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Trypsin/α-Amylase Inhibitors Inactivate the Endogenous Barley/Malt Serine Endoproteinase SEP-1

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Barley (*Hordeum vulgare* L.) malt contains endoproteinases belonging to all four of the commonly occurring classes, including serine proteinases. It also contains low molecular weight proteins that inhibit the activities of many of these endoproteinases, but it had never been shown that any barley or malt serine proteinases could be inhibited by any of these endogenous proteins. It is now reported that some proteins that were concentrated using an "affinity" method inhibited the activity of a malt serine endoproteinase. Two-dimensional electrophoretic and in vitro analyses showed that the inhibited enzyme was serine endoproteinase 1 (SEP-1) and that the inhibition could be quantified using a semipurified preparation of this enzyme. Amino acid sequencing and MALDI-TOF MS were used to identify the components of the partially purified inhibiting fractions. Only the "trypsin/α-amylase inhibitors" or chloroform/methanol (CM) proteins, most of which had truncated N and C termini, and one fragment of β-amylase were present in the inhibitory fractions. When a CM protein fraction was prepared from barley according to traditional methods, some of its component proteins inhibited the activity of SEP-1 and some did not. This is the first report of the purification and identification of barley malt proteins that can inhibit an endogenous serine proteinase. It shows that some of the CM proteins probably play a role in controlling the activity of barley proteinases during germination, as well as possibly protecting the seed and young plant from microbes or pests.

KEYWORDS: ***Hordeum vulgare*; barley; serine proteinases; inhibitors; CM proteins**

INTRODUCTION

During the germination process, as defined by maltsters, seeds produce a mixture of endoproteinases that, among other things, degrade their storage proteins to furnish amino acids that are utilized by the seedling until it begins autotrophic growth. Maltsters and brewers take advantage of this process to provide a source of amino acids in worts that are fermented by yeasts to produce beer. In the past, it has generally been accepted that most of the protein hydrolysis that occurred during malting and mashing was carried out by the cysteine class endoproteinases that are abundant in malt. However, our recent studies have shown that representatives of all four of the commonly occurring endoproteinase classes, including serine enzymes, occur in germinated barley (*1*). Carrying out mashes in the presence of specific inhibitors of the serine class proteinases, however, did not decrease the amounts of "soluble protein" (a mixture of amino acids, peptides, and proteins) in worts, indicating that the serine endoproteinases apparently did not directly contribute to the hydrolysis of barley storage proteins during mashing.

We recently purified and characterized one of the major serine endoproteinases of malt, which we called serine endoproteinase 1 (SEP-1) (*2*). As expected, the purified SEP-1 did not hydrolyze barley storage proteins (hordeins) in vitro, even though purified cysteine proteinases and metalloproteinases do (*3*, *4*). The other green malt serine endoproteinase that has been purified and studied, hordolisin, also did not appear to play any role in degrading the hordein storage proteins (*5*). It therefore seems likely that this and the other serine proteinases are present in malt to hydrolyze specific peptide bonds of a few special proteins, rather than simply to hydrolyze generic proteins to provide a mixture of amino acids for plant growth.

It was shown many years ago by Enari et al. (*6*) that certain barley proteins could inhibit the activities of some of the malt cysteine proteinases, and we have shown that these inhibitors are even more prevalent in malt (*7*). Some malt proteins also can inhibit the activities of serine proteinases from nonplant sources, but not those from either barley or malt. Østergaard et al. have shown, by using in vitro translated DNA, that wheat serpins (serine proteinase-inhibiting proteins) can inhibit some mammalian serine proteinases, but not those from either barley or muskmelon (*8*). No one has previously shown that any proteins extracted from a cereal grain could inhibit any cereal grain serine endoproteinases, including those that occur in either barley or malt.

We demonstrated recently that it was possible to concentrate malt endoproteinase inhibitors by using an "affinity" purification

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process that bound the inhibitors to malt proteinases and then disrupted this binding by heating (*2*). By using this method to concentrate the serine proteinase inhibitors from malt and using our purified SEP-1 enzyme as a test system, we have identified and partially purified a group of proteins from malt extracts that inhibit the activity of the SEP-1 enzyme in vitro. The inhibitors belonged to a group of proteins that are known as trypsin/α-amylase inhibitors or, alternatively, as chloroform/methanol (CM) proteins (*9*), due to the fact that they are readily soluble in mixtures of these organic solvents. Some of the barley CM proteins were known to inhibit either α-amylases from various sources, including those from insects that attack barley (but not those from barley or malt), or bovine trypsin. None inhibited both α-amylases and trypsin. When a mixture of the CM proteins was prepared according to traditional methods, some of its components also inhibited the activity of SEP-1.

MATERIALS AND METHODS

"Affinity" Purification Method. *Preparation of Malt.* Barley (cv. Morex, a high-quality six-rowed U.S. malting barley) was grown in Idaho in 1999, and 170-g (db) samples were malted using the methods described previously, including kilning to 85 °C (*10*). The resulting malt was very similar to that made commercially from Morex barley. After rootlets were removed, the malt was ground in a Brinkmann ZM-1 centrifugal grinding mill (Westbury, NY) to pass a 0.5-mm screen. The ground malt was stored at −20 °C until extracted.

Preparation of Malt Extract. The ground malt (100 g) was mixed with 300 mL of 50 mM, pH 5.5, ammonium acetate buffer. After the mixture had been stirred for 30 min at room temperature, it was centrifuged at 12100*g* for 10 min, and the supernatant was decanted through four layers of cheesecloth.

Bio-Gel P-30 (Gel Filtration) Separation of the Endoproteinase−Inhibitor Complexes from the Extract. A 190-mL portion of the extract supernatant was applied to a 5 cm × 85 cm Bio-Gel P-30 column (medium grade) and equilibrated with 50 mM, pH 5.5, ammonium acetate buffer. The column was eluted with the 50 mM ammonium acetate buffer, and 10.2-mL fractions were collected. The absorbance of the eluant was monitored at 280 nm. The fractions that contained 280-nm-absorbing material that voided the column (fractions 43−65) were pooled.

Splitting the Enzyme−Inhibitor Complexes by Boiling. The pooled solution was put into a round-bottom flask, heated to boiling in a heating mantle, and refluxed gently for 7 min. The boiled solution was cooled in an ice−water bath and centrifuged for 10 min at 12100*g*, and the supernatant was filtered through four layers of cheesecloth.

Ion Exchange Separation of the Boiled Extract. A 1 cm × 8 cm column of quaternary ammonium cellulose (QA 52, Whatman Bio-Systems Ltd., Maidstone, U.K.) was poured and washed thoroughly with 50 mM ammonium acetate, pH 5.5. The boiled extract supernatant (235 mL) was applied to the column, and the absorbance of the eluate was monitored at 280 nm. Fractions (400 drops each) were collected until the absorbance reached a maximum (∼0.87 A), after which the eluant was collected as a single fraction in a beaker. After all of the extract had been applied to the column, 400-drop fractions were again collected and the column was washed with the 50 mM ammonium acetate buffer until the absorbance reached baseline. The column was then eluted with a 50−300 mM linear ammonium acetate gradient, using 150 mL of each buffer concentration, and fractions (10.2 mL each) were collected as previously. Two fractions, one that contained the material that did not stick to the QA column and one with the protein that eluted as soon as the gradient was applied, were obtained. These were collected and freeze-dried.

Separation of the Serine Endoproteinase Inhibitors by Reverse Phase HPLC Chromatography. (1) QA-Separated Fractions. The two freeze-dried QA-separated fractions were each dissolved in 6 mL of water and filtered through a 0.45-μm Millex-HV membrane filter. Aliquots (3 mL) of the solutions were applied to a 150 × 4.6 mm Phenomenex (Torrance, CA) Luna C18, 5-μm HPLC column that was equilibrated with a solution of 5% solvent B (0.1% trifluoroacetic acid in acetonitrile) in solvent A (0.1% trifluoroacetic acid in water). After 1 min, the column was eluted with a linear 5−60%, 15 min, gradient of solvent B in solvent A. The column was then washed with 5% solvent B for 5 min, and the next sample was injected. The elution was monitored at 280 and 320 nm, and the 280-nm-absorbing material was collected as a series of fractions (see Results and Discussion). The collected fractions were freeze-dried twice to remove the trifluoroacetic acid.

(2) GPC-100 HPLC Gel Filtration Samples. Fractions that inhibited the serine endoproteinase and that had been separated by HPLC gel filtration on a GPC-100 column and freeze-dried (see below) were dissolved in water, filtered through 0.45-μm membrane filters, applied to the Luna C18 column, and eluted using the 5−60% solvent B gradient. The 280-nm-absorbing eluate was collected as a series of relatively pure fractions. The collected samples were freeze-dried twice and assayed for their abilities to inhibit the serine proteinase preparation.

Separation of the QA-C18 Fractions by HPLC Size Exclusion Chromatography. The freeze-dried extract fractions that had been subjected to QA ion exchange and reverse phase HPLC (RP-HPLC) separations were dissolved in ∼1 mL of 50 mM, pH 5.5, ammonium acetate buffer and filtered through a 0.45-μm membrane filter. Aliquots (250 μL) of the filtered samples were applied to a 300 × 7.8 mm Synchrome Synchropak GPC 100 size exclusion column that had been equilibrated with the 50 mM ammonium acetate buffer and eluted with the same buffer. The absorbance of the eluting material was monitored at 280 and 320 nm, and fractions that appeared to give the best separation of the eluting proteins were collected. The fractions were freeze-dried and reseparated by RP-HPLC (see above).

Chromatofocusing. Freeze-dried, partially purified, samples from either the CM (see below) or affinity purification method were dissolved/suspended in 25 mM imidazole−HCl buffer at pH 6.9. After centrifugation in a microfuge, the supernatant was applied to a 1.2 cm × 38 cm PBE-94 chromatofocusing column (Pharmacia Biotech, Uppsala, Sweden) that had been equilibrated with the 25 mM imidazole buffer. The column was eluted with Polybuffer 74−HCl, at pH 4.5, over a 600 mL gradient that ranged from pH 6.9 to ∼4.5. Fractions (∼6.7 mL each) were collected, and their absorbances at 280 and 320 nm, pH values, and abilities to inhibit the activity of SEP-1 were measured as specified below.

Chloroform/Methanol (CM) Purification Method. The basic method was modified from that of Salcedo et al. (*11*). Ground Morex barley (100 g) was mixed with 1 L of petroleum ether and held at room temperature for 1 h, with mixing every 10 min. The suspension was rested for 10 min, the liquid was decanted, and the solid remnant was dried. A 2:1 (v/v) chloroform/methanol solution was prepared, and 2 L was added to the defatted and dried barley. After 1 h of incubation at room temperature with frequent stirring, the supernatant was decanted and retained. The extraction was repeated with another 2 L of CM solvent, and this supernatant was added to the first. The CM solutions were dried under vacuum in a rotary evaporator at 40 °C.

The dried CM material was mixed with 2 L of 4 °C, 0.5 M, NaCl solution and held at 4 °C for 1 h, with mixing every 10 min. The solvent was decanted into a centrifuge bottle, and the solid remnant was treated with a second 2 L of 0.5 M NaCl. The second supernatant was added to the first, and the combined sample was centrifuged at 21600*g* for 25 min. The supernatant was then dialyzed three times versus 4 L of water with 3500 MWCO tubing and freeze- dried.

Reverse Phase HPLC Separation of the CM Preparation. The 0.9 g of solid from the CM extraction was dissolved in 50 mL of water, and 10-mL aliquots were filtered through a 0.45-μm filter and applied to a 150 × 4.6 mm Phenomenex Luna C18, 5-μm HPLC column that was equilibrated with 5% solvent B (see previous separations). After the 10 mL of sample was run onto the column, it was washed for 2 min with 5% solvent B and then with a linear gradient that ran from 5 to 50% solvent B over 12 min. The column was then returned to 5% solvent B in 1 min, and a new sample was loaded. The absorbance of the column eluant was monitored at 280 and 320 nm, because preliminary work had shown that many of the 280-nm-absorbing peaks also absorbed at 320 nm (unlike most proteins) and that the 320-nm-absorbing peaks did not cause inhibition. The two peaks and a trailing shoulder that eluted near the end of the run showed some inhibitory activity, so they were collected for mass spectrometric analysis and

further purification. They were labeled fractions cm1, cm2, and cm3, respectively.

Chromatofocusing of the CM Preparation. The three CM HPLC fractions were freeze-dried and separated by chromatofocusing as discussed previously. To remove the chromatofocusing ampholines, the 280-nm-absorbing fractions from the chromatofocusing step were subjected to RP-HPLC as described above, except that the elution gradient was held at 5% solvent B for 12 min, raised to 25% B in 1 min, then to 39% B between 13 and 23 min, and returned to 5% B in 1 min. The 280-nm-absorbing peaks that eluted after 17 min or later were collected and freeze-dried, and their inhibitory activities and mass spectra were measured.

Mass Spectrometric and Amino Acid Sequence Analysis of Fractions. The molecular masses of the purified proteins were determined by MALDI-TOF MS 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 selected 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.

Preparation of a Serine Endoproteinase (SEP-1) Fraction for Testing Inhibitors. A partially purified green malt (unkilned barley that was "steeped" and then germinated for 96 h) serine endoproteinase called SEP-1 was prepared using the initial steps of the method of Fontanini and Jones (*2*). The enzyme was extracted from green malt kernels from which the endosperm tissue had been excised. The kernels were extracted, dialyzed, and subjected to anion exchange chromatography, and the resulting activity was used as the serine endoproteinase source. It contained some contaminating proteins, including some metalloproteinase endoproteolytic activity, but by using a pH at which and a substrate against which the metalloproteinase was inactive, the activity of the SEP-1 enzyme was specifically measured.

Assaying for the Inhibition of the SEP-1 Activity. *In Vitro Assay.* A reaction mixture was prepared that normally contained 10 μL of inhibitor preparation, 75 μL of pH 6.5, 50 mM citrate−phosphate buffer, and 20 μL of the endoproteinase preparation (*2*). This inhibitor−enzyme−buffer solution was incubated for 10 min at room temperature, and the reaction was started by adding 2 μL of the synthetic substrate *N*-succinyl-alanyl-alanyl-prolyl-leucyl *p*-nitroanilide (sucAAPLpNA), which was dissolved at a concentration of 50 mM in dimethyl sulfoxide. The reaction was carried out at 50 °C in a temperature-controlled eight-cell cuvette in a Shimadzu BioSpec-1601 spectrophotometer. Readings were taken at 410 nm every 3 min, normally for 18 min. By carrying out the reaction at pH 6.5, there was no contribution to the reaction measurement from possible contaminating cysteine proteinases, which are active only at pH 5.5 and lower (*1*), and by using the substrate sucAAPLpNA, which is not hydrolyzed by metalloproteinases (*2*), there is no contribution to the activity from these enzymes.

When the inhibitory activities of freeze-dried samples were analyzed, the samples were normally dissolved in 300 μL of water, of which a 10-μL sample was initially analyzed. If the results thus obtained were ambiguous, or when column fractions were analyzed without freeze-drying, either larger or smaller volumes of inhibitor were added to the reactions.

2D IEF × PAGE Assay. The malt endoproteinases were separated by subjecting extracts to a two-dimensional separation on acrylamide gels, using isoelectric focusing (IEF) from pH 3.2 to 6.8 in the first dimension and polyacrylamide gel electrophoresis in 11% acrylamide gels that contained 0.1% azogelatin in the second, as reported by Zhang and Jones (*1*). The enzymatic activities were then analyzed by developing the gels in the presence or absence of inhibitor fractions, as described previously (*12*).

RESULTS AND DISCUSSION

We have been studying the protein-degrading systems of barley and malt for several years to gain a better understanding of how the levels of amino acids, peptides, and solubilized proteins in germinated (malted) seeds are controlled. This process is critical to the plant during the germination process, and it is also important industrially, because the amount of "soluble protein" that is formed during the malting process is crucial to the formation of acceptable malts, and thus for brewing. To understand this system in its entirety, we have studied both the endoproteinases of malt, most of which form during the malting process (*1*−*4*, *10*), and a group of low molecular weight proteins that can inhibit the activities of certain of the endoproteinases (*12*, *13*). During the study of the inhibitors of malt cysteine-class endoproteinases using a two-dimensional (2D) electrophoretic system (*12*), it was noticed that one of the serine class proteinase activity spots on the gel was also partially inactivated in the presence of a crude, boiled inhibitor preparation.

At the time, it was not possible to study this inhibition in depth, because no purified malt serine endoproteinase was available that could be used to carry out in vitro studies. However, we have recently purified and characterized a serine endoproteinase, SEP-1, from green malt (germinated barley) (*2*). This enzyme did not appear to be directly involved in the hydrolysis of storage proteins during the malting or mashing processes. This was not surprising, because we had previously shown that inhibiting the malt serine proteinases had no detectable effect on the production of soluble protein during mashing (*14*). The purpose of SEP-1 in the germinating grain is still unknown, but its presence in malt implies that it possibly plays some important role in the germinating seed.

Inhibition of Malt SEP-1 by Crude Affinity-Purified Barley and Malt Extracts. *Analysis by 2D Gel-Separated Endoproteinases*. We recently showed that endoproteinase inhibitors can be concentrated by an affinity method (*15*). In this procedure, the proteinase−inhibitor complexes that spontaneously form when ground barley or malt is dissolved in buffers are collected, and the inhibitors are then released by heating. The utility of this method for concentrating the serine endoproteinase inhibitor(s) was tested by preparing pH 5.5 buffer extracts from barley and from malt, isolating the enzyme−inhibitor complexes that voided from a Bio-Gel P-30 column and boiling the voided fraction. When the supernatants from this process were tested for their abilities to inhibit the activities of endoproteinases that had been separated by the 2D method (**Figure 1**), the activity of the major malt serine proteinase, which migrated as a strong activity spot (heavy arrow, **Figure 1A**), was essentially completely negated, as it was by the specific serine proteinase inhibitor phenylmethanesulfonyl fluoride (PMSF; **Figure 1B**). The SEP-1 activity was partially inhibited in the presence of 3 mL of the affinity-purified preparation (**Figure 1C**) and nearly completely inactivated with 10 mL of the inhibitor solution (**Figure 1D**). The gels depicted in **Figures 1C**,**D** were developed with inhibitor that was prepared from barley, but malt inhibitors gave the same effect.

Green malt contains a second fairly strong serine endoproteinase activity that migrates to the top right-hand corner of these 2D gels (light arrow, **Figure 1A**) and, unlike the SEP-1, was not strongly inhibited by the boiled extracts. There was also a weak activity that migrated to the left of the major serine proteinase and that was possibly a serine class enzyme (*1*). This minor activity was partially inhibited in the presence of the affinity-purified proteins. This ability to simultaneously observe the effects of the inhibitors on each of the separated green malt endoproteinases is one of the major advantages of the 2D inhibition method. The gels depicted in **Figure 1** were rinsed twice with, and then developed in, pH 6.5 buffer. However, the pH of the developing solution had increased to ~7.7 by the end of the overnight development incubation, due to the fact

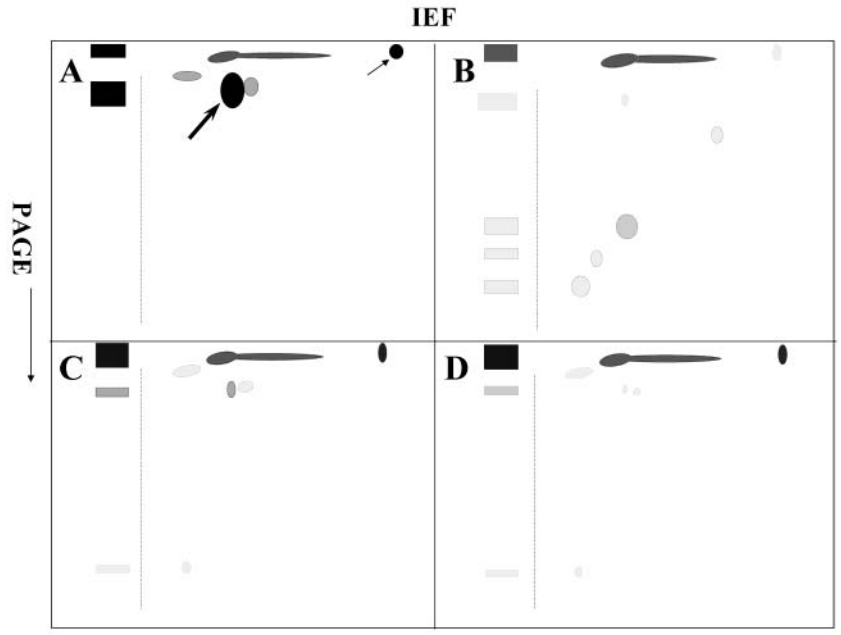

Figure 1. IEF × PAGE, pH 6.5, analysis of the activities of green malt endoproteinases in the presence and absence of inhibitors: (A) activities developed in the absence of inhibitors; (B, C, D) developed in the presence of PMSF, 3 mL of barley extract, and 10 mL of barley extract, respectively. The arrow indicates the SEP-1 serine endoproteinase activity. The sample on the left side of each gel was subjected only to the second dimension, PAGE, separation. The darker shadings indicate higher proteolytic activity levels.

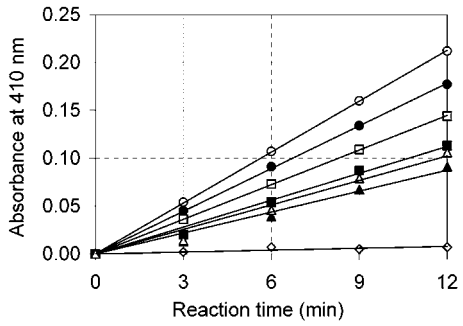

Figure 2. In vitro inhibition of the malt SEP-1 activity by barley and malt extracts. The semipurified green malt SEP-1 preparation was analyzed in the presence of (○) control, no inhibitor; (●) 1 μL of barley extract; (□) 2 μL of barley extract; (■) 4 μL of barley extract; (▲) 8 μL of barley extract; (△) 20 μL of malt extract; and (◇) no added enzyme.

that the pH inside the gels was 8.8 at the end of the separation process. Gels that were incubated in pH 5.5 buffer gave essentially identical results.

In Vitro Analyses. In vitro analyses were also carried out with a semipurified SEP-1 preparation. This enzyme extract was prepared from green malt from which endosperm had been removed, and it contained some metalloproteinase activities as well as the SEP-1 (*2*). The inhibition tests were carried out at pH 6.5 and with the synthetic substrate sucAAPLpNA. Neither cysteine nor aspartic barley endoproteinases are active at pH 6.5 (*1*), and the malt metalloproteinases do not hydrolyze the sucAAPLpNA substrate (results not shown), so that any activity differences that are measured under these conditions must be due to the serine proteases (mainly SEP-1).

This analysis system, like the 2D one, showed that both malt and barley extracts inhibited the serine proteinases to about an equal extent (**Figure 2**). In the absence of enzyme, no hydrolysis occurred, and the inhibition with the barley extract increased with the amount of inhibitor added, up to 8 μL. Above that level, to at least 20 μL, the inhibition remained constant, reducing the original activity by ∼60%. The inhibition with 20 μL of the malt inhibitor was also ∼60%. It is not clear why the inhibition with the crude inhibitor preparation never exceeded 60%, because partially purified inhibitor preparations often gave between 85 and 89% inhibition of the enzyme, compared to 89% inhibition by 10 mM PMSF. *o*-Phenanthroline, a specific inhibitor of metalloproteinases, did not inhibit the enzyme preparation, confirming that the metalloproteinases in the enzyme preparation did not interfere with the serine endoproteinase activity measurements.

Purification and Analysis of Affinity-Concentrated Inhibitors. To purify the inhibitor(s) for characterization, a malt sample was extracted with pH 5.5 buffer and passed through a Bio-Gel P-30 column. The solution that voided the column (which contained, among other things, a mixture of proteinases that were complexed with their inhibitors; *15*) was refluxed for 7 min (to destroy the enzyme−inhibitor complexes, release free inhibitors, and inactivate and precipitate the enzymes), clarified by centrifugation, and applied at pH 5.5 to a QA52 ion exchange column. Most of the 280-nm-absorbing material passed through the column without binding, and elution of the QA column with 20 mM, pH 6.5, ammonium citrate buffer displaced most of the material that had bound to it (results not shown). This QA-bound material was separated into eight fractions by RP-HPLC. Four of the separated fractions partially inhibited the green malt enzyme, but these fractions were not investigated further, because the proteins that did not bind to the QA column inhibited more strongly. The other four fractions did not inhibit the enzyme.

Proteins That Voided the QA Column. The material that did not adhere to the QA column at pH 5.5 was concentrated by freeze-drying, dissolved in ammonium acetate, and separated by RP-HPLC (**Figure 3A**). The absorbance of the eluant was monitored at both 280 and 320 nm, because preliminary studies had shown that there was a large amount of 320-nm-absorbing material in the extracts but that the fractions that absorbed strongly at 320 nm did not inhibit the serine proteinase. Several fractions from the RP-HPLC separation inhibited the enzyme

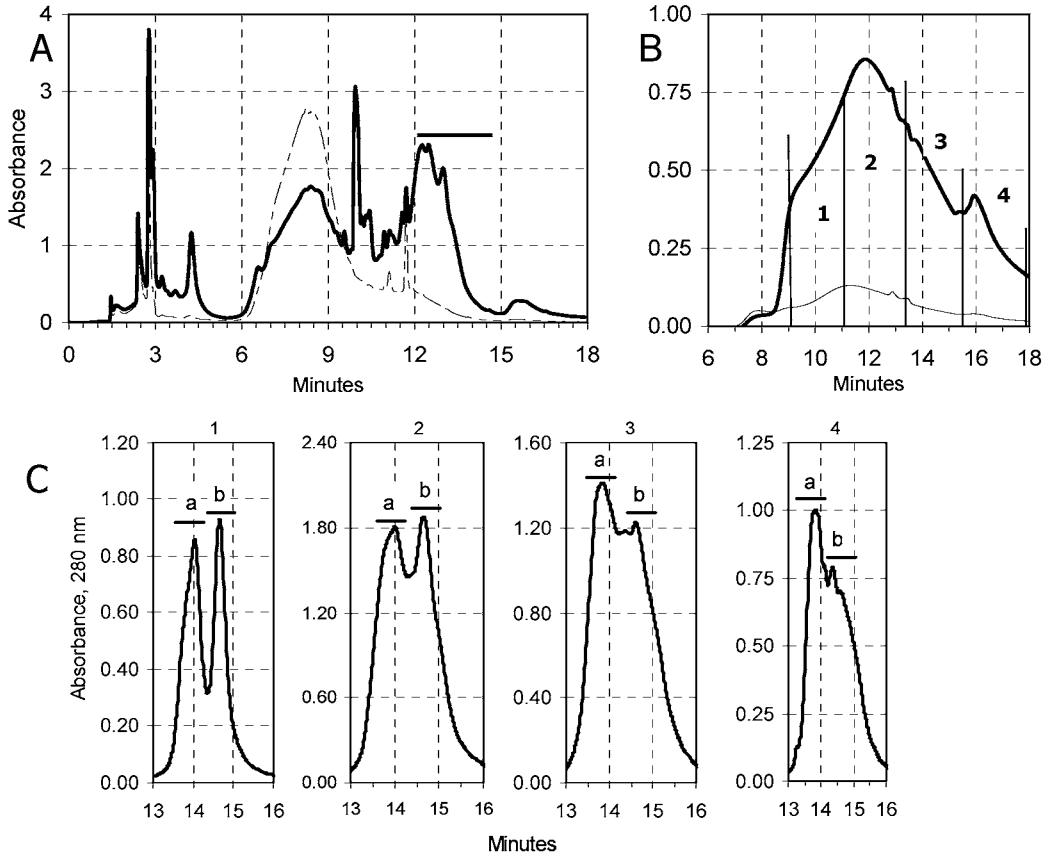

Figure 3. Partial purification of affinity-concentrated barley proteins that inhibited the activity of barley SEP-1: (A) separation of a buffer extract of barley by reverse phase HPLC [(—) Abs 280 nm; (—) Abs 320 nm; the horizontal bar indicates the fractions that inhibited the SEP-1 activity strongly and that were studied further]; (B) GPC-100 HPLC gel filtration of the fraction from (A) that is indicated by the bar; (C) reverse phase HPLC separations of fractions 1–4 that are indicated in (B). All of the collected samples were freeze-dried before being subjected to further purifications.

Table 1. Inhibition of the Malt Serine Endoproteinase by Partially Purified Inhibitors[a]

fraction	inhibition,[b] %	fraction	inhibition, %
1a	84	3a	73
1b	44	3b	81
2a	88	4a	81
2b	76	4b	82

[a] Extracts were separated by RP-HPLC, GPC-100 HPLC, and a second RP-HPLC step. [b] In the presence of 10 μL (of 300 μL total) of inhibitor solution. The addition of 20 μL of inhibitor solution gave essentially identical inhibition percentages.

preparation, but the majority of the inhibition occurred with the fraction that eluted at the end of the separation, as denoted by the bar in **Figure 3A**. This fraction was concentrated by freeze-drying, and its components were separated with a GPC-100 HPLC size separation column. The proteins eluted from the GPC-100 column as a broad peak that was collected as a series of four fractions, as indicated in **Figure 3B**. These fractions were individually freeze-dried and again separated with the RP-HPLC column (**Figure 3C**). Two major peaks, which varied in their relative amounts, were resolved from each of the GPC-100 fractions. These peaks were individually collected (see bars, **Figure 3C**) and freeze-dried, and their inhibitory activities were measured (**Table 1**). With the exception of fraction 1b, all of these samples inhibited the serine proteinase strongly.

Mass Spectrometric and Amino Acid Sequence Analyses of the Inhibitor Fractions. Samples were removed from each of the inhibiting fractions, freeze-dried, and subjected to MALDI-TOF MS analyses at the University of Wisconsin Biotechnology Center. Representative mass spectra of some of the fractions for which inhibitions are listed in **Table 1** are shown in **Figure 4**. Sample b2d corresponded to fraction 3a of **Table 1** and **Figure 3C**, but it originated from the 10.0−11.8-min section of the **Figure 3A** separation, whereas the other samples were all from the indicated 11.8−14.0-min section. Sample b2d caused 76% inhibition of the SEP-1 proteinase under the conditions used to obtain the data listed in **Table 1**. The mass spectra showed that each of the fractions contained a mixture of protein forms, the molecular masses of most of which fell between 12700 and 15700. Two protein populations were present; one covered the range from about 12700 to 13600 Da, the other from 15100 to 15700 Da. Some of the samples contained only the 13000 MW proteins (e.g., b2d, 1a, 2a, 4a) and others only the 15000 MW ones (1b, 2b, and 3b). Only sample 4b contained both 13000 and 15000 MW proteins.

Portions of four of these samples (1a, 2b, 4b, and b2d, substituting for 3a) were subjected to N-terminal amino acid sequencing. Because the MALDI MS patterns of fractions 1b, 2a, 4a, and 3b indicated they contained essentially the same proteins as samples 2b, 1a, 3a, and 2b, respectively, these samples were not sequenced. The sequencing indicated that various portions of four separate proteins were present (**Table 2**). One of these, which was present only in b2d in small amounts and was apparently a contaminant, was a small portion (residues 470−?) of barley β-amylase (Swiss-Prot P16098). The other three were all members of the CM (chloroform/methanol soluble) protein group:CMa, CMb, and CMd (Swiss-Prot P28041, P32936, and P11643). The N-terminal amino acid sequences of these sequenced proteins are listed in **Table 2**.

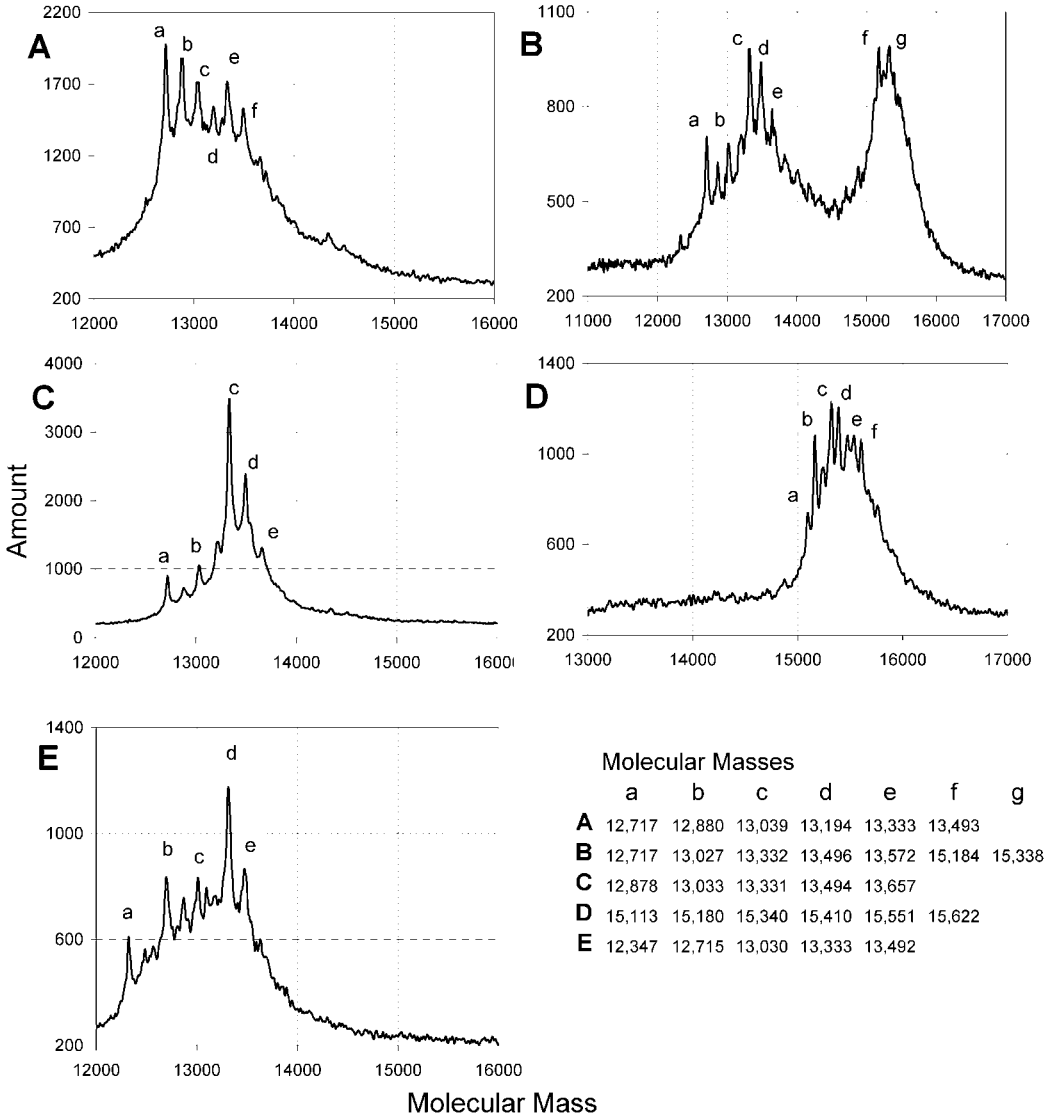

Figure 4. MALDI mass spectra of selected affinity-purified inhibitor fractions. The fractions analyzed were (A) b2d (equivalent to 3a of **Figure 3C**, but from the 10.0–15.8 min fraction of **Figure 3A**), (B) 4b, (C) 1a, (D) 1b, and (E) 4a from **Figure 3C**.

Table 2. Partial N-Terminal Amino Acid Sequences of Affinity-Purified Inhibitors of the SEP-1 Barley Serine Endoproteinase

sample		sequence	protein
b2d	1[a]	S-E-D-C-T-P-W-T-A-T-P-I-T-P-L-P-X-C-R	CMb, residues 27–
	2	T-X-Q-Y-C-Y-A-G-M-G-L-P-S-N-P-L-E-G-C-R-E-Y-V-A-Q-Q	CMa, residues 26–
	3	A-A-Q-X-K-L-Q-X-F-X-F-Q-E-H-T-D	β-amylase
1a	1	S-E-D-C-T-P-W-T-A-T-P-I-T-P-L-P-S-C-R-D-Y-V-X-Q-X-A-X-R-I	CMb, residues 27–
	2	X-G-Q-Y-C-Y-A-G-M-G-L-P-S-N-X-L	CMa, residues 26–
2b	1	A-E-D-C-S-P-G-X-A-F-P-T-N-L-L-G-H-C-R	CMd, residues 29–
	2	X-P-G-Q-P-Y-X-X-K-L-Y-X-X-X-X-L	CMd, residues 75–
4b	1	A-X-D-C-S-P-G-V-A-F-P-X-N-L-L-X-H-C-R-D	CMd, residues 29–
	2	Y-P-G-Q-P-Y-L-A-K-L-Y-X-X-Q-E-L-A-E-I-P	CMd, residues 75–
	3	S-E-D-C-T-P-X-T-A-T-P-I	CMb, residues 27–

[a] Each of the samples analyzed yielded more than one sequence. Samples b2d and 4b gave three and samples 1a and 2b two. The sequences are listed in order of the amount of each protein that was present. X = no detected amino acid. Because these proteins were not alkylated, their cysteine residues could not be detected and were, in each case, reported as X. In locations where such residues corresponded to C in Swiss-Prot, they have been recorded as C in the above sequences. In other cases, when residues could not be detected, they have been left as X.

As expected from their MALDI MS patterns, the proteins in fractions 1a and b2d were identical, except that b2d contained the β-amylase contaminant. Those of fraction 1b were different from the ones in 1a and b2d, and fraction 4b contained proteins from both the 1a (~13000 MW) and 1b (~15000 MW) classes.

The mass spectra of the fractions showed that there were more protein forms present in each of the samples (**Figure 4**) than were detected by the N-terminal protein sequencing. This indicated either that some of the proteins had partially and differentially degraded C termini (i.e., variable numbers of

amino acid residues cleaved off) or that some of the proteins had N termini that were blocked to sequencing. The former alternative seemed probable, because the affinity method used to purify the proteins made use of the fact that the inhibitors bind to proteinases as soon as malt is dissolved in pH 5.5 buffer (*15*). It seemed likely that, during this binding phase, some peptide bond hydrolysis had occurred. That such hydrolysis had occurred at the N-terminal ends of the proteins was supported by the fact that the N termini of the sequenced CMb forms began, in both cases, with amino acid residue 27, using the numbering system for its unprocessed precursor, whereas residue 25 is the accepted N terminus of the normal processed protein (Swiss-Prot P32936).

It appeared that the molecules had been trimmed at their C termini also, because no 14192 Da (residues 25−149; the entire CMb molecule) or 14036 Da (residues 27−149) proteins were detected. Also, no protein of MW 13113, the MW of the complete CMa protein, was present in either the b2d or 1a samples (**Figure 4A**,**C**). Two of the major peptides in preparation b2d had MWs of 12717 and 12880, which correspond very closely to the 12714 and 12884 masses expected of CMa molecules that were truncated at the C-terminal residues 141 and 143, respectively. The CMb molecule also apparently had a truncated C terminus, because it would otherwise (for residues 27−149) have had a MW of 14036 and none of the masses detected in the fractions that contained the CMb protein was greater than ∼13657. It appears that it was probably truncated at residue 143, because the two major components of the 1a sample had masses of 13331 and 13494, which correspond very well with the masses 13327 and 13483 calculated for CMb molecules having sequences that extend from residues 27 and 25 to 143. However, there was no detectable protein sequence that started with residue 25 of CMb, even though such sequences were detected in chloroform/methanol-purified CM protein extracts (see below). Alternatively, the mass 13494 protein could have been a residue 27−143 segment of CMb that was derivatized with a single sugar residue, because that would have a MW of 13489. However, this does not seem very likely, because there was no indication of the presence of any glycosylated CMb in the chloroform/methanol-extracted material (see below).

For the CMd samples, whose sequenced amino terminals started at residue 29, the MW of a protein that stretched from residue 29 to 171 would be 15818 and one comprising residues 25−171 (the complete molecule) would have a MW of 16103. None of the CMd-containing samples contained any such proteins. However, two of the major forms that were present in samples 1b (peaks Dd and Db of **Figure 4**), 2b, and 3b (not shown) had MWs of 15396 and 15174 (averages of the peaks in samples 1b, 2b, and 3b), which fit well with those expected for CMd proteins that extended from residue 29 to residues 168 (15406 Da) and 166 (15177 Da), respectively. The 2b and 4b samples both contained CMd protein with sequences starting at residue 75 (**Table 2**). Both also contained protein species with masses that averaged 5440 Da (not shown). Combining these observations, it seems likely that this peptide spanned residues 75−123 (MW = 5446) of the CMd molecule.

Many proteins whose N termini have not been truncated are resistant to amino acid sequencing. If this were the case with CMd, then some molecules that started at its normal N-terminal residue, 25, could have been present but not detected by sequencing. A complete molecule of this size (residues 25−171) would have had a MW of 16103. No molecules of MW >15600 were present in these affinity-purified inhibitor preparations, so apparently no complete CMd molecules were present. However, two of the major proteins in both preparations 1b and 4b had MWs that averaged 15182 and 15339, which correspond closely with those of CMd molecules having structures extending from the normal N-terminal residue 25 to residues 163 (MW 15165) and 165 (15335). It therefore seems probable that some CMd species were present having N termini beginning with residue 25 but that part of their C termini were missing, and they were resistant to sequencing using the Edman degradation method.

The first well-studied barley protein that was capable of inhibiting the activity of bovine trypsin was purified by Mikola and Suolinna in 1969 (*16*). Odani et al. then used the same method to purify a trypsin inhibitor, sequenced it, and showed that it was the protein that is now called CMe (*17*). This protein did not, however, inhibit the endogenous barley proteinase activities or any of the α-amylases against which it was tested. None of this protein was detected in any of the affinity-purified fractions that were subjected to sequencing during this study (**Table 2**), and this seemed strange because it is a known proteinase inhibitor. There were also no proteins in the mass spectrometric analyses that had masses of ∼13258, the MW of the full-length (residues 25−144) CMe (Swiss-Prot P01086). However, all of the samples that were analyzed contained small but significant amounts of a protein with an average mass of 13032. The mass of a CMe protein fragment running from residue 25 to 142 would be 13038, and it seems likely that this is what the observed mass peak was due to. Why was the amino acid sequence of CMe not seen, then? Although this protein was present in all of the ∼13000 mass fractions, it was never the main species present, and this may have led to its amino acids being overlooked during the sequencing of the mixtures of proteins. Also, its N terminus is that of the complete protein and may have been somewhat resistant to sequencing. When CMe was originally sequenced (*17*), only three residues of the full-length protein were determined, indicating that it did not sequence well. In any case, it seems probable that the fractions b2d, 4b, 1a, and 4a all contained some of this C-truncated trypsin inhibitor.

There were a few other minor proteins in these samples that are not accounted for in this discussion because it is not obvious what their structures were. It is obvious, however, that the components of these affinity-purified inhibitor fractions were mainly or totally composed of various CM protein forms and that these proteins really do function as inhibitors of the main endogenous serine endoproteinase(s) of barley.

Purification of, and inhibition by, CM Proteins. *Extraction and RP-HPLC Separation.* After it was determined that the malt affinity-purified SEP-1 inhibitors were various truncated forms of the CM proteins, those proteins were purified from barley, using classical methods, to test their inhibiting abilities. A crude preparation was readied by removing the lipids from ground barley, extracting the proteins with a chloroform/methanol solution, and then dissolving them in 0.5 M NaCl. This crude CM protein mixture was subjected to reverse phase HPLC, where it was separated into more than a dozen 280-nm-absorbing fractions (**Figure 5A**). Inspection of the UV absorption spectra of these fractions indicated that only five of them contained predominantly proteins. These fractions were collected, freeze-dried, and tested for their abilities to inhibit the activity of SEP-1. The 21-min fraction (chloroform/methanol fraction 1, or cm1; indicated in **Figure 5A**) inhibited strongly (84% inhibition); the cm2 and cm3 fractions inhibited weakly (37 and 29%, respec-

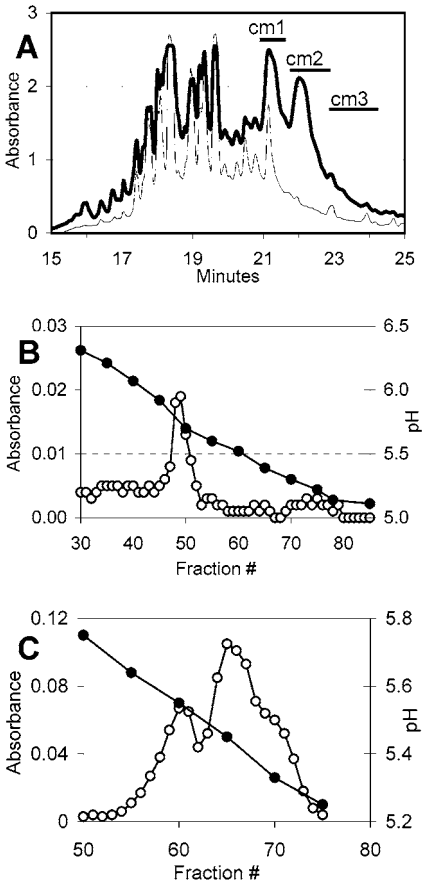

Figure 5. Reversed phase HPLC separation of a preparation of chloroform/methanol barley proteins (A) and chromatofocusing of the cm1 (B) and cm2 (C) RP-HPLC inhibitor fractions: (A) (—) Abs 280 nm and (–) Abs 320 nm; (B, C) (○) protein, Abs 280 nm, and (●) pH of the collected fractions.

tively). The other two fractions, which eluted prior to cm1, inhibited only marginally (17 and 18%) and were not studied further.

Chromatofocusing. To better partition the various CM proteins, the material in the cm1, cm2, and cm3 fractions was concentrated and separated by chromatofocusing with a pH gradient that covered the range from 5.0 to 6.8. The separation patterns of two of these are shown in **Figure 5B** (cm1) and **Figure 5C (**cm2**)**. Both cm1 and cm2 contained a large amount of 280-nm-absorbing material that did not bind to the column at the starting pH, but eluted in tubes 6−20 (cm1) or 6−13 (cm2). The "nonbound" material absorbed light at both 280 and 320 nm.

A single, symmetrical, protein peak, with a p*I* of 5.7, was separated from the cm1 fraction material that bound to the chromatofocusing column (**Figure 5B**). When their contaminating ampholines (from the chromatofocusing) were removed by RP-HPLC, the 280-nm-absorbing portions of fractions 47 and 48 separated into two subfractions. Fractions 49−51 each contained only a single protein. These are discussed below. The RP-HPLC fractions inhibited the SEP-1 activity, and the amount of inhibition was proportional to the absorbance of the fractions from 47 to 51. The cm1 chromatofocusing fraction 10 (not shown), which contained the maximum amount of material that **did not** bind to the column, did not inhibit the SEP-1 activity and was discarded. The nonbinding material from the cm2 and cm3 chromatofocusing separations also did not contain any inhibitory activities.

The portion of the cm2 fraction that bound to the chromatofocusing column contained a mixture of components, as shown in **Figure 5C**. Two peaks were separated that had p*I* values of about 5.54 and 5.45, with the second peak showing a prominent shoulder that eluted at pH ∼5.33. When these fractions were freed of ampholines by RP-HPLC and analyzed for their inhibitory activities, only minimal inhibition occurred. Nearly all of the material in cm3 voided the chromatofocusing column and absorbed at both 280 and 320 nm (not shown). The small amount of protein that did bind eluted from the column as a doubled peak at pH ∼5.7 (fractions 53−58) and ∼5.5 (fractions 66−69).

Mass Spectrometric and Amino Acid Sequence Analyses of the CM Chromatofocusing Protein Preps. (1) Cm1 Fractions. As indicated above, RP-HPLC purification of a pool of the chromatofocused cm1 fractions 47 and 48 yielded two proteins. The mass of the single protein that was present in fractions 49−51, which was also the major component of fractions 47 and 48, averaged 13480 for the six samples analyzed. The second, minor, protein, which was present only in the fraction 47−48 pool, had a mass of 14509. Amino acid sequencing of two separately purified samples of the 13480 MW material showed that its N-terminal sequence was V-G-S-E-D-X-T-P-W-T-A-T-P-I-T-P-L-P-S-X-R-D-Y-V-E-Q-Q-A-X-R. This sequence is identical [assuming that, as is normally the case, the unidentified (X) positions contained cysteine residues] with that of the CMb protein (Swiss-Prot P32936). The MW was smaller than that of the intact CMb molecule, 14192, but fit very well for that of a CMb fragment comprising residues 25−143, which would be 13483. It is notable that in this case, where the protein was isolated from barley using standard purification techniques, the molecule started at residue 25 of the precursor molecule, as expected (Swiss-Prot P32936), whereas when it was isolated from the enzyme−inhibitor complex (**Table 2**), the molecule started with residue 27 in every case. In addition, there was no size heterogeneity in the CMb molecules of the cm1 sample (**Figure 6A**), whereas the affinity-purified sample had contained molecules that extended from residue 27 to residues 141, 143, and 144. It has been reported that BMAI-1, one of the CM proteins that inhibits insect α-amylases, was glycosylated (*18*), and there was, as pointed out above, one protein in the affinity-purified samples that might have been a glycosylated fragment of CMb. It does not appear that this classically purified CMb contained sugars, because the singly glycosylated form of CMb that would have the closest MW to the measured value, 13480, would have been that of the residue 25−142 fragment, which would have had a MW of 13531.

The second, mass 14509, protein that occurred together with the main, MW 13480, protein in fraction 47−48 was present in only small amounts, and it could not be completely purified from the smaller protein. A sample that contained a mixture of the two proteins inhibited the activity of the SEP-1 proteinase, but it was impossible to determine how much of that inhibition was due to the mass 13483 contaminant. Because there was little of this protein, and because it was not pure, it was not studied further. Its MW was too high for it to have been the complete (residues 25−149, mass 14192) CMb protein, but it could theoretically have been that protein derivatized with two sugar molecules, which would have a theoretical mass of 14516.

(2) Cm2 and Cm3 Fractions. (a) Mass Spectrometric Information. Analysis of the inhibitory activities of the cm2 and cm3 chromatofocusing fractions yielded ambiguous results. The fractions from the leading edge of the larger cm2 peak (fractions 64 and 65, **Figure 5C**) seemed to inhibit weakly in some cases,

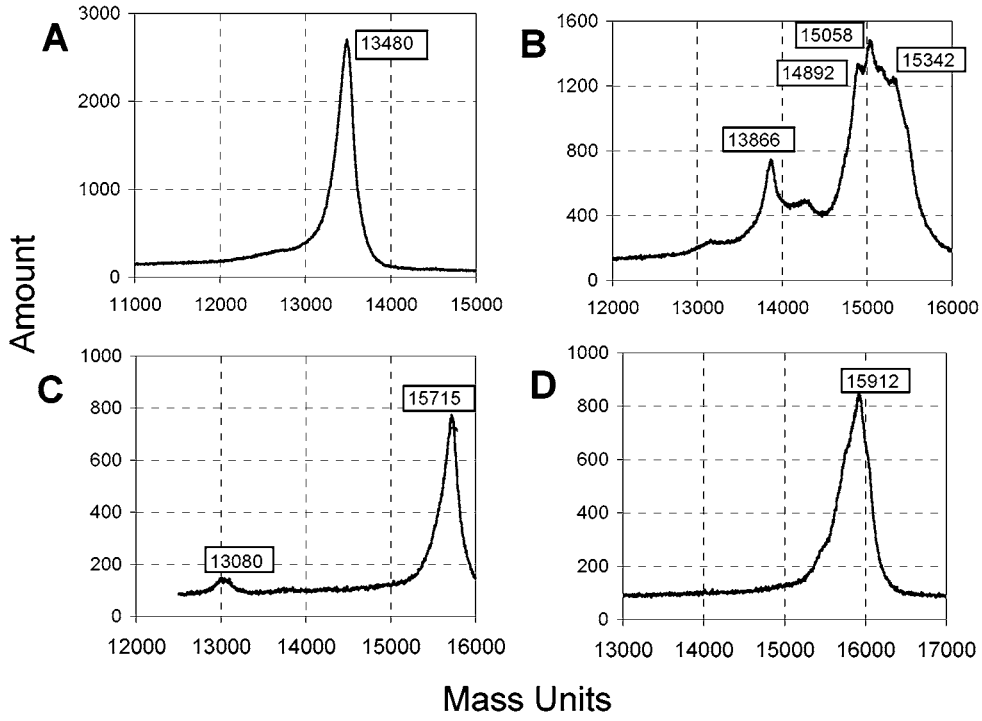

Figure 6. MALDI mass spectra of selected chromatofocused CM protein fractions: (A) cm1–48, (B) cm2–57, (C) cm2–66, and (D) cm2–71 from **Figure 5B** (cm1) and **Figure 5C** (cm2).

but this inhibition was not always repeatable. It appears that any inhibition by the proteins in these fractions is, at best, questionable. This inhibition variability may be tied to the fact that the polymerization states of the various CM proteins reportedly strongly affect their inhibitory properties and that in some cases inhibition occurs only when a mixture of inhibitor forms is present (*19*).

When RP-HPLC was used to remove the polybuffer ampholytes from the cm2 fractions 57−73 (**Figure 5C**), fractions 57−59, 63−68, and 72−73 each yielded a single 280-nm-absorbing peak. Mass spectrometric analyses, however, indicated that fractions 57−61 contained a protein of MW 13866 and varying amounts of a mixture of molecules—presumably proteins—having MWs ranging from about 14800 to 15350 (**Figure 6B**). Fractions 62 and 63 contained small amounts of this ∼15000 MW material, together with large amounts of another protein of MW 13092 (not shown). This latter protein comprised the vast majority of the protein in fraction 64, but by fraction 66 it had been replaced by a 15715 MW species (**Figure 6C**). In fractions 69 and 70 the 15715 MW protein was joined by two proteins with MWs of 15466 and ∼15912. The 15912 MW protein was the main component in fractions 71 and 72 (**Figure 6D**). The pattern of fraction 72, not shown, was the same as that of fraction 71. These relationships are diagrammed in **Figure 7**. The proportions shown are only approximations, however, because they are only rough comparisons of the mass spectrophotometric peak areas and the various components do not necessarily yield equivalent mass spectrophotometric molar intensities when analyzed by this method.

Mass spectrometric analyses of the cm3 HPLC-purified fractions indicated that fractions 57 and 58 contained primarily a protein of MW 13055, whereas the main protein in fractions 68 and 69 had a MW of 8794.

(b) Amino Acid Sequencing. From a comparison of the mass spectrometric patterns of the cm1−cm3 fractions, it was clear that all of the proteins that occurred in significant amounts could

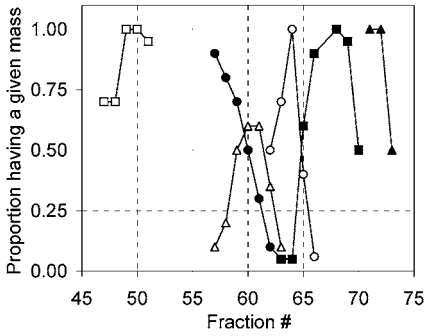

Figure 7. Fractions in which proteins of different masses eluted from the chromatofocusing columns. The eluted proteins had masses of (○) 13092, (□) 13494, (△) 13866, (●) "15000"—the wide peak from ∼14800 to 15300, (■) 15715, and (▲) 15912. All of these proteins were from the cm2 separation except 13494, which was from cm1.

be analyzed by sequencing the amino acids of fraction 48 of cm1, fractions 57, 64, 66, and 67 from cm2, and fractions 57 and 68 from cm3. Analyses of these fractions indicated that they contained only four distinct proteins that could be sequenced (**Table 3**). These proteins were CMb and CMd, both of which also were present in the affinity-purified inhibitors, and BMAI-1 (barley monomeric amylase inhibitor) and BDAI-1 (dimeric amylase inhibitor), which were not present in the affinity-purified sample. Like the CMa-e proteins, these latter proteins are members of the cereal trypsin/α-amylase inhibitor family (*18*−*22*). They show fairly strong homology with the CM proteins (*22*), and although both inhibited *Tenebrio molitor* α-amylases [BMAI-1 strongly (*20*) and BDAI-1 weakly (*19*)], neither affected the activity of trypsin (*22*).

Even though there was a large amount of material in fraction cm2-57 that had a broad range of molecular masses between 14800 and 15300, only the 13866 mass material yielded any sequence data. This sequenced protein was BMAI-1, and its N terminus started at residue 15 of its precursor form, indicating that this terminus was not truncated (Swiss-Prot P16968).

Table 3. Partial N-Terminal Amino Acid Sequences and Molecular Masses of Classically Purified CM Inhibitors of the SEP-1 Barley Serine Endoproteinase

sample	measured mass	sequence[a]	residues	protein	theoretical mass
cm1–48[b]	13491	V-G-S-E-D	25–143[c] (25–149)[d]	CMb	13494
cm2–57	13866	S-P-G-E-W-X-W	15–141 (15–146)	BMAI-1	13853
cm2–57	14800–15300	none	?		
cm2–64	13092	S-G-P-W-M-W	31–152 (31–152)	BDAI-1	13101
cm2–66	15714	A-A-A-A-T-D[e]	26–169 (25–171)	CMd	15732
cm2–66	13080	S-G-P-W-M-W	31–152 (31–152)	BDAI-1	13101
cm2–71	15912	A-A-A-A-T-D[e]	26–170 (25–171)	CMd	15918
cm2–71	16040?[f]		26–171?	CMd?	16031
cm2–71	15450?[f]	T-D-X-S-P-G-V	30–169 (25–171)	CMd	15448
cm2–71	15745?[f]		30–171	CMd?	15747
cm3–58	13055	S-P-G-E-W-X-W	15–135 (15–146)	BMAI-1	13048
cm3–68	8800	S-P-G-E-W-X-W	15–96 (15–146)	BMAI-1	8817

[a] Measured N-terminal amino acid sequence. [b] Chromatofocusing separation of cm1, fraction 48. [c] Calculated from N-terminal sequence and measured mass. [d] Sequence of the entire protein, as listed in Swiss-Prot. [e] Samples cm2–66 and cm2–71 yielded two sequences each. The sequences are listed in order of the amount of each protein that was present. [f] Shoulders on the main peak.

BMAI-1 was the only protein detected (**Table 3**) in each of the two fractions (cm3-58 and cm3-68) that were collected when the portion cm3 of the initial RP-HPLC separation (**Figure 5A**) was subjected to chromatofocusing (result not shown). As in the cm2-57 fraction, the N termini of both the cm3-58 and cm3-68 proteins also began with residue 15, indicating that neither was N-terminally truncated.

The N-terminal ends of the MW 13092−13080 protein (BDAI-1) in fractions cm2-64 and -66 were also not truncated, starting at residue 31 of their precursor molecules (**Table 3**, Swiss-Prot P13691). Three sequences corresponding to CMd protein forms occurred in fractions cm2-66 and -71; two had N-terminal residues that started at residue 26, the other at residue 30. It thus appears that the BMAI-1 and BDAI-1 proteins are more resistant to proteolytic hydrolysis than the other CM proteins. This is consistent with the fact that they were not present in the affinity-purified samples. Apparently they did not bind to the endoproteinases when the ground barley was extracted and so were not cleaved by those enzymes.

(c) C-Terminal Truncations. A comparison of these mass spectrometric data with the published nucleic acid sequences of the genes coding for these proteins showed that the BDAI-1 proteins maintained their complete amino acid sequences (**Table 3**). The C termini of the three BMAI-1 fractions that were obtained were all truncated; instead of extending to residue 146, as expected for the full-length protein, they stopped at amino acids 141, 135, and 96 (**Table 3**).

The CMd protein was, in its two most prevalent forms, truncated at its C terminus, as well as at its N-terminal end. In the cm2-66 sample, the major protein species was CMd that extended from residue 26 to 169, having lost two amino acid residues from its C-terminal end (Swiss-Prot P11643). Fraction cm2-71 contained only CMd protein, the great majority of which started with amino acid residue 26 and had a mass of 15912, indicating that it ended at residue 170, having lost a single amino acid from each end of its structure. Cm2-71 also contained a small amount of CMd protein having an N-terminal sequence starting at residue 30 (**Table 3**). The mass spectrum of cm2-71 showed that, in addition to the major species of mass 15912, there were small "shoulders" of material with MWs of approximately 15450, 15745, and 16040 (**Figure 6D**). These most likely correspond to CMd protein forms having amino acid sequences running from residues 30 to 169 (MW 15448), from 30 to 171 (MW 15747), and from 26 to 171 (MW 16031). The first two of these would account for the experimentally measured sequence that started at residue 30, and the final one would have contributed to the sequence that began with residue 26.

With the exception of the 8800 MW protein in fraction cm3-68, which had lost ∼50 residues, or 38% of its mass, the CM proteins that were detected had only small portions of their structures cleaved. This study does not answer the question of whether these CM proteins were degraded during the several steps of the purification procedure or whether they were already truncated in the malt or barley from which they were purified. Because the addition of class-specific inhibitors to the extraction and purification steps might have interfered with the ability to detect the proteinaceous inhibitors being studied, no such additions were made.

Conclusions. It has long been presumed that, because the CM proteins could not be shown to inhibit the activities of any endogenous barley or malt endoproteinases, their functions in the grain was to protect it from attack by pests. The findings of this study indicate that, if so, this is not their only purpose, because some of the members of this group can inhibit the activity of the major barley and malt serine endoproteinase, SEP-1. One of the chloroform/methanol-purified samples contained only the CMb protein, the concentration of which correlated with inhibitory activity, indicating that it functions as an SEP-1 inhibitor. One of the affinity-purified inhibiting fractions, 2b, contained two different forms of the CMd protein, and probably some CMe. Either one or both of these proteins are thus also inhibitors. The third affinity-purified CM protein, CMa, always occurred together with the inhibitor CMb (**Table 2**) and probably CMe. It may, therefore, not inhibit SEP-1, although the fact that it did bind to enzymes strongly enough to allow its partial purification by the affinity method indicates that it is likely also an inhibitor.

The BMAI-1 and BDAI-1 molecules apparently did not form enzyme−inhibitor complexes that were strong enough to allow their purification by the affinity method. Also, their abilities to inhibit SEP-1 were, if present, very weak. The poor inhibitory properties of these highly purified proteins may have been due to their not being in an acceptable physical or biochemical state. It has been reported that the form of BDAI-1 that inhibited the α-amylase enzyme of the insect *T. molitor* was present as a dimer (*20*) and that the inhibitory form of BMAI-1 was glycosylated (*18*). During our investigations of the masses of the BDAI-1 and BMAI-1 proteins, there were no indications that they were either polymerized or glycosylated. It seems likely that these BDAI-1 and BMAI-1 proteinase inhibition results are

the same as those of the Spanish researchers. Although they did not report testing either BDAI-1 or BMAI-1 for its ability to inhibit bovine trypsin (*20*, *21*), it seems likely that they did, given that they have studied this inhibition in depth with the other CM proteins, and in their recent review paper (*22*) neither of these proteins is listed as inhibiting trypsin.

The affinity-purified sample 2b (**Figure 3)** inhibited the activity of SEP-1 strongly and apparently contained mainly various forms of the CMd protein, probably with smaller amounts of CMe (**Table 2**). The samples cm2-66 and cm2-71, which were purified by the classical CM method, also contained various forms of the CMd protein (**Table 3**) but inhibited the enzyme only very poorly, if at all. This may indicate that the inhibition with the affinity-purified sample was due to the CMe protein, or the difference might be because the CMd forms in 2b (N-terminal residues 25, 29, and 75) differed from those of the CM-purified fractions (N-terminal amino acids 26 and 30). Possibly one or more of these proteins (the ones starting at residue 25, the normal N terminus?) were responsible for the inhibition.

As expected, there was more degradation (truncation) of the inhibitors during the affinity purification process than there was with the classical purification. This degradation did not, however, generally appear to be due to hydrolysis of the inhibitor by the endoproteinases that are present and to which the inhibitors would have been expected to bind. This is shown by the fact that most of the affinity-purified inhibitor molecules, although they were usually truncated at one or both ends, had lost only short sections of their structures. From this, it appears that they were hydrolyzed by exopeptidases, not by the endoproteinases, which would have cleaved internal peptide bonds to release large peptides. If the β-amylase that was in fraction b2d, which seems to be a contaminant, is ignored, the only obvious cases of endo cleavages were the ones that released the 75−123 residue fragment of CMd (in the affinity-purified samples 2b and 4b, **Table 2**) and the 8800 MW BMAI-1 fragment (residues 15−96) that was in sample 11−68. To release these fragments, the cleavages would have been between the amino acid residues N and Y (CMd) and E and V (BMAI-1), and neither the SEP-1 enzyme nor hordolisin (*5*) would have been expected to catalyze either of these, because their hydrolytic characteristics are like those of subtilisin (cleaves bonds on the C-terminal side of very hydrophobic residues). It thus seems unlikely that any of these cleavages were due to a classical inhibitory process, in which the tightly binding proteinaceous inhibitors were hydrolyzed at the active site of the SEP-1 endoproteinase.

It has been reported that in some barley cultivars CMb, but not CMa or CMd, was sometimes glycosylated and that this derivatization had no effect on its ability to inhibit the *T. molitor* α-amylase (*23*). Four different truncated CMb forms were distributed among three of the affinity-purified preparations that were studied in detail (**Table 2**), but there was no good indication that any of these, or any of the other inhibitors, was glycosylated. It is possible that the glycosylated proteins did not form enzyme complexes and therefore were not collected, but that does not seem likely as both the glycosylated and native forms inhibited the α-amylase (*23*). No evidence was seen for the existence of any glycosylated BMAI-1 protein either, even though it has been reported that this protein, but not BDAI-1, is a glycoprotein (*18*). In studies with other glycosylated proteins their glycosylated forms have been readily identified by MALDI-TOF MS. This aspect of the molecular characteristics of the inhibitors needs to be studied in more detail.

It has been generally accepted that the barley trypsin inhibitor (CMe) that was studied by Mikola and Suolinna (*16*) was a trypsin/α-amylase inhibitor. Kirsi and Mikola showed that the activity of this inhibitor decreased during malting and nearly disappeared from the endosperm tissue (*24*). However, this study showed that CMa, CMb, CMd, and, probably CMe itself were present in barley malt and, after extraction from the malt and boiling, at least two of these proteins still inhibited SEP-1. Mikola's group did not detect any inhibition of the endogenous barley endoproteinases by CMe, but this could have been because they used the substrate benzoyl-DL-arginine-*p*-nitroanilide to test for proteinase inhibition and we have shown that this compound is a very poor substrate for SEP-1 (*2*). The fact that Mikola and Kirsi detected little CMe in malt probably does not indicate that it was destroyed during malting, but that it and the other CM proteins behaved like the barley cysteine endoproteinase inhibitors that we have studied previously. These inhibitory proteins bind tightly to the endogenous cysteine proteinases and, as the concentration of these proteinases increased strongly during germination, the free inhibitor that was present in the seed complexed with them and was thereby rendered unmeasurable (*15*). In view of the fact that the CMa, CMb, CMd, and probable CMe SEP-1 proteinase inhibitors also bound to high MW proteins, which allowed their purification by the affinity method, it seems reasonable that the CMe inhibitors studied by Kirsi and Mikola (*24*) were also not inactivated or destroyed during malting but were merely bound to the SEP-1, or possibly to some other proteinase, that was synthesized during malting (*2*) and thus were not detectable by their methods.

Whether or not the CMe trypsin inhibitor of Kirsi and Mikola is an inhibitor of SEP-1 is also of interest because they reported (*24*) that it was present only in the "two endospermal tissues" of barley seeds, the starchy endosperm and the aleurone. The other CM proteins also occur mainly or solely in the endosperm tissues (*22*). Fontanini and Jones have shown that the SEP-1 enzyme activity (its ability to degrade gelatin) was detectable in all of the seed tissues except the starchy endosperm and that it was present only in small amounts in the aleurone (*2*). This raises the question of whether the CM protein−SEP-1 interactions are physiologically important, if the inhibitor(s) and their target enzymes are located in separate parts of the grain and can interact only after they are dissolved. On the other hand, if the SEP-1 and inhibitors were both present in the endosperm, they could have formed inactive complexes and the SEP-1 would not have been detected. In light of these findings, it is important that further studies be carried out to determine exactly where the various CM proteins and SEP-1 are located within the barley cell. Even if the CM proteins and SEP-1 enzyme do not interact within the grain, the inhibition might still be important commercially, because all of these proteins are quite soluble and could interact during the mashing step of the brewing process.

In any case, it is apparent from these findings that the CMb, CMd, and/or CMe and, probably, CMa (because it binds during the affinity purification) proteins can inhibit at least one of the main endoproteinases (SEP-1) that occurs in barley and malt. In the past it has seemed that some of these CM proteins might possibly inhibit certain of the serine endoproteinases of barley, because CMc and CMe inhibited the activity of the bovine serine endoproteinase trypsin (*25*). However, none of them had actually been demonstrated to inhibit any endogenous barley or malt endoproteinase. Whether the BMAI-1 and BDAI-1 CM proteins can function as inhibitors of the barley/malt serine endopro-

teinases is still an open question, because the pure proteins inhibited very poorly and they did not bind to any endoproteinases strongly enough to allow their purification by the affinity method. However, the inhibition of α-amylase by these proteins is reportedly strongly affected by their polymerization states, so their lack of observed inhibition may simply have been due to their not being in the correct physical state after being subjected to extraction in the strongly denaturing chloroform/methanol solution. These systems still need further characterization, and this can now be done readily, using either a completely or partially purified SEP-1 preparation.

ABBREVIATIONS USED

IEF, isoelectric focusing; PAGE, polyacrylamide gel electrophoresis; sucAAPLpNA, *N*-succinyl-alanyl-alanyl-prolyl-leucyl *p*-nitroanilide; SEP-1, serine endoproteinase 1; CMx, chloroform/methanol soluble protein x; RP-HPLC, reverse phase high-performance liquid chromatography; MW, molecular weight; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

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