# Note on the Development of Proteolytic Enzymes in Germinating Barley

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It was reported recently (1-4) that water or buffer extracts of barley and malt contain an enzyme (BAPA-ase), which catalyzes the hydrolysis of the synthetic substrate, alpha-benzoyl-L-arginine-*p*-nitroanilide (BAPA) at an optimum pH of 8.6. Burger (3) purified BAPA-ase extracted from green malt approximately 150-fold and studied some of its properties. As part of an investigation of the proteolytic enzymes of barley and malt, the development of BAPA-ase in germinating barley was studied in conjunction with that of Hb-ase, an enzyme which was found to catalyze the hydrolysis of commercial bovine hemoglobin (Hb) at an optimum pH of 3.8 and substrate concentration of 1.5% (w./v.) in 0.05 *M* sodium acetate buffer. The object of this work was to find out whether the rates of formation of BAPA-ase and Hb-ase were different during germination of the grain.

## EXPERIMENTAL

Samples of Proctor barley (10 g.) of the 1965 harvest were steeped, in triplicate, at the germination temperature (14°C.) in tap water for 24 hr. with a change of steep after 12 hr. The grain was then air-rested at the same temperature for 12 hr. and resteeped in tap water for 12 additional hr. At the end of steeping, the moisture content of the grain, as determined by surface-drying of the whole grain at 100°C. for 2 hr., was 43%. The steeped grain was blotted to remove excess moisture and grown in a growth cabinet at 87-90% r.h. in 6 by 3 1/2-in. glass bottles with screw-on lids, into each of which a 1/4-in. hole had been drilled. At appropriate intervals, the bottles were removed and their contents homogenized separately for 3-4 min. in an MSE homogenizer with 30 ml. of cold 0.15*M* sodium chloride solution (5) containing 0.5% (w./v.) L-cysteine hydrochloride (pH 7.0); the homogenate was extracted for 2 hr. with mechancial shaking at 4°C. Addition of 0.5% L-cysteine hydrochloride to the extracting agent caused a small increase in the Hb-ase activity (Table I). After extraction, each homogenate was strained through two layers of cheesecloth to remove the debris, and the extracts were centrifuged at 19,000 x *g* for 20 min. in a refrigerated centrifuge at 2°C. The supernatant fractions were dialyzed at 5°C. for 16 hr. against running distilled water to remove the salt and low-molecular-weight compounds. The dialyzed extracts were recentrifuged to remove materials which had sedimented during dialysis. The clear supernatant fractions, constituting in each case about 60% of the original extract volume, were made to 50 ml. with distilled water. Aliquots (1 ml.) were taken from each extract of 10 g. of grain for the assay of Hb-ase and BAPA-ase and for the estimation of soluble protein by the biuret method (6), with bovine serum albumin as reference standard. When ungerminated barley was used, the grain was first ground finely by hand in a coffee mill and then extracted at 4°C. as described above. Hb-ase was assayed at 40°C. by incubating 4 ml. of the hemoglobin sub-

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TABLE I. EFFECT OF ADDITION OF 0.5% (w./v.) L-CYSTEINE-HYDROCHLORIDE ON Hb-ase ACTIVITY OF BARLEY EXTRACTED WITH 0.15 *M* SODIUM CHLORIDE SOLUTION

| | Hb-ase Activities[a] | |
|---|---|---|
| Assay Time min. | No L-Cysteine-HCl Added | 0.5% L-Cysteine-HCl Added |
| 10 | 0.06 | 0.07 |
| 20 | 0.14 | 0.14 |
| 40 | 0.22 | 0.26 |
| 60 | 0.31 | 0.32 |

[a]Activities are expressed per 1 ml. of the final volume (50 ml.) from 10 g. of grain.

strate with the enzyme solution (1 ml.); enzyme action was stopped after 1 hr. by the addition of 5 ml. of 0.6*M* trichloroacetic acid (TCA). An aliquot of the TCA-soluble filtrate was taken to read absorbance at 280 mμ (7). BAPA-ase was assayed by the method of Enari *et al.* (1) in which the release of *p*-nitroaniline is measured spectrophotometrically at 410 mμ. Hb-ase and BAPA-ase activities are expressed as absorbance units, for 1-cm. light path, at 280 and 410 mμ respectively per hr. per g. barley. Protein concentration is expressed as mg./ml. of the final extract volume (50 ml.) from 10 g. of barley.

## RESULTS AND DISCUSSION

Figure 1 shows the observed Hb-ase and BAPA-ase activities and the soluble protein content of the ungerminated, steeped, and germinating barley. In spite of a wide variation in their pH optima (Hb-ase, 3.8; BAPA-ase, 8.6), development of these enzymes in germinating barley was similar, enzyme activities being maximum in both cases on the 5th day of germination. Thereafter little change in enzyme activities occurred. Increases in activities of the Hb-ase and BAPA-ase on germination were also substantially the same. Previous reports (1,8) also have shown maximum development of proteolytic activity in malting barley on the 5th day of germination, although in these studies only single substrates were employed for assay of proteolytic activity. Burger (3) concluded from his studies that Hb-ase and BAPA-ase were separate enzymes, as on purification of BAPA-ase there was a consistent decrease in Hb-ase activity of the malt extract. Nevertheless, the formation of these enzymes in germinating barley, under the experimental conditions described, was parallel. Since BAPA does not possess free terminal amino or carboxyl groups, it is most likely that this substrate is hydrolyzed by endopeptidases and not by exopeptidases. A close pattern of development of BAPA-ase and Hb-ase, such as that obtained in the present study, therefore would suggest that endopeptidases probably develop concomitantly in germinating grain.

A decrease in soluble protein content of the grain after steeping until the second day of germination (Fig. 1), when there was little corresponding enzyme activity and hence no hydrolysis of the reserve proteins occurred, appeared to indicate *in situ* utilization of the soluble proteins by the germinating embryo. Thereafter, with the development of enzyme activity, a concomitant hydrolysis of reserve proteins occurred which reached a maximum on the 5th day of germination, after which it

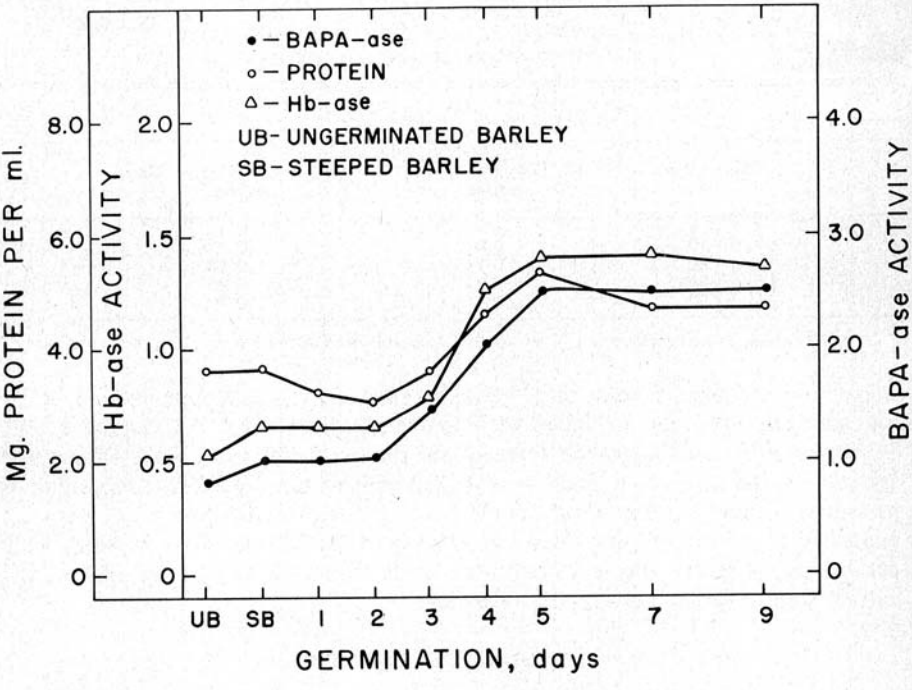


Fig. 1. Development of Hb-ase and BAPA-ase in germinating barley. (All results reported are means of three separate determinations.)

slightly decreased and then remained constant till the 9th day of germination. A later decrease in soluble protein content (after the 5th day of germination) suggested that at this stage the initial essentially degradative phase was replaced by one in which there was increasing use of the hydrolytic products for synthesis of cytoplasmic proteins. These results are consistent with the view (9) that during germination, hydrolysis predominating until about the 5th day when equilibrium between the two processes is established.

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