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LOCALIZATION OF AN ACID PROTEINASE IN HEMPSEED

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Abstract—An acid proteinase was detected in a solubilized preparation of dormant hempseed. It has an optimum pH of 3·2 with hemoglobin as substrate, and 4·3 with its natural substrate, edestin. The enzyme is temperature sensitive, the optimal temperature being 53°. Aleurone grains (protein bodies) are the principal subcellular site for the acid proteinase.

INTRODUCTION

THE SUBJECT of protein metabolism in seedlings was thoroughly reviewed by Chibnall[1] as early as 1939. Recently, Oota *et al.*[2] showed further evidence that reserve proteins of seedlings are enzymically hydrolyzed to peptides and amino acids. Since knowledge of the proteinases responsible for hydrolysis of reserve proteins is limited, a detailed study of the characterization and localization of these enzymes is needed.

In an earlier report,[3] we showed that edestin, the reserve protein of hempseed, *Cannabis sativa* L., is present as a crystalloid in subcellular particles, aleurone grains, or protein bodies. As part of this study, we have examined these seeds for the presence of a proteinase to obtain information on catabolic mechanisms of storage proteins as well as intracellular localization of the proteolytic enzymes. In this communication we report the presence of an acid proteinase associated with aleurone grains isolated from dormant viable seed.

RESULTS AND DISCUSSION

The homogenate, precipitate (primarily edestin), supernatant, and fatty layer fractions, prepared from 32 g of hempseed according to the procedure described in the Experimental section, were collected separately and analyzed for their protein content and for their enzymic activity. Each fraction, suspended in 0·01 M phosphate buffer, was adjusted to pH 3·4 with dilute phosphoric acid prior to determination of activity. The distribution of enzyme and protein, as seen in Table 1, shows that the supernatant fraction accounted for 55 per cent of the total activity present in the homogenate. This fraction represented a fourfold purification of the enzyme. Whereas the specific activity of the enzyme in the fatty layer was higher, the total recovery of enzyme was much lower.

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[1] A. C. CHIBNALL, *Protein Metabolism in the Plant*, p. 54, Yale University Press, New Haven (1939).
[2] Y. OOTA, R. FUJII and S. OSAWA, *J. Biochem.* (*Tokyo*) **40**, 649 (1953).
[3] A. J. ST. ANGELO, L. Y. YATSU and A. M. ALTSCHUL, *Archs Biochem. Biophys.* **124**, 199 (1968).

TABLE 1. DISTRIBUTION OF PROTEIN AND ENZYME

Fraction	Total protein (mg)	Protein distribution (%)	Total activity (units)	Enzyme distribution (%)	Specific activity (units/mg protein)
Homogenate	4,920	100	290,000	100	59
Precipitate	3,820	80	43,000	15	11
Fatty layer	225	5	87,000	30	386
Supernatant	728	15	158,000	55	214
Recovery	97%		99%		

The pH optimum for the enzyme in the supernatant fraction was approximately 3·2 with denatured hemoglobin as substrate. As the pH approached neutrality no activity was found. However, the enzyme was active at low pH, suggesting that it be classified as an acid proteinase according to the terminology of Hartley.[4] The enzyme had maximum activity at a temperature of 53°; no enzymic activity was found above 75°.

The time course of the reaction mixture, containing hemoglobin, enzyme, and citrate-phosphate buffer at pH 3·4, was studied (Fig. 1). Time of incubation began as soon as the

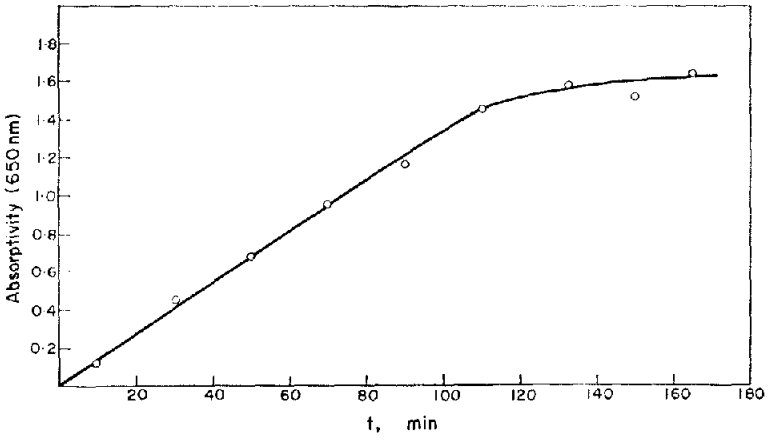

FIG. 1. TIME COURSE STUDY OF THE HYDROLYSIS OF 0·20 mM DENATURED HEMOGLOBIN BY THE ACID PROTEINASE AT 24° WITH SHAKING. THE REACTION MIXTURE CONTAINED 136 mg OF HEMOGLOBIN, 2 ml OF ENZYME PREPARATION, AND 8 ml OF 0·015 M CITRATE-PHOSPHATE BUFFER, pH 3·4.

enzyme was added to the substrate solution. At stated intervals from zero to 170 min, 1-ml portions of the reaction mixture were removed and assayed for absorbance at 650 nm. Results illustrated that the rate of reaction was linear for at least 90 min.

Since the reserve protein is stored within the aleurone grains,[3] it was of interest to determine whether proteinase was also associated with these organelles. Aleurone grains, which swell and eventually burst in aqueous media, were isolated by the method of Yatsu and Jacks.[5] Viable hempseeds, 5 g, were homogenized in 30 ml of glycerol, filtered through

[4] B. S. HARTLEY, *Ann. Rev. Biochem.* **29**, 45 (1960).
[5] L. Y. YATSU and T. J. JACKS, *Archs Biochem. Biophys.* **124**, 466 (1968).

cheesecloth, and centrifuged at 1100 × *g* for 5 min. The precipitate contained cell debris, some aleurone grains, and unbroken cells with their complement of aleurone grains, but the bulk of aleurone grains were contained in the supernatant fraction. A 4-ml portion of this supernatant liquid was removed for analysis, and the remainder of the supernatant liquid, containing 19,000 units of activity, was centrifuged at 35,000 × *g* for 20 min. The purified aleurone grains collected in the pellet were washed with glycerol and recentrifuged. Finally, they were homogenized with 0·01 M phosphate buffer, pH 7·4, adjusted to pH 3·2 with dilute phosphoric acid, and assayed for proteolytic activity. Approximately 75 per cent of the total

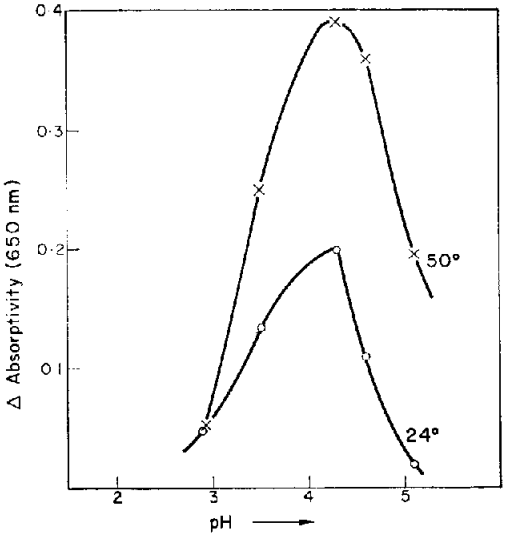

FIG. 2. EFFECT OF pH AT TWO DIFFERENT TEMPERATURES ON EDESTINASE ACTIVITY WITH 1·5% EDESTIN AS SUBSTRATE. EACH TUBE CONTAINED 15 mg OF EDESTIN, 0·2 ml OF ENZYME (4·8 mg PROTEIN/ml), 0·8 ml OF 0·1 M SODIUM ACETATE-ACETIC BUFFER.
Experiments were done in triplicate with mechanical shaking for those tubes at 24°. Tubes incubated at 50° were not subjected to shaking. Activity was reported as the change in absorptivity per hr of incubation.

enzymic activity was found in this homogenate, which suggested that the enzyme is associated with the aleurone grains. These findings are similar to recent reports of Yatsu and Jacks,[5] and Matile,[6] who attempted to show that plant aleurone grains were analogous to animal lysosomes. These authors reported finding various hydrolytic enzymes associated with aleurone grains of cottonseed and pea, respectively. Included in their observations were proteases. The enzyme from cottonseed was active over a broad range of pH in the acid region; there appeared to be two pH optima, the first at pH 2, and a second at pH 3·4. No attempt was made to elucidate the pH optimum of the pea protease. The hempseed proteinase, however, has an optimum pH at 3·2, and was virtually inactive below pH 2 or above pH 5.

In hempseed, edestin, the major storage protein that provides the amino acids needed by the germinated seedling, is located inside the aleurone grains (protein bodies).[3] In an attempt to better understand the catabolism of reserve proteins, we tested for enzyme activity on the natural substrate, edestin, in addition to the exogenous substrate, hemoglobin. Results, shown in Fig. 2, demonstrated that the enzyme was indeed capable of catalyzing the hydrolysis of edestin.

[6] P. MATILE, *Z. Pflanzenphysiol.* **58**, 365 (1968).

Enzyme activity was found over a pH range from 3 to 5, but optimum activity was found at pH 4·3, a shift from 3·2, the optimum observed with hemoglobin as substrate. Optimum activity was found at pH 4·3 whether the reaction was run at 24 or at 50°; the latter is the optimal temperature for activity of the enzyme. When the reaction was run at 50° and pH 4·3, activity doubled. The optimum pH observed with edestin as substrate is probably closer to the natural conditions for hydrolysis *in situ*.

In summary, it may be concluded that acid proteinase, which is associated with the same subcellular sites as reserve protein, namely in the aleurone grains, is probably the enzyme that hydrolyzes edestin to peptides and amino acids for utilization by the germinating seedling.

EXPERIMENTAL

Materials

Viable hempseed was purchased from the Philadelphia Seed Company, Philadelphia, Pennsylvania, with permission obtained under U.S. Treasury Department Marihuana Order No. A 5064. Hemoglobin and bovine serum albumin were purchased from Sigma Chemical Company, St. Louis, Missouri. Edestin was prepared from isolated aleurone grains as described previously.[3]

Enzyme Preparation

The acid proteinase of viable hempseed was isolated and partially purified as follows. 32 g of viable hempseed were homogenized in an Omnixer with 150 ml of 0·01 M sodium phosphate buffer, pH 7·4, 0°. After homogenizing for 120 sec with 20-sec alternating intervals, the homogenate was strained through four layers of cheesecloth and, after withdrawing a 4-ml portion for subsequent assaying, the remaining homogenate was centrifuged at 20,000 × *g* for 20 min. The temperature was controlled between 0 and 4° throughout all operations. The precipitate and fatty layer fractions were collected and stored separately at 4° until analyzed. The supernatant was recentrifuged. The small precipitate and fatty layer fractions were added to those fractions obtained after the first centrifugation step and each fraction, including the homogenate, was then assayed for protein content as well as enzymic activity. Quantitative data shown in Table 1 suggested that the supernatant contained approximately 55 per cent of the enzymic activity. Hence, the supernatant fraction was used as the source of enzyme for all subsequent experiments.

Assay Methods

Protein analysis was accomplished by use of the Lowry Method.[7] Bovine serum albumin was used as the protein standard.

Determination of proteinase activity in fractions tested was accomplished by the method of Anson,[8] with minor modifications. The reaction consisted of 0·2 ml of enzyme fraction, 0·1 ml of hemoglobin (0·001 M), and 0·7 ml of 0·01 M phosphate buffer adjusted to pH 3·4 with dilute phosphoric acid. Time of incubation was 30 min at 25°. Zero time samples contained, in addition to the above, 2 ml of 5% cold trichloroacetic acid (TCA). After incubation 2 ml of TCA was added to each tube to stop enzymic activity. Tubes were centrifuged twice at 1100 × *g* for 5 min. The clear supernatant, in 1-ml portions, was removed; 2 ml of 0·5 M NaOH were added to each tube. Finally, 0·6 ml of Folin-Ciocateau phenol reagent (diluted 1:2 with water) was added to each tube and the absorbance of each tube was read at 650 nm. One unit of activity was defined as a change of 0·001 absorptivity unit at 650 nm per hr.

[7] O. H. LOWRY, N. H. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[8] M. L. ANSON, *J. Gen. Physiol.* **22**, 79 (1938).