $\alpha$ 1 was slightly larger than  $\gamma$ -hordothionin and had a quite different amino acid composition.

The inhibition of insect  $\alpha$ -amylase activity by  $\gamma$ -hordothionin or by other barley proteins related to  $\gamma$ -thionins has not been reported previously, even though inactivation of  $\alpha$ -amylase activity by wheat purothionins has been reported (Jones and Meredith 1982). This study found that both BI $\alpha$ 1 and BI $\alpha$ 2 ( $\gamma$ -hordothionin) inhibited the  $\alpha$ -amylase activity of the yellow meal worm, an insect that attacks barley flour. This is consistent with the results reported for sorghum proteins, which are also plant defensins and whose amino acid sequences are highly homologous with that of  $\gamma$ -hordothionin (Bloch and Richardson 1991, Nitti et al 1995).

The members of another group of barley proteins, designated CM-proteins, also inhibit insect  $\alpha$ -amylase activities (Gutierrez et al 1990). However, neither BI $\alpha$ 1 nor BI $\alpha$ 2 showed any homology with the amino acid consensus sequence of the CM-proteins. BI $\alpha$ 1 and BI $\alpha$ 2 were less potent inhibitors of *T. molitor*  $\alpha$ -amylase than were the barley CM-proteins. BI $\alpha$ 1 and BI $\alpha$ 2 had IC50 values of 80  $\mu$ g/mL (12.5  $\mu$ M) and 34  $\mu$ g/mL (6.8  $\mu$ M), respectively, while one of the CM-proteins had an IC50 value of only 2  $\mu$ g (*unpublished data*). Studies correlating the  $\alpha$ -amylase inhibitor (CM-protein) contents of eastern soft wheats with the development of rice weevils growing on those wheats indicated that the  $\alpha$ -amylase inhibitors had little practical effect on reducing the initial weevil populations, but that the mean development times of the insects were significantly different when they were fed wheat cultivars with low and high inhibitor levels (Baker et al 1991). Whether BI $\alpha$ 1 and BI $\alpha$ 2 function as defensive proteins in barley seeds is still an open question, but the similarities to the other plant defensive proteins indicate they may well constitute a part of the barley seed defense mechanism.

#### ACKNOWLEDGMENT

We thank the American Malting Barley Association for their financial support and Christopher H. Martens for his excellent technical assistance.

#### LITERATURE CITED

BAKER, J. E., WOO, S. M., THRONE, J. E., and FINNEY, P. L. 1991. Correlation of  $\alpha$ -amylase inhibitor content in eastern soft wheats with development parameters of the rice weevil (*Coleoptera:Curculionidae*). *Environ. Entomol.* 20:53-60.

BARBER, D., SANCHEZ-MONGE, R., GARCÍA-OLMEDO, F., SALCEDO, G., and MENDEZ, E. 1986. Evolutionary implications of sequential homologies among members of the trypsin/ $\alpha$ -amylase inhibitor family (CM-proteins) in wheat and barley. *Biochim. Biophys. Acta* 873:147-151.

BERNFELD, P. 1955. Amylases,  $\alpha$  and  $\beta$ . *Methods Enzymol.* 1:149-158.

BLOCH, C., JR., and RICHARDSON, M. A. 1991. A new family of small (5 kDa) protein inhibitors of insect  $\alpha$ -amylases from seeds of sorghum (*Sorghum bicolor* (L.) Moench) have sequence homologies with wheat  $\gamma$ -purothionins. *FEBS* 279:101-104.

BOHLMANN, H. 1994. The role of thionins in plant protection. *Crit. Rev. Plant Sci.* 13:1-16.

BOHLMANN, H., and APEL, K. 1991. Thionins. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 42:227-240.

BOWLES, D. J. 1990. Defense-related proteins in higher plants. *Ann.*

*Rev. Biochem.* 59:873-907.

BRADFORD, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.

BUONOCORE, V., PETRUCCI, T., and SILANO, V. 1977. Wheat protein inhibitors of  $\alpha$ -amylase. *Phytochemistry* 16:811-820.

BUONOCORE, V., POERIO, E., GRAMENZI, F., and SILANO, V. 1975. Affinity column purification of amylase on protein inhibitors from whole wheat kernel. *J. Chromatogr.* 114:109-213.

CARRASCO, L., VAZQUEZ, D., HERNANDEZ-LUCAS, C., CARBONERO, P., and GARCÍA-OLMEDO, F. 1981. Thionins: Plant peptides that modify membrane permeability in cultured mammalian cells. *Eur. J. Biochem.* 116:185-189.

FERNANDEZ DE CALEYA, R., GONZALEZ-PASCIAL, B., GARCÍA-OLMEDO, F., and CARBONERO, P. 1972. Susceptibility of phytopathogenic bacteria to wheat purothionins in vitro. *Appl. Microbiol.* 23:998-1000.

GARCÍA-OLMEDO, F., CARBONERO, P., HERNANDEZ-LUCAS, C., PAZ-ARES, J., PONZ, F., VICENTE, O., and SIERRA, J. M. 1983. Inhibition of eucaryotic cell free protein synthesis by thionins from wheat endosperm. *Biochim. Biophys. Acta* 740:52-56.

GARCÍA-OLMEDO, F., CARBONERO, P., and JONES, B. L. 1982. Chromosomal locations of genes that control wheat endosperm proteins. Pages 1-47 in: *Advances in Cereal Science and Technology*, Vol. 5. Y. Pomeranz, ed. Am. Assoc. Cereal Chem.: St. Paul, MN.

GARCÍA-OLMEDO, F., RODRIGUEZ-PALENZUELA, P., HERNANDEZ, C., and CARBONERO, P. 1989. The thionins: A protein family that includes purothionins, viscotoxins and crambins. *Plant Mol. Cell Biol.* 6:31-60.

GARCIA-OLMEDO, F., SALCEDO, G., SANCHEZ-MONGE, R., HERNANDEZ-LUCAS, C., CARMONA, M. J., LOPEZ-FANDO, J. J., FERNANDEZ, J. A., GOMEZ, L., ROYO, J., GARCÍA-MAROTO, F., CASTAGNARO, A., and CARBONERO, P. 1992. Trypsin/ $\alpha$ -amylase inhibitors and thionins: Possible defense proteins from barley. Pages 335-350 in: *Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology*, P. R. Shewry, ed. CAB Int.: Oxford.

GUTIERREZ, C., SANCHEZ-MONGE, R., GOMEZ, L., RUIZ-TAPIADOR, M., CASTAÑERA, P., and SALCEDO, G. 1990.  $\alpha$ -Amylase activities of agricultural insect pests are specifically affected by different inhibitor preparations from wheat and barley endosperms. *Plant Sci.* 72:37-44.

JONES, B. L., and MEREDITH, P. 1982. Inactivation of  $\alpha$ -amylase activity by purothionins. *Cereal Chem.* 59:321.

JONES, B. L., and POULLE, M. 1990. A proteinase from germinated barley. II. Hydrolytic specificity of a 30 kDa cysteine proteinase from green malt. *Plant Physiol.* 94:1062-1070.

LOOKHART, G. L., JONES, B. L., COOPER, D. B., and HALL, S. B. 1982. A method for hydrolyzing and determining the amino acid compositions of picomole quantities of proteins in less than 3 hours. *J. Biochem. Biophys. Methods* 7:15-23.

MENDEZ, E., MORENO, A., COLILLA, F., PELAEZ, F., LIMAS, G., MENDEZ, R., SORIANO, F., SALINAS, M., and HARO, C. 1990. Primary structure and inhibition of protein synthesis in eukaryotic cell-free system of a novel thionin,  $\gamma$ -hordothionin, from barley endosperm. *Eur. J. Biochem.* 194:533-539.

NITTI, G., ORRÙ, S., BLOCH, C., MORHY, L., MARINO, G., and PUCCI, P. 1995. Amino acid sequence and disulphide-bridge pattern of three  $\gamma$ -thionins from *Sorghum bicolor*. *Eur. J. Biochem.* 228:250-256.

SHEWRY, P. R. 1993. Barley seed proteins. Pages 131-197 in: *Barley, Chemistry and Technology*. A. W. MacGregor and R. S. Bhatti eds. Am. Assoc. Cereal Chem.: St. Paul, MN.

STUART, L. S., and HARRIS, T. H. 1942. Bactericidal and fungicidal properties of a crystalline protein isolated from unbleached wheat flour. *Cereal Chem.* 19:288-300.

TERRAS, F. R. G., EGGERMONT, K., KOVALEVE, V., RAIKHEL, N. V., OSBORN, R. W., KESTER, A., REES, S. B., TORREKENS, S., LEUVEN, F. V., VANDERLEYDEN, J., CAMMUE, B. P. A., and BROEKAERT, W. F. 1995. Small cysteine-rich antifungal proteins from radish: Their role in host defense. *Plant Cell* 7:573-588.

TERRAS, F. R. G., SCHOOF, H. M. E., DE BOLLE, M. F. C., LEUVEN, F. V., REES, S. B., VANDERLEYDEN, J., CAMMUE, B. P. A., and BROEKAERT, W. F. 1992. Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sativus* L.) seeds. *J. Biol. Chem.* 267:15301-15309.

[Received September 27, 1996. Accepted December 3, 1996.]