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PEPTIDE HYDROLASE IN WHEAT EMBRYO

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Abstract—Commercial wheat embryo and embryo excised by hand from a hard red spring wheat (Selkirk) and a durum wheat (Leeds) were devoid of peptide hydrolase B. The embryo from all sources had an enzyme similar to peptide hydrolase A from germinated barley with respect to pH stability, thermal stability, and to the ratio of activity with α-*N*-benzoyl-L-arginine ethyl ester (BAEE) and α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPA) substrates. The enzyme from the commercial embryo was stimulated by metal ions in the same way as the barley enzyme and hydrolyzed the same simple peptides. K_m values for the three embryo enzymes were essentially the same and were about three times that of the enzyme from germinated barley.

INTRODUCTION

GERMINATED wheat contains peptide hydrolases which appear to be very similar to, if not identical with, the peptide hydrolases A and B (PHA and PHB) of barley.[1,2] These enzymes increase in the embryo during germination.[3] In addition, germinated wheat contains a third hydrolase which has not been separated from PHA, yet has properties similar to barley PHB.[2]

Earlier work[4] with commercial wheat embryo (CWE) showed that this material possesses one or more enzymes not adsorbed by carboxymethyl cellulose (CMC) at pH 5·5 which will hydrolyze BAPA and BAEE but no peptide hydrolase B. In fact, the adsorbed proteins contained no peptide hydrolase activity as measured with these substrates.

The following report describes the extraction, purification and properties of PHA from CWE and from embryos excised by hand from Selkirk hard red spring wheat and Leeds durum wheat.

RESULTS AND DISCUSSION

CMC Chromatography and Sephadex G-100 Filtration

The elution patterns for the extract of Selkirk embryo upon CMC chromatography are shown in Fig. 1. The pattern for the CWE preparation (not shown) is essentially the same and suggests that there is little difference in the enzymes due to source. Enzyme activity is eluted by the buffer in the dilute portion of the salt gradient. The pattern for the Leeds embryo extract (Fig. 2) differs in the shape of the activity peak, although its position relative to the initial increase in absorbance is the same. The activity peak here is broad and it is felt that this is a reflection of the properties of the enzyme or impurities associated with it and not an

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[1] W. C. BURGER, N. PRENTICE, J. KASTENSCHMIDT and M. MOELLER, *Phytochem.* 7, 1261 (1968).
[2] N. PRENTICE, W. C. BURGER and M. MOELLER, *Phytochem.* 7, 1899 (1968).
[3] N. PRENTICE, W. C. BURGER and E. WIEDERHOLT, *Physiol. Plantarum* 22, 157 (1969).
[4] N. PRENTICE, W. C. BURGER, J. KASTENSCHMIDT and J. D. HUDDLE, *Physiol. Plantarum* 20, 361 (1967).

artifact. In no case was PHB present, which would have been eluted in the concentrated portion of the gradient.

Elution patterns for PHA with Sephadex G-100 filtration were all very similar; that for the CWE peptide hydrolase A is shown in Fig. 3. The purification data for the enzymes from the three sources treated with CMC and G-100 are shown in Table 1. The ratio of specific

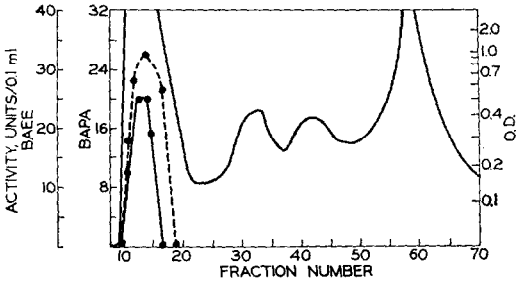

FIG. 1. CMC CHROMATOGRAPHY OF SELKIRK EMBRYO PEPTIDE HYDROLASE A.
—— O.D. 280 nm ●——● BAPA substrate ●----● BAEE substrate

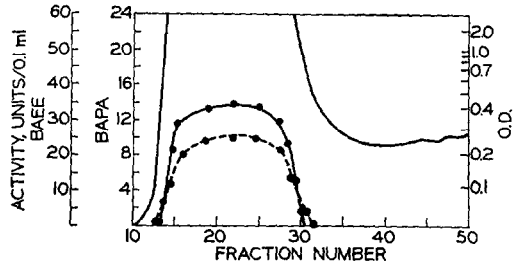

FIG. 2. CMC CHROMATOGRAPHY OF LEEDS EMBRYO PEPTIDE HYDROLASE A.
—— O.D. 280 nm ●——● BAPA substrate ●----● BAEE substrate

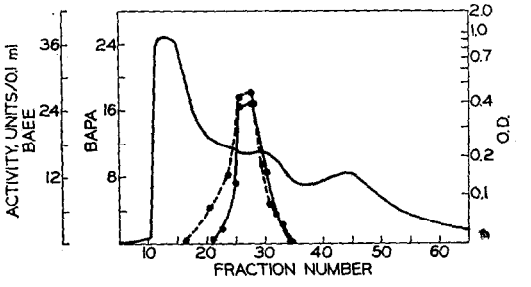

FIG. 3. SEPHADEX G-100 FILTRATION OF CWE PEPTIDE HYDROLASE A.
—— O.D. 280 nm ●——● BAPA substrate ●----● BAEE substrate

activity with the two substrates remains approximately 1·2–2·4 throughout the entire purification. This value for the corresponding fraction from whole germinated barley is about 1[1] and from whole germinated wheat about 14.[2] The high ratio for germinated wheat was caused by the presence of a second hydrolase whereas in germinated barley preparations only one, i.e. barley PHA, was present. Table 1 shows that a very high specific activity could be obtained with the Leeds preparation.

TABLE 1. PURIFICATION OF PEPTIDE HYDROLASE A FROM CWE, SELKIRK EMBRYO AND LEEDS EMBRYO

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	
CWE									
Dialyzed extract	27	12·4	280	400	100	100	23	32	1·4
Unadsorbed fraction CMC column	17·5	4·5	243	580	56	94	54	128	2·4
G-100	8·2	1·3	460	710	50	54	355	545	1·5
Selkirk embryo									
Dialyzed extract	29	12·2	213	397	100	100	17	33	1·8
Unadsorbed fraction CMC column	22	1·3	237	296	84	57	183	227	1·2
G-100	12·2	0·54	194	370	38	39	352	685	1·9
Leeds embryo									
Dialyzed extract	55	6·1	127	172	100	100	21	29	1·3
Unadsorbed fraction CMC column	19	5·0	280	400	76	80	56	80	1·4
G-100	12	0·25	340	600	58	76	1380	2400	1·7

* BAPA = α-*N*-benzoyl-DL-arginine *p*-nitroanilide; BAEE = α-*N*-benzoyl-L-arginine ethyl ester.

Stability of Enzymes at Various pH Values

The pH effect given in Table 2 shows that the hydrolase from all embryos reacts to this treatment in a manner analogous to that of barley PHA,[1] i.e. it is inactivated at low pH. There is no evidence for the presence of the enzyme from germinated wheat that showed stability to low pH.[2]

TABLE 2. THE EFFECT OF pH ON PEPTIDE HYDROLASE A

		Substrate					
		Commercial wheat embryo		Selkirk embryo		Leeds embryo	
Buffer (0·05 M)	pH	BAPA	BAEE	BAPA	BAEE	BAPA	BAEE
		% original activity					
Tartarate	3·5	0	0	0	0	0	0
Acetate	5·0	78	34	64	59	80	35
Tris	8·0	72	84	89	84	83	95
Borate	9·2	72	88	96	100	82	70

Stability of Enzymes with Elevated Temperature

Elevated temperature affected activity as shown in Table 3. Here again the pattern was similar to the germinated barley enzyme[1] except that some activity remained after treatment at 45° with the Selkirk embryo enzyme.

TABLE 3. THE EFFECT OF TEMPERATURE ON PEPTIDE HYDROLASE A

	Substrate					
	Commercial wheat embryo		Selkirk embryo		Leeds embryo	
Temperature (deg.)	BAPA	BAEE	BAPA	BAEE	BAPA	BAEE
	% original activity					
35	78	71	55	59	79	79
45	0	0	5	11	0	0
55	0	0	0	7	0	0

The Effect of Metal Ions on PHA from CWE

In the presence of Ca, Mg, Mn, Ni and Zn ions and BAPA substrate the activity of the CWE enzyme was altered as shown in Fig. 4. Essentially the same pattern was obtained with BAEE as substrate (not shown). The effect shown is similar to that displayed with this system by the corresponding germinated barley[1] and wheat enzymes.[2] As with enzymes from germinated barley and wheat, a high concentration of metal ions is required for stimulation of activity, which suggests a labile association between the ion and one or more components of the system. Limited supplies of the enzymes from Selkirk and Leeds embryos did not allow this experiment to be done with these enzymes.

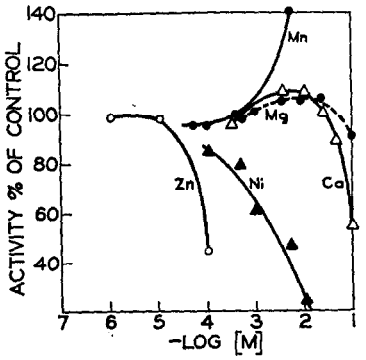

FIG. 4. EFFECT OF METAL IONS ON CWE PEPTIDE HYDROLASE A WITH BAPA SUBSTRATE.

pH Optimum for Activity

With BAPA as the substrate the variation in activity of CWE enzyme with pH was similar to that shown previously with wheat,[2] i.e. there was a broad plateau between pH 8 and 9. With BAEE substrate the maximum activity of this enzyme occurred over the range pH 7·5–8·0. Since BAEE undergoes alkaline hydrolysis at and above pH 8, the experiment was limited to this pH.

Kinetic Constants with BAEE Substrate

These values are shown in Table 4. The K_m values are all similar and support the view that the three embryo enzymes are similar. K_m for the CWE enzyme was not altered by removal of α-naphthyl acetate-ase (ANA-ase) and L-leucyl-β-naphthylamide-ase (LNA-ase) by gel electrophoresis. The value for the corresponding enzyme from germinated barley was $4{\cdot}4 \times 10^{-5}$ M.[1] Thus the wheat embryo enzymes are somewhat less active than the one from barley. The V_{max} values indicate the activities of the particular preparations. These constants for the enzyme acting on BAPA could not be determined because of the inhibitory effect of D-BAPA. Also, comparison cannot be made with the corresponding preparation from germinated wheat since two BAEE-ase enzymes are known to be present.

TABLE 4. KINETIC CONSTANTS WITH BAEE SUBSTRATE

	Commercial wheat embryo	Selkirk embryo	Leeds embryo
K_m Molar	$1{\cdot}13 \times 10^{-4}$ ($1{\cdot}02 \times 10^{-4}$)*	$1{\cdot}3 \times 10^{-4}$	$1{\cdot}02 \times 10^{-4}$
V, U/0·1 ml	72 (31)*	53	60

* Preparation from gel electrophoresis free of ANA-ase and LNA-ase.

Hydrolysis of Peptides

The substrates examined were as shown in Table 5. Those hydrolyzed are in general the same as the ones hydrolyzed by barley PHA.[6] This suggests a striking similarity between

[5] W. C. BURGER, N. PRENTICE, M. MOELLER and J. KASTENSCHMIDT, *Phytochem.*, 9, 33 (1970).
[6] M. MOELLER, W. C. BURGER and N. PRENTICE, *Phytochem.*, 8, 2153 (1969).

these enzymes. There was insufficient PHA from Selkirk and Leeds embryos to permit the experiment to be done with these.

TABLE 5. HYDROLYSIS OF PEPTIDES BY CWE PEPTIDE HYDROLASE A

Ala-asn	−	Gly-trp	+slow
Ala-gly-gly	+	Gly-tyr	+slow
Ala-phe	−	Hippuryl-arg	−
Ala-ser	−	Hippuryl-gly	−
Gln-asn	−	Leu-gly	+slow
Gly-glu	−	Leu-gly-gly	+
Gly-gly	+slow	Lys-ala	−
Gly-gly-gly	+slow	Lys-gly	−
Gly-leu	+slow	Lys-lys	+
Gly-lys	+slow	Phe-leu	+
Gly-phe	+slow	Trp-gly	+
Gly-thr	−	Trp-leu	+
		Trp-ethylester	+

EXPERIMENTAL

Plant Material

Unheated CWE was supplied by the Bay State Milling Co., Winona, Minn. The wheat from which it was separated by conventional roller milling procedures was a mixed lot of spring and winter wheats (*Triticum aestivum* L.) of the 1965 crop. Selkirk hard red spring wheat and Leeds durum wheat (*T. durum*) were grown in North Dakota in 1967 and 1966, respectively. All plant materials were stored at −25° until used.

Separation of Embryo Tissue

After the Selkirk and Leeds kernels had been soaked in water at 4° for about 30 min the embryo was excised with a small spatula. The embryo tissue and the remaining endosperm portions were frozen until used.

Moisture Determination

About 1 g of the tissue was heated at 105° for 90 min for moisture determination.

Assay for Protein and for Peptidase Activity

These were performed as described previously.[1]

Extraction of Enzymes

The embryo tissue (21 g CWE, 9·7 g Selkirk embryo, 8·0 g Leeds embryo, dry basis) was extracted by thoroughly grinding in a mortar at 4° with 4 ml buffer (0·05 Tris chloride, 0·005 M EDTA Na salt, pH 7·5) and 0·5 g acid washed quartz sand per g tissue. The suspension was centrifuged 30 min at 30,000 × *g* at 4°. The supernatant was removed for storage at −25° or used immediately.

Preparation of Carboxymethyl Cellulose (CMC) Columns

The ion exchanger Whatman CM 52 was suspended in successive 15 vol. of 0·5 N NaOH and, after standing 30 min, the supernatants were decanted until all fines were removed. The exchanger was then washed with distilled water until the slurry was about pH 8. It was then treated similarly with successive 15 vols. of 0·5 N HCl and washed with distilled water until the pH was about 7. Acetate buffer (0·5 M, pH 5·5) was added and the slurry, equilibrated at pH 5·5, was centrifuged in 250-ml bottles at 1500 rev/min. The exchanger was then equilibrated similarly with 0·005 M acetate, pH 5·5. A slurry in this buffer was used for packing columns 2·5 cm in dia. and 40 cm in length.

Chromatography with CMC Columns

Tissue extract was dialyzed against 0·005 M acetate, pH 5·5, and centrifuged at 30,000 × *g* for 30 min. About 25–27 ml of this solution representing 8–10 g of embryo tisuse and containing about 12 mg protein per ml was pumped at 15–20 ml per hr on the column by the upward flow technique. The column was then

developed with the acetate concentration gradient at pH 5·5 as described previously.[5] Fractions of 4–5 ml were collected. Active fractions were pooled and concentrated by ultrafiltration for further purification with Sephadex G-100.

Filtration with Sephadex G-100; Treatment of Enzymes with Buffers of Various pH's; Treatment of Enzymes at Various Temperatures

These operations were done as previously described.[1, 2]

Effect of Metal Ions upon Enzyme Activity

The procedure has been described previously.[1] Metal ion solutions (0·1 ml) of suitable concentrations were added to substrate solution so that the final metal ion concentration was 10^{-6}–10^{-1} M. Upon addition of enzyme the reaction rate was followed.

Hydrolysis of Peptides

A preparation from CWE (22 ml containing 0·84 mg protein per ml and 140 units per ml of activity with BAPA substrate) was gel electrophoresced as described previously[6] to remove α-naphthyl acetate-ase (ANA-ase) and L-leucyl-β-naphthylamide-ase (LNA-ase), and reacted with peptide substrates.[1, 6] The enzyme solution used for the reactions had 0·05 mg protein per ml, 106 units activity per ml with BAPA and 278 units activity per ml with BAEE.

Determination of Kinetic Constants

K_m and V were determined as described previously.[1] The constants for the CWE enzyme were determined before and after removal of ANA-ase and LNA-ase by gel electrophoresis.