Purification and Some Properties of Malted-Wheat BAPA-ase[1]

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ABSTRACT

A peptide hydrolase from malted wheat, hydrolyzing the synthetic substrate benzoyl-arginine-*p*-nitroanilide, has been purified by ammonium sulfate fractionation, DEAE-cellulose chromatography, and preparative disc electrophoresis. The isoelectric point, molecular weight, pH optimum, thermal stability, and effects of various chemicals on the activation or inhibition of this enzyme were determined. The enzyme had negligible ability to degrade azocasein, hemoglobin, or gelatin.

The peptide hydrolase BAPA-ase, which hydrolyzes the artificial substrate *a*-benzoyl-L-arginine-*p*-nitroanilide (BAPA), has been studied quite extensively in barley and barley malt (1-6). In wheat and germinated wheat the enzyme has been partially purified and characterized by Prentice et al. (7,8,9). Characterization of partially purified enzymes, however, is often complicated by the presence of contaminating protein and other biochemical impurities. The present paper describes a procedure that has been found successful for obtaining the BAPA-ase from malted wheat in a highly purified state. A comparison of the properties of this enzyme with the previously partially purified enzyme from germinated wheat has been made and further properties of the enzyme reported.

MATERIALS AND METHODS

Canadian hard red spring wheat (var. Marquis) was malted in this laboratory. It contained 14.1% protein and 1.29% ash on a 13.5% moisture basis after malting. The malted wheat was stored at 3°C. and ground into whole-wheat flour by a Wiley mill (equipped with a 1-mm. mesh sieve) just prior to use.

BAPA-ase Assay

BAPA-ase activity was determined by the method of Erlanger et al. (10) as modified by Burger (3). Substrate solution was prepared by dissolving 43.5 mg. BAPA (Mann Research Laboratories) in 1 ml. of dimethyl sulfoxide and adding 0.05M Tris-HCl, pH 8.6, containing 0.02M $CaCl_2$, to a total volume of 100 ml. To 2 ml. of the BAPA substrate was added 0.1 to 0.3 ml. of enzyme solution and 0.7 to 0.9 ml. of the above Tris-HCl buffer, such that the final volume was 3 ml. The reaction was maintained at 35°C. and terminated by the addition of 1 ml. of 30% acetic acid. The quantity of *p*-nitroaniline liberated was then estimated spectrophotometrically at 410 nm. with a Beckman DU spectrophotometer. One unit of BAPA-ase activity was defined as that amount which catalyzed the hydrolysis of a 1-nm. mole of BAPA per min. at 35°C. and pH 8.6.

For inhibitor and activation studies, the above reaction was modified by following the reaction with a Gilford Model 2000 spectrophotometer equipped

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with an absorbance converter with recorder, and with thermospacer plates through which water of 30°C. was circulated. The reaction was started by adding the enzyme to the reaction mixture containing the inhibitor or activator at the appropriate concentration.

For studies on the effect of pH on BAPA-ase activity, the reaction was carried out in the same manner except that the pH of the 0.05M Tris-HCl was varied between pH 7.0 and 9.0.

BAEE-ase Assay

The ability of enzyme preparations to hydrolyze *a*-benzoyl-L-arginine ethyl ester (BAEE) was determined by adding 0.1 ml. of enzyme solution to 2.5 ml. of 3×10^{-4} BAEE dissolved in 0.05M phosphate buffer, pH 7, and incubating at 30°C. The increase in absorbance was followed with time at 252 nm. with a Gilford Model 2000 spectrophotometer.

Protein Estimation

Protein was determined according to Lowry et al. (11) using bovine plasma albumin (Calbiochem. Co.) as the standard.

Proteinase Assays

Azocasein as Substrate. The use of azocasein as substrate provides a sensitive method for determination of proteolytic enzymes (12). The procedure adopted in this laboratory is as follows. Azocasein (2.4 g., Mann Research Laboratories) is dissolved in 75 ml. of 0.05M McIlvaines citric acid-disodium phosphate buffer, pH 6.0, and the volume brought to 100 ml. Two milliliters of substrate solution and 2 ml. of enzyme solution are then incubated at 40°C. The reaction is terminated by addition of 5 ml. of 10% trichloroacetic acid. The mixture is filtered and 5 ml. of 0.5N sodium hydroxide (NaOH) is added to 5 ml. of the filtered solution. After 20 min., the absorbance of the solution is measured at 440 nm.

Hemoglobin as Substrate. Assay was made by the method of Anson (13). The substrate contained 1% hemoglobin (Difco Laboratories) dissolved in 0.05M sodium acetate buffer, pH 4.5. One milliliter of enzyme solution was incubated at 35°C. with 3 ml. of substrate solution. The reaction was terminated by addition of 5 ml. of 0.3M trichloroacetic acid. After filtering, 5 ml. of the filtrate was added to 10 ml. of 0.3N NaOH containing 3% sodium carbonate, followed by 3 ml. of diluted Folin phenol reagent (Fisher Scientific Co.). The absorbance of the solution was measured after 10 min. at 650 nm.

Gelatin as Substrate. The viscometric method of Massart (14), as modified by Enari et al. (1), was used to study the ability of BAPA-ase to degrade gelatin. A pH of 5.2 and reaction temperature of 35°C. were used.

Chromatography

Ion-exchange chromatography was carried out on microgranular N,N-diethylaminoethyl cellulose (Whatman DE-32), as described previously (15).

Preparative Polyacrylamide-Disc Electrophoresis

Preparative disc electrophoresis was carried out with a Canalco Prep-Disc apparatus (Canal Industrial Corp.) with a PD 2/320 upper column. The experimental conditions are described in the next section.

Analytical Polyacrylamide-Disc Electrophoresis

The procedure of Davis (16) was followed. The pH of the small-pore gel was 8.9; and of the large pore, 6.7. Electrophoresis was carried out at 2°C. at 3 ma. per tube and continued until the bromphenol-blue marker had progressed to the bottom of the gel. Following electrophoresis, protein bands were detected by staining the gels with amido schwarz and removing the excess dye with a Canalco Quick Gel destainer (Canal Industrial Corp.). Duplicate gels were sliced into sections 1 mm. thick by a commercially obtained gel slicer (Canal Industrial Corp.). The gel sections were each incubated with 1 ml. of BAPA solution, and the yellow color of liberated *p*-nitroaniline indicated which slices contained the enzyme. These active slices could then be used to determine which of the protein bands on the stained gels was the BAPA-ase.

Electrofocusing

Electrofocusing experiments were as described by Vesterberg and Svenson (17) with column, ampholytes, and gradient mixer purchased from LKB Products. A 110-ml. column and temperature of 11°C. were used in all experiments. The sample (4 to 6 ml.) was first dialyzed against distilled water and mixed into the light solution used to form the sucrose density gradient. Electrofocusing was carried out for 64 hr. at 300V in a pH 3 to 10 gradient, or for 48 hr. at 525V in a pH 4 to 6 gradient. Following electrofocusing, 1.5-ml. fractions were collected from the column in a refrigerated fraction collector, and pH measurements were made at 11°C. on a Sargent Model DR pH meter.

Molecular Weight Determination

The molecular weight (MW) of purified BAPA-ase was determined by gel filtration on Sephadex G-100 (Pharmacia Ltd.). Many workers (18–22) have shown that the MW of a protein is inversely correlated with its elution volume. The procedure of Whitaker (19) was followed in which a linear correlation is found between the logarithm of the MW of a protein and the ratio of its elution volume, V_e, to the void volume, V_o. Experiments were carried out on a 2.5 × 100-cm. Sephadex K25/100 column (Pharmacia Ltd.) equipped with upward-flow adapters and filled to a height of 90 cm. with Sephadex G-100. The buffer used was 0.5M sodium acetate, pH 6.0, containing 0.1M sodium chloride (NaCl). Protein solutions were loaded in a volume of 2 ml., and an upward-flow rate of 16.8 ml. per hr. maintained by a peristaltic pump. The collection and detection of proteins were made with a LKB Recycyrom system, 2.3-ml. fractions being collected. The V_o of the column was determined with Blue Dextran (Pharmacia Ltd., MW 2×10^6). The column was calibrated with the following proteins: Ribonuclease (beef), trypsin (beef), chymotrypsinogen A (beef), pepsin (pork), peroxidase (horseradish), plasma albumin (beef), gamma-globulin (human). The resulting calibration curve is shown in Fig. 1 (with the exception of gamma-globulin).

EXPERIMENTAL

This section describes the procedures used in extracting and purifying malted-wheat BAPA-ase.

Extraction of Malted-Wheat BAPA-ase

The extraction medium was distilled water adjusted to pH 8.0 with dilute

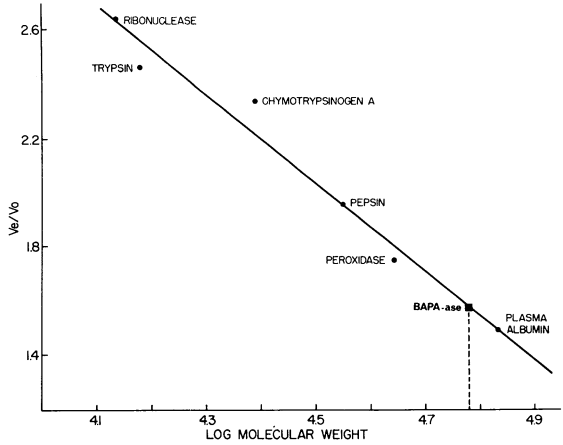

Fig. 1. Plot of log of MW vs. V_e/V_o for determination of MW on Sephadex G-100. Circles: calibrating proteins; square: malted-wheat BAPA-ase.

NaOH. Ground malted wheat (600 g.) was slowly added to 1,200 ml. of the extracting solution and the mixture stirred for 4 hr., centrifuged at 4°C. at 9,500 r.p.m. (12,000 × g) for 15 min., and the resulting supernatant stored at 3° to 4°C. All centrifugations were carried out under these conditions.

Purification of Malted-Wheat BAPA-ase

The enzyme was purified by ammonium sulfate fractionation, double DEAE chromatography, and preparative disc electrophoresis. The individual steps are described in detail below.

Ammonium Sulfate Fractionation. The operation was carried out at 4°C. The pH of the extract was first lowered to 5.9 with cold dilute HCl. The extract was then fractionated with saturated ammonium sulfate, with the precipitate formed between 0.4 and 0.8 saturation being retained. This precipitate was then dissolved in a small amount of cold distilled water.

DEAE Chromatography I. The previous extract was dialyzed against several changes of 0.1M Tris-HCl, pH 7.1. Any precipitate which formed was removed by centrifugation. The extract was then passed through a 4.8 × 13-cm. bed of DEAE cellulose which had been equilibrated with 0.1M Tris-HCl, pH 7.1. The BAPA-ase was retained on the column and eluted with a linear gradient prepared from a two-chambered gradient device. The first chamber consisted of 100 g. of stirring 0.1M Tris-HCl, pH 7.1, into which flowed the contents of the second vessel consisting of 100 g. of 0.1M Tris-HCl, pH 7.1, containing 0.5M NaCl. The effluent was collected in a refrigerated fraction collector and the tubes containing BAPA-ase activity combined.

DEAE Chromatography II. The active tubes from the above chromatography were combined and dialyzed against 0.1M Tris-HCl, pH 7.1. The extract was then passed through a 2.1 × 40-cm. bed of DEAE cellulose which had been equilibrated with 0.1M Tris-HCl, pH 7.1. The BAPA-ase was eluted from this column with a linear gradient prepared from a two-chambered gradient device. The first chamber

consisted of 400 g. of stirring 0.1M Tris-HCl, pH 7.1, into which flowed the contents of the second vessel, consisting of 400 g. of 0.1M Tris-HCl, pH 7.1, containing 0.2M NaCl. The effluent was collected in a fraction collector and the tubes having the largest BAPA-ase activity were combined and concentrated to a small volume with a LKB Ultrafilter (LKB-Producter).

Preparative Disc Electrophoresis. This final purification step was carried out using a Canalco Prep-Disc apparatus with a PD 2/320 upper column containing a 2.3-cm. (8 $cm.^3$) separating gel, a 1.15-cm. (4 $cm.^3$) spacer gel, and a 2.3-cm. (8 $cm.^3$) sample gel. Buffer and gel formulations were prepared as described by Davis (16) for analytical disc electrophoresis, except that a 10% separating gel was used. The system was maintained at 5°C. by a cooling bath. The elution buffer consisted of 0.087M imidazole and 0.01M N,N,N^1,N^1-tetramethylethylene diamine (TEMED) adjusted to pH 7.0 with 1N HCl, and a flow rate of 60 ml. per hr. was maintained. The sample was applied to the column in the form of a sample gel and electrophoresis was carried out at 12 to 15 ma. The eluted proteins were collected in a fraction collector and the fractions were assayed for BAPA-ase.

RESULTS AND DISCUSSION

The peptide hydrolase BAPA-ase has been purified from malted wheat by a process involving ammonium sulfate precipitation, double chromatography on DEAE cellulose, and preparative disc electrophoresis.

The recovery of protein and enzyme activities from a typical purification are shown in Table I. The enzyme recovery after preparative disc electrophoresis was

TABLE I. PURIFICATION OF BAPA-ase FROM MALTED WHEAT

Procedure	Volume	BAPA-ase Units/mg.	BAPA-ase Total Units	Protein mg./ml.	Specific Activity Units/mg. Protein	Yield %	Purification
Initial extract	820	90.88	74,520	12.25	7.4		
Ammonium sulfate fractionation	112	727.04	81,428[a]	19.62	37.1	100	5
DEAE chromatography I	122	465.60	56,803	2.50	186.2	69.8	25
DEAE chromatography II	155	135.12	20,944	0.60	225.2	25.7	30
Preparative disc electrophoresis	293	37.20	10,090	0.033	1127.3[b]	12.4	152

[a]Removal of inhibitor.

[b]Some fractions were of considerably higher activity than the combined fractions after preparative disc electrophoresis. The most active fraction had a specific activity of 2,445 units per mg. protein, which corresponded to a 330-fold purification.

12.4% with a 152-fold purification. Analysis of the fractions from preparative disc electrophoresis prior to pooling indicated that some were of very high specific activity with the purest found constituting a 330-fold purification. Figure 2

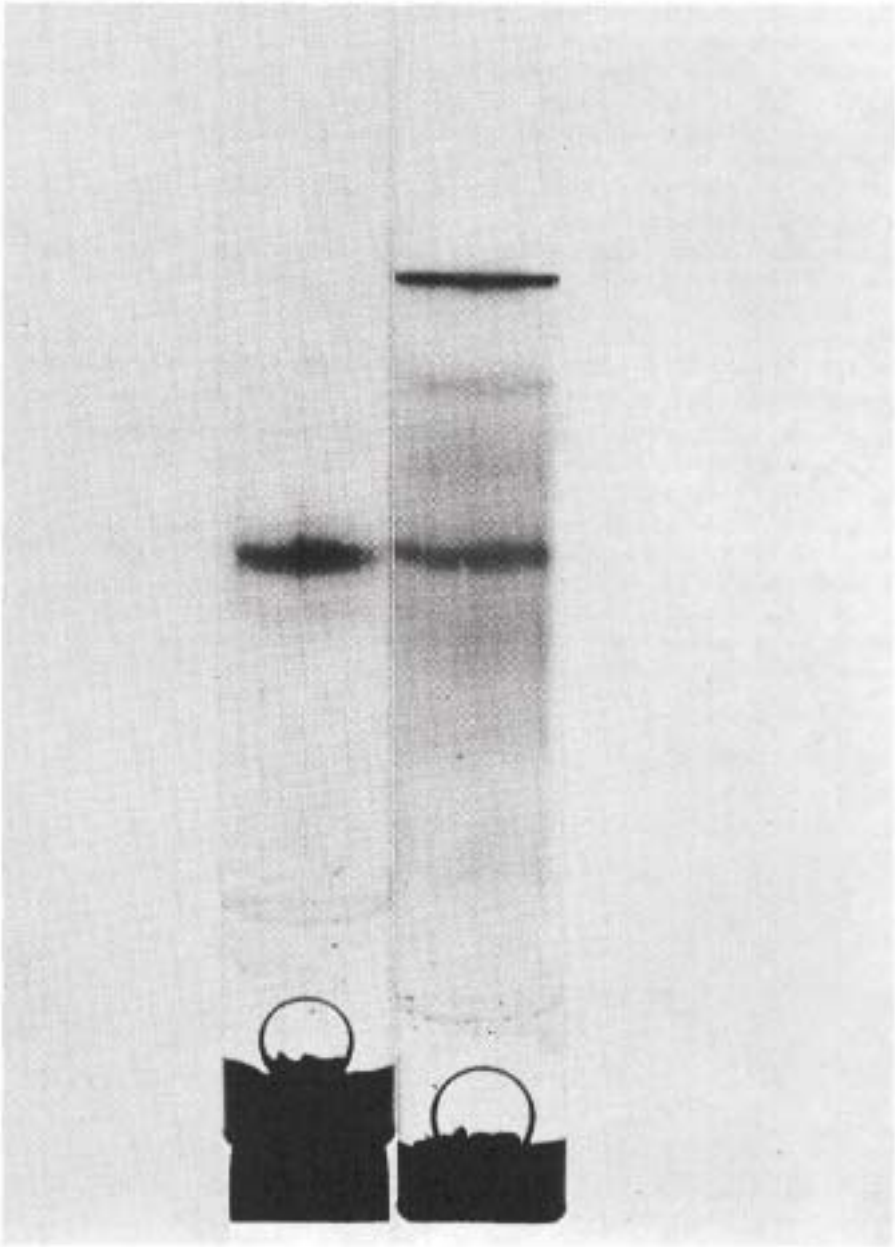

Fig. 2. Analytical disc electrophoresis before and after preparative disc electrophoresis. Left gel: Extract after DEAE-cellulose chromatography. Right gel: Extract after preparative disc electrophoresis.

illustrates the purity of enzyme preparations before and after preparative disc electrophoresis as shown by analytical disc electrophoresis. The gel on the left indicates the protein components present after DEAE-cellulose chromatography; two major components and many minor ones are present. The gel on the right indicates the protein components present after preparative disc electrophoresis; one major protein component and two minor components are present. By gel slicing and incubation with BAPA-ase of duplicate gels, the major protein band was found to be the malted-wheat BAPA-ase.

Germinated wheat is believed to contain three peptidases hydrolyzing the substrate BAEE (7). One of these enzymes was thought to be related to and possibly identical with the enzyme hydrolyzing BAPA. In this study BAEE-ase activity was found in all extracts prior to preparative disc electrophoresis. No BAEE-ase activity was found, however, in the purified BAPA-ase enzyme following preparative disc electrophoresis. As a consequence, kinetic constants such as K_m and V could not be determined with BAEE as substrate. Kinetic constants using BAPA as substrate were also not determined, as the values obtained are felt to be untrustworthy owing to the inhibitory effect that D-BAPA has on the catalysis of the L-isomer (6,10). The enzyme was therefore characterized by its isoelectric point, MW, pH optimum, stability to heat, and behavior toward various organic chemicals, oxidizing and reducing agents, and metal ions.

Isoelectric focusing of the purified enzyme was first carried out in a pH 3 to 10 gradient and indicated a pI of approximately 4.6. Electrofocusing in a pH 4 to 6 gradient indicated a more precise value of 4.46 (Fig. 3). An initial extract of the

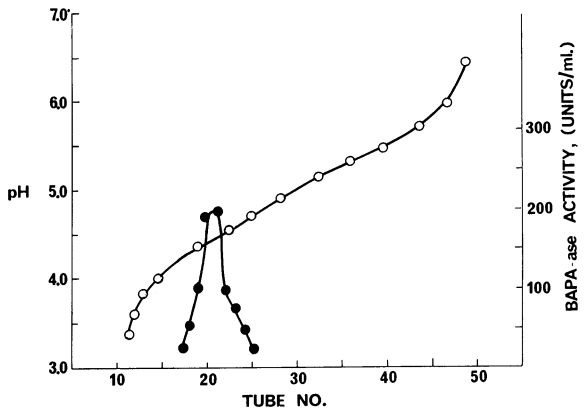

Fig. 3. Electrofocusing of malted-wheat BAPA-ase in a pH 4 to 6 gradient. Open circles: pH gradient; closed circles: BAPA-ase activity.

enzyme was also focused between pH 4 and 6 to determine if BAEE-ase activity would remain associated with the BAPA-ase activity. The results indicated that two BAEE-ase enzymes were present with pI values of 4.78 and 5.07. BAPA-ase activity remained in a single peak with a pI of 4.46, and had negligible BAEE-ase activity associated with it.

Gel filtration on Sephadex G-100 indicated a V_e/V_o value of 1.57, which corresponded to a MW of 59,000 (Fig. 1). The enzyme eluted as a single symmetrical peak from the gel column. This, coupled with the fact that a single enzyme peak was also obtained on DEAE-cellulose chromatography, preparative disc electrophoresis, and isoelectric focusing, indicates that the enzyme is probably not present in multiple forms, as has been found for many wheat enzymes.

The effect of pH on the ability of this enzyme to degrade BAPA is shown in Fig. 4. Maximal activity is found between pH 8.6 and 9.1. Prentice et al. (7) have found a broader pH optimum, extending from 7.5 to 9.0.

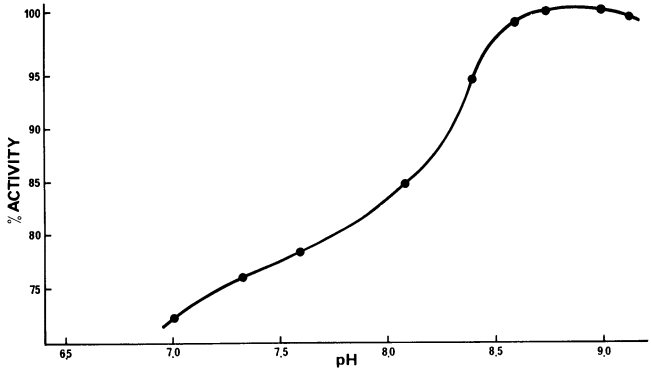

Fig. 4. Effect of pH on the activity of malted-wheat BAPA-ase.

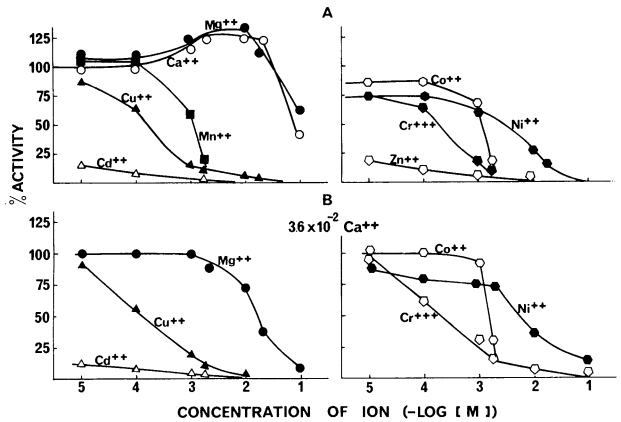

Fig. 5. Effect of metal ions on the activity of malted-wheat BAPA-ase. A, Without added Ca^{++} ions; B, with 3.6×10^{-2} Ca^{++} ion present.

Various metal ions (Ca^{++}, Mg^{++}, Mn^{++}, Co^{++}, Ni^{++}, Zn^{++}, Cr^{+++}, Cu^{++}, and Cd^{++}) were tested for their activation or inhibition of malted-wheat BAPA-ase (Fig. 5, A). The anion used was Cl^- with exception of Ni^{++} and Cd^{++} which had $SO_4^=$ and $C_2H_3O_2^-$ anions, respectively. With Ca^{++} and Mg^{++} the enzyme activity increased with increasing metal ion concentration and then decreased quite sharply

at high ionic concentration. All other metals caused inhibition of activity upon increase of concentration, with Zn^{++} and Cd^{++} being particularly effective. The behavior of this enzyme toward metal ions is similar to the acidic peptidase hydrolyzing BAPA that has been partially purified from germinated wheat (7) in that both are stimulated by increasing concentration of Ca^{++} and Mg^{++} ions. Unlike the formerly described enzyme, however, there is no stimulation found with increasing concentration of Mn^{++}, whereas Zn^{++} had a much larger inhibitory effect, being effective at very low concentrations. Increasing concentrations of Mg^{++}, Co^{++}, Ni^{++}, Cd^{++}, and Cr^{+++} ions were also added in combination with 3.6×10^{-2} Ca^{++} ions. As shown in Fig. 5, B, no further activation is found with Ca^{++} and Mg^{++} ions acting in combination once the maximum activation has been reached. Inhibitory effects with Co^{++}, Ni^{++}, Cd^{++}, or Cr^{+++} ions are basically the same as without Ca^{++} ions, indicating that a high concentration of Ca^{++} does not stabilize the enzyme against inhibition.

The effect on the inhibition of malted-wheat BAPA-ase at 30°C. of adding various organic chemicals and oxidizing and reducing agents at a concentration of 0.01M is shown in Table II. Large reductions in activity of 68 and 56% were caused

TABLE II. EFFECT OF CHEMICALS AT A CONCENTRATION OF 0.01M ON THE INHIBITION OF MALTED-WHEAT BAPA-ase

Compound	% Inhibition
N-Ethylmaleimide	68
Iodoacetic acid	0
p-Hydroxychloromercuribenzoate	56
Potassium bisulfite	13
Potassium bromate	0
Oxidized glutathione	0
Glutathione	5
Cysteine hydrochloride	0
Diisopropylfluorophosphate (DFP)	0

by N-ethylmaleimide and *p*-hydroxychloromercuribenzoate, respectively, indicating that sulfhydryl groups may be involved in the reactivity of this enzyme. It was found, however, that iodoacetic acid and potassium bromate caused no inhibition and that potassium bisulfite caused only a small inhibition. It has formerly been believed (23,24,25) that proteolytic enzymes were involved in the improver reaction of doughs. The present results indicate that the dramatic change in dough properties found by adding oxidizing and reducing agents to dough is not caused by the inhibition or activation of the action of this enzyme. Glutathione caused a small reduction in activity, whereas oxidized glutathione and cysteine hydrochloride had no effect. Although malted-wheat BAPA-ase was similar to trypsin in that it degraded BAPA and was activated by calcium, DFP, a well-known trypsin inhibitor, had no effect. As similar studies have not been previously made with germinated-wheat BAPA-ase, no comparisons can be made. The effects of adding chemicals on the activation or inhibition of malted-wheat BAPA-ase are similar, however, to those found for malted-barley BAPA-ase. Malted-barley BAPA-ase is not inhibited by potassium bromate (1,2,3,5) or iodoacetic acid (1), but is inhibited by N-ethylmaleimide (3,4) and *p*-hydroxychloromercuribenzoate. Malted-barley

BAPA-ase was also found to be activated by cysteine (3) but this activation was time dependent, explaining why a similar effect may not have been seen in malted-wheat BAPA-ase.

The thermal stability of malted-wheat BAPA-ase was determined at pH 5.0 and 7.0 by heating portions of the enzyme for 30 min. at temperatures of 20°, 30°, 40°, and 50°C. The temperature was then lowered and the activity of the enzyme determined. The pH values of dough systems normally fall between the range of 5.0 and 7.0 that was used in this study. The results (Fig. 6) indicated that the enzyme was relatively heat-labile, being almost completely inactivated by a temperature of 50°C. for 30 min. Preparations of BAPA-ase from germinated wheat and barley have been described in previous studies (7,8) as being heat-labile, but differences in condition do not permit exact comparisons.

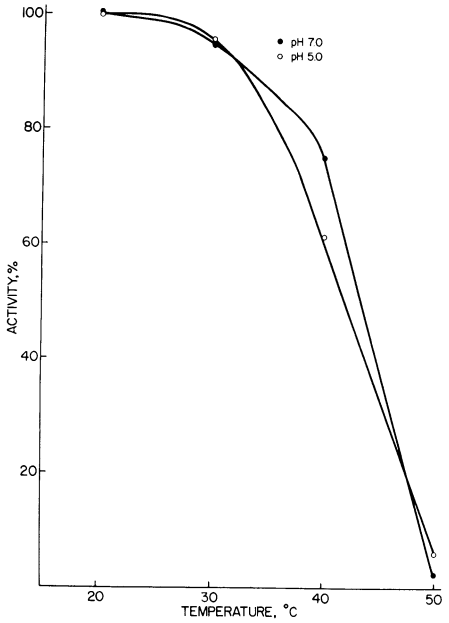

Fig. 6. Thermal stability of malted-wheat BAPA-ase at pH 5.0 and 7.0.

Portions of the purified enzyme (400 to 600 BAPA units) were incubated with azocasein, hemoglobin, and gelatin and the amount of substrate degradation assessed after various intervals of time. The results indicated that no hydrolysis occurred with reaction times of up to 8 hr. This confirms that malted-wheat BAPA-ase is not a proteinase but a peptidase, hydrolyzing small peptides. As such, the presence of this enzyme would not greatly influence the breadmaking process through alteration of the gluten proteins. Its role, however, in the metabolic pathways of the plant, and possibly in the synthesis or breakdown of storage proteins, could be quite important, and remains to be elucidated.

Acknowledgment

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