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PURIFICATION OF A BARLEY PEPTIDE HYDROLASE BY DISC ELECTROPHORESIS

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Abstract—Analytical and preparative disc electrophoresis have been used for further purification of peptide hydrolase A from germinated barley. Its substrate specificity has been reexamined. Peptide bonds with an aromatic amino acid on the carboxyl side of the bond or lysine on the amino side were readily hydrolyzed.

INTRODUCTION

GERMINATED barley contains peptide hydrolase A (PHA) which has been partially purified and characterized.[1] This enzyme hydrolyzes α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPA) and α-*N*-benzoyl-L-arginine ethyl ester (BAEE). Tests of PHA solutions with other synthetic substrates revealed that additional enzymes were present.[2] Two of these enzymes hydrolyze α-naphthyl acetate (ANA) and another hydrolyzes L-leucyl-β-naphthylamide (LNA). It was therefore desirable to reexamine the substrate specificities of PHA after the removal of these enzymes.

DISCUSSION AND RESULTS

General Technique

Disc electrophoresis as developed by Ornstein and Davis[3] was selected as the method for removal of the contaminating enzymes because of the resolution obtainable through differences in mobility and molecular size. When PHA was electrophoresced on gels that contained traces of persulfate, only faint activity was recovered. It was therefore necessary to clear the gels of persulfate by applying voltage for several hours prior to addition of sample. The same buffer that had been used in the polymerization of the gel was employed for clearing to avoid any change in the pH and ionic characteristics of the gel. There was no difference between stain patterns from experiments with cleared and uncleared gels. Stacking gels were not added until immediately before electrophoresis of the enzyme sample to prevent diffusion of the pH front and impairment of the unique effects of the discontinuous buffer system. It was not necessary to clear the stacking gels polymerized with riboflavin; PHA could be recovered in good yield from separating gels polymerized with riboflavin, but very poor resolution was obtained.

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[2] W. C. BURGER, N. PRENTICE, M. MOELLER and J. KASTENSCHMIDT, *Phytochem.*, in press.
[3] L. ORNSTEIN and B. J. DAVIS, *Ann. N. Y. Acad. Sci.* **121**, 404 (1964).

Gels could be quickly and easily frozen by immersing in a dry ice–acetone bath in either glass or plastic containers, but it was then necessary to wait until the gels softened somewhat in order to cut them into slices for removal of the region containing the enzyme. It was preferable to lay the gels on a glass plate and to freeze them by sprinkling with powdered dry ice. The moist gels frozen to the glass plate were held firmly for convenient cutting. Furthermore the gels froze more slowly than by immersion and, by removing the dry ice after a short interval, the gels could be sliced before they became too hard.

Elution of the gel slices was carried out by allowing the slices to stand in the cold at least 20 hr. With 1 mm slices it was not necessary to homogenize the gel before elution. 95 per cent of the total recovery was found in the first 1 ml wash with 5 per cent in the second ml wash and none in the third. In general, only the first wash was saved.

Results of Small-Scale Electrophoresis with 6 mm Diameter Columns

Protein separations were quite sharp. About 70–80 per cent of the enzyme activity recovered from each column was in the eluates from one or two adjacent 1 mm slices. Activities with BAPA and BAEE substrates were found in the same slices. The ratio of activities with BAPA and BAEE in all eluates was the same which supports previous evidence that both activities are associated with one enzyme, PHA. The R_f for PHA with respect to the tracking dye front was approximately 0·6. The tracking dye did not bind to any of the proteins in the gels.

The purity of the enzyme sample could be further increased by a second electrophoretic treatment. Since a third electrophoretic treatment did not remove four faint bands (two on either side of the band containing the enzyme), these bands may arise from the decomposition of the enzyme during electrophoresis.

Preparative Scale Electrophoresis

In order to purify enough enzyme to test for hydrolysis of peptide substrates and similar compounds, large size columns (10 mm i.d.) were used. The current was kept low so that no heating was apparent during the run. A gel slice containing the enzyme from the first treatment was loaded directly on a second column without elution and immediately electrophoresced again. Recoveries of PHA from the large columns were quite variable ranging from 6 to 48 per cent, with the applied samples of highest purity giving the best recoveries. LNA-ase and ANA-ase activities were completely removed. Specific activity increased from 2·5- to 15-fold with the purer applied samples again giving the best results.

Hydrolysis of Peptide Substrates

When the substrates previously examined with PHA[1] were treated with the highly purified enzyme, the results shown in Table 1 were obtained. The data show that the enzyme is more specific in its substrate requirements than was originally reported. Probably part of the activity reported previously was due to impurities present in the partially purified preparation.

The presence of an aromatic residue on the carboxyl side of the peptide link (Table 1) tended to promote hydrolysis. The presence of lysine on the amino side also resulted in hydrolysis. Most of the other peptides examined were hydrolyzed only very slowly or were not attacked.

TABLE 1. HYDROLYSIS OF PEPTIDE AND ESTER SUBSTRATES BY PHA

Ala-asn	−	Lys-gly	−
Ala-lys	+	Lys-lys	+
Ala-phe	+slow	Phe-leu	+
Ala-ser	−	Trp-gly	+
Ala-val	−	Trp-leu	+
Gly-glu	−	Tyr-gly	+
Gly-leu	+slow	Ala-gly-gly	+slow
Gly-lys	+	Gly-gly-gly	+slow
Gly-phe	+slow	Leu-gly-gly	+
Gly-thr	−	Hippuryl-arg	−
Gly-trp	−	Hippuryl-gly	−
Gly-tyr	−	Tyr ethyl ester	+
Leu-gly	−		

MATERIALS AND METHODS

Barley

Hordeum vulgare, variety Trophy, 1965 crop, which had been stored at −25°.

Reagents

Reagents for electrophoresis gels and buffers were obtained from the Canalco Co., Rockville, Md.; peptides and related compounds for substrate hydrolysis were the same as previously;[1] α-naphthyl acetate was recrystallized from ethanol, m.p. 95°. All solutions used in this study were prepared in glass-distilled water.

Germination and Extraction of Barley: Preliminary Purification

Germination and extraction were carried out as previously described.[1] The extract was purified with columns of carboxymethyl cellulose and Sephadex G-100.[2]

Assay Methods

Protein and PHA activities with BAPA and BAEE substrates were assayed as previously described[1] with 8×10^{-4} M BAEE and 259 nm for the assay with BAEE. ANA-ase and LNA-ase were assayed by a similar procedure:[2] 3 ml of $5{\cdot}38 \times 10^{-4}$ M ANA in 0·05 M phosphate buffer, pH 7·0, was treated with 0·1 ml of appropriately diluted enzyme and the increase in absorptivity at 310 nm was recorded for the initial 5–6 min at 35°; 3 ml of $3{\cdot}42 \times 10^{-3}$ M LNA in 0·05 M succinate buffer, pH 6·5, was treated with 0·1 ml of enzyme of suitable activity and the increase in absorptivity at 335 nm was recorded for the initial 5–6 min at 35°.

Disc Electrophoresis

Electrophoresis was carried out using a modification of the method and apparatus described by Davis.[3] All operations except polymerization were carried out at 4–10°. Stock solutions and gels were prepared according to Davis with the following exceptions: (A) 1 N HCl 48 ml; TRIS 36·3 g; TEMED 0·46 ml; water to make a total volume of 100 ml. (C) Acrylamide 30·0 g; BIS 0·8 g; water to make a total volume of 100 ml. (F) Distilled water. Clearing buffer for reservoirs: 12 N HCl 10·25 ml; TRIS 90·8 g; TEMED 1·15 ml; water to make a total volume of 2 l. Use without dilution. Tracking Dye Solution: Bromphenol blue 1 mg; sucrose 15 g; water to make a total volume of 100 ml. BAPA Solution: BAPA 14·5 mg dissolved in 0·166 ml dimethyl sulfoxide; water to make a total volume of 25 ml.

Small-scale separations were made with 6 × 100 mm i.d. glass columns and preparative ones with 10 × 100 mm i.d. glass columns (the largest which would fit the apparatus). A 7 cm column of separating gel was polymerized in each tube and cleared by running at 10 mA per tube for 4 hr with clearing buffer in both buffer compartments. Gels were stored under fresh clearing buffer until used (up to 24 hr). A 1 cm column of stacking gel was added to the top of the separating gel column just before use.

Each small column was loaded with 20 μl of tracking dye solution and PHA in concentrated sucrose solution. This enzyme solution contained 0·4–0·8 mg protein and 50–100 units of activity with BAPA and had been concentrated to 40–100 μl by ultrafiltration followed by exosmosis with sucrose. Electrophoresis was carried out for 50–70 min at 3–5 mA per tube. Gels were removed from the tubes and partially frozen on

a glass plate by sprinkling with powdered dry-ice. The region from R_f 0·45 to 0·75 was cut with a scalpel into discs approximately 1 mm thick. Each slice was eluted 20–48 hr with 1 ml distilled water. The supernates were assayed and the most active fractions pooled.

Preparative columns were each loaded with about 25-μl tracking dye solution and PHA in concentrated sucrose solution. This enzyme solution contained 5–6 mg protein and 475 to 800 units of activity with BAPA which had been concentrated to 130 to 170 μl as described for the sample for the small columns above. These were electrophoresced at 10 mA per tube until the tracking dye was about 1 cm from the bottom (1·25–1·5 hr). The gels were removed and painted with BAPA solution in a streak along the gel. The yellow *p*-nitroaniline produced by the enzyme revealed its location. After 1–2 min the gels were frozen as above. The yellow band (at about R_f 0·6) was cut out and immediately placed on top of another previously cleared gel column. Tracking dye was added and 10 mA per tube was applied until the tracking dye was about 1 cm from the bottom (1–1·5 hr). The *p*-nitroaniline from the band of gel containing the enzyme stayed near the top of the column and was therefore separated from the migrating enzyme. The gels were removed, cut into slices, and eluted with distilled water as above. The most active eluates were pooled and concentrated by ultrafiltration.

Hydrolysis of Peptide Substrates

The concentrated enzyme solution (3 ml) obtained from electrophoresis was dialysed against four 150-ml changes of 0·05 M phosphate buffer, pH 7·0, over a period of 48 hr before using. Substrates in 0·05 phosphate, pH 7·0, were treated with the enzyme as described previously.[1] The enzyme solution used contained 13 units activity per 0·1 ml with BAPA and had a specific activity of 214 units per mg protein. There was no ANA-ase or LNA-ase activity.