The Hydrolysis of Alpha Naphthyl Acetate and L-Leucyl-Beta-Naphthylamide by Enzymes from Wheat Embryo[1]

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ABSTRACT

Commercial wheat embryo, and embryo excised by hand from Selkirk HRS wheat and Leeds durum wheat, contained two enzymes that hydrolyzed alpha naphthyl acetate, and one enzyme that hydrolyzed L-leucyl-beta-naphthylamide (LNA). Their behavior during extraction and purification, and when exposed to various pH values and temperatures, showed some similarities to the behavior of the corresponding enzymes in germinated barley. K_m values for the Leeds enzymes suggested that these differ significantly from the corresponding enzymes from the other embryos. K_m values also showed that differences existed between the wheat and barley enzymes. Wheat embryo LNA-ase yielded highly variable results in treatments at pH 5 and 8.

Wheat embryo contains peptide hydrolase A (PHA) but no peptide hydrolase B (PHB) (1,2), whereas germinated wheat has both enzymes and another acidic enzyme that catalyzes the hydrolysis of alpha-N-benzoyl-L-arginine ethyl ester (BAEE). Unlike PHA, however, this acidic enzyme is stable at low pH (3).

Germinated barley contains PHA and PHB, but is devoid of the enzyme stable at low pH (4). It also has several enzymes that promote the hydrolysis of alpha naphthyl acetate (ANA) and an enzyme that promotes the hydrolysis of L-leucyl-beta-naphthylamide (LNA) (5). This report describes ANA-ase and LNA-ase in a sample of commercial wheat embryo, and in embryos excised from Selkirk HRS wheat and Leeds durum wheat.

MATERIALS AND METHODS

Plant Material

Unheated commercial wheat embryo was supplied by the Bay State Milling Co., Winona, Minn. The wheat from which it was obtained was a mixture of spring and winter wheats (*Triticum aestivum* L.) of the 1965 crop. Selkirk HRS wheat and Leeds durum wheat (*T. turgidum* ssp. durum) were grown in North Dakota in 1967 and 1966, respectively. All plant materials were stored in the freezer until used.

Methods

Separation of embryo tissue; moisture determination; extraction of enzymes; CMC chromatography; Sephadex G-100 filtration; treatment of enzymes at various temperatures and pH values; determination of kinetic constants; assays for protein and PHA; and hydrolysis of protein substrates were done as described previously

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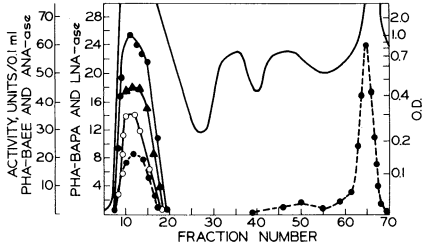

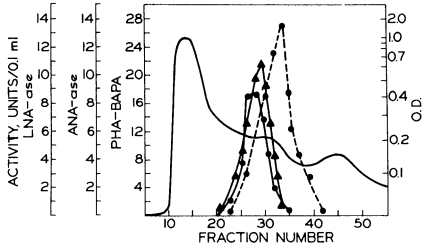

Fig. 1 (left). CMC chromatography of extract of commercial wheat embryo. Legend: solid line, absorbance 280 mμ; black triangles, LNA-ase; black circles, PHA with BAPA; white circles, PHA with BAEE; black circles and dashed line, ANA-ase.

Fig. 2 (right). Sephadex G-100 filtration of peptide hydrolase A, LNA-ase, and CMC-unadsorbed ANA-ase from commercial wheat embryo. Legend, same as for Fig. 1.

(2,4,5). The quantities (dry basis) of embryo tissues used were 21 g. commercial embryo, 9.7 g. Selkirk, and 8.0 g. Leeds.

Assays for LNA-ase and ANA-ase

These were performed as previously described (5). Coefficient of variability values were 2.5 for LNA-ase and 4.7 for ANA-ase. These determinations were made with the LNA-ase and ANA-ase IV fractions obtained by CMC gradient elution chromatography of extracts from germinated Dickson barley with the use of a range of enzyme sample volumes.

RESULTS

Carboxymethyl Cellulose (CMC) Ion-Exchange Chromatography and Sephadex G-100 Filtration

Figure 1 shows the location of the ANA-ase and LNA-ase from an extract of commercial wheat embryo which was dialyzed against 0.005M succinate, pH 5.5, and chromatographed on a CMC column with a 0.005M to 0.5M sodium succinate gradient, pH 5.5. The location of PHA is shown by its reaction with the substrates BAEE and alpha-N-benzoyl-DL-arginine-*p*-nitroanilide (BAPA). This pattern is similar to that obtained with germinated Trophy barley (5) in which PHA, LNA-ase, ANA-ase I, and ANA-ase II were present in the initial peak, i.e. in the fraction unadsorbed on CMC. However, upon filtration of the unadsorbed proteins (fractions 9 to 16, Fig. 1) on Sephadex G-100, only one ANA-ase peak was evident (Fig. 2). Its position relative to PHA suggests that this enzyme is similar to ANA-ase II from Trophy barley (5). Upon filtration of germinated barley extracts on Sephadex G-100 the ANA-ase I immediately preceded ANA-ase II from the column but was not completely separated from ANA-ase II. However, there is in Fig. 2 no evidence for ANA-ase I from commercial wheat embryo.

Figure 1 shows the location of the ANA-ase enzymes that were adsorbed on the CMC and eluted at high ionic strength (fractions 40 to 55 and 56 to 70). The enzyme in fractions 40 to 55 is ANA-ase III which is present in small amount, and

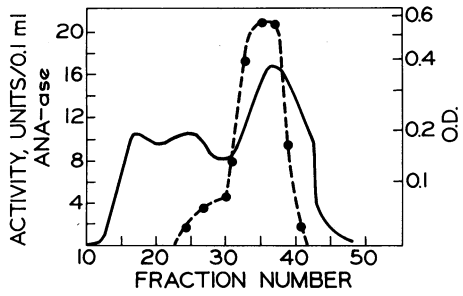

Fig. 3. Sephadex G-100 filtration of ANA-ase IV from commercial wheat embryo. For legend refer to Fig. 1.

the enzyme in fractions 56 to 70 is ANA-ase IV. This pattern is similar to the one with extracts of germinated Trophy barley (5).

Figure 3 shows the pattern obtained by filtration of the ANA-ase IV (fractions 60 to 68, Fig. 1) through Sephadex G-100. The behavior of this enzyme during this filtration appears to be the same as that of the ANA-ase IV from germinated barley.

Essentially the same elution patterns were obtained for the enzymes from embryos of Selkirk and Leeds wheats.

Purification

The purification data obtained by the CMC and Sephadex G-100 treatments of commercial wheat embryo enzymes are shown in Table I. The results for the Leeds and Selkirk tissues were similar to these, except that the specific activities of the LNA-ase and ANA-ase II were somewhat higher. The specific activities of LNA-ase, ANA-ase II, and ANA-ase IV from commercial wheat embryo (Table I) are all 1.5 to 2 times as great as the corresponding values for preparations from germinated Trophy barley (5). Perhaps wheat embryo tissue is a better source material for the enzymes than the germinated whole kernels of barley, the enzyme source used in previous work.

Stability of Enzymes to pH and Temperature

The stability of the enzymes to pH and elevated temperature is shown in Table II. In general, ANA-ase II from all three sources appeared stable at alkaline pH but was sensitive to low pH. ANA-ase IV, however, had a broader range of pH stability. Except for the Leeds preparation at pH 3.5, good stability was shown over the pH range 3.5 to 9.2.

Inexplicably high and irreproducible recoveries of LNA-ase from all embryos were obtained in repeated dialysis experiments in which the enzyme preparations were exposed for 16 hr. to pH 5 and 8 at 2°C. The variation in replicated LNA-ase values shown in Table II is greater than could be caused by errors in the assay, which has a relatively low coefficient of variability. There were no unusual changes in activity of LNA-ase during its purification (Table I) in which 0.005M to 0.5M succinate buffers, pH 5.5, were used for CMC chromatography, and 0.1M succinate, pH 5.9, was used for Sephadex G-100 treatment. It is possible that succinate may have a stabilizing effect. Furthermore, LNA-ase exhibited a marked, inexplicable difference with respect to sample source: at pH 3.5 the enzyme from commercial

TABLE I. PURIFICATION OF ANA-ase II AND IV AND LNA-ase; COMMERCIAL WHEAT EMBRYO

Purification Step	Vol. ml.	Protein mg./ml.	Activity ANA-ase U/ml.	Activity LNA-ase U/ml.	Recovery ANA-ase %	Recovery LNA-ase %	Specific Activity ANA-ase U[a]	Specific Activity LNA-ase U[a]	Purification Fold ANA-ase	Purification Fold LNA-ase
CMC treatment										
Sample applied:										
Extract dialyzed vs. 0.005M acetate	38	11.6	780	450	100	100	67	39		
Products:										
ANA-ase II, LNA-ase	25	4.1	330	340	27	49	80	84		2.2
ANA-ase III	18	0.6	66		4		110			
ANA-ase IV	21	3.8	470				124			
Sephadex G-100 treatment										
Sample applied:										
ANA-ase II, LNA-ase	14.5	4.5	480	446	100	100	107	99		
Products: ANA-ase II	9.5	1.2	460		62		370			
LNA-ase	8.0	1.3	130	180	15	22	100	138		1.4
Sample applied: ANA-ase IV	24.8	3.5	418		100		119			
Product: ANA-ase IV	22.5	1.1	352		77		322		2.7	

[a]Per mg. protein.

TABLE II. pH AND TEMPERATURE STABILITY

Buffer	pH	Enzyme Recovery %[a] Commercial Wheat Embryo ANA-ase II	ANA-ase IV	LNA-ase	Selkirk Embryo ANA-ase II	ANA-ase IV	LNA-ase	Leeds Embryo ANA-ase II	ANA-ase IV	LNA-ase
Tartarate	3.5	0	81	91	0	85	0	18	49(43)	0
Acetate	5.0	70	84	192(196)(73)	32	94	242(155)	65	90(85)	36(135)
Tris	8.0	71	84	139(200)(61)	105	87	118(204)	103	85(87)	100(156)
Borate	9.2	83	85		103	108		84	81(82)	
	Temp. (°C.)									
	35	120	113	119	92	81	121	91	98	108
	45	60	102	117	76	36	91	58	61	93
	55	0	56	0	0	0	21	0	0	15

[a]Values in parentheses are replicates.

wheat embryo was stable, but those from Selkirk and Leeds were completely inactivated. LNA-ase was not dialyzed at pH 9.2 because of nonenzymatic hydrolysis at this high pH value.

Several substances were tested in attempts to stabilize, or to stimulate, LNA-ase as a possible means of determining the cause of the variable results shown in Table II. Because Mg^{++} and Mn^{++} are known to stimulate leucine aminopeptidases from several sources (6), these ions were tested at 10^{-4} and 10^{-2}M concentrations. No stimulation was observed. The following substances were tested for possible effects with no stimulation or significant stabilizing effects after the enzyme was stored in them for 5 days at 2°C.: cysteine (0.005M), LNA ($1.7X10^{-3}$M), sucrose (12.5% w./v.), and acetate buffer, pH 5.5 (0.25M). Further study is required to explain the variability observed with LNA-ase. This great variability was not obtained in replicates with barley LNA-ase (5).

ANA-ase II from all sources lost some of its activity at 45°C. and was completely inactivated by exposure to 55°C. Similarly, LNA-ase from all sources appeared to resist exposure to high temperatures. Stability of ANA-ase IV at these temperatures varied according to source. The enzyme from Leeds and, particularly, Selkirk seemed sensitive to temperature, but the enzyme from commercial wheat embryo was resistant at 45°C. and to an appreciable extent at 55°C.

Kinetic Data

The results of determinations of the Michaelis constants (K_m) for the nine enzyme preparations are summarized in Table III. Close agreement in the values for the corresponding ANA-ases of Selkirk and commercial wheat embryo is evident. However, the preparations from Leeds differ significantly. The values for the two ANA-ases from Leeds are about four times those found for Selkirk and commercial wheat embryo, and the K_m for LNA-ase from Leeds is only 4% of those obtained with this enzyme from the other two sources.

In all cases, K_m for ANA-ase II is smaller than that for ANA-ase IV. This is the converse of the data obtained for these enzymes in germinated barley (5). Also, barley LNA-ase has a much smaller K_m ($1.6 X10^{-4}$M) than do any of the corresponding wheat enzymes.

DISCUSSION

The chromatographic behavior of corresponding enzymes from the embryo tissues of wheat and from germinated Trophy barley suggests that they are similar in gross properties. However, as might be expected, corresponding enzymes from various tissues differ in some details, perhaps in configuration near the active sites

TABLE III. MICHAELIS CONSTANTS[a]

Enzyme	Commercial Wheat Embryo K_m Molar	Selkirk Embryo K_m Molar	Leeds Embryo K_m Molar
ANA-ase II	1.3×10^{-5}	1.4×10^{-5}	5.6×10^{-5}
ANA-ase IV	2.2×10^{-5}	2.5×10^{-5}	9.5×10^{-5}
LNA-ase	4.0×10^{-2}	3.0×10^{-2}	1.4×10^{-3}

[a]ANA was used as substrate for ANA-ase II and IV, and LNA was used as substrate for LNA-ase assays.

on the molecules, which is reflected in differences in K_m values of the individual preparations.

The ANA-ase and LNA-ase enzymes appear to be present in diverse cereal tissue and deserve additional study to evaluate their substrates and their function in the germinating seed. LNA-ase, by its action on an amino acid amide, is suggestive of an exopeptidase. Less can be said about the two ANA-ases. Numerous exo- and endopeptidases are known to have esterolytic activity. The partially purified ANA-ase II had no endopeptidase activity on casein at pH 6 or on hemoglobin at pH 3.8, and its peptidase activity remains to be demonstrated.

Literature Cited

1. PRENTICE, N., BURGER, W. C., KASTENSCHMIDT, JEANNINE, and HUDDLE, J. D. The distribution of acidic and neutral peptidases in barley and wheat kernels. Physiol. Plantarum 20: 361 (1967).
2. PRENTICE, N., BURGER, W. C., KASTENSCHMIDT, JEANNINE, and MOELLER, MARY. Peptide hydrolase in wheat embryo. Phytochem. 9: 41 (1970).
3. PRENTICE, N., BURGER, W. C., and MOELLER, MARY. Partial purification and characterization of peptide hydrolases from germinated wheat. Phytochem. 7: 1899 (1968).
4. BURGER, W. C., PRENTICE, N., KASTENSCHMIDT, JEANNINE, and MOELLER, MARY. Partial purification and characterization of barley peptide hydrolases. Phytochem. 7: 1261 (1968).
5. BURGER, W. C., PRENTICE, N., MOELLER, MARY, and KASTENSCHMIDT, JEANNINE. Hydrolysis of alpha naphthyl acetate and L-leucyl naphthylamide by barley enzymes. Phytochem. 9: 33 (1970).
6. SMITH, E. L., and HILL, R. L. Leucine aminopeptidase. In: The enzymes, ed. by P. D. Boyer, H. Lardy, and K. Myrback; vol. 4, chap. 3, p. 37. Academic Press: New York (1960).

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