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# PURIFICATION OF ACID PROTEINASE FROM *CANNABIS SATIVA* L.

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**Abstract**—Edestinase, an acid proteinase, was found in the supernatant fraction obtained by centrifuging a homogenate of resting seeds of *Cannabis sativa* L. in phosphate buffer. When 50% ammonium sulfate was added to the supernatant at pH 7·5 the enzyme was found in a fatty layer that formed after high-speed centrifugation. However, if the supernatant were acidified to pH 3·4, then made to 50% ammonium sulfate, the enzyme precipitated upon centrifugation. After solubilization of this precipitate in pH 3·4 phosphate buffer, the enzyme was purified 25-fold. The proteinase contains only one component when examined by the analytical ultracentrifuge; the sedimentation coefficient is 1·34 and the estimated molecular weight, 20,000. By gel electrophoresis, there are at least three protein components present.

## INTRODUCTION

DURING germination, the reserve proteins of seeds are hydrolyzed by proteolytic enzymes into peptides and amino acids which are then translocated to growing parts of the embryo for subsequent synthesis of new protein.[1] Knowledge of these enzymes is limited and virtually nothing is known about their regulation. In a preliminary report,[2] we have shown that dormant hempseed contains an active proteinase associated with the aleurone grain fraction, the organelle in which the reserve protein, edestin, is also located.[3] This proteinase exhibited a pH optimum of 3·2 with hemoglobin as substrate and showed highest activity at 50°. The pH optimum shifted to 4·3 when its natural substrate edestin was used. Recent reports on aleurone grains of cottonseed[4] and pea[5] showed these seeds to contain a number of enzymes, e.g. $\beta$-amylase, $\alpha$-amylase, $\alpha$-glucosidase, esterase, acid phosphatase, and acid proteinase; however, a detailed study of them has not been reported.

As part of a study on edestin, the heat-stable major storage protein of *Cannabis sativa* L., and the auxiliary enzymes which provide amino acids needed by the growing seedling, we report here the purification and physical properties of an acid proteinase from viable hempseed.

## RESULTS

The flow sheet for the purification procedure is presented in Fig. 1. The quantitative data from a representative purification of acid proteinase of *Cannabis sativa* L. starting with 64 g

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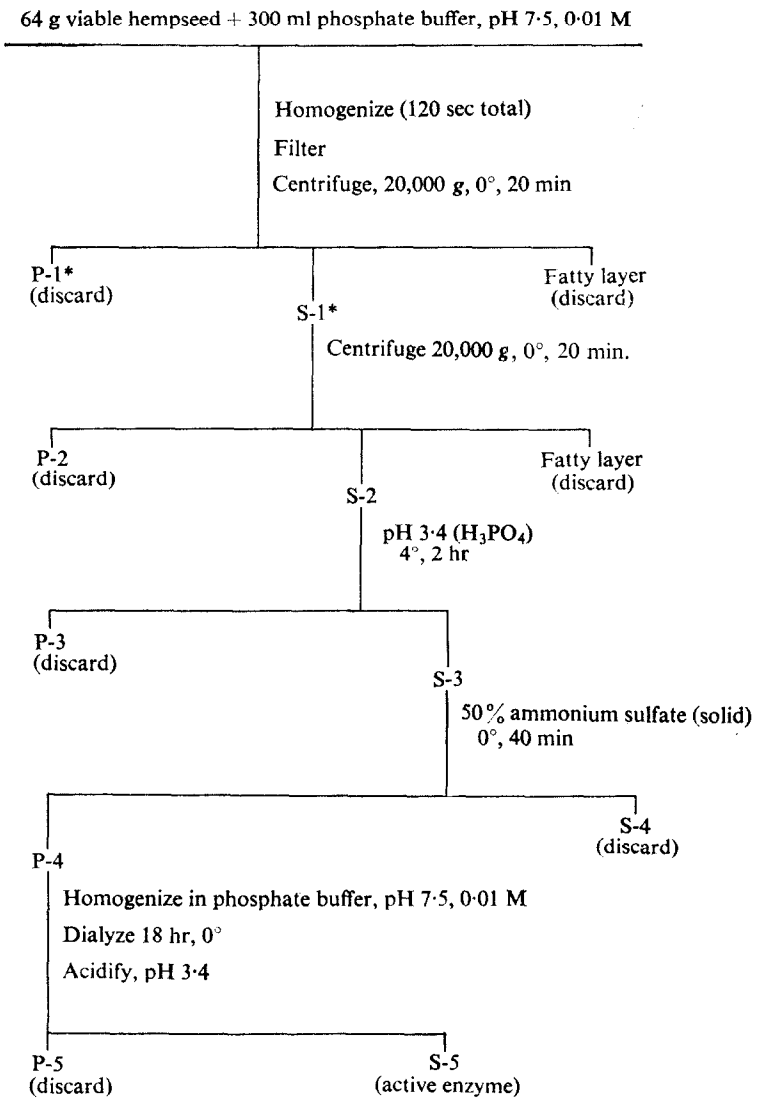


FIG. 1. PURIFICATION OF ACID PROTEINASE FROM HEMPSEED.
* (P = precipitate, S = supernatant.)

TABLE 1. FRACTIONATION OF EDESTINASE

| Fraction | Total activity (units × $10^{-3}$) | Specific activity (units/mg protein) | % Recovery |
|---|---|---|---|
| Homogenate | 468 | 38 | 100 |
| S-2 | 297 | 99 | 64 |
| S-3 | 292 | 207 | 62 |
| S-5 | 277 | 790 | 59 |

of whole ungerminated hempseed is shown in Table 1. The acidic supernatant, S-3, contained 62 per cent of the total activity, and 11 per cent of the protein. The final supernatant, S-5, contained 59 per cent of the proteinase activity. The specific activity of this preparation was 790 units per mg of protein, which represented a 25-fold purification, from the original homogenate. The S-5 fraction was used as the source of enzyme in the ultracentrifugation and electrophoretic studies.

In an earlier attempt to isolate the acid proteinase the S-2 supernatant fraction at pH 7·5 was made to 50 per cent saturation with crystalline ammonium sulfate, stirred, incubated for a total of 40 min, then centrifuged at 20,000 *g* for 20 min. Surprisingly, no precipitate appeared but a thick fatty layer above the supernatant was found to contain all of the enzymic activity. This fatty layer was resuspended in the same buffer and homogenized in a Potter–Elvejhem tube. The enzyme remained in suspension and appeared to be either particulate-bound or lipid-bound since a strong Tyndall effect was evident. The specific activity of the acid proteinase prepared in this manner increased twofold. Using a sucrose density gradient, the density of the suspended enzyme appeared to be between 1·01 and 1·03 suggesting that it might be bound to lipid or to a membrane. Attempts to completely solubilize the suspended enzyme were unsuccessful. Therefore, the procedure shown in Fig. 1 was employed to prepare the enzyme.

To determine if edestinase, fraction S-5, contained any lipid, 43 mg of enzyme preparation were dialyzed against deionized water and lyophilized. The dry, white, fluffy powder was extracted with methanol/chloroform (1:2) and analyzed for lipid composition by paper chromatography.[6,7] The results showed slightly more neutral lipids than phospholipids. In either instance, the small quantities present (less than 3 per cent combined) were considered insufficient to classify the enzyme as a lipoprotein. The neutral lipids included triglycerides, free fatty acids, and a third component whose $R_f$ was indicative of a fatty alcohol. The phospholipids were primarily phosphatidyl inositol, ethanolamine, and lecithin, all in trace quantities.

A recent report of a diglyceride-bound lipoprotein[8] stated that its solubility was effected by changes in either pH or ionic strength of the buffer. The diglyceride was believed to be conjugated to a single protein. There are other reports on the effect of pH change on the removal of lipid from albumin.[9,10] William and Foster[10] demonstrated the removal of lipid material from bovine serum albumin by lowering the pH to 3·0 and allowing the solution to stand for 2–3 days at 5°. The insoluble lipid material was filtered off and the protein recovered in the filtrate. It therefore seems possible that the acid proteinase of hempseed, which is found in fraction S-2, pH 7·5, may be similarly bound to lipid matter, and that this complex was broken by incubation in an acid medium. After the complex was broken, the enzyme could be removed by salting out with ammonium sulfate.

*Ultracentrifugation*

The acid proteinase appeared as a single component when examined in the analytical ultracentrifuge. Approximately 33 min after the centrifuge reached top speed the enzyme began to move away from the meniscus. The rate of sedimentation suggested that the

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proteinase is a small molecule; the sedimentation coefficient was 1·34. When this value was substituted into Svedberg's formula,[11] a molecular weight of approximately 20,000 was obtained for the enzyme. This value is comparable to molecular weights of a number of other proteolytic enzymes; e.g. 21,000 for papain, 23,000 for chymotrypsin, 34,000 for trypsin, and 35,000 for pepsin.[12]

*Electrophoresis*

20 ml of enzyme preparation, S-5, were lyophilized overnight and the dry powder (79·5 mg) solubilized in 3 ml of deionized water. The preparation was examined by polyacrylamide gel electrophoresis and showed three bands after staining with amido black. Two were very prominent bands and the third a minor constituent. However, a photometric tracing of the gel showed that the broadest band is composed of at least two components with very similar electrophoretic mobilities.

## DISCUSSION

The results presented here are primarily concerned with the purification and physical properties of a proteolytic enzyme that is associated with aleurone grains of hempseed.[2] Only recently have acid hydrolases been discovered in higher plant tissue, and in only a few cases have their subcellular localization been investigated, e.g. Yatsu and Jacks,[4] found the occurrence of acid proteinase in aleurone grains of dormant cottonseed, and Matile[5] reported acid proteinase activity in aleurone grains of germinating pea. Indeed, aleurone grains in seed tissues may be the equivalent to lysosomes[13, 14] with respect to degradative enzyme activity.

The acid proteinase in hempseed appeared to be associated with some lipid material, presumably a phospholipid membrane that surrounds the aleurone grain as reported in wheat endosperm.[15, 16] Depending upon the pH of the supernatant (S-2) prior to ammonium sulfate treatment, the enzyme was recovered in either the precipitate, P-4, or in a fatty layer obtained after high-speed centrifugation. The enzyme isolated by the scheme shown in Fig. 1 contained very little lipid, but preliminary studies showed that the enzyme in the fatty layer contained an appreciable amount of lipid. Research on the nature of the latter enzyme system has been initiated and will be reported separately.

## EXPERIMENTAL

*Materials*

Viable hempseed was purchased from the Philadelphia Seed Company, Philadelphia, Pennsylvania, with permission obtained under U.S. Treasury Department Marihuana Order No. A5064. Bovine serum albumin and hemoglobin were purchased from Sigma Chemical Co.,* St. Louis, Missouri.

* Trade names are given in reporting exact experimental conditions; no recommendations of the products over others of similar manufacture are implied.

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*Assay Procedure*

Enzymic activity was determined by a modification of the method of Anson:[17] the reaction mixture consisted of 0·2 ml of enzyme, 0·1 ml of 0·001 M hemoglobin as substrate, and 0·7 ml of 0·01 M Na phosphate buffer, then adjusted to pH 3·4 with dil. $H_3PO_4$. Zero time samples contained, in addition to the above, 2 ml of 5% cold trichloroacetic acid (TCA). After incubation for 30 min at 25°, 2 ml of the TCA solution was added to stop enzymic activity and the tubes were centrifuged twice at 1100 *g* for 5 min. The clear supernatant was removed and 2 ml of 0·05 M NaOH was added followed by 0·6 ml of Folin–Ciocateau phenol reagent (diluted 1:2 with water). The absorbance of the solutions were read at 650 nm. One unit of enzymic activity is defined as a change of 0·001 absorbance unit at 650 nm/hr. Protein concentrations were determined according to the method of Lowry *et al.*,[18] using bovine serum albumin as the standard. Appropriate blanks using only hemoglobin or enzyme showed no activity. This enzyme did not require any known activators, cofactors or other additives for optimum activity.

*Ultracentrifugation*

Sedimentation velocity measurements were made on a 1% solution of the acid proteinase at 24° in a Spinco model E ultracentrifuge at 59,780 rev./min, bar angle 60°. The sedimentation coefficient reported in Svedberg units corrected to $S_{20,w}$, was determined by the moving boundary method, and the molecular weight determined according to the formula of Svedberg.[11]

*Electrophoresis*

Electrophoretic patterns of acid proteinase solutions were run on polyacrylamide gel sheets using a vertical cell, manufactured by E. C. Apparatus Company, by the method of Evans *et al.*[19] Photometric tracings of electrophoretic patterns were scanned and recorded with a Photovolt Densitometer.

*Purification of Acid Proteinase from Viable Hempseeds*

See Fig. 1.

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