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DETECTION AND PROPERTIES OF L-HISTIDINOL DEHYDROGENASE IN WHEAT GERM

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Key Word Index—*Triticum aestivum*; Gramineae; wheat germ; biosynthesis; histidine; histidinol; dehydrogenase.

Abstract—The presence and partial characterization of the properties of L-histidinol dehydrogenase (EC 1.1.1.23), the enzyme catalysing the last step in the pathway of histidine biosynthesis, has been described in higher plants for the first time. The activity has been found in cell-free extracts from wheat germ, turnip root, radish root and squash fruit. The enzyme has been partially purified and characterized from extracts of acetone powders of wheat germ. DEAE-cellulose chromatography revealed two peaks of histidinol dehydrogenase activity. In one there was a rapid reduction of NAD^+ in the absence of histidinol; however, the rate was stimulated by the addition of histidinol. The rate in the absence of substrate became quite low after several min and the histidinol-dependent rate was then easily observed. The second peak of activity did not reduce NAD^+ unless L-histidinol was present in the assay mixture. The K_ms for L-histidinol and NAD^+ were determined for this latter enzyme. The values obtained at saturating concentrations of the other substrate were L-histidinol, 8.8 μM and NAD^+, 0.14 mM. The product of the dehydrogenase reaction was histidine as determined by paper chromatography.

INTRODUCTION

Histidine biosynthesis has been studied most extensively in bacterial systems. The biosynthetic pathway in *Salmonella typhimurium* consists of ten enzymic steps from phosphoribosylpyrophosphate to histidine [1]. The biosynthetic pathway in higher plants is still obscure. Activities corresponding to phosphoribosyl-ATP synthetase, imidazole glycerol phosphate dehydratase, and L-histidinol phosphate phosphatase have been demonstrated in crude extracts from young shoots of higher plants [2]. These activities correspond to the first, seventh and ninth steps of histidine biosynthesis in the *Salmonella* pathway. The present report is the first published account characterizing the final enzyme in the sequence, L-histidinol dehydrogenase (EC 1.1.1.23), in higher plants. Active preparations have been obtained from wheat germ, turnip root, radish root and squash fruit. The partial purification of the histidinol dehydrogenase from wheat germ (*Triticum aestivum*) and some of its properties are also described.

RESULTS

Possible isozymes in wheat germ

Histidinol dehydrogenase activity was partially purified by the procedure described in the Experimental. A summary of a typical purification is shown in Table 1.

Table 1. Summary of a typical purification and recovery of the wheat germ enzyme

Fraction	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg protein)	Recovery	Purification
1. Original extract*	505	177	0.35	100	1.0
2. Protamine treatment	391	172	0.44	97	1.3
3. 30–60% $(NH_4)_2SO_4$ precipitate	236	163	0.69	92	2.0
4. Gel filtration eluate	101	157	1.55	89	4.4
5. DEAE-cellulose eluate					
Peak I	7.6	25	3.3	14	9.4
Peak II	8.4	40	4.8	23	13.7

* 3.5 g acetone powder extracted in 50 ml buffer.

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Two peaks of activity were recovered after DEAE-cellulose chromatography (Fig. 1). This may indicate the presence of two histidinol dehydrogenases or isozymes. The first peak had a strong background activity, i.e. an increase in absorbance in the presence of NAD^+ prior to the addition of histidinol. This background activity decreased markedly after several min, but on the addition of L-histidinol a further continuing increase in absorbance at 340 nm was observed. The peak I fraction has been rechromatographed on DEAE-cellulose in the presence of β-mercaptoethanol and the detergent, Triton X-100. No dissociation of the background activity and the histidinol dehydrogenase activity was obtained. The dehydrogenase activity in peak I is much less stable on storage than that in peak II.

Michaelis constants

The peak II fraction was used for kinetic studies since it had no activity in the absence of added histidinol. The Michaelis constants for the substrates NAD^+ and L-histidinol were determined by varying each substrate independently in the presence of a saturating concentration of the other, using the standard assay conditions. The K_m for each substrate was then determined by the use of a regression analysis of the data as a fit for the Lineweaver–Burk straight line formulation of the Michaelis–Menten equation. The K_m for L-histidinol was 8.8 μM and for NAD^+ 0.14 mM.

Effects of divalent cations

The presence of various divalent cations had a significant effect on the reaction rate. As summarized in Table 2 it appears that most divalent cations at quite low concentrations inhibit significantly and in fact Cu^{2+} at 0.5 mM concentration completely inhibits the reaction. Mg^{2+} stimulates slightly and Mn^{2+} gives a significant boost in the rate of the reaction.

Product identification

It was necessary to determine the final product of the reaction obtained from the histidinol. A reaction mixture composed of 100 mM L-histidinol, 10 mM NAD^+, 50 mM CHES (2[*N*-cyclohexylamino]ethane sulfonic acid), pH 10, and peak II enzyme was incubated for 15 hr at room temperature. A control reaction mixture without histidinol was also incubated. The reaction was stopped by the addition of ethanol to a final concentration of 70%.

Table 2. The effect of divalent metal ions on the activity of histidinol dehydrogenase

Cation (0.5 mM)	Relative activity (%)
None	100
Ba^{2+}	78
Ca^{2+}	46
Cd^{2+}	75
Cu^{2+}	0
Mg^{2+}	113
Mn^{2+}	135
Zn^{2+}	52

The anion was Cl^- in every case but Cd^{2+} and Cu^{2+} where the sulfate salt was used.

The precipitate which formed was removed by centrifugation and aliquots of the supernatant solutions of each reaction mixture were analysed by descending paper chromatography for imidazole-containing products. Known standards of histidine and histidinol were run alongside. The solvent systems used and spray reagents are given in Table 3. In the reaction mixture containing histidinol, only one imidazole-containing compound other than histidinol was found and this had the same R_f as histidine in both solvent systems.

Distribution of the enzyme

A brief survey of the distribution of the enzyme was made. The results are given in Table 4. This very cursory study encompassed only five families. The enzyme was found in representatives of three of the families and in several different tissues. Within the Cruciferae, the enzyme was quite active in roots, but it was not detectable in buds of some varieties of *Brassica oleracea*. The enzyme also appeared in representatives of both monocotyledonous and dicotyledonous species.

DISCUSSION

The results above give further reinforcement to the belief that histidine biosynthesis in higher plants utilizes the same or a very similar pathway to that which has been carefully worked out in *Salmonella typhimurium* [1]. Of

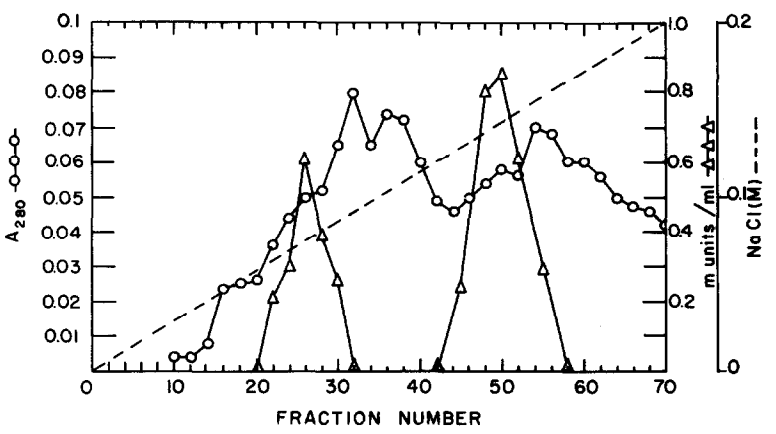

Fig. 1. Elution of two dehydrogenase activities from DEAE-cellulose column.

Table 3. Comparison of R_f values for histidinol, histidine and enzymic reaction product*

	R_f (*t*-BuOH–pyridine–H_2O, 14:3:6)	R_f (*t*-BuOH–pyridine–H_2O–formic acid, 14:3:3:1)
Histidinol diHCl†	0.55	0.34
Histidine†	0.31	0.18
Enzymic reaction product	0.31	0.18

* The chromatograms were developed on Whatman No. 1 paper by descending method for 18 hr. Spots were visualized by spraying with diazotized sulfanilic acid (mixed equal parts of 1% sulfanilic acid in 1 N HCl with 5% $NaNO_2$) followed by 15% Na_2CO_3. Both histidinol diHCl and histidine spots give red colors at once.

† Both the standard solutions contained 50 mM CHES, pH 10.

the ten enzymatic steps starting from phosphoribosyl-pyrophosphate, enzyme activities catalysing the first, seventh and ninth steps had been reported previously [2], and now the enzyme responsible for the final reaction in the sequence has been described in higher plants and partially characterized.

The enzyme from *Salmonella* has been purified to homogeneity and crystallized [3]. The K_m of this enzyme for histidinol at pH 9.4 was 11.5 μM and for NAD^+ was 1.3 mM [4]. The wheat germ enzyme has a very similar K_m for histidinol but the K_m for NAD^+ was an order of magnitude smaller. The stimulation of NAD^+ reduction by histidinol in the presence of Mn^{2+} has been observed in the wheat germ studies. A similar activation had been previously reported as a characteristic of the bacterial enzyme, but no absolute requirement for Mn^{2+} could be established [4].

The final product of the enzyme reaction in both the bacterial and wheat germ preparations was histidine. In the bacterial system the reaction apparently proceeds via 2 sequential NAD^+-linked reactions in which histidinol is first converted to histidinal and this latter compound then further oxidized to histidine. No effort was made to determine whether histidinal was a substrate for the wheat germ system; however, it appears a reasonable presumption that the same route of conversion would be followed.

The presence of two easily separated fractions having dehydrogenase activity in the wheat germ extract gives rise to a number of speculations as to whether these are isozymes or whether peak I is merely a polymeric aggregate of peak II, or an association of peak II with other proteins. Efforts to resolve peak I were not successful. Peak I can reduce NAD^+ in the absence of histidinol for several mins. On dialysis or gel filtration, this activity is not lost. One possible explanation is that this may represent a fraction of the enzyme with enzyme-bound intermediate such as histidinol or histidinal. Under these conditions, it might change the elution properties of the protein from DEAE-cellulose to a considerable extent. A final alternative is that this is a true isozyme which is spatially and functionally distinct from the peak II enzyme in the plant cell.

EXPERIMENTAL

Acetone powder preparation. The wheat germ was a gift from General Mills, Inc., Vallejo, California. Usually 100 g of wheat germ was blended with 500 ml pre-chilled (−15°) Me_2CO for 30 sec. The suspension was filtered by suction and air-dried on the filter. The blending was repeated once. After being air-dried overnight, the Me_2CO powder was stored at −15°.

Enzyme purification. All purification procedures were carried out in the cold (4°). In a typical purification 3.5 g of Me_2CO powder was suspended in 50 ml 10 mM NaPi buffer, pH 7.2 and stirred for 20 min. The extract was filtered through cheesecloth and the filtrate centrifuged at 24000 *g* for 30 min. To the supernatant soln 10 ml of 1% protamine sulfate was added per 100 ml of supernatant. The mixture was stirred for 20 min and centrifuged at 24000 *g* for 20 min. The supernatant soln was decanted and solid $(NH_4)_2SO_4$ added with constant stirring to a concn of 30%. After 20 min stirring the ppt. was removed by centrifugation and discarded. The $(NH_4)_2SO_4$ concn was then brought to 60%. After centrifugation the pellet was dissolved in 10 mM NaPi, pH 7.2.

Five ml of this fraction was applied to a Sephacryl 300 column (2.6 × 35 cm) which had been equilibrated with the NaPi buffer. The column was eluted with 10 mM NaPi buffer, pH 7.2, and 6 ml fractions were collected. The fractions with dehydrogenase activity were pooled and placed on a DEAE-cellulose column (2.0 × 22 cm) previously equilibrated with the 10 mM NaPi buffer. The protein was eluted by use of a linear gradient of NaCl using 250 ml of 10 mM Pi buffer, pH 7.2 in the mixing vessel and an equal vol. of 10 mM buffer containing 0.2 M NaCl. The elution pattern obtained is shown in Fig. 1. The eluate was collected in 7 ml fractions. Fractions with the dehydrogenase activity were pooled from each peak and kept at −15°. The pooled peak II

Table 4. Detection of histidinol dehydrogenase activity in some representative plant families

Family	Species	Organ	Activity (mU/mg protein)
Gramineae	Wheat (*Triticum aestivum*)	Germ	0.69
Cruciferae	Turnip (*Brassica rapa*)	Root	0.10
	Cauliflower (*B. oleracea* var. *botrytis*)	Bud	0
	Broccoli (*B. oleracea* var. *pompejana*)	Bud	0
	Radish (*Raphanus sativus*)	Root	0.31
Cucurbitaceae	Squash (*Cucurbita pepo*)	Fruit	0.11
Solanaceae	Potato (*Solanum tuberosum*)	Tuber	0
Umbelliferae	Carrot (*Daucus carota*)	Root	0

fraction was concentrated 7 to 10-fold by ultrafiltration prior to use in kinetic studies.

Distribution studies. All plant materials were purchased from local markets. After peeling (except for the bud materials) 100 g of tissues was blended in 100 ml 10 mM NaPi buffer, pH 7.2. In the case of carrot and potato, 5 mM dithioerythritol and 5 mM Na metabisulfite were added to the blending buffer. After homogenization the slurry was filtered through cheesecloth and centrifuged as above. The supernatant was treated with protamine sulfate soln as usual and the $(NH)_2SO_4$ fraction precipitating between 30 and 75% satn used as the enzyme test material. The 30–75% $(NH_4)_2SO_4$ fraction was dialysed against the 10 mM Pi buffer before assay.

Enzyme assay. The dehydrogenase activity was assayed by measuring the A increase at 340 nm due to the reduction of NAD^+ to NADH. The standard complete reaction mixture contained 0.1 M CHES, pH 10; 2 mM NAD, 5 mM L-histidinol, and enzyme in a final vol. of 1 ml. The reaction was started by the addition of histidinol and the A_{340} increase was monitored by use of a recording Gilford 250 spectrophotometer. In the assay of peak I which had the high background, the reaction was pre-incubated for 10–15 min prior to the addition of histidinol. A control cuvette minus histidinol was run in each instance and the enzyme activity based on the difference in A between the two cuvettes. One enzyme unit (U) is defined as that amount of enzyme which will reduce 1 μmol NAD^+ per min.

Protein was determined by the method of ref. [5].

Chemicals. L-Histidinol dihydrochloride, NAD^+, dithioerythritol and CHES were products of Sigma. Sephacryl 300 was purchased from Pharmacia. Enzyme grade $(NH_4)_2SO_4$ was obtained from Schwarz-Mann.

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REFERENCES

1. Brenner, M. and Ames, B. N. (1971) in *Metabolic Regulation* (Vogel, H. J., ed) Vol. 5, pp. 349–387. Academic Press, New York.
2. Wiater, A., Krajewska-Grynkiewicz, K. and Klopotowski, T. (1971) *Acta Biochim. Polon.* **18**, 299.
3. Martin, R. G., Berberich, M. A., Ames, B. N., Davis, W. W., Goldberger, R. F. and Yourno, J. D. (1971) in *Methods of Enzymology* (Tabor, H. and Tabor, C. W., eds.) Vol. 17B, pp. 3–44. Academic Press, New York.
4. Loper, J. C. and Adams, E. (1965) *J. Biol. Chem.* **240**, 788.
5. Bradford, M. (1977) *Analyt. Biochem.* **72**, 248.