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HYDROLYSIS OF ALPHA NAPHTHYL ACETATE AND L-LEUCYL NAPHTHYLAMIDE BY BARLEY ENZYMES

W. C. BURGER, NEVILLE PRENTICE, MARY MOELLER and JEANNINE KASTENSCHMIDT

Barley and Malt Laboratory, Crops Research Division, Agricultural Research Service, U.S. Department of Agriculture, Madison, Wisconsin, U.S.A.*

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Abstract—Germinated barley contains four enzymes which hydrolyze α-naphthyl acetate (ANA) and one which hydrolyzes L-leucyl-β-naphthylamide (LNA). These enzymes were purified by treatment of dialyzed extracts with carboxymethyl cellulose and Sephadex G-100. Two ANA-ases (ANA-ase II and IV) and LNA-ase were characterized. ANA-ase IV was resistant to extremes of pH and temperature. ANA-ase II and LNA-ase were inactivated at low pH and elevated temperature. K_m values for these enzymes are given.

INTRODUCTION

GERMINATED barley and wheat contain two peptide hydrolases, A and B, which have been purified and partially characterized.[1-3] Peptide hydrolase A (PHA) was found in the more acidic salt-soluble proteins that are not adsorbed on carboxymethyl cellulose (CMC) by batch treatment at pH 5·5; peptide hydrolase B (PHB) is less acidic and is adsorbed on CMC at pH 5·5.

A significant advance in separation methods was made when a cross-linked CMC became available which could be used in columns without compacting significantly under pressure at high ionic strengths. Examination of chromatographic fractions obtained in the separation of barley peptide hydrolases A and B with other synthetic substrates revealed that additional enzymes were present. Four of these enzymes hydrolyzed α-naphthyl acetate (ANA) and another hydrolyzed L-leucyl-β-naphthylamide (LNA). The separation and some of the properties of three of these enzymes are described.

RESULTS AND DISCUSSION

Assays for ANA-ase and LNA-ase

The spectra for ANA and α-naphthol (AN) are shown in Fig. 1. Since it is convenient to assay when the absorbance is increasing rather than decreasing, 310 nm was chosen. The pH is not critical (Fig. 2) but pH 7·0 was chosen. Figure 3 shows that 335 nm is a suitable wavelength for the LNA-ase assay and Fig. 4 that pH 6·5 is appropriate. These procedures for assay are simple to perform, so the numerous determinations needed for monitoring chromatographic fractions can be done with ease.

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[1] N. PRENTICE, W. C. BURGER, J. KASTENSCHMIDT and J. D. HUDDLE, *Physiol. Plantarum* **20**, 361 (1967).
[2] N. PRENTICE, W. C. BURGER and M. MOELLER, *Phytochem.* **7**, 1899 (1968).
[3] W. C. BURGER, N. PRENTICE, J. KASTENSCHMIDT and M. MOELLER, *Phytochem.* **7**, 1261 (1968).

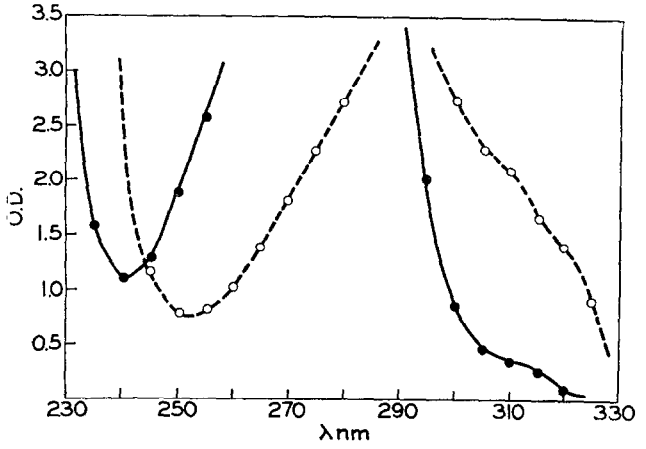

FIG. 1. SPECTRA FOR α-NAPHTHYL ACETATE (ANA) AND FOR α-NAPHTHOL (AN).
—— ANA ---- AN

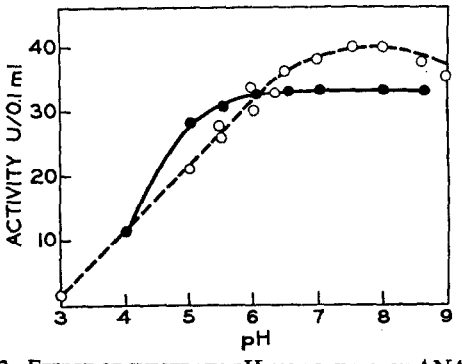

FIG. 2. EFFECT OF SUBSTRATE pH ON ACTIVITY OF ANA-ASE.
—— ANA-ase IV, Conquest barley, 34 units, 0·55 mg protein.
---- ANA-ase II, Trophy barley, 32 units, 0·44 mg protein.
Substrate concentration $5{\cdot}38 \times 10^{-4}$ M; 35°.

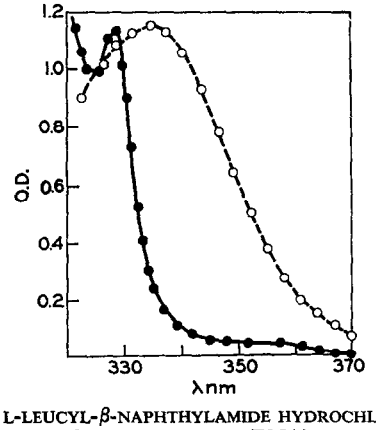

FIG. 3. SPECTRA FOR L-LEUCYL-β-NAPHTHYLAMIDE HYDROCHLORIDE (LNA) AND FOR β-NAPHTHYLAMINE (BNA).
—— LNA ---- BNA

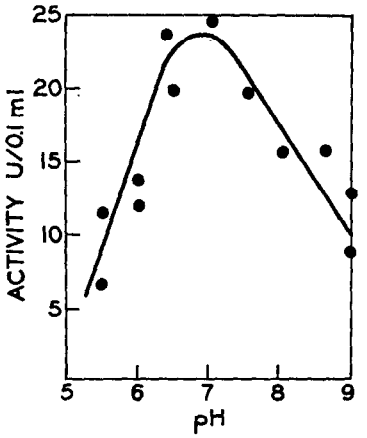

FIG. 4. EFFECT OF SUBSTRATE pH ON LNA-ASE.
Trophy barley, 38 units, 0·44 mg protein. Substrate concentration $3{\cdot}42 \times 10^{-3}$ M; 35°.

CMC Chromatography

The pH of the fractions from the CMC column (Fig. 5) varied from 5·7 to 4·8 as the gradient was pumped through. The column was therefore not in equilibrium with pH even when the acetate buffer of the gradient approached 0·5 M.

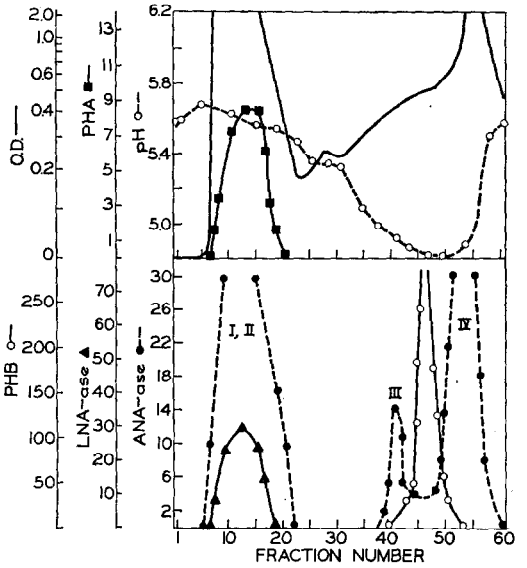

FIG. 5. CMC CHROMATOGRAPHY OF GERMINATED TROPHY BARLEY EXTRACT.
Activities are in units/0·1 ml.

The PHA, indicated by its hydrolysis of α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPA) (Fig. 5), is in the unadsorbed fraction and was washed through the column by the dilute portion of the buffer gradient. This would be expected from previous work with batchwise CMC treatment.[1–3] Figure 5 shows also that the unadsorbed fraction contains LNA-ase and

ANA-ase activity. Gel electrophoretic treatment of this unadsorbed fraction removes LNA-ase and ANA-ase activity from PHA.[4] Therefore the LNA-ase and ANA-ase activity of this unadsorbed fraction are not caused by PHA.

The adsorbed enzymes, eluted by the more concentrated portion of the buffer gradient, consist of two ANA-ase enzymes: ANA-ase III which has little activity, and ANA-ase IV. PHB, which hydrolyzes α-*N*-benzoyl-L-arginine ethyl ester (BAEE) but not BAPA, is located between ANA-ase III and IV.

Sephadex G-100 Filtration

When the unadsorbed peak in Fig. 5 (fractions 6–20) was filtered through G-100, a partial separation into two ANA-ase enzymes occurred. This is represented by two areas designed as ANA-ase I and II (Fig. 6). PHA is in the same fractions as ANA-ase I. ANA-ase II could be obtained free of PHA by isolating fractions 35–45. Figure 6 shows also the locations of LNA-ase which is not separated from PHA or from ANA-ase I. The results of G-100 filtration of the ANA-ase IV from the CMC adsorbed fraction (fractions 50–56, Fig. 5) are shown in Fig. 7. PHB is separated well from ANA-ase IV.

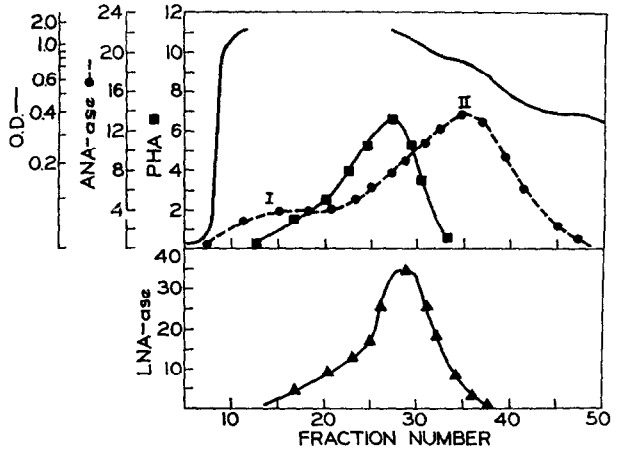

FIG. 6. G-100 FILTRATION OF THE UNADSORBED FRACTION FROM CMC.
Activities are in units/0·1 ml.

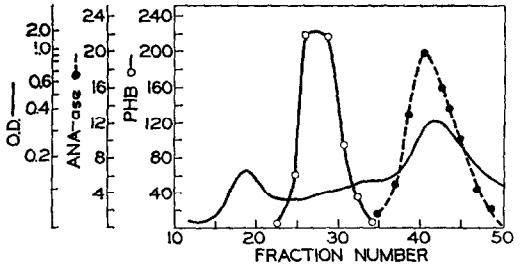

FIG. 7. SEPHADEX G-100 FILTRATION OF CMC ADSORBED FRACTION.
Activities are in units/0·1 ml.

[4] M. MOELLER, W. C. BURGER and N. PRENTICE, *Phytochem*, 8, 2153 (1969).

Purification

The activities of representative samples and products involved in the purification are shown in Tables 1 and 2. The ANA-ase III fraction from CMC (Table 1) was not gel filtered and was

TABLE 1. PURIFICATION OF ENZYMES ANA-ASE II AND IV

Purification step*	Vol. (ml)	Protein (mg/ml)	ANA-ase activity (units/ml)	Recovery (%)	Specific activity (units/mg protein)
CMC treatment					
Sample applied:					
Extract dialyzed vs. 0·005 M acetate	48·0	9·2	610	100	66
Products:					
ANA-ase I and II, LNA-ase	18·5	3·9	430	27	111
ANA-ase III	9·0	0·7	135	4	193
ANA-ase IV	12·5	14·0	830	34	59
G-100 treatment					
Sample applied:					
ANA-ase I and II, LNA-ase	26·0	8·2	460	100	56
Products:					
ANA-ase II	40·0	0·65	125	42	193
Sample applied:					
ANA-ase IV	15·0	2·4	470	100	31
Products:					
ANA-ase IV	26·5	0·77	200	76	260

* ANA = α-naphthyl acetate; LNA = L-leucyl-β-naphthylamide.

TABLE 2. PURIFICATION OF THE ENZYME LNA-ASE

Purification step	Vol. (ml)	Protein (mg/ml)	LNA-ase activity (units/ml)	Recovery (%)	Specific activity (units/mg protein)	Purification fold
CMC treatment						
Sample applied:						
Extract dialyzed vs. 0·005 M acetate	48·0	9·2	220	100	24	
Products:						
ANA-ase I and II, LNA-ase	18·5	3·9	520	88	134	5·6
G-100 treatment						
Sample applied:						
ANA-ase I and II, LNA-ase	26·0	8·2	600	100	73	
Products:						
LNA-ase	44·0	0·90	240	68	258	3·5

not examined further since this enzyme was present at a low level. ANA-ase II was isolated from the G-100 column as the trailing portion of the ANA-ase peak (fractions 35 to 45, Fig. 6).

CMC and G-100 provide a good separation of ANA-ase IV. The overall purification cannot be calculated since the relative activities of the three ANA-ase enzymes in original extracts is not known. For LNA-ase (Table 2) CMC provided a 5·6-fold purification from dialyzed extracts, and G-100 provided a 3·5-fold purification of the product from the CMC treatment.

pH Stability

The stabilities of ANA-ase II, ANA-ase IV and LNA-ase over the range pH 3·5–9·2 are shown in Table 3. There is a marked difference in the data from ANA-ase II, which is labile at pH 5 and below, and for ANA-ase IV which is relatively stable at these pH's but labile at pH 8 and above.

TABLE 3. pH STABILITY

Buffer (0·05 M)	pH	Enzyme recovery (%)		
		ANA II	ANA IV	LNA
Tartrate	3·5	3	65	0
Acetate	5·0	15	71	84
Tris	8·0	89	50	100
Borate	9·2	67	46	

Temperature Stability

ANA-ase IV is remarkably stable, as shown in Table 4; even at 55° there was no decrease in activity. There appears to be significant but inexplicable stimulation at 35° and particularly at 45°.

TABLE 4. TEMPERATURE STABILITY

Temperature (deg)	Enzyme recovery (%)		
	ANA II	ANA IV	LNA
35	130	114	100
45	117	124	104
55	52	107	0

Kinetic Constants

The K_m and V values are given in Table 5. ANA-ase IV, with $K_m = 2{\cdot}3 \times 10^{-5}$ M, is a somewhat more active enzyme than ANA-ase II with $K_m = 3{\cdot}4 \times 10^{-5}$ M. These values are of the same order of magnitude as the K_m for peptide hydrolase A, $4{\cdot}4 \times 10^{-5}$ M, but somewhat less than that for peptide hydrolase B, $3{\cdot}8 \times 10^{-4}$ M.[4] The LNA-ase K_m of $1{\cdot}6 \times 10^{-4}$ M is closer to that of peptide hydrolase B.

TABLE 5. KINETIC CONSTANTS

	ANA-ase II	ANA-ase IV	LNA-ase
K_m, M	$3{\cdot}4\times10^{-5}$	$2{\cdot}3\times10^{-5}$	$1{\cdot}6\times10^{-4}$
V, U/0·1 ml	11	9·8	26

EXPERIMENTAL

Assay for ANA-ase

α-Naphthyl acetate (ANA) was recrystallized from ethanol before use. A 1·0% solution in ethanol was diluted with 0·05 M phosphate, pH 7·0, to a final concentration of $5{\cdot}3\times10^{-4}$ M. α-Naphthol (AN) was vacuum distilled (m.p. 95°). A 1% solution in ethanol was diluted with 0·05 M phosphate, pH 7·0, to a final concentration of $6{\cdot}86\times10^{-4}$ M. The spectra (Fig. 1) of these solutions were determined at 35° with a Beckman DU spectrophotometer equipped with thermospacer plates and a Gilford optical density converter. The wavelength chosen for the assay was 310 nm.

For the assay of ANA-ase the following procedure was adopted: 3 ml of $5{\cdot}38\times10^{-4}$ M ANA in 0·05 M phosphate, pH 7·0, was treated with 0·1 ml of the appropriately diluted enzyme and the increase in absorptivity at 310 nm was recorded for the initial 5–6 min at 35°. The rate of hydrolysis of the ester under these conditions was calculated by the procedure of Cammarata and Cohen[5] with molar absorptivities of 344 for ANA and 3170 for AN. A unit of ANA-ase activity is defined as that amount of enzyme which catalyzes the hydrolysis of 1 mμmole of ANA per min under the above conditions.

The pH optimum is approximately 7 (Fig. 2). Buffers were 0·05 M as follows: tartrate, pH 3·0 and 4·0; acetate, pH 5·0 and 5·5; succinate, pH 6·0 and 6·5; phosphate, pH 7·0; and tris chloride, pH 8·0 and 8·6. The substrate concentration was $5{\cdot}3\times10^{-4}$ M. The reaction temperature was 35°. The extinction coefficients were not materially affected by these solutions. The ANA-ase IV used was obtained from germinated Conquest barley.

To determine a suitable substrate concentration, ANA solutions in 0·05 M phosphate, pH 7·0, were prepared at $10{\cdot}7\times10^{-4}$ M, $5{\cdot}38\times10^{-4}$ M and $1{\cdot}08\times10^{-4}$ M. To these solutions was added ANA-ase II from germinated Trophy barley having 32 units of activity and 0·44 mg protein. This gave a linear increase in absorbance at 35° for 30, 25 and 10 min respectively. Thus $5{\cdot}38\times10^{-4}$ M was chosen as an appropriate concentration.

Assay for LNA-ase

A commercial sample of L-leucyl-β-naphthylamide (LNA) was used as received. A $7{\cdot}8\times10^{-2}$ M solution in 0·05 M succinate, pH 6·5, was prepared. β-Naphthylamine (BNA) was vacuum distilled (m.p. 110°) and a $1{\cdot}18\times10^{-3}$ M solution was prepared in 0·05 M succinate, pH 6·5. The spectra (Fig. 3) for these solutions obtained as described for the ANA-ase assay, revealed that a suitable wavelength is 335 nm.

For the assay of LNA-ase the following procedure was adopted: 3 ml of $3{\cdot}42\times10^{-3}$ M LNA in 0·05 M succinate, pH 6·5, was reacted with 0·1 ml of enzyme of suitable activity. Hydrolysis was followed at 35° and calculated as described for the ANA-ase assay. The molar extinction coefficients were 1750 for BNA and 66 for LNA. A unit of LNA-ase activity is defined as that amount of enzyme which catalyzes the hydrolysis of 1 mumole of LNA per minute under the above conditions.

The pH optimum for the reaction is 6·5 (Fig. 4). Buffers were 0·05 M: acetate, pH 5·0 and 5·5; succinate, pH 6·0 and 6·5; and phosphate, pH 7·0. Substrate concentration was $5{\cdot}44\times10^{-5}$ M and the reaction temperature was 35°. Extinction coefficients were not materially affected by these conditions.

For the determination of a suitable substrate concentration, activities were determined with substrate levels at $3{\cdot}42\times10^{-3}$ M and $6{\cdot}9\times10^{-4}$ M. To 3 ml of these solutions 24 units of LNA-ase containing 0·05 mg protein were added. A linear increase in absorbance resulted for more than 30 min for the high concentration and for 12 min for the low concentration. $3{\cdot}42\times10^{-3}$ M was chosen for the LNA-ase assay.

Plant Material

The Trophy and Conquest barleys (*Hordeum vulgare*) were from the 1965 and 1967 crops respectively and were stored at −25° until used.

Germination and Extraction of Barley; Assays for Protein and Peptide Hydrolases A and B

These operations have been described.[3] 30 g (dry basis) of germinated barley were extracted.

[5] P. S. CAMMARATA and P. P. COHEN, *J. Biol. Chem.* **193**, 45 (1951).

Carboxymethyl Cellulose Ion-Exchange Columns

185 g (47 g dry matter) of moist carboxymethyl cellulose (CM 52) was washed with 0·5 N NaOH, distilled water, 0·5 N HCl–0·5 N NaCl, and finally with water. The exchanger was equilibrated with 0·005 M acetate, pH 5·5, and used to form a column 2·5 × 40 cm. The enzyme solution, 20–40 ml containing approximately 500 mg protein, which was previously equilibrated by dialysis against 0·005 M acetate, pH 5·5, was pumped on the column by the upward flow technique at the rate of 17 ml per hour. An acetate concentration gradient at pH 5·5 was then pumped on the column at the same rate. 200 ml of 0·5 M acetate was drawn into 200 ml of 0·005 M acetate as the latter was pumped on the column. The effluent was monitored for adsorption at 280 nm. Fractions were collected at 15-min intervals and were examined for activity of peptide hydrolases A and B, ANA-ase and LNA-ase. Fractions were pooled according to the enzyme contained by them.

Purification with Sephadex G-100 Columns

Pooled fractions from one or more of the CMC columns were concentrated to 15–30 ml by ultrafiltration and dialyzed against the gel filtration buffer. They were then gel filtered on a 2·5 × 90 cm column as described previously.[3] The appropriate fractions were pooled and concentrated by ultrafiltration.

Treatment of Enzymes with Solutions of Various pH's; Treatment of Enzymes at Various Temperatures

These operations were done as described previously.[2]

Determination of Kinetic Constants

Initial reaction rates for 7–10 concentrations of substrate were used to determine K_m and V.[3] The pH and buffer molarities were as described for the standard assays. ANA concentrations ranged from $2{\cdot}7 \times 10^{-4}$ M to $2{\cdot}7 \times 10^{-5}$ M. LNA concentrations were from $3{\cdot}5 \times 10^{-3}$ M to $3{\cdot}5 \times 10^{-5}$ M.