Phytochemistry, 1968, Vol. 7, pp. 1899 to 1905. Pergamon Press. Printed in England

# PARTIAL PURIFICATION AND CHARACTERIZATION OF PEPTIDE HYDROLASES FROM GERMINATED WHEAT

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(*Received* 29 *May* 1968)

**Abstract**—Peptide hydrolases from germinated wheat were purified by methods developed previously for barley peptide hydrolases. Comparisons of the wheat enzymes for their thermal stability, pH optima, stability at various pH values, kinetic constants, and effects of metal ions revealed that wheat possesses a neutral peptide hydrolase with properties very similar to those of barley hydrolase B. After considerable purification the acidic peptide hydrolase fraction of wheat appeared to contain two peptide hydrolases, one that was similar to barley peptide hydrolase A and a second which was more resistant to low pH and thermal denaturation.

## INTRODUCTION

GERMINATED barley contains barley peptide hydrolases A and B which hydrolyze alpha-*N*-benzoyl-L-arginine ethyl ester (BAEE).[1,2] Barley peptide hydrolase A, but not hydrolase B, hydrolyzes also alpha-*N*-benzoyl-DL-arginine *p*-nitroanilide (BAPA). The hydrolase A is an acidic protein and hydrolase B a neutral protein based on their separation on carboxymethyl cellulose (CMC) at pH 5·5.[2] Previous work[3] has shown that germinated wheat contains similar enzymes but there are some differences in their distribution within the kernel. In addition, a second wheat acidic enzyme capable of hydrolyzing BAEE increases by relatively large amounts during germination and subsequent growth of the embryo. It was therefore of interest to examine some of the properties of the wheat peptide hydrolases in a more purified state and this is the subject of the present paper.

## RESULTS AND DISCUSSION

### *Purification*

Table 1 shows the purification accomplished when CMC-treated fractions of germinated wheat were cycled through Sephadex G-100 five times. Previous work with germinated barley has shown that this procedure accomplishes a greater degree of purification with somewhat higher yields than the methods used in earlier experiments.[1,2]

Previous results indicated that at least two enzymes capable of hydrolysing BAEE are present in the acidic protein fraction of germinated wheat extracts.[3] In the present work we were not able to separate them by 5 cycles through Sephadex G-100. Moreover, the ratio of

* The work of the Barley and Malt Laboratory is supported in part by a research grant from the Malting Barley Improvement Association and is published with the permission of the Wisconsin Agricultural Experiment Station. Mention of a trade product, equipment, or commercial company does not imply its endorsement by the U.S. Dept. of Agriculture.

W. C. BURGER, N. PRENTICE, J. KASTENSCHMIDT and J. D. HUDDLE, *Cereal Chem.* **43**, 547 (1966).
[2] W. C. BURGER, N. PRENTICE, J. KASTENSCHMIDT and M. MOELLER, *Phytochem.* **7**, 1261 (1968).
[3] N. PRENTICE, W. C. BURGER, J. KASTENSCHMIDT and J. D. HUDDLE, *Physiol. Plantarum* **20**, 361 (1967).

TABLE 1. PURIFICATION OF ACIDIC AND NEUTRAL PEPTIDE HYDROLASES

| Purification step | Vol. (ml) | Protein (mg/ml) | Activity (U/ml) | | Recovery (%) | | Specific activity (U/mg protein) | | Ratio of specific activity BAEE/BAPA |
|---|---|---|---|---|---|---|---|---|---|
| | | | BAPA | BAEE | BAPA | BAEE | BAPA | BAEE | |
| Dialyzed extract | 450 | 8·5 | 28 | 305 | 100 | 100 | 3·3 | 36 | — |
| *Acidic hydrolases* | | | | | | | | | |
| CMC supernatant | 82 | 6·5 | 60 | 950 | 39 | 56 | 9 | 145 | 16 |
| 5th Cycle G-100 | 57 | 0 5 | 57 | 810 | 26 | 33 | 114 | 1600 | 14 |
| *Neutral hydrolase* | | | | | | | | | |
| CMC eluate | 41 | 27·2 | 14 | 830 | 5 | 26 | 0·5 | 31 | |
| 2nd Cycle G-100 | 60 | 5·4 | 1 | 500 | 0 5 | 22 | 0 2 | 92 | |
| 5th Cycle G-100 | 39 | 0 77 | 0 | 720 | 0 | 20 | 0 | 930 | |

activity with BAPA substrate to that with BAEE substrate in the final product (Table 1), was similar to that obtained with this fraction which was not gel filtered. It is therefore presumed that this acidic fraction contains two peptide hydrolases, one which hydrolyzes both BAEE and BAPA and is similar to barley peptide hydrolase A, and another which hydrolyzes BAEE but not BAPA. In contrast to barley peptide hydrolase A (which undergoes little, if any, loss in activity upon treatment with CMC) approximately 60 per cent of the acidic peptide hydrolase fraction of wheat (BAPA substrate) is lost at this step.

The purified neutral peptide hydrolase of wheat appears to be similar to barley peptide hydrolase B in that it is inactive with BAPA substrate.

*Stability of Enzymes at Various* pH *Values*

Table 2 shows that the activity of the acidic fraction after exposure to low pH remains high with BAEE substrate but drops to zero with BAPA substrate. The activity of the corresponding barley fraction drops to zero with both substrates after exposure to low pH.[2] This

TABLE 2. THE EFFECT OF pH ON PEPTIDE HYDROLASES

| Buffer (0·05 M) | pH | Acidic peptide hydrolase | | Neutral peptide hydrolase |
|---|---|---|---|---|
| | | BAPA | BAEE | BAEE |
| | | % of original activity | | |
| Tartrate | 3·0 | 0 | 107 | 95 |
| Tartrate | 3·5 | 8 | 98 | 95 |
| Acetate | 4·0 | 49 | 98 | 89 |
| Acetate | 5·0 | 82 | 94 | 86 |
| Acetate | 5·5 | 88 | 102 | 100 |
| Phosphate | 8·0 | 100 | 96 | 78 |
| TES | 8·0 | 100 | 35 | 72 |
| Tris-chloride | 8·0 | 100 | 35 | 14 |
| Tris-chloride | 8·2 | 100 | 22 | 8 |
| Tris-chloride | 8·4 | 90 | 17 | 0 |
| Tris-chloride | 8·6 | 82 | 13 | 0 |
| Tris-chloride | 8·8 | 93 | 15 | — |
| Borate | 8·8 | 117 | 42 | — |
| Borate | 9·2 | 117 | 12 | — |

behavior of the acidic fraction from wheat can be explained by the presence of two enzymes, one similar to the hydrolase A of germinated barley which is sensitive to low pH, stable at high pH, and hydrolyzes both substrates, and another enzyme which is stable at low pH but labile to high pH and which hydrolyses BAEE but not BAPA.

The neutral fraction from wheat is stable to low pH, labile to high pH, and thus appears similar to peptide hydrolase B from barley.[2]

*The Effect of Elevated Temperature, Lyophilization and Low-Temperature Storage*

The stability of these enzymes when exposed to elevated temperature is shown in Table 3. It is apparent that the neutral peptide hydrolase fraction is quite resistant to the treatments employed. There is a clear separation of the activity in the acidic fraction upon heat treatment, depending upon which of the two substrates is used. The hydrolase activity with BAEE substrate is fairly heat-resistant but the BAPA substrate activity is eliminated by heating for

TABLE 3. HEAT STABILITY OF PEPTIDE HYDROLASES

| Temp (°C) | Acidic peptide hydrolase | | Neutral peptide hydrolase |
|---|---|---|---|
| | BAPA | BAEE | BAEE |
| | % of original activity | | |
| 35 | 75 | 97 | 105 |
| 45 | 0 | 78 | 100 |
| 55 | 0 | 64 | 81 |

1 hr at 45° or higher. In these respects the neutral enzyme is again very similar to barley peptide hydrolase B, and the activity of the acidic fraction of wheat with BAPA substrate resembles closely the peptide hydrolase A of barley. However, wheat appears to possess a second acidic enzyme capable of hydrolyzing BAEE which is moderately resistant to denaturation by heat and low pH.

Small decreases in activity (10–20 per cent) were found to occur with both acidic and neutral hydrolases upon storage for 3 months in the lyophilized and frozen states. Similar losses occurred in solutions of these enzymes in the gel filtration buffer (see Experimental) held at 4° for the same length of time. Alternate freezing and thawing caused no appreciable loss of activity.

*The Effect of Metal Ions on Peptide Hydrolase Activity*

When calcium, magnesium, manganese, nickel and zinc ions were added to the BAPA substrate immediately prior to enzyme addition, the activity of the acidic fraction was altered, as shown in Fig. 1. However, in the presence of BAEE substrate the acidic fraction showed

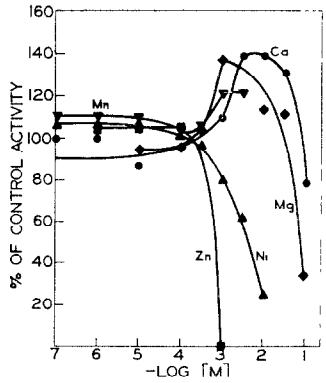


FIG. 1. EFFECT OF METAL IONS ON ACTIVITY OF ACIDIC PEPTIDE HYDROLASE. BAPA SUBSTRATE.

the activity pattern given in Fig. 2. No stimulation occurred in the latter case. The rather unusual stimulation with BAPA as substrate is quite similar to that reported for barley peptide hydrolase A.[2] The lack of stimulation observed with the acidic preparation with BAEE as substrate differs from the results obtained with the corresponding preparation from barley and reflects the large preponderance of the BAEE-splitting enzyme as compared to that which acts on BAPA (Table 1). The pattern obtained with the neutral peptide hydrolase is shown in Fig. 3. There is no marked stimulation with any of the ions.

The results of these experiments with metal ions were not materially altered by the incubation of substrate and metal for 1 hr prior to addition of enzymes, or by similar incubation of metal ion and enzyme prior to the addition of substrate. The relatively high concentrations of calcium, magnesium and manganese required for enhanced activity conform with those observed with the barley peptide hydrolase A and indicate a loose association between metal and either enzyme, substrate, or enzyme-substrate complex in the cases where stimulation of activity occurred.

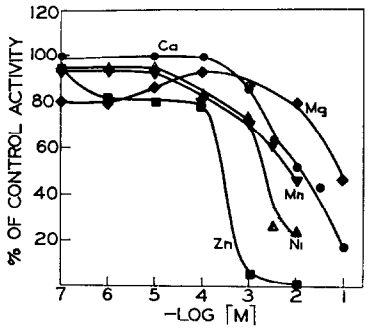


FIG. 2. EFFECT OF METAL IONS ON ACTIVITY OF ACIDIC PEPTIDE HYDROLASES. BAEE SUBSTRATE.

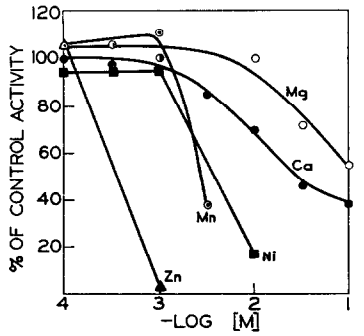


FIG. 3. EFFECT OF METAL IONS ON ACTIVITY OF NEUTRAL PEPTIDE HYDROLASE. BAEE SUBSTRATE.

pH *Optima for Activity*

The activity of acidic peptide hydrolase acting on BAPA varied with pH as shown in Fig. 4. The curve consists of a broad region from pH 7·5 to approximately 9 in which activity is uniformly maximal. The neutral peptide hydrolase has a rather well-defined optimum at pH 6·5 to 6·7 (Fig. 4). The above values agree closely with those found for the corresponding enzymes from barley.

The pH optimum for the acidic hydrolase fraction with BAEE substrate was not determined because more than one enzyme capable of hydrolyzing BAEE are believed to be present.

*Kinetic Constants*

Neutral peptide hydrolase from germinated wheat was found to have a $K_m = 2{\cdot}6 \times 10^{-4}$ M and $V = 53$ U. These values compare with $3{\cdot}8 \times 10^{-4}$ M and 80 U, respectively, for the corresponding enzyme from barley.[2] Thus hydrolase B from barley is a somewhat less active enzyme

than that from wheat. Kinetic constants for the enzymes hydrolyzing BAPA could not be determined because this material was available as the racemic mixture and the D-isomer is known to have an inhibitory effect on the hydrolysis of the L-form by trypsin.[4]

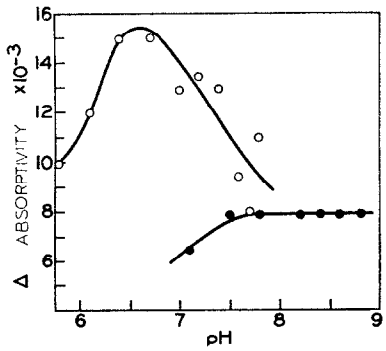


FIG. 4. THE EFFECT OF pH ON THE ACTIVITY OF ACIDIC PEPTIDE HYDROLASE WITH BAPA SUBSTRATE (CLOSED CIRCLES), AND ON THE ACTIVITY OF NEUTRAL PEPTIDE HYDROLASE WITH BAEE SUBSTRATE (OPEN CIRCLES).

A comparison of the present results with those of the limited number of earlier studies of wheat peptide hydrolases[5] is complicated by the presence of mixtures of hydrolases in the extracts used in these early studies and by differences in methods of assay. Therefore it is felt that no worthwhile comparison can be made at this time.

The neutral peptide hydrolase of wheat has been shown to be similar in many of its properties examined here to the nuetral hydrolase of barley called barley peptide hydrolase B. It would be advantageous from the standpoint of simplicity to refer to the wheat enzyme as wheat peptide hydrolase B. This is in accord with the recommendations of the International Union of Biochemistry.[6] Because the acidic peptide hydrolase fraction contains more than one enzyme, a formal designation of these enzymes should await further study.

## EXPERIMENTAL

### *Materials and Methods*

*Wheat.* A mixture of hard red spring varieties of wheat (*Triticum aestivum* L.) from the 1963 crop grown at Madison, Wisconsin.

*Potassium gibberellate* (*Gibrel, Mark* 60830). This was a gift from Merck and Co., Rahway, N.J.

*Moisture determination.* 1½ g of wheat ground finely in a Labconco mill, was dried at 105° for 90 min.

### *Extraction of Germinated Wheat; Protein Assay; Peptide Hydrolase Assays*

These operations were performed as described previously.[2]

### *Germination of Wheat*

This was done as described previously[2] except that the germination period was 5 days. The germinated product was lyophilized directly to approximately 10 per cent moisture and stored at −25° until used.

### *Purification of Enzymes with Carboxymethyl Cellulose and Sephadex G-100*

The procedures for treatment with CMC have been described.[1,2]

For gel filtration a Sephadex G-100 bed 5 cm in dia. and 90 cm in length was equilibrated with the gel filtration buffer.[2] The acidic hydrolase fraction from CMC (Table 1) was concentrated by ultrafiltration to

[4] B. F. ERLANGER, N. KOKOWSKY and W. COHEN, *Arch. Biochem. Biophys.* **95**, 271 (1961).

[5] G. REED and J. A. THORN, *Wheat Chemistry and Technology* (edited by I. HLYNKA), Chapter 9, American Association of Cereal Chemists, St. Paul (1964).

[6] International Union of Biochemistry, *Enzyme Nomenclature*, p. 34, Elsevier ,Amsterdam (1965).

80 to 100 ml, applied to the column and eluted with the buffer at the rate of 40 ml per hr. Four equal volumes of the eluate were collected per hr. Those solutions containing most of the activity were pooled and the volume reduced to 30–40 ml by ultrafiltration. This was then reapplied to the gel column as before. By this procedure the preparation was passed through the column five times. The neutral hydrolase fraction from CMC (Table 1) was treated similarly. The products so obtained were concentrated until the specific activities were as shown in Table 1.

*Treatment of Enzymes with Solutions of Various* pH's

One ml of the enzyme solutions obtained from gel filtration was dialyzed against two 125-ml portions of 0·05 M buffers of pH 3–10 (Table 2) for about 18 hr at 0–4°. Activities were compared with the undialyzed sample after allowances for volume changes were made.

*Treatment of Enzymes at Various Temperatures*

One ml of the enzyme solutions obtained from gel filtration (i.e. in 0·1 M acetate, 0·2 M NaCl, 0·004 M EDTA buffer pH 5·9) was held at 35°, 45° and 55° for 1 hr. Activities were compared with the corresponding unheated enzymes.

*Alternate Freezing and Thawing of Enzyme Solutions*

One ml of each enzyme solution from gel filtration purification was frozen at −25° and thawed alternately five times over a period of about 4 hr, and the activity compared with corresponding enzyme stored at about 4°.

*Storage of Enzymes at Low Temperatures*

One ml of the enzyme solutions from gel filtration purification were stored for 3 months at 4° and at −25°. One ml was lyophilized and stored at −25°.

*Effect of Metal Ions upon Enzyme Activity*

The effect of metal ions at concentrations of $10^{-1\cdot5}$ to $10^{-6}$ M was measured by three procedures:[2] (*a*) substrate (BAEE or BAPA), enzyme and metal ion were combined and the activity determined immediately; (*b*) substrate and metal ion were equilibrated 1 hr at 35° before addition of enzyme and measurement of reaction rate; (*c*) enzyme and metal ion were equilibrated 1 hr at 35° before addition of substrate. In this case enzyme and metal ion were dissolved in the appropriate buffer and after 1 hr at 35° 0·1 ml of $2{\cdot}7 \times 10^{-2}$ M BAEE in 0·05 M TES (N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid), pH 7·0 or 0·1 ml 0·01 M BAPA in dimethyl sulfoxide was added. For each procedure the reaction rates were measured and compared with those obtained by the corresponding procedure but without the metal ion.

pH *Optima for Activity*

BAEE ($8 \times 10^{-4}$ M) in 0·05 M phosphate buffer at pH 6–8 and BAPA ($6{\cdot}67 \times 10^{-4}$ M) in 0·05 M Tris at pH 7 to 8·9 were used to determine pH optima. The extinction coefficients of the products of these two enzyme reactions do not vary significantly over the respective ranges in pH employed.

*Determination of Kinetic Constants for Neutral Peptide Hydrolase*

$K_m$ and $V$ were determined for neutral peptide hydrolase as described previously.[2]