Interactions of Malt and Barley (*Hordeum vulgare* L.) Endoproteinases with Their Endogenous Inhibitors

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For producing worts that are optimal for beer production, some, but not all, of the barley proteins must be degraded during malting and mashing. This protein hydrolysis is controlled by endoproteinases, and, in turn, is partially regulated by the presence of low-molecular-weight (LMW) proteinaceous inhibitors. This paper reports studies of the interactions between the proteinases and inhibitors and an "affinity" method for concentrating the inhibitors. The malt inhibitors (I) and proteinases (E) quickly formed strong (E−I) complexes when dissolved together, and all of the I was complexed. Heating at 100 °C, but not 70 °C, dissociated the complex, even though the enzyme activities were destroyed at 70 °C. The released I readily recomplexed with fresh E. Barley, however, contained insufficient E to complex all of its I complement. The E−I complex was treated with salts, detergents, and reducing agents to release active E molecules, but none disrupted the complex. By removing the LMW proteins from a malt E−I extract and dissociating the complex by heating, the concentration of I molecules was greatly increased. This "affinity" method can thus be used to concentrate the I molecules for further purification.

Keywords: *Hordeum vulgare; protease; inhibitors; protein hydrolysis; brewing; soluble protein*

INTRODUCTION

In order for brewers to produce a wort that is optimal for producing beer, some, but not all, of the barley proteins must be degraded to "soluble protein" (a mixture of amino acids, peptides, and dissolved protein) during the malting and mashing processes. In addition to performing the critical function of serving as yeast food, the components of the soluble protein mixture contribute to brewing characteristics such as beer foam formation and stability, and beer haze formation. The rate of protein hydrolysis during malting and mashing is regulated by the activities of the endoproteinases (E), which are known to be the rate-limiting enzymes (*1, 2*). Although members of all four of the standard proteinase classes are present in malt, the cysteine-class proteases apparently play the biggest role in solubilizing proteins (*3, 4*). The presence of these endoproteinases is not, however, sufficient to ensure that the barley proteins are rendered soluble, because there are low-molecular-weight (LMW) proteins in both barley (*5*) and malt (*6*) that, when present in solution, interact with the cysteine-class endoproteinases to form enzyme−inhibitor complexes (E−I) and thus inhibit their activities (*6, 7, 8*). Two of these endogenous inhibitors (I) have been purified and characterized and were the proteins previously named lipid transfer protein 1 (LTP1) and lipid transfer protein 2 (LTP2). There are additional inhibitors in malt and barley that have not yet been purified and characterized.

It is imperative that newly developed malting barleys produce intermediate, optimal levels of soluble protein during malting and mashing. To ensure that this happens, we need to know how the proteolytic activities are controlled, and thus how the enzymes and their inhibitors interact. For example, in our laboratory we analyze the malting quality of over 4000 newly developed malting barley lines each year in order to select the dozen or so lines that have quality good enough to warrant further testing by industry. During all of this testing, we are unable to obtain an accurate indication of the total proteolytic capacities of the lines because their malts contain inhibitors that selectively inactivate unknown amounts of the overall endoproteolytic activities. If we could devise some method whereby the inhibitors could be removed from the proteinases without the enzymes being inactivated, then we could obtain a more realistic measure of the proteolytic potentials of the various lines. This study was carried out to partially characterize the interactions of the proteinases and their inhibitors and to dissociate the enzyme−inhibitor complexes so that we can get a more accurate measure of the total proteolytic activities of the various lines and cultivars. In addition, the fact that the enzyme−inhibitor complexes and the component enzymes are large molecules, while the inhibitors are small, suggested that it might be possible to use an "affinity" method to more easily and efficiently purify some of the previously unstudied proteolytic inhibitors. None of the methods investigated resulted in providing inhibitor-free proteinases for malting quality analyses, but the affinity method does readily concentrate the inhibitors for further purification.

MATERIALS AND METHODS

Preparation of Malts. Malts were prepared from the good malting quality 6-rowed barley (*Hordeum vulgare* L.) cultivar Morex using the methods described earlier (*9*), with steeping to 45% moisture, a 4-day, 16 °C germination, and kilning to 85 °C.

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10.1021/jf010611q
Published on Web 10/31/2001

Preparing an Endoproteinase Fraction (Crude Extract, CE). After its 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 samples (8 g) were extracted by stirring for 30 min with 24 mL of Na acetate (NaAc) buffer (0.1 M, pH 4.7). The extracts were centrifuged at 12100*g* for 20 min, and the supernatant was strained through two layers of cheesecloth, frozen, and stored at −20 °C until analyzed. Immediately prior to being used, the preparations were thawed and centrifuged for 5 min at 11500*g* to remove any residual haziness.

Extraction of E−I Complexes and I from Malt/Barley. A ground malt or barley sample was mixed with 3 volumes (v/w) of 50 mM ammonium acetate (NH_4Ac) solution, pH 5.5, and stirred for 30 min at room temperature. The suspension was centrifuged at 10000*g* for 5 min, and the supernatant was collected. In most cases, the supernatant contained a mixture of E and E−I. To release I from the E−I complex so that it could be extracted, the 10000*g* supernatant was heated at 100 °C. To prepare large volumes of I solution (more than 10 mL), the supernatant was heated to boiling in a microwave oven, immediately placed in a boiling water bath, and held there for 10 min. For volumes smaller than 10 mL, the supernatant solutions were put into test tubes, placed in a boiling water bath, and incubated for 10 min with frequent mixing. After heating, the solutions were centrifuged at 10000*g* for 5 min, and the supernatants were used for carrying out experiments.

Separation of E−I and I by P-30 Gel Permeation Chromatography. After the extracts had been subjected to the various experimental treatments, they (normally 15-mL samples) were loaded onto a 2.5 × 46 cm column that was packed with medium grade Bio-Gel P-30 gel filtration material (Bio-Rad Laboratories, Hercules, CA) that had been equilibrated with 50 mM, pH 5.5, NH_4Ac solution. The column was eluted with the same buffer, and 5.0-mL fractions were collected. The elution of the protein was monitored by following its absorbance at 280 nm, and the 280 nm absorbance of each fraction was also measured with a spectrophotometer. Under these conditions, the E−I complex eluted early, in approximately fractions 17−20, and the smaller I molecules normally eluted in fractions 21−30. When appropriate, the P-30 separation was scaled up by using larger sample volumes and columns and collecting bigger fractions.

Analyzing Fractions for Proteolytic or Inhibitory Activities. The proteolytic or inhibitor activities of the fractions eluted from the P-30 and carboxymethyl cellulose (CMC) ion-exchange columns were analyzed using the "in solution" azogelatin proteolysis method of Jones et al. (*10*). Lyophilized azogelatin was dissolved in warm 0.1 M NH_4Ac, pH 4.8, to make a 2% (w/v) substrate solution. The substrate solution was stored at 4 °C and heated at 40 °C prior to use to liquify the azogelatin for pipetting into the assay tubes.

The assays were conducted in the presence of 4 mM cysteine. A typical assay was carried out by mixing 59 μL of 200 mM cysteine with 0.25 mL of malt enzyme extract (CE, see above), 1.20 mL of P-30 eluate (the fraction being evaluated), and 0.54 mL of 0.1 M NH_4Ac, pH 4.8, buffer. The mixtures were incubated at 40 °C for 10 min, after which the reactions were started by adding 1.80 mL of the azogelatin solution. The reactions were terminated by removing 0.74-mL aliquots of the reaction mixtures and adding them to 0.50 mL of 25% (w/v) trichloroacetic acid. Typically, aliquots were removed after 0, 14, and 28 min of reaction. After sitting for 15 min in an ice−water mixture, the tubes were centrifuged for 10 min at 11500*g,* and the absorbances of the supernatants were measured at 440 nm. The inhibitor and proteinase activity assays were both carried out in the presence of added malt proteinase (CE). Thus, if no proteinase or inhibitor was present in a series of fractions that were being analyzed, each fraction would show a constant level of proteolytic activity; that which was due to the hydrolysis of azogelatin by the added CE. If active proteinase (E) was present, the proteolytic activity was enhanced above that of the control, and when free I was present the activity was decreased.

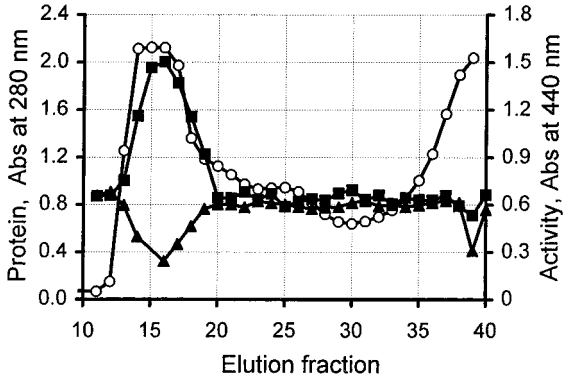

Figure 1. An analysis of the proteinase and proteinase inhibitor activities of a P-30 gel-filtration separated malt extract. The fractions were analyzed without heating (to detect enzyme and free inhibitor activities) and after being heated at 100 °C for 10 min (to detect any inhibitory activities that were released from E−I complexes). (○) Protein absorbance at 280 nm; (■) activity, unheated sample; (▲) activity, heated sample.

"Affinity" Concentration of Inhibitors. Unheated inhibitor extracts were prepared as described above, and 35 mL of the centrifugation supernatant was applied to a 340-mL (2.5 cm × 69 cm) Bio-Gel P-30 chromatography column. The column was eluted with pH 5.5, 20 mM, NH_4Ac buffer, and 5.2-mL fractions were collected. The fractions that contained the initial peak of highly absorbing material (fractions 14−19) were pooled. A second, identical, extract was prepared and separated in the same way. The two pooled solutions were combined, heated at 100 °C for 10 min, and centrifuged for 10 min at 11500*g*, and the supernatant was freeze-dried. The freeze-dried material was dissolved in 11 mL of 20 mM buffer and applied to a 210-mL (2.0 × 67 cm) P-30 column. The column was eluted with the 20 mM NH_4Ac buffer, and 5.2-mL fractions were collected. The fractions were tested for their abilities to inhibit the activities of a Morex malt extract, and the inhibiting fractions were pooled and freeze-dried.

CMC Separations of the Affinity-Concentrated Inhibitors. The freeze-dried affinity-concentrated sample was dissolved in 19.5 mL of 10 mM NH_4Ac, pH 5.0, and applied to a 1 cm × 7 cm chromatography column that was packed with CM-52 CMC ion exchange material (Whatman, Maidstone, England) that had been equilibrated with 10 mM, pH 5.0, NH_4Ac buffer. The column was eluted with a 10−250 mM NH_4Ac, pH 5.0, linear gradient (100 mL of each concentration) and 5.2-mL fractions were collected. The inhibitory activities of the fractions were measured, and three areas of inhibition were apparent. The fractions comprising these areas were individually pooled and freeze-dried.

RESULTS AND DISCUSSION

Separating E, I, and the E−I Complex and Analyzing their Activities. *Analysis of a Crude Extract.* To ensure that a P-30 separation system would suffice for analyzing the malt E − I and E + I system, a crude Morex malt extract was prepared and separated with the P-30 column. After the separation was completed, the resulting fractions were analyzed for their abilities to hydrolyze azogelatin (activity) and to inhibit the hydrolysis of azogelatin by a malt proteinase extract (inhibition). Preliminary studies had indicated that, in these extracts, most of the I molecules were probably complexed with the E enzymes and that there were more E molecules present than I molecules. For this reason, it was expected that essentially all of the I would be bound, while much of the E would remain uncomplexed. As shown in Figure 1, this was the case; there was no low-molecular-weight (LMW) inhibitory activity

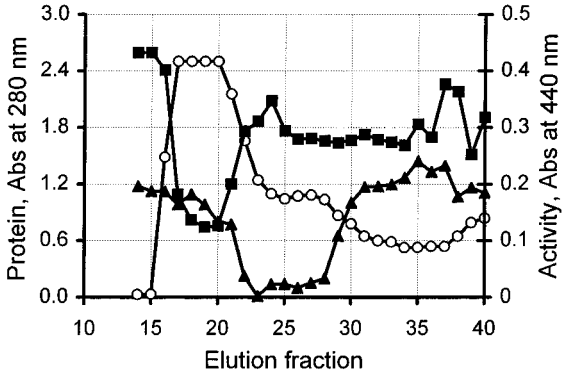

Figure 2. Analysis of the proteinase and proteinase inhibitor activities of P-30 gel filtration-separated unheated and heated malt extracts. (○) Protein absorbance at 280 nm; (■) activity, unheated sample that was passed through the P-30 column and then heated; (▲) activity, sample heated at 100 °C prior to separation on the P-30 column.

(i.e., I, which would have eluted in tubes 20−28) present in the unheated eluant, but there was strong enzymatic activity (E) that coeluted with the high-molecular-weight (HMW) proteins (fractions 14−19). All of the analyses in these experiments were carried out with reaction mixtures to which a constant amount of malt endoproteinase preparation was added. Under these conditions, if neither "free" (uncomplexed) enzyme or inhibitor was present in the samples being tested, the baseline activity (about 0.6 OD 440 nm in this experiment) was seen. When free E was present in the fractions being analyzed, the activity was raised above this baseline level, and when uncomplexed inhibitor was present it fell below it. Throughout this paper the term "heated", used without qualification, is used to designate samples that have been heated to 100 °C for 10 min. When the separated fractions were heated prior to analysis, there was no proteolytic activity left (because of the proteinases being inactivated) but there was inhibitory activity that had coeluted with the E and the other HMW proteins (Figure 1, fractions 14−19). The large molecular size (i.e., voided on the P-30 column) of this inhibitory fraction indicated that it was eluted from the column as part of a large E−I complex that was broken down into inactive E and still-active I by the heating process that was applied after the separation had occurred.

Heating of the E−I Complex at 100 °C Released Free I. A malt extract was prepared and divided into two aliquots. One aliquot was applied directly to the P-30 column and the other was heated at 100 °C for 5 min prior to being clarified by centrifugation and applied to the column. After the elutions were completed, the collected fractions from the separation of the unheated sample were heated prior to analysis. The fractions from each of the separations were then analyzed for their enzymatic or inhibitory activities. The results (Figure 2) indicted that the unheated fraction contained only HMW inhibitory activity and no free I, confirming the results seen previously in Figure 1. The sample that was boiled prior to its P-30 separation, however, contained no HMW inhibitory activity; its inhibitors had eluted as if they were LMW proteins. This experiment demonstrated that the P-30 method could readily separate the E and E−I complex from free I, and that the separated I could still strongly inhibit the activities of malt proteinases after it had been heated at 100 °C for several minutes. It also reinforced the data of Figure 1,

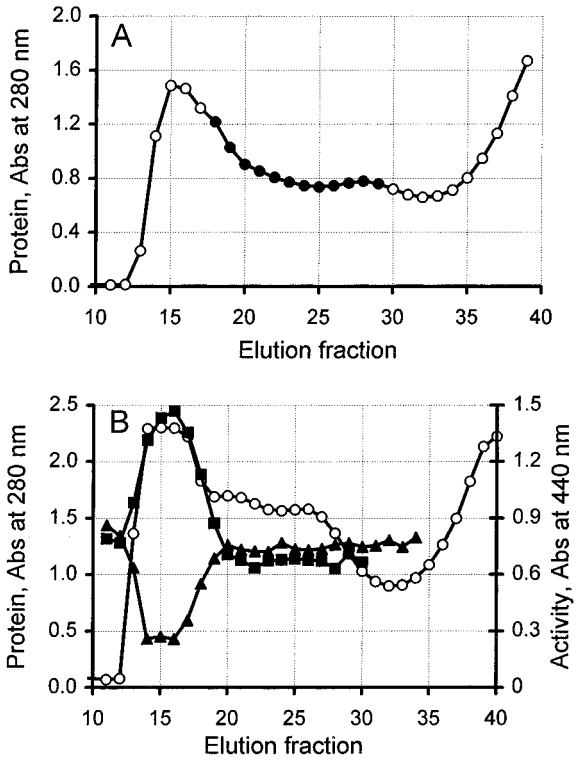

Figure 3. The binding of inhibitor from heat-disrupted E−I complex to endoproteinases. Heat-treated extract was separated on a P-30 gel filtration column (A), and the protein absorbance was measured at 280 nm: (○) fractions that did not contain inhibitor activity; (●) the inhibitory fractions. The inhibiting fractions were pooled, added to a fresh extract and incubated. The supplemented, incubated extract was then separated on the P-30 column (B) and the inhibiting activities of the eluant were analyzed: (■) as collected and (▲) after being heated to 100 °C. (○) Protein absorbance at 280 nm.

showing that all of the I was tightly bound to HMW material (presumably E) as soon as it was extracted from ground malt.

I can be Dissociated from the E−I Complex and Separated, and Can Then Reassociate with E. A malt extract was prepared, heated at 100 °C, and passed through the P-30 column (Figure 3A). Analyses of the collected fractions indicated, as before, that no proteolytic activity was present in the boiled preparation, and that the I molecules eluted from the column as LMW proteins, in fractions 18 to 29. These fractions were pooled and freeze-dried. A second malt sample was then extracted and centrifuged, and the freeze-dried material from the first extract was dissolved in its supernatant. This mixture was stirred for 30 min and separated on the same P-30 column. As in previous experiments, this nonheated sample contained enzymatic activity that eluted from the column together with the HMW proteins (Figure 3B). When the eluted fractions were heated and then tested for inhibitory activity, there was strong inhibitory activity in the early-eluting fraction (where E−I eluted) and none in the LMW elution area where the free I from Figure 3A would have eluted, had it not bound to E (Figure 2). These findings indicated that the free I that had been released from E−I by the heating of the initial sample (Figure 3A) had recombined with the free E present in the unheated preparation to form E−I that eluted together with the HMW material (Figure 3B). When the E−I complex is thus dissociated into its components by heating to 100 °C, the activities of the E enzymes are destroyed, but

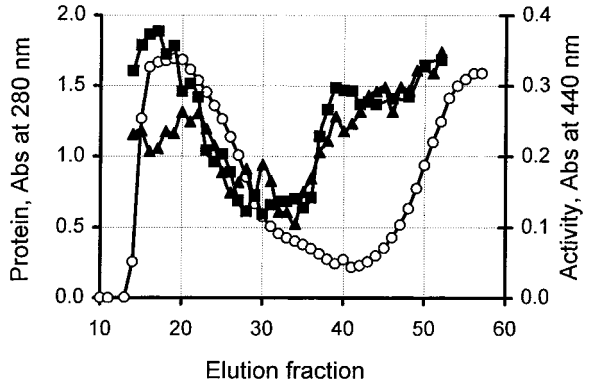

Figure 4. Analysis of the proteinase and proteinase inhibitor activities of P-30 gel filtration-separated barley extracts. (○) Protein absorbance at 280 nm; (■) activity, unheated sample; (▲) activity, sample heated at 100 °C before separation. The activity in the absence of separated proteinase or inhibitor was approximately 0.3 absorbance units.

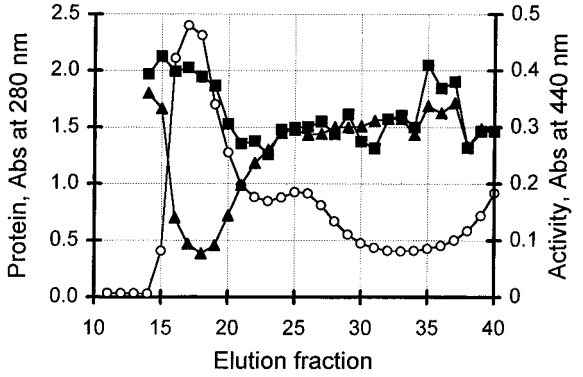

Figure 5. Analysis of the proteinase and proteinase inhibitor activities of an extract that was heated at 70 °C prior to P-30 gel filtration. (○) Protein absorbance at 280 nm; (■) activity, sample not heated further after the P-30 separation; (▲) activity, sample heated at 100 °C after the P-30 separation but before analysis.

the ability of the I molecules to inhibit the activities of endoproteinases is apparently unimpaired by this process.

Proteinases and Inhibitors of Barley. The experiments reported above were all carried out with extracts prepared from malts, which are known to contain relatively large amounts of inhibitors (*6*) and high levels of endoproteinases (*11*). Under these conditions, all of the inhibitors present in the malt were clearly complexed with the proteinases as soon as the two components dissolved during the extraction. Unmalted barley, however, contains little endoproteolytic activity (*12*) and less than half as much inhibitory activity (*6*) as malt. In addition, the proteinases that are present in barley are apparently mainly aspartic and serine class enzymes (*12*, *13*), and not the cysteine ones that are inhibited by, and thus presumably bind to, the endogenous inhibitors. To determine how these differences affected the formation of the E−I complex, barley was ground and an extract like those previously used to study malt was prepared. It was divided in half; one of the halves was subjected to the normal heating process, the other was not heated.

When these extracts were separated by P-30 chromatography and analyzed (Figure 4), the unheated sample contained a low level of enzymatic activity that eluted with the HMW proteins (in fractions 15−19 of this column) and it also contained some free LMW I molecules that eluted in fractions 22−38. These unheated barley extract results fit well with our previous knowledge; there was only a low level of proteolytic activity present and it did not form a complex with the majority of the inhibitor that was present. Two of the barley endoproteinase inhibitors have been purified and characterized, and both of these inhibited only members of the cysteine proteinase class (*7*, *8*). Considering there is little or no cysteine class proteinase activity in barley, it makes sense that most of the inhibitors remained uncomplexed.

The results obtained with the heated extract (Figure 4), indicated that, as expected, the heating inactivated all of the low-level endoproteinase activity that was originally present in the barley. It appears that a small portion of the unheated barley E was complexed with inhibitor, as there was a distinct, but low, level of inhibition in fractions 13−18 of the heated fraction separation, indicating that some of the inhibitor was still bound in a HMW form when the P-30 separation occurred. The majority of the inhibitor eluted in fractions 23−42, however, indicating that it was not bound to E prior to the heating step.

Inactivating Malt Endoproteinases at 70 °C does not Dissociate the E−I Complex. To make good brewing worts, about 45% of the barley protein needs to be converted into what is termed "soluble protein" by the end of the mashing process (*14*). This soluble protein fraction contains amino acids, peptides, and dissolved proteins, and a large proportion of these are formed by the action of the malt proteinases on the barley proteins. This means that it is necessary to understand how the endoproteinases (the rate-limiting enzymes for this protein degradation) and their inhibitors interact to control the hydrolysis that occurs during the malting and mashing processes. One problem associated with such studies is that it is impossible to get a correct measure for the endoproteolytic activities of malt samples because they always contain unknown amounts of inhibitors that quickly interact with the dissolved cysteine proteinases to lower their overall activities. This means that the results obtained on measuring the extract proteinase activities always reflect lower amounts of proteinases than are actually present. For developing and selecting improved malting barley lines, it would be helpful to be able to accurately measure the proteinase activities of the malts. Researchers and breeders, therefore, need a method whereby they can gently dissociate the malt E−I complex into its components so that its total E and I levels can be measured easily and reliably.

As a start toward this end, it was necessary to ascertain whether the E−I complex could be easily dissociated. It had been determined previously that the mash endoproteinases were quickly inactivated when the temperature was raised to 70 °C (*3*), and it seemed possible, considering heating to 100 °C destroyed the complex, that the E−I complex might be dissociated as soon as the enzymes were inactivated. To test this, an extract was incubated at 70 °C for 30 min and divided into two aliquots. Both were passed through a P-30 column without heating. The eluted fractions from both separations were collected and those from one of the separations were heated at 100 °C for 10 min. The heated and unheated fractions were analyzed for their effects on a malt enzyme preparation (Figure 5). There was considerable variation in the protease activities of

Table 1. Protein-Inhibitor Disruption Tests that Did Not Dissociate the E−I Complex

method	effect on enzyme activity
70 °C, 30 min	destroyed the proteolytic activity of E
2.0 M NaCl	no effect
5.0 M NaCl	destroyed the enzyme activity of the analysis system
4.0 M urea	no effect as assayed
6.9 M urea	no effect as assayed
CHAPS detergent	no effect as assayed
10 mM cysteine	cysteine increased the enzymatic activity
20 mM cysteine	cysteine increased the enzymatic activity
SDS	destroyed the enzyme activity of the analysis system
pH lowered to 3.0	pH of eluate adjusted to 4.8 before analysis
pH 3.0 + 1 M NaCl	pH of eluate adjusted to 4.8 before analysis

the fractions that were not heated after chromatography but little, if any, enzymatic activity was present in the HMW area of the 70 °C treated fractions, and no inhibition was detected in the LMW fractions, indicating that even though the proteolytic activity was destroyed by the heat treatment the E−I complex was not dissociated. The inhibitors were still present and active after the treatment, because they were readily detected in the HMW area of the fractions that were heated to 100 °C after chromatography (Figure 5).

From the results of this experiment, it is obvious that the proteinase enzymatic activities are inactivated at considerably lower temperatures than are needed to dissociate the E−I complexes. Destroying the activities of the endoproteinases is not sufficient to cause disruption of the bonds that hold the E−I complex together. Other experiments (results not shown) have shown that the E−I complex was not dissociated unless the temperature was raised to very nearly 100 °C.

Attempts to Dissociate the E−I Complex with Various Treatments. Several methods were tested for dissociating the E−I complex, and these are listed in Table 1. The extracts were treated with the "dissociating" reagent being tested, and the treated sample was passed through a P-30 column that was equilibrated with the dissociating reagent. As far as could be ascertained, none of the treatments dissociated the complex, although it was impossible to test the effect of SDS, because it destroyed the activity of the analysis system. The presence of 5.0 M NaCl also inactivated the analysis system, but in that case the activity could be approximated by treating the sample with 5 M NaCl and then diluting its concentration to 2 M and analyzing the activity. The salt, urea, and detergents were tested because these reagents are known to sometimes dissociate protein complexes.

The effect of cysteine was tested because our earlier studies had shown that the activity of malt proteinase extracts increased strongly in the presence of cysteine, and that there seemed to be two distinct phases to this activation: one that operated between 0 and 5 mM cysteine and the other that operated between 5 and 20 mM (results not shown). Increasing the cysteine concentration also caused a strong increase in the solubilization of protein during mashing (*3*). It seemed possible that one of these activation phases might be due to the activation of inert cysteine proteinases and the other due to the cysteine displacing the I from the E−I, freeing the active E to hydrolyze protein. As neither 10 nor 20 mM cysteine released any LMW inhibitors in this study, this does not appear to be the case.

When an E−I sample was incubated at pH 3.0 and passed through a P-30 column that had been equilibrated with pH 3.0 buffer, the inhibitory activity eluted over a slightly wider area than when the pH was maintained at 5.5, indicating that a portion of the E−I complex was smaller than normal. However, even this late-eluting material eluted earlier than free I, implying that the E−I complex may have been loosened, but that it was not dissociated into its two components. The experiment was then repeated, except that the incubation and P-30 separation were carried out in the presence of pH 3.0 buffer that contained 1 M NaCl, to ascertain whether the addition of the salt would further decompose the "weakened" E−I complex. The NaCl had no effect; the elution pattern was the same whether the NaCl was present or absent.

To date it has not been possible to dissociate the E and I under conditions that release the E in an active state, so that the total, uninhibited, activities of extracts can be measured. Only heating to 100 °C has resulted in dissociation, but this treatment totally destroyed all of the proteolytic activity.

"Affinity" Method for Concentrating the Endogenous Endoproteinase Inhibitors of Barley and Malt. We have purified and characterized two endogenous endoproteinase inhibitors from barley, and at least one of these is also present in malt (*7, 8*). These, and other endoproteinase inhibitors that we are still studying, are LMW proteins that range in size from about 7000 to 14000 Da. One of the problems associated with purifying these inhibitors is that they must be separated from relatively large amounts of other LMW proteins. These LMW proteins are present in barley and at even higher amounts in malt, where they have been formed by the protein hydrolysis that occurred during the malting process. It would simplify the purification process if the inhibitory proteins could be quickly and easily separated from these contaminating peptides. Theoretically, this can be done by taking advantage of the fact that the peptide inhibitors bind to E to form large complexes and that they can then be released from the complexes, returning to their LMW forms, with their inhibiting abilities unchanged. We have tested the practicality of this method.

Two identical protein extracts were prepared from ground malt samples with pH 5.5 buffer, and the proteins from each extract were separated on a 340-mL Bio-Gel P-30 column. One of these two fractionations is illustrated in Figure 6A (open circles). The HMW protein fractions of the two separations (tubes 14−19) were collected, pooled into a single sample, and freeze-dried. The protein levels of these fractions were very high, exceeding the range of the spectrophotometer. The freeze-dried fractions were dissolved, heated at 100 °C for 10 min, and applied to a second, smaller P-30 column. The elution pattern of this fraction (Figure 6A, closed circles) showed that it contained much less LMW protein than either of the original extracts. An analysis of the separated fractions (Figure 6A) indicated that the inhibitory activities of the heated, "affinity concentrated" extract eluted from the column late (fractions 22−38), as expected of LMW proteins, and that little protein eluted during that time, indicating that a good purification of the inhibitors had been obtained. The inhibitors eluted over a rather wide volume, making it appear that several different inhibitor forms were

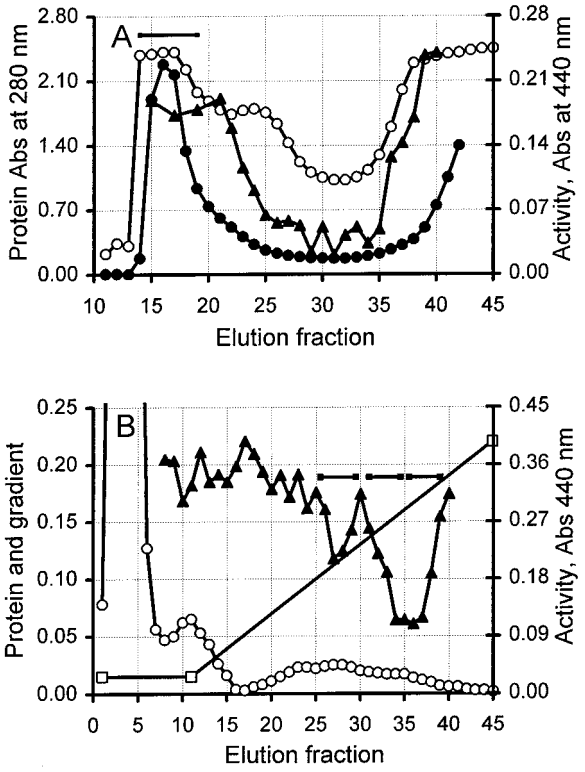

Figure 6. An "affinity" method for concentrating and partially purifying endoproteinase inhibitors from extracts. A. (○) the elution of the proteins of a large extract from P-30 chromatography. The fractions indicated by ▪—▪ were collected and heated. Material from two of these separations was pooled, heated, and applied to a smaller P-30 column from which proportionally smaller fractions were collected; (●) eluted protein; (▲) protease inhibition. B. CMC ion exchange chromatography of the pooled inhibiting fractions from A. (○) Protein elution, absorbance at 280 nm; (▲) protease inhibition; (□) elution gradient. The fractions indicated by ▪—▪ were collected and used for other studies.

present and that these were partially separated on the P-30 column.

When the inhibiting fractions from the P-30 column were pooled, freeze-dried, dissolved, applied to a CMC ion exchange column and eluted, the separation shown in Figure 6B was obtained. The inhibitors were well separated from the contaminating 280-nm absorbing material and were separated into two areas of inhibition. The protein absorbances of the fractions in which the inhibitors eluted was only about 0.02−0.03, so there was little contamination of the inhibitors with other proteins. This inhibitor elution pattern was very similar to that found when crude malt extracts were separated by CMC chromatography in previous studies (*6*), indicating that few, if any, of the inhibitors were lost during the "affinity" concentration steps.

The CMC-separated fractions were pooled as indicated by the bars in Figure 6B and freeze-dried. The first inhibition area was collected as a single pool; the second was divided into two. Preliminary indications are that each of the collected fractions contained one or more endoproteinase inhibitors and these are now being purified by HPLC chromatography for further characterization. Not all of the proteins that were separated by using this method would be expected to be proteinase inhibitors, because any materials that originally were bound to proteins and that were released by the heating step would have been present in the solution that was applied to the CMC column. For example, several small proteins that inhibit the activities of carbohydrate-degrading enzymes such as α-amylase (*15*, *16*) and limit dextrinase (*17*) by binding to them are known to occur in malt and might well have been carried through the P-30 separations. There are probably also some nonenzyme malt proteins to which LMW proteins were bound and these would presumably also have been concentrated by this method.

SUMMARY

Both barley and malt contain LMW proteins that can inhibit the activities of many of the endoproteinases that form in barley while it is undergoing malting. These are important to the brewing process because they interact with the cysteine-class proteinases to strongly inhibit their abilities to hydrolyze proteins during the mashing, and possibly malting, processes. While it has not yet been possible to determine whether the endoproteinases and their endogenous inhibitors are localized together in the grain during the malting process, this report demonstrates that they form tightly bound complexes as soon as they are dissolved together (Figures 1 and 2). These E−I complexes and the unbound I molecules can be readily differentiated using BioGel P-30 gel filtration columns (Figures 1 and 2). It has not yet been possible to gently dissociate the E and I portions of the complex to obtain active proteinases that are free of inhibitor. Free I molecules can be obtained by heating the complex to 100 °C for 10 min, when the enzyme part of the complex is inactivated, dissociates, and precipitates (Figure 2). Heating at 70 °C, which inactivates the proteinases, did not dissociate the E−I complex (Figure 5). The heat-freed I proteins are capable of rebinding to other proteinase molecules to reform E−I complexes (Figure 3). Barley extracts, which contain little or no cysteine endoproteinase activity, do not contain very much E−I but, in contrast to malt extracts, do contain free I molecules (Figure 4). This is presumably due to the fact that barley does not contain sufficient cysteine class E to complex all of the I that is present. An "affinity" method has been designed and tested that utilizes the size differences between the E−I and I molecules to prepare extracts that contain relatively large amounts of the inhibitors from which most of the contaminating LMW proteins have been removed (Figure 6). Extracts prepared with this method are being used as starting material to purify additional barley and malt endogenous proteinase inhibitors for characterization.

ABBREVIATIONS USED

E, uncomplexed endoproteinase enzymes; I, uncomplexed endogenous endoproteinase inhibitors; E−I, complexes between proteinases and their endogenous inhibitors; HPLC, high-performance liquid chromatography; NaAc, sodium acetate; NH_4Ac, ammonium acetate; LMW, low molecular weight; HMW, high molecular weight; CMC, carboxymethyl cellulose.

ACKNOWLEDGMENT

I thank Dr. D. Wesenberg for supplying the barley that was used, Christopher Martens and Laurie Marinac for carrying out the enzymatic assays, and Eddie Goplin for preparing the malt.

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Received for review May 11, 2001. Revised manuscript received August 22, 2001. Accepted September 25, 2001. I thank the American Malting Barley Association for partially supporting this research. Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

JF010611Q