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Activity Patterns of Three Peptide Hydrolases and an Amidase during Malting and Brewing¹

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ABSTRACT

The activities of three peptide hydrolases (PHA, PHB, and PHC) and of an amidase which hydrolyzes L-leucyl- β -naphthylamide (LNA-ase) were followed in Dickson and Piroline barley throughout the malting process and the mashing stage of brewing. Activities of the three peptide hydrolases increased after steeping until the fourth day of germination. The LNA-ase decreased slightly in steep, increased during the first two days of germination, then declined thereafter. Most activities decreased during kilning. Under conditions of practical brewing, the enzymes, while present in the malt tissue, were at a low level in the mash liquid at the protein-rest stage.

Barley malt has held an eminent place throughout the world as the prime ingredient in beer production. Some reasons for this are: a) the ease with which barley germinates; b) the presence of husk, which facilitates filtration of wort; c) the presence of appropriate flavor and color precursors; and d) the provision of water-soluble components suitable for yeast nutrition. The increased use of carbohydrate adjuncts by the U.S. brewing industry has placed a greater requirement on barley malt as the source of constituents other than carbohydrate.

While yeast will grow in a medium containing an ammonium salt as its sole source of nitrogen, in practical brewing it is the malt which supplies the nitrogenous

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requirements of the yeast, mainly as amino acids and low-molecular-weight peptides. Free amino acids in barley and malt have been the subject of extensive studies (1-4). Some of the peptide hydrolases and peptidyl peptide hydrolases thought to be responsible for the release of amino acids and peptides from the endosperm proteins have been characterized (5-9). A site for the synthesis of the latter type of enzyme appears to be the aleurone cells (10).

Our recent work (7,8) has characterized three peptide hydrolases of barley malt which hydrolyze synthetic substrates useful for measuring activity. These enzymes are peptide hydrolase A (PHA), which hydrolyzes α -N-benzoyl-D-L-arginine-*p*-nitroanilide (BAPA); peptide hydrolase B (PHB), which hydrolyzes α -N-benzoyl-L-arginine ethyl ester (BAEE); and peptide hydrolase C (PHC), which hydrolyzes *a*-naphthyl acetate (ANA). In addition an amidase (LNA-ase) which hydrolyzes L-leucyl- β -naphthylamide (LNA) and several esterases which hydrolyze ANA (ANA-ases) have been described (9). PHA is likely the same enzyme as the BAPA-ase described by Enari and Mikola (5).

PHA and PHB activities in Himalaya barley tissues incubated on Petri plates have been followed (11), but the fate of these enzymes, PHC, and LNA-ase in malting and brewing procedures is unknown. To this end, the activity patterns were measured for partially purified preparations of these four enzymes in two barley varieties at five stages during the malting process and at three stages of the brewing procedure.

MATERIALS AND METHODS

Barley (*Hordeum vulgare*)

The six-row variety, Dickson, was grown in Fargo, N.D., and a two-row variety, Piroline, was grown in Burley, Idaho. Both were of the 1968 crop. The six-row variety, Trophy, was grown in Madison, Wis., in 1963.

Malting

One-hundred-and-seventy-gram (dry basis) portions of the Dickson and Piroline barleys were steeped to 45% moisture at 16°C. and germinated in the dark for up to 5 days at 16°C. in a manner similar to that described by Shands et al. (12). A portion of the barley was removed after steeping; after 2, 4, and 5 days' germination; and after kilning. The kilning program was: 6 hr. at 35°C., 7 hr. at 43°C., 5 hr. at 55°C., 5 hr. at 65°C., 2 hr. at 74°C., and 2.5 hr. at 85°C. All samples except the completely kilned malt were frozen and lyophilized to 4 to 8% moisture.

The Trophy barley was germinated and lyophilized as described previously (7). Briefly, the grain was steeped in water at 16°C. until the moisture content was 45%. To each 225 g. dry weight of barley, 2.25 mg. Gibrel (Merck & Co., Rahway, N.J.) in 10 ml. of water was applied. The barley was germinated for 6 days in the dark at 16°C., and lyophilized to approximately 10% moisture. It was stored at about -25°C. until used.

Brewing

Dickson and Piroline malts were used for brewing. The double-mash microbrewing technique of Burkhart et al. (13) was used with the malt ground in the usual way with a Miag mill (Miag North America Inc., Minneapolis, Minn.).

Briefly, the usual microbrewing procedure is as follows. The mash consists of 70% malt and 30% corn grits. A "cooker mash" is prepared with 10% of the malt and all of the corn grits by mashing at 45°C. for 10 min. This mash is then brought to boiling in 35 min. and boiled for 15 min. A "malt" mash is prepared with the remainder (90%) of the malt. This mash is agitated at 38°C. for 8 min. and then "rested" at this temperature for 50 min. — the so-called protein rest. The cooker mash is then combined with the malt mash and the temperature adjusted to 72°C. When the iodine test for starch is negative, i.e., after "conversion", the mash is heated to 75°C. and transferred to the lauter. After the mash has rested in the lauter at 75°C. for 15 min., the wort is filtered off and poured back into the lauter for re-filtration. The grain in the lauter is washed (sparged) with water. The combined wort and washings are boiled. Hops are added during this period. The filtered, hopped wort is then ready for inoculation with yeast.

The only variation in the above procedure was the use of 84% of the cooker-mash ingredients, as 16% of the malt mash was removed after the protein rest for enzyme-activity determinations. Samples during the brewing were taken as follows: 150 g. of the malt mash after the protein rest, which contained 36 g. malt, dry basis; 300 g. of the combined mash after conversion, which contained 47 g. malt contributed by the malt mash; and 612 ml. of the sparged wort from the lauter, equivalent to 53 g. of malt from the malt mash. The mash samples were cooled rapidly to 4°C. and centrifuged at 14,000 × g for 20 min. The resulting supernatants and the wort sample were concentrated to about 20 ml. by ultrafiltration. They were then dialyzed at 4°C. against 3 × 1,300 ml. 0.005M succinate, pH 5.5.

In a separate brewing experiment the Dickson malt was ground finely with a Labconco mill (Laboratory Construction Co., Kansas City, Mo.) at the No. 8 setting, instead of on the Miag mill.

Extraction of Barley and Malt

The plant tissue was ground finely with the Labconco mill and extracted as previously described (7,9). Briefly, 50 g. of ground barley or malt was extracted for 1 hr. at 4°C. with 150 ml. of 0.05M *tris*-chloride buffer which was 0.005M with respect to EDTA and which contained 12.5% sucrose and 0.5% ascorbic acid, pH 7.5. The mixture was centrifuged at 24,000 × g for 20 min., and the supernatant dialyzed against 3 × 1,300 ml. of 0.005M succinate, pH 5.5. It was concentrated by ultrafiltration to about 40 ml. and dialyzed again for about 5 hr. against the succinate buffer.

Purification of Enzymes with Carboxymethyl Cellulose

One-half of the solution from the extraction of barleys and malts, representing about 25 g. of material, and the solutions as described from the brewing procedure, were applied to 2.5 × 40-cm. columns of carboxymethyl cellulose (CMC) (7,9) in equilibrium with 0.005M succinate, pH 5.5. A succinate concentration gradient at pH 5.5 was pumped on the column at 20 ml. per hr. Two hundred grams of 0.5M succinate was drawn into 200 g. of 0.005M solution as the latter was pumped on the column. The effluent was monitored at 280 nm. and fractions of approximately 4 ml. were collected and examined for enzyme activity. Fractions containing the resolved enzymes were pooled, and the pooled lots assayed for total activity.

Assays for Peptide Hydrolase and Protein

The assays have been described in detail (7,9). The activity of PHA was measured by the hydrolysis of BAPA which produces *p*-nitroaniline and increased absorption at 410 nm. PHB was measured by observing the increased absorption at 259 nm. when BAEE is hydrolyzed. Similarly, hydrolysis of LNA increases absorption at 335 nm., and hydrolysis of ANA increases absorption at 310 nm. The units of enzyme activity are expressed as the number of n moles of products formed per min. under the conditions of the assay. The protein assay was by the conventional Lowry method (13), with purified bovine plasma albumin used as a standard.

Electrofocusing of PHA and LNA-ase

Finely ground Trophy malt was extracted at 0°C. with 0.05M *tris*-chloride buffer, pH 7.5, which contained 12% sucrose, as described previously (7). This extract was dialyzed against 3 × 1,300 ml. of 0.005M acetate, pH 5.5, and concentrated 3.5-fold by ultrafiltration. Just prior to electrofocusing, an aliquot portion was dialyzed against 1% glycine for 16 hr., and 5.3 ml. of this solution, containing 85 mg. protein, 3,700 units LNA-ase, and 133 units PHA was applied to the electrofocusing column. The electrofocusing was done with an LKB column of 110-ml. capacity, as described previously (8). A pH 4 to 6 carrier ampholyte was used at 0.83% (w./w.) concentration in a sucrose-density gradient of 0 to 47% (w./w.) at 2°C. A potential of 600 v. was applied for 66 hr. After development of the column, the contents were removed by gravity flow at about 1 ml. per min. and collected in 1-ml. aliquots.

RESULTS AND DISCUSSION

The separation of enzymes obtained with the CMC column is shown in Fig. 1 for Dickson barley germinated 5 days. Essentially the same pattern resulted for Piroline germinated for 5 days and, except for the total quantity of enzymes present, the same pattern was obtained for both barleys at the various germination periods, including the kilned malts. The pattern is also similar to that described previously for Trophy malt (7).

The enzymes which are not adsorbed on CMC at the pH and ionic strength at the start of the gradient appear in fractions 3 to 13. These are PHA, LNA-ase, and, as shown previously (9), at least two ANA-ases, which in the previous work were designated as ANA-ase I and ANA-ase II. These ANA-ase enzymes have not been completely separated from each other. PHA, however, has been separated from LNA-ase and the ANA-ase enzymes by gel electrophoresis which inactivated all but PHA (15). Electrofocusing the enzymes from Trophy malt that are unadsorbed on CMC (Fig. 2) gave a partial separation of LNA-ase from PHA and several ANA-ases. The front of the LNA-ase peak (fractions 25, 26, 27) had no PHA or ANA-ase activities. While the separation is not complete, it shows that LNA-ase does not hydrolyze BAPA or ANA.

The enzymes which are adsorbed on the CMC and which are eluted by the high-ionic-strength portion of the gradient, as shown in Fig. 1, are PHB (fractions 18 to 29) and PHC, which in earlier work (9) was designated as ANA-ase IV

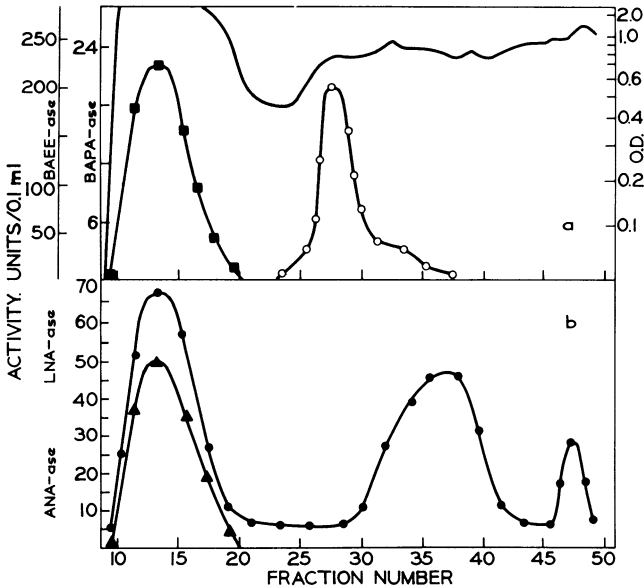


Fig. 1. CMC chromatography of enzymes from Dickson malt. Plain line = absorbance; squares = PHA; open circles = PHB; closed circles = ANA-ase; and triangles = LNA-ase.

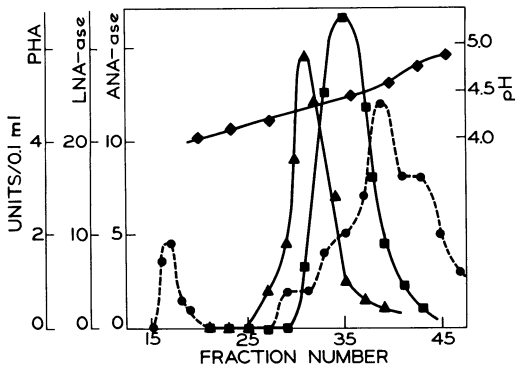


Fig. 2. Electrofocus pattern of enzymes unadsorbed on CMC. Diamonds = pH; circles = ANA-ase; squares = PHA; and triangles = LNA-ase.

(fractions 26 to 37). In some experiments there were small quantities of an enzyme corresponding to ANA-ase III described previously (9) which is absent in Fig. 1, but which would be expected to appear in fractions 20 to 25. Another ANA-ase that appeared only in some of the experiments is the one contained by fractions 40 to 45. However, this enzyme, when present, occurred in small quantity. These and other minor ANA-ase enzymes that appear inconsistently suggest that they are labile under our experimental conditions. Their properties have yet to be studied.

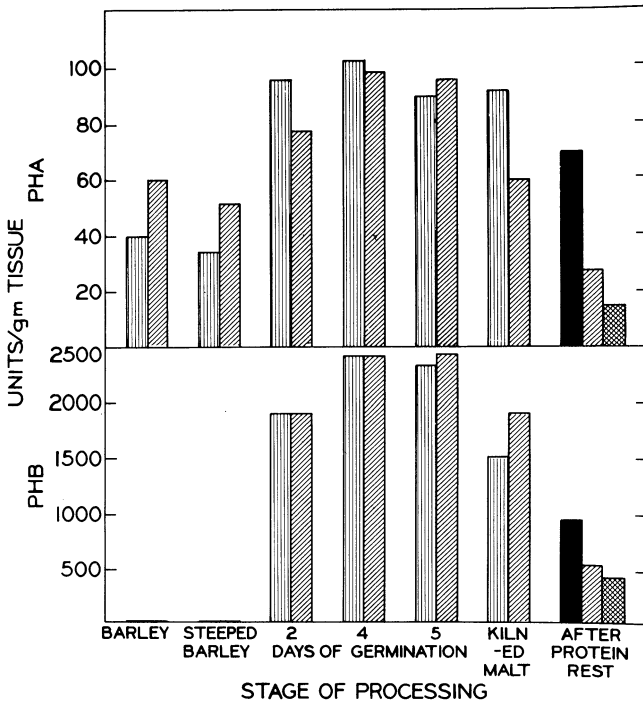


Fig. 3. PHA and PHB activities at stages of malting and brewing. Slanted lines = Dickson malt, finely ground; vertical lines = Piroline malt, finely ground; crossed lines = Dickson malt, coarsely ground; and black = Piroline malt, coarsely ground.

Figure 3 shows the change in activity of PHA and PHB, and Fig. 4 shows the change in LNA-ase and PHC throughout the malting period and after the protein-rest stage of brewing. For both barley varieties little change occurred during steeping, except for LNA-ase, which showed a pronounced decrease. PHA activity increased during the first 4 days of germination, and decreased slightly thereafter. PHB, on the other hand, was not detected in the ungerminated barley, but increased significantly during germination and reached maximal activity by day 4. The two barley varieties had essentially the same levels of PHB activity during this period. This behavior of PHA and PHB during the germination period is in general agreement with the results of earlier experiments (11) in which these enzymes were followed in Himalaya barley during 70-hr. incubation.

PHC in both Dickson and Piroline barleys increased during the first 2 days of germination, and remained essentially constant for the remainder of the germination period. LNA-ase activity for both varieties increased sharply during the first 2 days of germination and decreased moderately during the next 2 days.

Thus, except for LNA-ase, the activities of the enzymes increased for 2 to 4 days with little change through the 5th day of germination. This is in agreement with changes in overall peptide hydrolysis as measured by levels of free amino acids during germination (1,2).

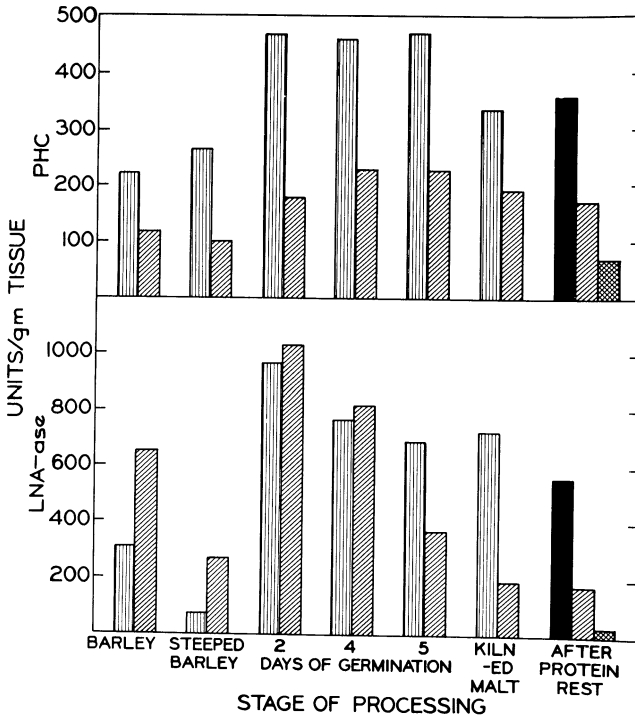


Fig. 4. PHC and LNA-ase activities at stages of malting and brewing. (Symbols as for Fig. 3.)

Figures 3 and 4 show that activities of all enzymes except Pirolinase PHC were diminished in the mash liquids after protein rest when the malt for these mashes had been ground coarsely with the Miag mill in the normal procedure for brewing. These low levels of enzymatic activity seemed surprising in view of the quantities of amino acids produced during mashing (1,2). Also, the decreases in the enzymes during protein rest are not consistent with the known stability of these enzymes to pH and heat (7,9). It therefore seemed that if the enzymes were not inactivated during mashing-in and during protein rest, they may not have been thoroughly extracted under normal mashing conditions. Visual inspection of the coarsely ground malt grist normally used for brewing revealed only superficial disintegration of the embryo. This malt had been ground with the Miag mill. The relatively gentle agitation used for mashing most likely did not contribute appreciably to further disintegration of the embryo parts and solubilization of their cellular contents. Since the bulk of PHB and approximately one-third of PHA were previously shown to reside in the embryo residue (scutellum + scutellar node + coleorhiza) (11), it seemed likely that during the mashing-in process of practical brewing these two enzymes remained in the embryo tissue.

That this was indeed the case became apparent upon examination of the mash made with the Dickson malt ground finely with the Labconco mill (Figs. 3 and 4). In the liquid of this mash after the protein rest, the activities were increased by factors of 4.6 for LNA-ase, 1.8 for PHA, 1.2 for PHB, and 2.5 for PHC. Thus the

peptide hydrolases examined in our work are probably some of the enzymes about whose presence there has been speculation based on amino acid production during mashing (2).

For malt ground with either mill, the mash samples taken after conversion had little activity. Samples from the lauter were inactive.

Literature Cited

1. JONES, MARGARET, and PIERCE, J. S. Amino acids in brewing. The development of individual amino acids during malting and mashing. *Eur. Brew. Conv., Proc. Cong.*, 1963, p. 101.
2. ROBBINS, G. S., CHESTERS, JOAN, and DICKSON, A. D. Quantitative changes in free amino acids during malting of two barley varieties. *Amer. Soc. Brew. Chem., Proc.* 1963, p. 141.
3. JONES, MARGARET, and PIERCE, J. S. Malt peptidase activity. *J. Inst. Brew. (London)* 73: 347 (1967).
4. JONES, MARGARET, and PIERCE, J. S. Malt peptidases. *J. Inst. Brew. (London)* 73: 349 (1967).
5. ENARI, T. M., and MIKOLA, J. Characterization of the soluble proteolytic enzymes of green malt. *Eur. Brew. Conv., Proc. Cong.*, 1967, p. 9.
6. ten HOOPEN, H. J. G. The proteolytic enzymes of barley and malt. I. Extraction of peptidyl peptide hydrolases (endopeptidases) with activity at pH 5 from malt. *Cereal Chem.* 45: 19 (1968).
7. BURGER, W. C., PRENTICE, N., KASTENSCHMIDT, JEANNINE, and MOELLER, MARY. Partial purification and characterization of barley peptide hydrolases. *Phytochemistry* 7: 1261 (1968).
8. BURGER, W. C., PRENTICE, N., and MOELLER, MARY. Peptide hydrolase C in germinating barley. *Plant Physiol.* 46: 860 (1970).
9. BURGER, W. C., PRENTICE, N., MOELLER, MARY, and KASTENSCHMIDT, JEANNINE. Hydrolysis of alpha naphthyl acetate and L-leucyl naphthylamide by barley enzymes. *Phytochemistry* 9: 33 (1970).
10. JACOBSEN, J. V., and VARNER, J. E. Gibberellic acid induced synthesis of protease by isolated aleurone layers of barley. *Plant Physiol.* 43: 1596 (1967).
11. PRENTICE, N., BURGER, W. C., and WIEDERHOLT, ELIZABETH. Distribution of acidic and neutral peptidases in germinating barley. *Physiol. Plant.* 22: 157 (1969).
12. SHANDS, H. L., DICKSON, A. D., DICKSON, J. G., and BURKHART, B. A. The influence of temperature, moisture, and growth time on the malting quality of four barley varieties. *Cereal Chem.* 18: 370 (1941).
13. BURKHART, B. A., OTIS, O. J., and DICKSON, A. D. A microbrewery for the early quality evaluation of hybrid barley selections. *Amer. Soc. Brew. Chem., Proc.* 1960, p. 123.
14. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265 (1951).
15. MOELLER, MARY, BURGER, W. C., and PRENTICE, N. Purification of a barley peptide hydrolase by disc electrophoresis. *Phytochemistry* 8: 2153 (1969).
16. ROBBINS, G. S., FARLEY, MARY, and BURKHART, B. A. Quantitative changes in free amino acids during the protein rest of mashing of barley malts. *Amer. Soc. Brew. Chem., Proc.* 1963, p. 124.

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