# Purification and Partial Characterization of a Second Cysteine Proteinase Inhibitor from Ungerminated Barley (*Hordeum vulgare* L.)

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It was previously shown that ungerminated barley contains inhibitors that suppress the activities of green malt cysteine proteinases. This paper reports the purification and partial characterization of a second barley cysteine endoproteinase inhibitor, a protein called lipid transfer protein 2 (LTP2). The chromatographically purified inhibitor had a molecular mass of 7112. The amino acid composition and sequence data of the purified inhibitor indicated that it was a protein whose gene, but not the protein itself, was isolated earlier from barley aleurone tissue. The purified protein inhibited the activities of electrophoretically separated green malt cysteine proteinases but not the activities of the serine- or metalloproteinases. The purified LTP2 inhibited the same proteases as the LTP1 that was characterized previously but was present in the mature seed in much smaller amounts. Neither LTP1 nor LTP2 has been proven to transport lipids in vivo, and it seems possible that both serve to keep cysteine endoproteinases that are synthesized during barley seed development inactive until the plant needs them. The small amount of LTP2 in the seed made it impossible to determine whether it, like LTP1, is involved in beer foam formation. Because of its proteinase-inhibiting ability and its resistance to heat inactivation, some of the LTP2 may persist in beer.

**Keywords:** *Hordeum; LTP; cereal; protease; lipid transfer protein 2*

## INTRODUCTION

We are in the process of identifying, purifying, and characterizing the important endoproteinases of germinating barley and malt, to define how they operate to transform barley storage proteins into low molecular weight compounds that, during mashing, will yield a wort that is optimally suited for brewing. To understand how this enzyme system functions, it is important that we also understand the effects of any compounds that might enhance or reduce the activities of the endoproteinases.

Previously, we reported that both ungerminated barley seeds and kilned malt contained multiple, low molecular weight, proteinaceous, proteinase inhibitors (Jones and Marinac, 1991, 1995). These preferentially inhibited the activities of the cysteine endoproteinases extracted from green malt (Jones and Marinac, 1995) and are therefore referred to as endogenous proteinase inhibitors. Such inhibitors are probably important to seed germination and to the malting process, because they affect the cysteine endoproteinases. The cysteine endoproteinases are apparently the most important enzymes involved in transforming the large, insoluble barley storage proteins into small nitrogenous compounds that can be utilized by the growing barley plant following germination or by yeasts during the brewing process (Hammerton and Ho, 1986; Mikola, 1983). The inhibitors may affect the rate and extent of protein breakdown that occurs during germination by regulating the cysteine proteinase activities. If so, they would be important factors in the determination of the malting and brewing quality of barley cultivars.

It was shown over 30 years ago (Enari et al., 1964) that inhibitors present in ungerminated barley that was used as adjunct sometimes interfered with protein breakdown during mashing to the extent that wort fermentation was impossible, but at that time it was believed that the inhibitors were destroyed during malting (Mikola and Enari, 1970). Our work has shown, however, that kilned malt contains even more endogenous inhibitory activity than barley (Jones and Marinac, 1995). On the basis of their elution patterns from carboxymethyl cellulose (CMC) ion exchange columns, there are four groups of endogenous inhibitors in Morex barley seeds and three groups in kilned Morex malt (Jones and Marinac, 1995), and it is probable that all but one of these inhibitor groups contain multiple inhibitory compounds. We have purified and studied some of these inhibitors and identified and characterized one of them, called lipid transfer protein 1 (LTP1; Jones and Marinac, 1997). LTP1 appears to play a very important role in brewing; it has been shown that it is one of the major proteins that stabilizes beer foam (Lusk et al., 1995; Sorensen et al., 1993), as well as a proteinase inhibitor.

This paper reports the purification of a second inhibitor (originally called LTP, now LTP2) from ground barley seeds and some of the characteristics of this protein. The gene coding for LTP2 was cloned from barley aleurone layers and described by Jakobsen et al.

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in 1989. They showed that the LTP2 mRNA level was present in the aleurone tissue 20 days postanthesis, and Kalla et al. (1994) showed that the mRNA was present in newly formed aleurone cells, reached a maximum at grain mid-maturity, and was absent from mature grains. Messenger RNA coding for a homologous protein from rice, however, accumulated in the dry seeds (Garcia-Garrido et al., 1998). Since LTP2 proteins have been isolated from wheat (Monnet, 1990; Castagnaro and Garcia-Olmedo, 1994) and barley (this paper), it is apparent that the protein is stable enough that it is present even after its messenger has disappeared.

Prior to this study, the LTP2 protein had not, to our knowledge, been purified from barley grain. A homologous protein, however, has been purified from wheat by Monnet (1990) and by Castagnaro and Garcia-Olmedo (1994), who called it W-FABP. Castagnaro and Garcia-Olmedo (1994) have emphasized that the characteristics of wheat LTP2 are quite different from those of LTP1 and that the probability of homology existing between the two proteins is very low. If sufficient LTP2 amino acid deletions are allowed, however, the two proteins can be shown to share a conserved cysteine residue motif (Marion and Douliez, 1999). It should be noted that in the work published by Garcia-Olmedo's group [for example, Molina and Garcia-Olmedo (1997) and Caaveiro et al. (1997)], the protein that they have called LTP2 is not the one discussed in this paper, but a 9 kDa protein related to barley LTP1.

## MATERIALS AND METHODS

**Chemicals.** Azocasein, cysteine, gelatin, TRIS, glycine, ethylenediaminetetraacetic acid (EDTA), *trans*-epoxysuccinyl L-leucyclamido(4-guanidino)butane (E-64), and gel filtration molecular size standards were obtained from Sigma Chemical Co., St. Louis, MO. Bio-Gel P-30, acrylamide, and *N,N'*-methylenebis(acrylamide) (bis) were from Bio-Rad, Richmond, CA. Ampholines were obtained from Pharmacia LKB, Uppsala, Sweden, and the CMC cation exchanger (CM52) was from Whatman BioSystems, Maidstone, U.K. Sequanal grade HCl, phenylisothiocyanate (PITC), trifluoroacetic acid, and amino acid analysis standard mix H were from Pierce, Rockford, IL. Acetonitrile and methanol were obtained from the Burdick & Jackson Division of Baxter Healthcare Corp. (Muskegon, MI). All other chemicals were the purest available from commercial sources.

**Plant Material**. Barley grain (*Hordeum vulgare* L. cv. Morex) was kindly supplied by Dr. D. Wesenberg, USDA/ARS, Aberdeen, ID. Green malt was prepared from barley kernels (in 170 g aliquots) that were steeped to 45% moisture (~36 h) at 16 °C. During steeping, the samples were subjected to two 1-h air rests, at 12-h intervals, prior to germination for 96 h, at 16 °C with 100% humidity, in slowly rotating perforated metal cans. The conditions were chosen to simulate the procedures used by commercial maltsters. The green malt was frozen and stored at −20 °C until needed. Ungerminated barley was ground to a powder using a Retsch-Brinkmann mill that was fitted with a 0.5 mm screen and stored at −20 °C until needed.

**Preparation of Green Malt Proteinase Extract**. All enzyme preparation steps were performed at 4 °C. Typically, 100 g of green malt, containing 45% moisture, was macerated with 200 mL of extraction buffer [0.1 M sodium acetate (NaAc), pH 4.8, containing 2 mM cysteine and 0.1 mM EDTA] using a Waring blender for 1 min at high speed. The mixture was then further homogenized, using a Brinkmann Polytron, with three 1-min homogenization cycles separated by 1-min rest periods. The preparation was stirred slowly for 30 min and centrifuged (13000*g*, 20 min). The supernatant was dialyzed against 4 L of pH 5.0, 5 mM, NaAc for 16 h and centrifuged as before, and the final supernatant was stored at −20 °C until needed.

**Crude Inhibitor Preparation.** All purification steps were performed at 20 °C. Finely ground barley, 100 g, was mixed with 300 mL of extraction buffer (0.1 M $NH_4Ac$, pH 5.5, containing 2 mM cysteine and 0.1 mM EDTA) and stirred slowly for 45 min. The slurry was centrifuged (13000*g*, 20 min), and the supernatant was boiled for 15 min to destroy any endoproteolytic activity. After a second centrifugation to remove any proteins that were denatured by the boiling, the supernatant was lyophilized and stored until needed.

**Assay for Proteinase Inhibition.** Endoproteinase inhibitory activity was determined using azocasein as the protein substrate. Reaction mixtures were prepared by mixing 10 $\mu$L of 147 mM cysteine with 125 $\mu$L of crude green malt proteinase extract. After incubation for 5 min at room temperature, 300 $\mu$L of the inhibitor preparation was added to the mixture, which was incubated for an additional 10 min. Three hundred microliters of substrate [1% (w/v) azocasein in 0.1 M ammonium acetate ($NH_4Ac$), pH 5.5] was added to start the hydrolysis reactions. The final cysteine and substrate concentrations were thus 2.0 mM and 0.41%, respectively. The reactions were stopped at appropriate times (typically 30 and 60 min) by the addition of 0.5 mL of 15% (w/v) trichloroacetic acid (TCA), and the reaction mixtures were held in an ice−water bath for 15 min to allow any remaining undigested substrate to precipitate. After centrifugation (2000*g*, 15 min), the amount of hydrolyzed substrate in the supernatant was measured by spectrophotometry [absorbance at 440 nm ($A_{440nm}$)]. The inhibitor reduces the activity of the proteinase preparation and is thus measured as a decrease in the amount of peptide solubilized by the enzymes. Controls were prepared by adding the TCA to the reaction mixtures prior to the substrate.

**Bio-Gel P30 Chromatography.** Lyophilized crude inhibitor preparation was dissolved in elution buffer (0.1 M $NH_4Ac$, pH 5.5), and the solution was clarified by centrifugation at 2000*g* for 5 min and was loaded onto a Bio-Gel P30 (5 × 74 cm) size exclusion column that had been equilibrated with elution buffer. The proteins were eluted and fractions collected and assayed for their abilities to inhibit a crude green malt proteinase preparation. The fractions showing inhibitory activity were pooled and lyophilized. All of the column chromatography and HPLC elutions were monitored at 280 nm.

**CMC Ion Exchange Chromatography.** Inhibiting material from the P30 chromatography step was lyophilized twice to remove the volatile $NH_4Ac$, dissolved in 0.02 M $NH_4Ac$, pH 5.5, and applied to a 1.2 × 12 cm CMC column that had been equilibrated with the same buffer. After the nonbound material was removed by washing the column with 0.02 M $NH_4Ac$, the column was eluted with a linear 0.02−0.3 M gradient of $NH_4Ac$ adjusted to pH 5.5 (500 mL total volume). The eluted fractions were collected and assayed for inhibition activity, and the inhibiting fractions were pooled and lyophilized.

**Reversed Phase High-Performance Liquid Chromatography (RP-HPLC).** The HPLC system used consisted of two Shimadzu LC-600 pumps controlled by an SIL-6B autoinjector, a CR5A Chromatopac Shimadzu integrator, and a Gilson model 116 UV column monitor. Purification of the inhibitor fraction was achieved using a Beckman C-18 column (Ultrasphere ODS, 4.6 × 45 mm, 5 $\mu$m particles, 80 Å pore size). The lyophilized sample was dissolved in 0.3% (v/v) trifluoroacetic acid (TFA) and subjected to RP-HPLC at a flow rate of 2 mL/min. After the HPLC column was equilibrated with a solution containing 75% solvent A [0.3% (v/v) TFA in $H_2O$] and 25% solvent B [0.3% (v/v) TFA in acetonitrile], the sample was applied. After 3 min, the concentration of solvent B was increased linearly to 75% over 13 min. Following a 1-min wash with 75% solvent B, its concentration was returned to 25% and held for 3 min until the next injection. As individual absorbing fractions eluted, they were collected and assayed for inhibitory activity and the active fractions were lyophilized.

**Amino Acid Composition Analysis.** Protein samples, including the purified inhibitor, were sealed under vacuum in glass ampules with 200 $\mu$L of 6 N HCl and heated at 155 °C for 55 min (Lookhart et al., 1982). The resulting amino acids were derivatized with PITC and separated and quantified

according to a modification of the HPLC method of Heinrikson and Meredith (1984). The hydrolysis mixture was lyophilized, dissolved in 75 $\mu$L of drying solution, lyophilized, dissolved in 50 $\mu$L of PITC derivatizing reagent, incubated at room temperature for 30 min, and lyophilized again. The derivatized amino acids were dissolved in 220 $\mu$L of amino acid analysis solvent A [14.4 mL of acetic acid (HAc) and 1 mL of triethylamine (TEA) were dissolved in 900 mL of $H_2O$, the pH was adjusted to 6.05 with concentrated $NH_4OH$, 64 mL of acetonitrile was added, and the volume was adjusted to 1 L with $H_2O$] and subjected to analysis by RP- HPLC. A Phenomenex C-18 column (IB−SIL, 4.6 × 250 mm, 5 $\mu$m packing) maintained at either 55 or 52 °C was equilibrated with solvent A at a flow rate of 1.5 mL/min with detection at 254 nm. The column was eluted with a linear gradient that ran from 3% solvent B (100% acetonitrile)−97% solvent A to 100% solvent B. The gradient started at 3% B, went to 14% B in 4 min, from 14 to 20% B in 3.5 min, from 20 to 30% B in 3.5 min, and from 30 to 100% in 1.5 min. After a 5.5-min wash with 100% B, the column was returned to 3% B in 0.5 min and was ready for the next injection after 5.5 min. A Pierce Chemical Co. standard amino acid mix H, hydrolyzed native $\beta$-purothionin and hydrolyzed reduced and pyridylethylated α-hordothionin (Mak and Jones, 1978) were used collectively as standards to calculate the amino acid compositions of the analyzed samples. The purothionin and hordothionin samples were extracted from unbleached commercial durum semolina and ground Morex barley preparations, respectively, using the "purothionin extraction" method from Lecomte et al. (1982). The purified purothionin and hordothionin samples were then reduced and pyridylethylated using the method of Mak and Jones (1978).

**N-Terminal Amino Acid Sequence Analysis**. An N-terminal amino acid sequence analysis was performed commercially at the University of Wisconsin Biotechnology Center, Madison, WI.

**Mass Spectrometric Analysis.** Mass analysis of the purified protein was performed commercially by MassMetrics, Madison, WI, with a matrix-assisted laser desorption/ionization (MALDI) analyzer.

**Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE) and Isoelectric Focusing (IEF).** SDS−PAGE and IEF were performed using a Pharmacia PhastSystem electrophoresis unit. Homogeneous 20% and high-density precast electrophoretic gels were utilized for SDS−PAGE. The electrophoresis method was that suggested in the operator's manual, file 111, as was the optimized silver staining method. The IEF separation was carried out using precast gels that covered a pH gradient of 3−9. The separation file 100 method was used, together with the optimized silver staining method that is recommended by Pharmacia for IEF analysis.

**CMC × PAGE and IEF × PAGE Two-Dimensional Separation Analyses.** Green malt proteinases fractionated by CMC chromatography (first-dimension separation) and then by PAGE (second dimension) were used to study the effect of the purified inhibitor on the individual endoproteinase activities, using a modification of the method of Wrobel and Jones (1993). The studies were conducted with gelatin substrate incorporated into the PAGE gels.

Green malt proteinases that had been separated by tube gel IEF in a first dimension and then by native PAGE on slab gels that contained incorporated gelatin substrate (Zhang and Jones, 1995) were also used to study the effect of the inhibitor on the endoproteolytic activities. This initial separation of the endoproteinases on the basis of their p*I* values, followed by PAGE separation, gave a more clearly defined separation of the individual enzyme activities than CMC × PAGE did.

## RESULTS

**Purification of the Inhibitor Protein.** The purification of the inhibitor protein to homogeneity was accomplished by applying four sequential chromatography steps to the initial extract. The first (Bio-Gel P30) separation step (not shown) yielded two major inhibitor fractions previously named $I_a$ and $I_b$ (Jones and Marinac, 1991). The $I_a$ fraction comprised proteinaceous inhibitors, whereas $I_b$ contained nonproteinaceous, low molecular weight organic compounds. The pooled, lyophilized, $I_a$ fraction was rechromatographed on the same P30 column, and the collected fractions were assayed for inhibitory activity, using azocasein substrate and a 1-h hydrolysis period (Figure 1). Fractions 39−86 showed inhibitory activity and were retained for further purification. After the inhibitory P30 fractions were pooled and lyophilized twice to remove the excess $NH_4Ac$, the sample was applied to a CMC column and eluted with a linear $NH_4Ac$ concentration gradient. Analysis of the eluted fractions showed one major and two minor inhibition areas (Figure 2). The inhibitor fractions, designated 1, 2, and 3, were pooled as indicated on Figure 2 and lyophilized. The main inhibitor present in fraction 2 was the LTP1 that we had purified and characterized previously (Jones and Marinac, 1997), so we purified and studied the inhibitor in fraction 1.

When the lyophilized fraction 1 material was dissolved in 0.3% TFA solution and applied to a C-18 RP-HPLC column, elution with an acetonitrile gradient separated the proteins into seven distinct fractions (Figure 3) that were individually collected and lyophilized. Analysis of these seven fractions for inhibitory activity at pH 5.5, using the substrate azocasein, showed that only fraction D contained significant inhibitory activity (Table 1). The fraction D material was collected and lyophilized, and SDS−PAGE and isoelectric focus-

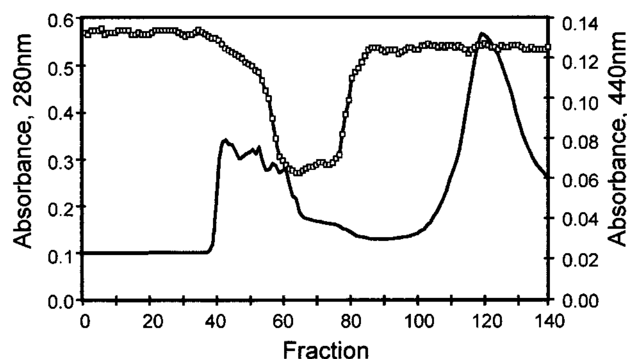


**Figure 1.** Bio-Gel P-30 chromatographic separation of the proteinase inhibitors extracted from barley seed: (−) protein, $A_{280nm}$; (□) proteinase activity, $A_{440nm}$. The hydrolysis of azocasein by a green malt extract in the presence of the separated fractions was monitored at 440 nm.

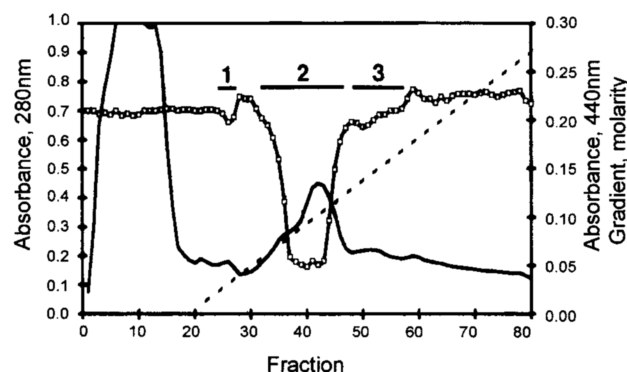


**Figure 2.** CMC separation of the inhibiting fractions from Figure 1: (−) protein, $A_{280nm}$; (□) proteinase activity, $A_{440nm}$; (- - -) $NH_4Ac$ gradient. 1−3 represent fractions that were pooled for further purification.

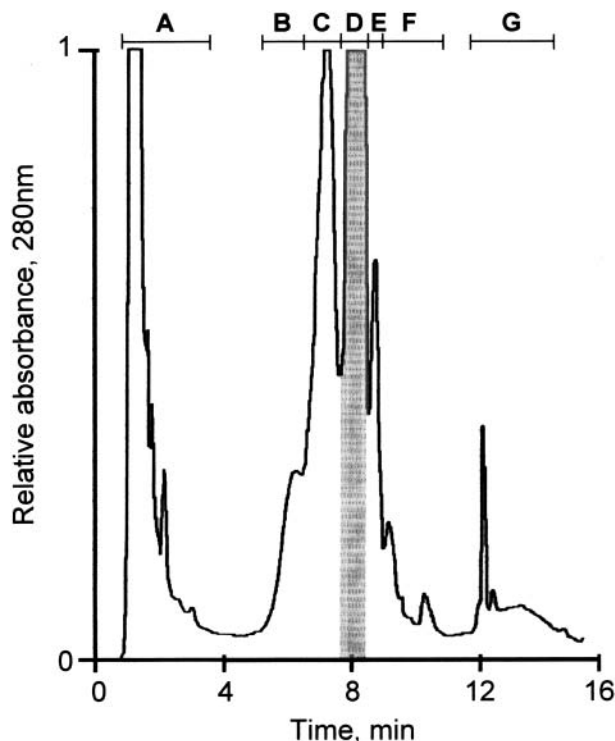


**Figure 3.** RP-HPLC separation of fraction 1 from Figure 2. A−G represent fractions pooled, lyophilized, and analyzed for inhibitory activity. The shaded area, fraction D, contained the inhibitor.

**Table 1. Inhibition of the Proteolytic Activity of a Green Malt Extract by RP-HPLC Purified Inhibitor Protein**

| HPLC fraction | proteinase activity[a] 30 min[b] | 60 min | % inhibition[c] |
|---|---|---|---|
| none | 0.078 | 0.140 | |
| A | 0.088 | 0.141 | 0 |
| B | 0.088 | 0.137 | 1 |
| C | 0.087 | 0.132 | 3 |
| D | 0.033 | 0.047 | 62 |
| E | 0.083 | 0.128 | 4 |
| F | 0.093 | 0.139 | 0 |
| G | 0.089 | 0.140 | 0 |

[a] Absorbance of the supernatant at 440 nm. [b] Length of proteinase assay. [c] Average of 30 and 60 min reactions.

ing were used to confirm that the fraction contained only a single protein (Figure 4). SDS−PAGE of the reduced and nonreduced inhibitor each produced a single band when either 20% or high-density precast Pharmacia PhastGels were used. Figure 4A shows the high-density SDS−PAGE separation of the nonreduced protein sample. The inhibitor migrated to a position corresponding to a molecular weight of ~14500. This may indicate that the inhibitor normally assumes a dimeric conformation, because the reduced protein migrated as if it had a molecular weight of ~7000 (not shown). Figure 4B shows the results obtained when the inhibitor was subjected to IEF on a PhastGel covering the pH range 3−9. The inhibitor (lane 3) migrated as a single band. Calculations using IEF p*I* standards (lanes 1, 2, and 4) indicated the inhibitor had a p*I* value of ~7.2.

MALDI mass spectrophotometric analysis of the inhibitor supported the electrophoretic data, indicating that the purified protein had a mass of 7112 ± 7 amu. This value corresponds almost perfectly with the calculated molecular weight of the B11E gene protein product, 7117.

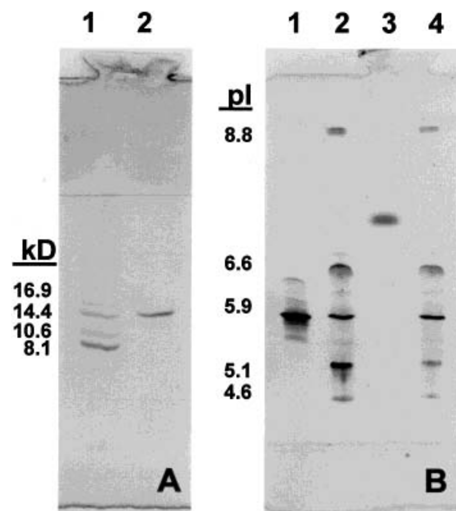


**Figure 4.** SDS−PAGE and IEF analyses of the purified inhibitor protein: (A) SDS−PAGE of the purified inhibitor protein [(lane 1) peptide standards; (lane 2) unreduced inhibitor protein]; (B) IEF analysis [(lanes 1, 2, and 4) IEF standards; (lane 3) purified protein].

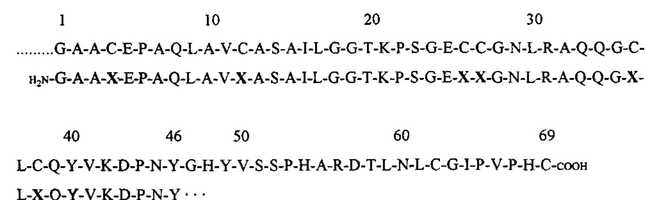


**Figure 5.** Partial amino acid sequences of the purified inhibitor protein (lower sequence) and the protein that would be coded by the LTP2 gene (upper sequence). X, unidentified amino acids.

**Amino Acid Composition and Sequence Analyses.** Jakobsen et al. (1989) had previously cloned and sequenced a gene from barley aleurone tissue that they called B11E. Kalla et al. (1994) studied this gene and pointed out that it coded for a protein having an amino acid sequence very similar to that of a wheat protein that was thought to be a lipid transfer protein (Monnet, 1990), so they named the barley gene product LTP2. When the amino acid sequence of the first 46 residues of the purified inhibitor protein was determined (Figure 5, lower sequence), the sequence was identical with that of the proposed LTP2 protein (Figure 5, upper sequence), if the assumption is made that all unidentified amino acids (X residues in Figure 5) were cysteine residues. This assumption is probably valid, because the sequenced protein was not reduced and alkylated, and nonalkylated cysteine residues are notoriously hard to detect during sequencing, compared to the other amino acids.

The amino acid composition of the inhibitor was determined by hydrolyzing the protein, derivatizing the amino acids with PITC, and using HPLC to separate the derivatives for quantification. The individual amino acid concentrations were calculated by comparing their peak sizes to those of a commercial hydrolyzed protein amino acid standard mix and also by using hydrolyzed native $\beta$-purothionin and reduced and pyridylethylated $\alpha$-hordothionin preparations as standards. The purothionin standards allow a calculation that takes into account the degradation of some of the amino acids that are not totally stable to hydrolysis under the conditions used.

**Table 2. Amino Acid Composition of the Purified Inhibitor**

| amino acid | calcd from protein[a] | deduced from LTP gene[b] | amino acid | calcd from protein[a] | deduced from LTP gene[b] |
|---|---|---|---|---|---|
| D/N | 4.9 | 5 | Y | 3.2 | 3 |
| E/Q | 5.7 | 6 | V | 4.8 | 4 |
| S | 3.8 | 4 | M | 0 | 0 |
| G | 9.2 | 8 | PC[c] | –[d] | 8 |
| H | 2.1 | 3 | I | 2.3 | 2 |
| R | 3.2 | 2 | L | 5.2 | 6 |
| T | 2.5 | 2 | F | 0.5 | 0 |
| P | 9.6 | 6 | K | 1.2 | 2 |
| A | 6.6 | 8 | W | 0 | 0 |

[a] Average of six analyses. [b] From Kalla et al. (1994). [c] Pyridylethylcysteine. [d] Not determined.

The results are summarized in Table 2. These results were obtained by averaging the values obtained using the various standards. The table also shows the amino acid composition of the protein that would be coded by the LTP2 gene (Kalla et al., 1994). Our inhibitor protein had an amino acid composition very similar to that of the postulated LTP protein. This, in turn, implied that the amino acid sequence of the inhibitor beyond residue 46, where our analysis stopped, was likely identical with that of LTP2. The inhibitor composition appeared to contain four more proline residues than the LTP, but this difference was probably due to the presence of a PITC contaminant peak that coeluted with the proline derivative peak during the amino acid analyses.

**Inhibition of Green Malt Endoproteinases by Purified Inhibitor.** The effect of the purified inhibitor on CMC × PAGE separated green malt endoproteinase activities that hydrolyze gelatin is shown in Figure 6. Enzyme preparation (12.5 μL) was loaded onto each lane of each of these gels. Lane 1 was loaded with crude green malt endoproteinase preparation, lane 2 was loaded with green malt endoproteinase preparation material that did not bind when passed through a CMC column, and lanes 3–15 were loaded with sequential fractions that were eluted with a salt gradient from a CMC column that had been loaded with green malt extract.

Figure 6A is a control gel that was loaded with green malt proteinase extract. As previously reported (Zhang and Jones, 1995), there are ~20 endoproteolytic gelatin-hydrolyzing activities that are obvious at pH 4.8 in the control gels containing the gelatin substrate. When the endoproteinase activities that had been separated on the gels were developed in the presence of 10 μM E-64 (Figure 6B), a compound that specifically inhibits cysteine class proteinases, nearly all of the activities that migrated very far into the gel were inactivated, except for one large activity spot on lane 1. Little or none of the activity that migrated only a short distance into the gels was inhibited. Most of the activities that moved a significant distance into the gel were, therefore, due to cysteine class proteinases. When a similar experiment was carried out in which the E-64 was replaced by 16 μg/mL (2.25 μM) of the purified inhibitor protein, the results were as shown in Figure 6C. In the presence of this low level of inhibitor, the activities of all of the proteinases that were sensitive to E-64 were reduced, whereas the E-64-resistant enzymes were not affected by the purified inhibitor. These findings provide strong evidence that the purified inhibitor specifically inhibited the activities of the cysteine class proteinases of green

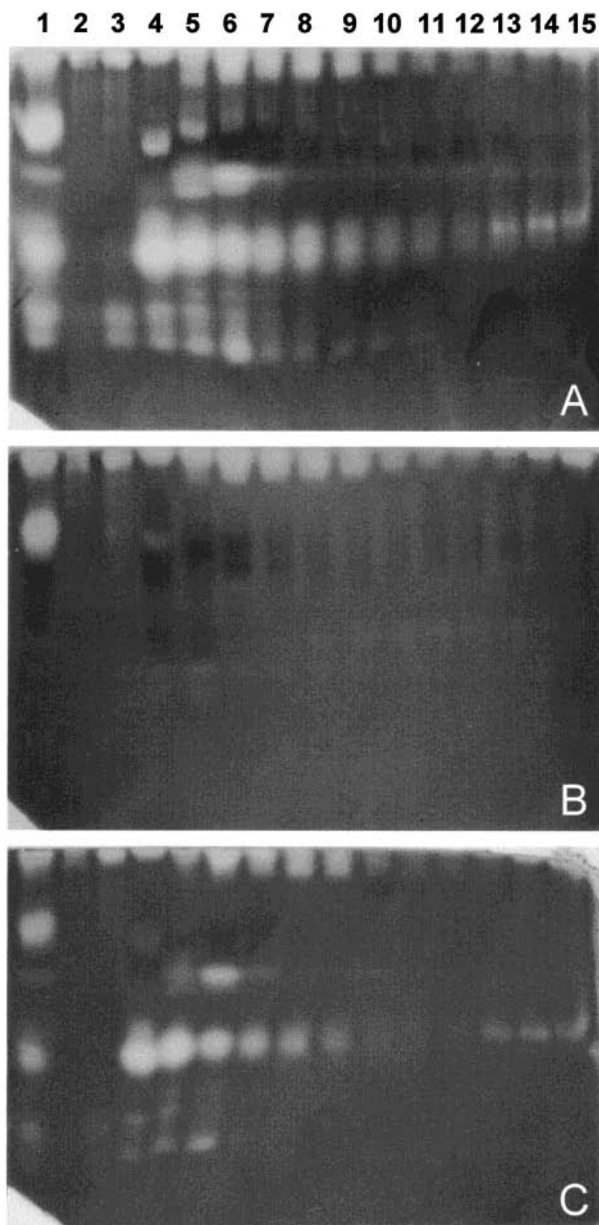


**Figure 6.** 2-D, CMC × PAGE separations of green malt proteinase activities developed in the presence or absence of inhibitors: (A) no inhibitor; (B) same as (A) except the incubation buffer contained E-64, a class-specific cysteine inhibitor; (C) same as (A) except gel was developed in the presence of the purified inhibitor protein; (lane 1) unfractionated green malt proteinase extract; (lane 2) material from the green malt extract that did not bind to the CMC column; (lanes 3–15) fractions that were sequentially eluted from the CMC column with a salt gradient. The PAGE gels contained gelatin substrate. Development was at pH 4.8.

malt and did not inhibit endoproteinases of the other classes. Although the cysteine proteinase activities were only reduced in this experiment, it seems likely that they would have been totally inhibited in the presence of increased amounts of the inhibitor, because previous experiments with unpurified inhibitor fractions indicated that proteinases that were partially inhibited by low amounts of inhibitors were totally inhibited in the presence of increased inhibitor levels (Jones and Marinac, 1991). All of the gels shown in Figure 6 were developed in pH 4.8 buffer. The endosperm tissue of germinating barley has an overall pH of 4.8, so these results likely reflect the hydrolytic events that occur in

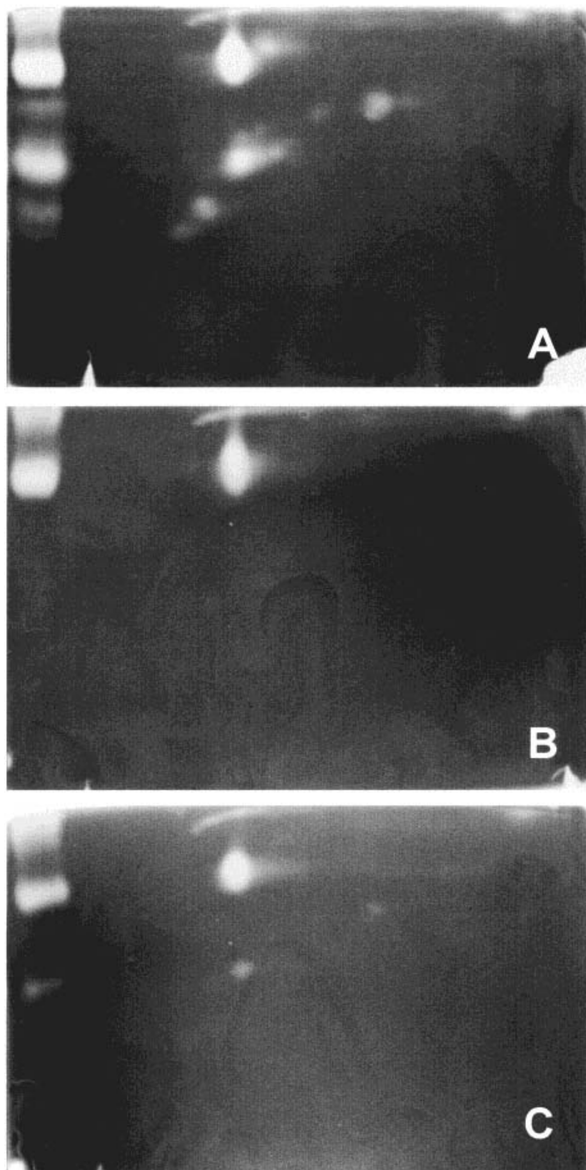


**Figure 7.** Inhibition of 2-D, IEF × PAGE separated green malt proteinases by E-64 and purified inhibitor: (A) no added inhibitor; (B) E-64 added to the incubation buffer; (C) purified barley inhibitor protein added to incubation buffer. The PAGE gels contained gelatin substrate. The enzymatic activities were developed at pH 4.8 in the presence or absence of inhibitors. The left lane was loaded with crude green malt extract that was not separated by IEF.

the germinating barley endosperm, where the majority of storage protein hydrolysis occurs during seed germination.

Figure 7 also shows that the purified inhibitor specifically inhibited cysteine class endoproteinases. In this experiment, IEF × PAGE 2-D gels were used to separate the endoproteinases prior to inhibition, because this gives a clearer indication of which individual proteinase activities are affected by the inhibitor. The substrate protein used was gelatin because it reveals more endoproteinase activities than any of the other substrates tested (results not shown), and the pH of development was 4.8. On these gels, the left-hand lane was loaded with 15 $\mu$L of green malt endoproteinase extract after the IEF separation had been carried out, and the rest of the gel was occupied with the IEF × PAGE 2-D separation carried out with an equal amount of extract. The control gel (Figure 7A) shows that, at this pH, there were ~23 activity spots. This agrees with our previous findings with pH 4.8 gelatin gels (Zhang and Jones, 1995). Those studies had also shown that the observable proteinases that migrated more than a quarter of the way into the PAGE gel were cysteine class enzymes, whereas those near the top of the gel belonged to the serine- or metalloprotease classes. This is confirmed in Figure 7B, which was developed in the presence of E-64. All of the activities that migrated very far into the gel were inhibited by this cysteine-class-specific proteinase inhibitor. Figure 7C shows a gel having endoproteinase activities that were developed in 200 mL of buffer containing 7.5 mL of the purified inhibitor. The pattern is essentially identical with that of Figure 7B, except that there was still a trace of cysteine protease activity. This indicates once again that the inhibitor specifically inhibited all of the cysteine proteinases, and only cysteine proteinases. In this case, the inhibitor concentration was high enough that it caused almost complete inhibition of the cysteine activities.

## DISCUSSION

We have purified and partially characterized a second endogenous inhibitor of cereal endoproteinases; that is, a compound from a cereal that inhibits the activity of proteinases extracted from the same cereal. In this case, the inhibitor was a polypeptide from ungerminated barley that inhibited the activities of certain specific endoproteinases extracted from green malt. This polypeptide, together with the other endogenous barley seed and malt inhibitors, may play a large part in determining which barley storage proteins are hydrolyzed during the malting and mashing processes and to what extent and how quickly those proteins are degraded. The U.S. brewing industry presently prefers that malt for brewing should have a soluble protein level of between 4.6 and 5.6%. Currently, breeders must make many crosses and then laboriously select out those lines that meet these specifications. After we deduce how the overall barley protein hydrolyzing system operates, it should be possible to use either classical breeding or genetic engineering methods to more efficiently manipulate the endoproteinases and their endogenous inhibitors to design barleys that have exactly the right soluble protein levels for their intended final uses.

The inhibitor purified, which apparently comprised all of the inhibitory material present in the inhibitor 1 region of the CMC elution (Figure 2), was from ungerminated barley. From our earlier work (Jones and Marinac, 1995), it appears that this inhibitor is not present in kilned malt, although it occurs in such small amounts, even in barley seed, that it may have been overlooked in malt, where the overall inhibitor level is considerably higher (Jones and Marinac, 1995). Studies by Jakobsen et al. (1989) showed that the mRNA corresponding to the clone called B11E, which specifies the synthesis of this inhibitor protein, was present in aleurone cells during the development of the barley seed (20−30 days after anthesis). It was, however, present in only very low levels in mature aleurone cells. However, the low mRNA level in mature aleurone tissue would not necessarily mean that the LTP2 protein was present in low quantities in barley seeds, if it was stable to degradation.

It does appear from this work, however, that LTP2 is a fairly potent endoproteinase inhibitor that is present in relatively low levels in the barley, because the

inhibitory activity of the CMC inhibitor separation fraction 1 amounted to <5% of the total activity (Figure 2), whereas the results of Figure 7 show that the concentrated inhibitor suppressed about half of the total activity present on the IEF × PAGE 2-D gel. These activity differences may be due, in part, to the fact that the in vitro analyses (Figure 2) were conducted with azocasein substrate, whereas the Figure 7 IEF × PAGE analyses measured the hydrolysis of gelatin.

It was not surprising that the inhibitor inhibited only the cysteine class proteinases because all of our previous studies (Jones and Marinac, 1995, 1997) had shown that the great majority of the unpurified endogenous inhibitor activity was directed toward the cysteine proteinases.

The barley LTP2 inhibitor protein may serve several functions. It was originally studied because its gene, pulled at random from a mixture of genes synthesized in large amounts in the aleurone tissue of developing grain, showed strong homology with other previously studied "lipid transfer proteins" (Kalla et al., 1994). Genes that could code for similar proteins have also been found in the other cereal grains maize and wheat (Linnestad et al., 1991; Castagnaro and Garcia-Olmedo, 1994), and a protein encoded from a wheat LTP gene has reportedly been isolated by use of the in vitro lipid transfer assay (Monnet). It may therefore serve a lipid-transferring function in the barley grains. It has not been shown to do so, however.

In addition, a second barley−beer LTP protein, LTP1, has been reported by two different research groups (Sorensen et al., 1993; Lusk et al., 1995) to be one of the main proteins present in beer foam and to be one of the proteins primarily responsible for foam formation and retention in beer. It seems possible that the LTP2 protein may play a similar role, although it is present at much lower levels than LTP1. It would not be surprising that the LTP2 protein was not degraded during brewing, because this work shows that it is an inhibitor of the cysteine proteinases, which have long been recognized as being the main ones responsible for degrading proteins during the malting and mashing processes.

It also seems likely that it might survive wort boiling, because we subject the inhibitor to boiling as one of the first steps in purifying it and have seen no evidence that this boiling precipitates or destroys any of the inhibitor. We have evidence, which will be presented elsewhere, that the short boiling step used frees the barley and malt inhibitors from bound material with which they have become complexed, probably during the extraction process. If the LTP2 behaves like barley LTP1, this short boiling period would have little effect on its structure, since it takes an hour of vigorous boiling to change half of the "barley" LTP1 to its "foam" form during brewing (Bech et al., 1995). However, as pointed out by Castagnaro and Garcia-Olmedo (1994), the LTP1 and LTP2 proteins are really not very similar in their characteristics and, therefore, may not react similarly to boiling. Essentially nothing is known about the physical and biochemical characteristics of barley LTP2, so the question of how it behaves when boiled for a short time remains open. However, if, as has been indicated (Jakobsen et al., 1989), it is present in beer after a ∼1 h wort boil, it is apparently quite stable to boiling.

Why is this protein present in barley? Obviously, it did not evolve so that beer would have a good, stable head. Because the protein has never really been shown to transfer any lipids in barley, it seems quite possible that its real purpose in the grain is to inactivate any cysteine endoproteinases that form during grain maturation until such time as their activities are needed in the mature seed or during seed germination.

## ABBREVIATIONS USED

LTP, lipid transfer protein; CMC, carboxymethyl cellulose; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; E-64, *trans*-epoxysuccinyl L-leucyclamido(4-guanidino)butane; PITC, phenylisothiocyanate; RP-HPLC, reversed phase high-performance liquid chromatography; $NH_4Ac$, ammonium acetate; TCA, trichloroacetic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; IEF, isoelectric focusing; TFA, trifluoroacetic acid.

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