PARTIAL PURIFICATION OF PROTEASES FROM GERMINATED BARLEY[1]

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ABSTRACT

Proteases from germinated barley have been separated by treatment with carboxymethyl cellulose and by filtration of products therefrom through a dextran gel. These consist of (a) an acidic peptidase which hydrolyzes alpha-N-benzoyl-DL-arginine-*p*-nitroanilide (BAPA-ase), together with an acidic peptidase which hydrolyzes alpha-N-benzoyl-L-arginine ethylester (BAEE-ase) and an acidic proteinase; (b) two separated acidic proteinases; (c) a neutral BAEE-ase together with a neutral proteinase; and (d) a separated neutral proteinase.

Barley and malt contain several proteolytic enzymes which hydrolyze gelatin and hemoglobin, as well as peptidases which hydrolyze a variety of peptides and other amino acid derivatives (1–5). Two of the peptidases can be distinguished from one another by their specificity for BAPA[4] or BAA. A third activity, which splits BAEE, is known to be distinct from the BAA-ase, but its relationship to BAPA-ase has not been established (1).

The present study concerns the development of a comprehensive procedure for separating the soluble proteases of malt on a scale that

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[4]The following abbreviations for chemical names are used: BAPA (alpha-N-benzoyl-DL-arginine-*p*-nitroanilide); BAA (alpha-N-benzoyl-L-arginine amide); BAEE (alpha-N-benzoyl-arginine ethylester); CMC (carboxymethyl cellulose); EDTA (ethylenediamine tetraacetic acid); Hb-ase (hemoglobinase).

is sufficient for subsequent purification and study of the various enzymes obtained.

Materials and Methods

Materials. Malt: Trophy barley grown in 1963 was germinated with the application of 10 p.p.m. of Gibrel[5] (Merck & Co., Inc., Rahway, N.J., Mark 60830, potassium salt of gibberellic acid) as described by Burger (1), except that the time of germination was 6 days.

Carboxymethyl cellulose: Whatman Powder, CM-70, Lot No. 519-527, 100- to 200-mesh (H. Reeve Angel & Co., Inc., Clifton, N.J.).

Sephadex G-100 (Pharmacia Fine Chemicals Inc., Piscataway, New Market, N.J.).

Substrates: Benzoyl-L-arginine ethylester hydrochloride and benzoyl-DL-arginine-*p*-nitroanilide (Mann Research Laboratories, New York, N.Y.); gelatin, U.S.P. (Fisher Scientific Co., Chicago, Ill.); bovine hemoglobin enzyme substrate powder and bovine plasma albumin Fraction V (Pentex Inc., Kankakee, Ill.).

Methods. All solutions were prepared with glass-distilled water. All enzyme separations were carried out between 0° and 8°C.

1. *Extraction of freeze-dried germinated barley.* This was done as previously described (1).

2. *Enzyme assays.* BAPA-ase, BAEE-ase, and Hb-ase were assayed as previously described (1), except that 0.1 ml. of enzyme was used in each case and the reaction temperature for the BAEE-ase was 35°C.

Gelatinase was determined viscometrically with 5 ml. of substrate solution and 1 ml. of enzyme solution in Ostwald ASTM No. 100 viscometers. The substrate solution consisted of 50 g. gelatin in 500 ml. distilled water and 200 ml. of acetate buffer (1 part 0.1*M* acetic acid plus 2 parts 0.1*M* sodium acetate). The solution was diluted to 1 liter with distilled water. Relative viscosities were calculated as $(t\text{-}t_0)d/t_0d_0$, where t = flow time for the reaction mixture; t_0 = flow time of a standard solution consisting of 5 ml. gelatin and 1 ml. of 0.02*M* acetate buffer; d = density of the reaction mixture and d_0 = the density of the standard solution, both at 40°C.

Enzyme units under the conditions of the assays are: (a) BAPA-ase, the production of 1 mμmole *p*-nitroaniline per min.; (b) BAEE-ase, Δ O.D. (absorbance) of 0.001 per min.; (c) gelatinase, change in relative viscosity of 0.01 per 30 min.; and (d) Hb-ase, release of 1 γ of acid-soluble nitrogen per hr.

Protein was determined by the method of Lowry *et al.* (6) with

[5]Mention in this publication of a trade product, equipment, or a commercial company does not imply its endorsement by the U.S. Department of Agriculture over similar products or companies not named.

bovine plasma albumin as the standard.

Concentration of enzyme solutions was done by ultrafiltration under reduced pressure with 8/32-in. Visking casing at 0°–4°C.

3. *Preliminary treatment of CMC.* To 1,500 ml. of 0.5*M* sodium hydroxide solution which was 0.5*M* with respect to sodium chloride, 40–50 g. of CMC was added with stirring. After 30 min. the CMC was recovered by filtration and washed with four 1-liter portions of distilled water. The CMC was dispersed in 0.1*N* hydrochloric acid, filtered immediately, and washed as before. The treatment with base and acid was repeated. The CMC was stored in the damp condition in the cold.

4. *Batch treatment with CMC at pH 5.5.* Fifty-milliliter portions of malt extract (Fig. 1) were dialyzed against 0.005*M* acetate pH 5.5. The dialysate was added to 69 g. wet cake (12 g. dry weight) CMC equilibrated with this buffer and allowed to stand 10 min. The supernatant was removed and the CMC was washed with three 50-ml. portions of this buffer to completely remove the BAPA-ase, acidic BAEE-ase, and acidic proteinases. The neutral BAEE-ase and neutral proteinases were then eluted from the CMC with four 50-ml. portions of 0.5*M* acetate, pH 5.5.

5. *Gel filtration with Sephadex G-100.* Columns of Sephadex G-100 (2.5 × 32 cm. or 2.5 × 90 cm.) were equilibrated with a 0.1*M* acetate solution, pH 5.9, which was 0.2*M* in sodium chloride and 0.004*M* in EDTA. Columns were loaded with sample concentrated to 12–18 ml. containing 10–15 mg. of protein. The columns were developed with an upward flow rate of 9–13 ml./hr. and eluates were collected in 3- to 4.5-ml. fractions.

Results and Discussion

Preliminary experiments to establish the essential conditions for the batchwise separation of acidic proteases from the neutral and basic enzymes on CMC revealed that BAEE-ase, BAA-ase, and a considerable portion of the Hb-ase activity are adsorbed to the exchanger at pH 5.5 in 0.005*M* acetate buffer. In addition to BAPA-ase, a large amount of Hb-ase and a very low but consistent level of BAEE-ase were not adsorbed. These are the acidic enzymes. An increase of 50 to 100% in BAPA-ase activity after the CMC treatment indicated that the ion exchanger also adsorbs some of the BAPA-ase inhibitor(s).

The adsorbed BAEE-ase and Hb-ase were eluted by increasing the concentration of pH 5.5 acetate buffer to 0.5*M*. Because the cationic proteins are elutable at pH 5.5, they are classified herein as neutral rather than basic proteins. The more-basic BAA-ase was eluted only by increasing the pH to 8 or higher. For this reason it is believed that

the basic proteins are not eluted at pH 5.5 with 0.5*M* sodium acetate buffer and that they require a considerably higher pH for elution.

Purification of Proteases. As a consequence of the investigations with CMC, the procedure outlined in Fig. 1 was developed. For purpose of reference, Roman numerals are assigned to fractions containing the various Hb-ases.

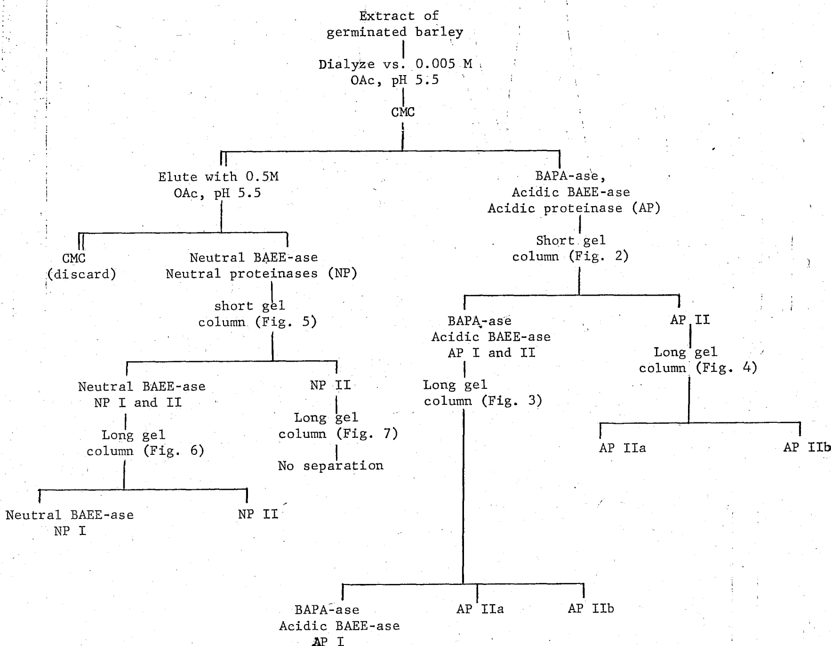

Fig. 1. Flow diagram for the purification of barley proteases.

Gel filtration of the acidic enzymes on the short column (2.5 × 32 cm.) of G-100 Sephadex gave the separation shown in Fig. 2. The resulting fractions were pooled in two groups, one containing the BAPA-ase, acidic BAEE-ase, and acidic proteinases I and II (Fig. 2, fractions 20–27), the other containing acidic proteinase II (Fig. 2, fractions 30–42).

Further gel filtration of the former group on a larger (2.5 × 90 cm.) column of G-100 showed that the acidic proteinase II may consist of two enzymes which are referred to as acidic proteinase IIa (Fig. 3, fractions 64–68) and acidic proteinase IIb (Fig. 3, fractions 70–80).

Also separated as a group on this column were BAPA-ase, acidic BAEE-ase, and acidic proteinase I (Fig. 3, fractions 51–62).

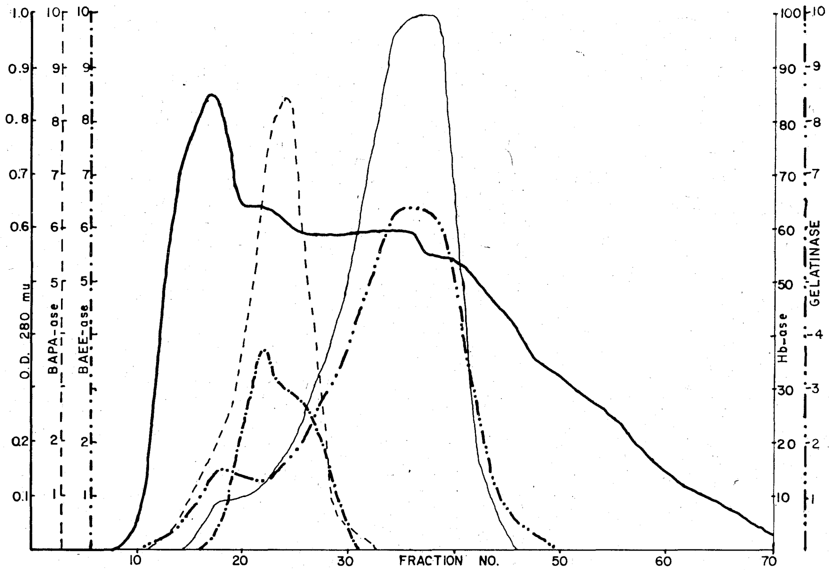

Fig. 2. Gel filtration of BAPA-ase, acidic BAEE-ase, and acidic proteinases; 2.5 × 32-cm. column of Sephadex G-100. Sample: 18 ml. containing 846 units of acidic BAEE-ase and 193 units of BAPA-ase. Flow rate: 9 ml./hr.

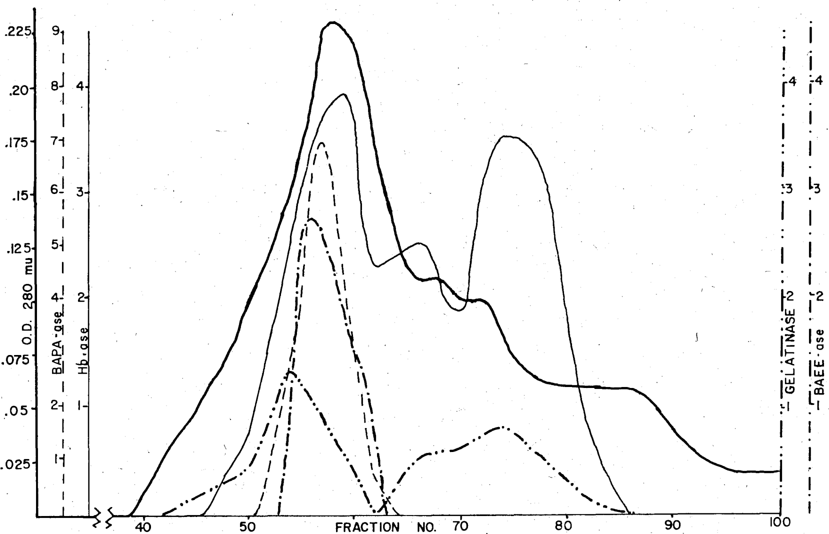

Fig. 3. Gel filtration of BAPA-ase, acidic BAEE-ase, and acidic proteinases I and II; 2.5 × 90-cm. column of Sephadex G-100. Sample: 13.3 ml. containing 14.6 mg. protein, 745 units of BAEE-ase, 2,050 units of BAPA-ase, 3,900 units of Hb-ase, and 77 units of gelatinase. Flow rate: 10.5 ml./hr.

What appear to be acidic proteinases IIa and IIb were again obtained when the acidic proteinase II peak (Fig. 2, fractions 30–42) was subjected to filtration on the long column. Two partially resolved regions of Hb-ase and gelatinase activity were obtained (Fig. 4).

Similar treatment of the neutral proteins that were eluted from CMC provided the pattern shown in Fig. 5 for the short-column treatment. The neutral BAEE-ase appears in fractions containing neutral proteinase I (fractions 10–23). There appears to be another proteinase (neutral proteinase II) that follows these enzymes off the column (fractions 24–40).

Refiltration of the neutral BAEE-ase-neutral proteinase fraction on the long column afforded the separation shown in Fig. 6. The neutral proteinase appears to be separated into two enzymes, possibly consisting of neutral proteinase I (fractions 38–54) and neutral proteinase II (fractions 55–66) with which neutral proteinase I was contaminated. Neutral BAEE-ase is only partially separated from neutral proteinase I.

The refiltration of neutral proteinase II (Fig. 5, fractions 23–29) achieved no further separation of activity (Fig. 7), although considerable extraneous protein was removed.

The above procedure involving CMC and G-100 has separated some of the proteases of germinating barley into five fractions which are suitable for further study of activity and purification. These fractions are (Fig. 1): 1) BAPA-ase, acidic BAEE-ase, and acidic proteinase I; 2) acidic proteinase IIa; 3) acidic proteinase IIb; 4) neutral BAEE-ase, neutral proteinase I; and 5) neutral proteinase II.

The specific activities for some of these enzymes are listed in Table I. In general, the acidic enzymes responded better to purification than the neutral enzymes, but both types of proteinases showed poor recoveries. The lack of any purification of neutral proteinase II likely resulted from its denaturation during processing.

TABLE I
SUMMARY OF SPECIFIC ACTIVITIES, RECOVERIES, AND PURIFICATION

ENZYME FRACTION	SPECIFIC ACTIVITY	RECOVERY [a]	PROTEIN	PURIFICATION [a]
	units/mg. protein	*%*	*mg.*	*-fold*
BAPA-ase	623	34	2.1	178
Acidic BAEE-ase	300	38	2.1	103
Acidic proteinases IIa and IIb	6,270 [b]	4	2.2	133
Neutral BAEE-ase	887	37	7.4	30
Neutral proteinase II	133 [b]	2	54.0	0

[a] Based on activity of pH 5.5 dialysate (see Fig. 1).
[b] Hb-ase data.

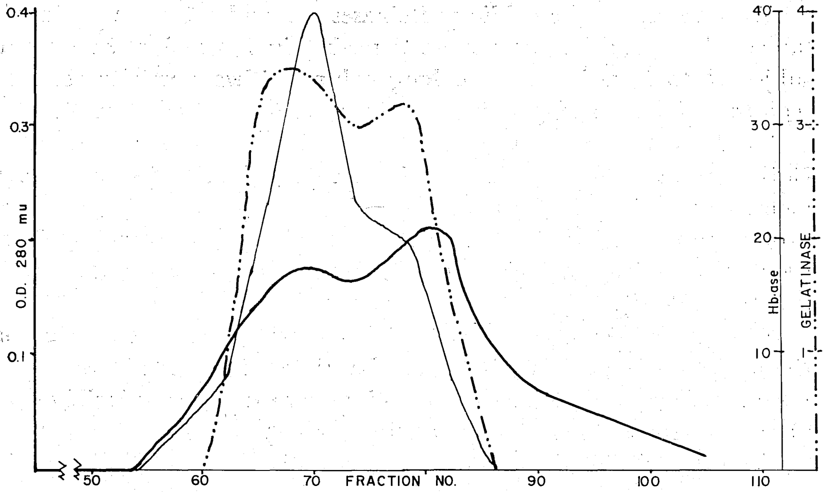

Fig. 4. Gel filtration of acidic proteinase II; 2.5 × 90-cm. column of Sephadex G-100. Sample: 16 ml. containing 10.5 mg. protein, 28,000 units of Hb-ase, and 218 units of gelatinase; no BAEE-ase or BAPA-ase. Flow rate: 10 ml./hr.

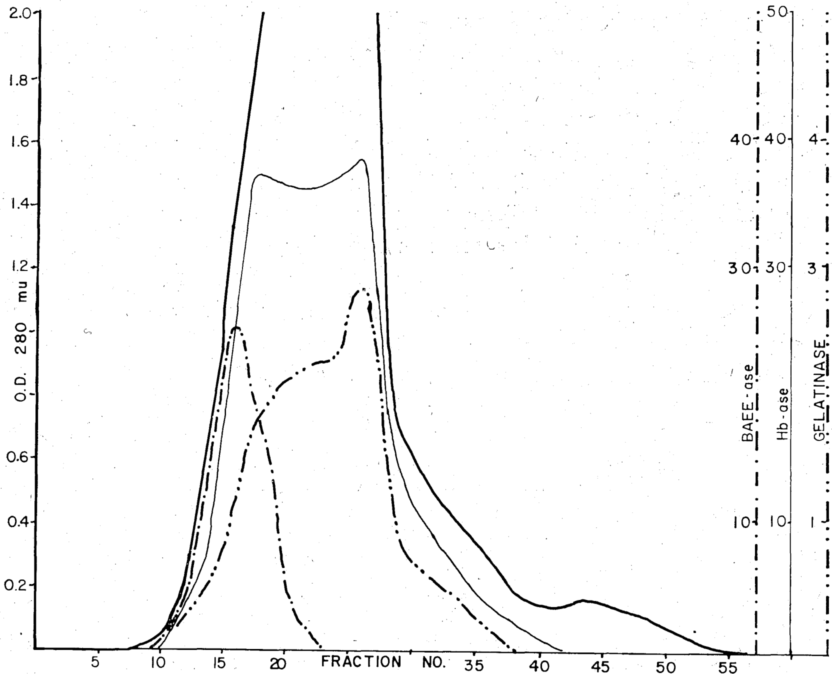

Fig. 5. Gel filtration of neutral proteases; 2.5 × 32-cm. column of Sephadex G-100. Sample: 21.5 ml. containing 5,000 units of BAEE-ase, 38,500 units of Hb-ase, and 282 units of gelatinase. Flow rate: 13.2 ml./hr.

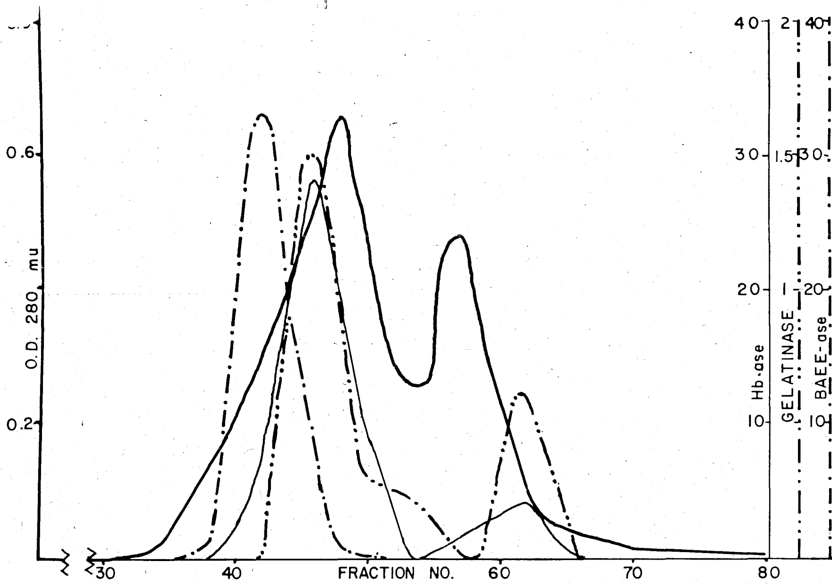

Fig. 6. Gel filtration of neutral BAEE-ase and neutral proteinases I and II; 2.5 × 90-cm. column of Sephadex G-100. Sample: 15 ml. containing 30 mg. protein, 4,590 units of neutral BAEE-ase, 9,750 units of neutral Hb-ase, and 57 units of gelatinase. Flow rate: 13.5 ml./hr.

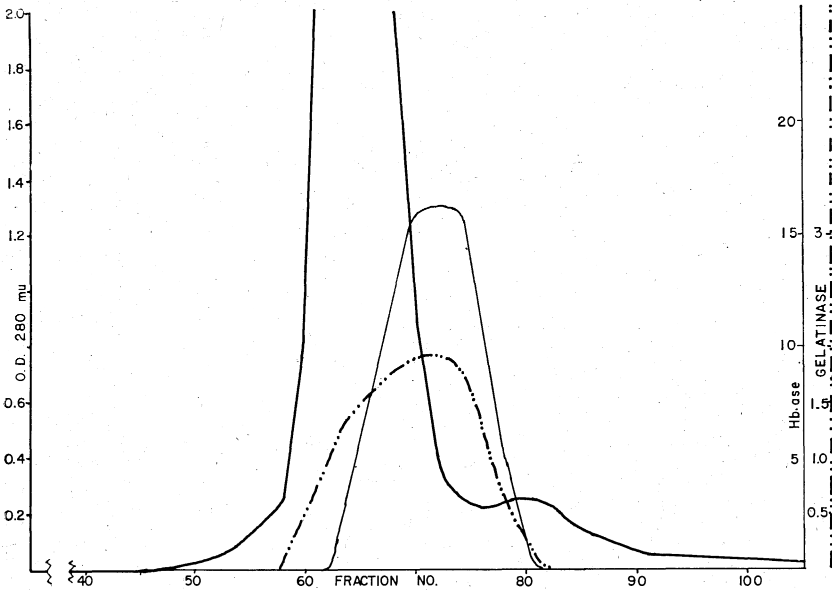

Fig. 7. Gel filtration of neutral proteinase II; 2.5 × 90-cm. column of Sephadex G-100. Sample: 19 ml. containing 70.3 mg. protein, 1,330 units of Hb-ase, and 91 units of gelatinase. Flow rate: 12 ml./hr.

None of these fractions is pure, but the specific activities shown in Table I indicate that except for neutral proteinase II they are attractive preparations for further study, and the procedures involved are such that larger amounts of starting material can be employed.

The two methods used for determining proteinase – namely, the determination of acid-soluble nitrogen with hemoglobin as substrate and the decrease in viscosity of gelatin solutions – can detect quite different types of attack on the protein substrates. In the present work both methods showed essentially the same pattern of proteinase distribution in the ion exchange and gel filtration fractions, indicating that the two substrates were hydrolyzed by the same or similar enzymes.

The acidic BAEE-ase has not, to our knowledge, been previously described. BAPA-ase and neutral BAEE-ase have not previously been shown to be separate enzymes. There is a copious literature concerning proteases from germinated cereals (3), but differences in methods of preparation and, more importantly, methods of assay prevent at the present time the identification of any of our active fractions with those described in the earlier literature.

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