Isoenzymes of Acid Phosphatase in Germinating Peas

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Summary. Acid phosphatase activity in pea cotyledons increases during germination. Gel filtration separates three isoenzymes with pH optima of 4.9, 5.6 and 6.0. The relative activities of these isoenzymes change during germination. The increase in activity is confined to two of the three isoenzymes. Density-labelling experiments show that these are synthesised de novo. Activity of the third isoenzyme, which is present in dry seeds, does not change significantly during germination, and is not affected by cycloheximide but is lost on freezing. The possible localization of each isoenzyme is discussed in the light of these findings and in relation to histochemical studies.

Introduction

Acid phosphatase (orthophosphoric monoester phosphorylase, EC 3.1.3.2) is widely distributed in plant tissues. Histochemical procedures have shown that the enzyme is localized both in the cell wall and in the cytoplasm (Atkinson and Polya, 1967; Flinn and Smith, 1967; Gilder and Cronshaw, 1973; Hall, 1971; Poux, 1970; Sexton et al., 1971). Fractionation of plant extracts by gel filtration (Atkinson and Polya, 1967; Ikaya et al., 1964; Sexton and Sutcliffe, 1969) and gel electrophoresis (Bessemmer and Claus, 1968; Meyer et al., 1971; Reid and Bieleski, 1970) reveals a number of isoenzymes of acid phosphatase but the subcellular distribution of the isoenzymes is not known. Changes in enzyme activity are associated with many stages of development, including germination (Flinn and Smith, 1967; Meyer et al., 1971), differentiation (Hall, 1971; Sexton et al., 1971; Sexton and Sutcliffe, 1969; Robards and Kidwai, 1969), and wounding (Jones and Villiers, 1972). Little is known, however, about the regulatory mechanisms governing acid phosphatase activity. We have studied the changes in activity of isoenzymes of acid phosphatase in cotyledons of germinating seeds of *Pisum sativum* cultivar Alaska. Evidence concerning the subcellular distribution and regulation of acid phosphatase activity has
been obtained from a study of the properties of the isoenzymes and from density-labelling experiments.

**Materials and Methods**

*Preparation of Acid Phosphatase Extracts.* Seeds of *Pisum sativum* cultivar Alaska were surface sterilized by immersing them for 10 minutes in 1% sodium hypochlorite solution. They were then washed several times in sterile distilled water and soaked for 24 hours in sterile distilled water. The imbibed seeds were planted in vermiculite and grown at 25°C for the required period with daily watering. Some seeds were soaked in, and watered with, 1 M phosphate buffer pH 7.0. Seeds for density-labelling experiments were grown on sterile H₂O or sterile ²H₂O in boiling tubes containing autoclaved sand. Cotyledons were excised immediately prior to use and their testas removed. Twenty cotyledons were weighed and then homogenised with a mortar and pestle in 0.02 M acetate buffer pH 5.5 with a little acid washed sand. The homogenate was filtered through two layers of muslin and then centrifuged at 4000 ×g for 10 minutes. The supernatant was used for enzyme assay.

*Gel Filtration.* For most experiments a column 20 × 300 mm containing Sepharose 4 B was used. For the preparation of larger aliquots of the isoenzymes for density-labelling experiments a column 50 × 250 mm was used. The columns were maintained at 4°C and were equilibrated with 0.02 M acetate buffer pH 5.5. With the smaller columns 0.3 ml of enzyme extract was applied and fractions of 4 drops (0.2 ml, approx) were collected. In the case of the larger column 1.5 ml of extract was used and 40 drop fractions collected.

**Assay of Acid Phosphatase**

(a) With p-nitrophenyl phosphate as substrate. To each fraction to be assayed was added 0.5 mg p-nitrophenyl phosphate dissolved in 1.0 ml 0.2 M acetate buffer pH 5.5. The reaction mixture was incubated for a suitable time (between 15 and 120 min) and then the reaction was stopped with 2.0 ml of 10% sodium carbonate solution. The absorbance at 410 nm was measured using a Pye Unicam SP 1800 spectrophotometer.

(b) With other substrates. Activity was determined by measuring the release of inorganic phosphate. The method of Ames (1966) was used. After incubation of the enzyme preparation with the substrate for a suitable time the reaction was stopped using 1.0 ml of a mixture of one part 10% ascorbic acid and six parts of freshly prepared 0.42% ammonium molybdate in sulphuric acid. The absorbance at 810 nm was measured using a Pye Unicam SP 1800 spectrophotometer.

*Equilibrium Density Gradient Centrifugation.* Gradients were prepared and ultracentrifugation carried out using the method of Johnson et al. (1973). 0.1 ml of extract was layered over 5.0 ml of Caesium chloride of buoyant density 1.32 g ml⁻¹ and centrifuged at 45000 rev/min in a Spincce Type 65 fixed-angle rotor and a Beckman L2-65 or 65 B ultracentrifuge for 40–60 hours. Fractions were collected by gently lowering a narrow gauge needle to the bottom of the centrifuge tube and removing the solution with a perstaltic pump. Three drop fractions were collected. The refractive index of every tenth fraction was measured using an Abbé 60 refractometer and these values were converted to buoyant density units using the formula of Ifft, Voet and Vinograd (1961).