ATPase and Acid Phosphatase Activities Associated with Vacuoles Isolated from Storage Roots of Red Beet (Beta vulgaris L.).

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Abstract. Phosphatase activities were measured in preparations of vacuoles isolated from storage roots of red beet (Beta vulgaris L.). The vacuoles possessed both acid phosphatase and ATPase activities which could be distinguished by their susceptibility to inhibition by low concentrations of ammonium molybdate [(NH₄)₆Mo₇O₂₄·4H₂O]. The acid phosphatase was completely inhibited by 100 μM ammonium molybdate but the ATPase was unaffected. The acid phosphatase was a soluble enzyme which hydrolysed a large number of phosphate esters and had a pH optimum of 5.5. In contrast, the ATPase was partially membrane-bound, had a pH optimum of 8.0 and hydrolysed ATP preferentially, although it was also active against PP_i, GTP and GDP. At pH 8.0 both the ATPase and PPase activities were Mg²⁺-dependent and were further stimulated by KCl. The ATPase and PPase activities at pH 8.0 may be different enzymes. The recovery and purification of the ATPase during vacuole isolation were determined. The results indicate that the Mg²⁺-dependent, KCl-stimulated ATPase activity is not exclusively associated with vacuoles.

Key words: Acid phosphatase – ATPase – Beta – Pyrophosphatase – Vacuoles.

Introduction

Plant vacuoles contain high concentrations of ions and other solutes which are accumulated against their chemical potential gradients (MacRobbie 1971; Matile 1978). The mechanisms responsible for the active transport of these solutes at the tonoplast have not been identified but it has been suggested that an ATPase might be involved (Runnie and Wiskich 1973; Hodges 1976; Lin et al. 1977; Doll et al. 1979; Guy et al. 1979). Histochemical studies have shown that the tonoplast apparently possesses ATPase activity (Hall 1971) but detailed biochemical investigations have been limited by the difficulty of identifying tonoplast in conventionally-prepared membrane fractions (Quail 1979). Nonetheless the conclusion that tonoplast must possess ATPase activity has been supported by the demonstration of membrane-bound ATPase activity in vacuole-like structures (lutoïds) isolated from Hevea brasiliensis latex (d’Auzac 1975). This has been further substantiated following the development of methods for the isolation of intact vacuoles (e.g. Wagner and Siegelman 1975; Leigh and Branton 1976; Nishimura and Beevers 1978; Saunders and Conn 1978; Boller and Kende 1979). Lin et al. (1977) found membrane-bound, salt-stimulated ATPase activity associated with vacuoles isolated from petal and leaf protoplasts. Recently Doll et al. (1979) have shown that mechanically isolated beet vacuoles possess Mg²⁺-dependent, KCl-stimulated ATPase activity.

Acid phosphatase activity is also detectable in isolated vacuoles (d’Auzac 1975; Butcher et al. 1977; Nishimura and Beevers 1978; Leigh et al. 1979; Boller and Kende 1979; Mettler and Leonard 1979). When studying ATPase activities of vacuoles it is important to establish that acid phosphatase is not interfering with the measurement of specific ATPase activity. For instance, Doll et al. (1979) measured ATPase...
activity in isolated beet vacuoles and found highest activity at pH 6. Unfortunately they did not determine whether the acid phosphatase, that is also present in beet vacuoles (Leigh et al. 1979), was contributing to the activity measured at this pH. Mettler and Leonard (1979), working with tobacco suspension cell vacuoles, have shown that in their preparations the majority of ATPase activity measured at pH 6 was due to non-specific acid phosphatase activity. Doll et al. (1979) have suggested that the ATPase activity which they measured in beetroot vacuoles is involved in sucrose transport at the tonoplast. In order to verify the validity of this suggestion it is important to determine whether the activity measured by these authors was a specific ATPase or a non-specific acid phosphatase, and whether the ATPase activity is membrane-bound.

In the work described in this paper we attempted to distinguish non-specific acid phosphatase and specific ATPase activities associated with isolated beet vacuoles by inhibiting the former enzyme with low concentrations of ammonium molybdate. This compound has been used previously to distinguish these two activities in preparations of lutoïdes from *Hevea brasiliensis* (d’Auzac 1975). Our results indicate that the majority of ATPase activity measured below pH 7 is due to a non-specific acid phosphatase activity, but above this pH a more specific enzyme operates. The specific ATPase activity is partially membrane-bound.

**Materials and Methods**

**Plant Material.** Storage roots of red beet (*Beta vulgaris* L.) were obtained locally and were used at once or after storage for up to six months in moist vermiculite in covered bins on the roof of the Botany School.

**Isolation and Subfractionation of Vacuoles.** Vacuoles were isolated by the method of Leigh and Branton (1976) as modified by Leigh et al. (1979) except that the unfractionated homogenate was centrifuged at 3,500 g for 15 min. The collection medium contained 25 mM 2-mercaptoethanol and all other solutions contained 5 mM 2-mercaptoethanol. Purified vacuoles were removed from the Mitrazamide gradient with a Pasteur pipette and the other fractions were usually discarded. For the experiment described in Table 4 all fractions were retained. The nomenclature for these fractions is that used previously (Leigh et al. 1979). For the experiments described in Table 3 vacuoles were separated into soluble and membrane fractions. Vacuoles were lysed by pipetting one volume of vacuoles into two volumes of ice-cold dilution buffer (1 mM Na₂EDTA, 5 mM 2-mercaptoethanol, 10 mM Tris-MES, pH 7.6). Examination with a light microscope showed that all vacuoles were disrupted by this procedure. A sample of the lysed vacuoles was retained for assays and the remainder was centrifuged at 100,000 g for 2.5 h in a Beckman Type 42.1 rotor operated at 4°C. The supernatant was removed and retained and the pellet was resuspended in vacuole resuspension medium (Leigh et al. 1979) diluted with dilution buffer so that the final concentration of sorbitol was the same in all fractions. This was necessary because sorbitol, which is the osmoticum used to stabilise vacuoles (Leigh and Branton 1976), interfered with the Pi assay used but this interference could be overcome over particular sorbitol concentration ranges (see below; Leigh and Walker, in press).

**Enzyme and Chemical Assays.** The ATPase and acid phosphatase assays described below were those used routinely. In some experiments the assay conditions were varied and these deviations are noted in the appropriate figure and table legends. Non-enzymic breakdown of substrates was determined from samples incubated with boiled vacuoles or with boiled subfractions obtained from vacuoles.

ATPase was assayed in 1.5 ml capped microcentrifuge tubes. The assay medium contained 37.5 mM Tris-MES, pH 8.0, 3 mM Tris-ATP, 3 mM MgSO₄, 50 mM KCl and 100 μM ammonium molybdate [(NH₄)₆MoO₄·4H₂O] in a final volume of 0.3 ml. Reaction was started by the addition of a sample of vacuoles (or vacuole subfraction) containing 2-10 μg of protein. Samples were incubated at 30°C for up to 30 min and reaction was stopped by the addition of 0.7 ml of precipitation mixture (7.15 mM ammonium molybdate in 5% [w/v] TCA) and 0.1 ml of 10 mg ml⁻¹ BSA. Samples were left on ice for 30 min to ensure complete protein precipitation. There was no significant breakdown of ATP during this period. Pi release was measured by the method of Leigh and Walker (in press). Precipitated samples were centrifuged for 3 min in a Beckman Microfuge B. The clear supernatant was discarded and the yellow-green pellet, which contained all the Pi present in the original sample (Leigh and Walker, in press), was washed with 1 ml of 7% [w/v] TCA. The washed pellet was then dissolved in 0.1 ml of 0.5 M Tris by leaving at 4°C overnight or by vigorous agitation at room temperature. Protein was reprecipitated by adding 0.5 ml of water then 0.5 ml of 35% [w/v] TCA and centrifuging as above. Pi remained in the supernatant under these conditions. One ml of the supernatant was transferred to a test tube and was mixed with 0.6 ml of assay mixture (6 parts of 50 mM ammonium molybdate in 0.5 M H₂SO₄ mixed with 1 part of 10% [w/v] ascorbic acid immediately before use, see Ames 1966). The samples were incubated at 37°C for 1 h and the absorbance at 820 nm was measured. Pi release was determined from a calibration curve prepared from sorbitol-containing Pi standards that were precipitated and assayed at the same time as the unknown samples.

Acid phosphatase was measured in a medium containing 50 mM acetic acid-Tris, pH 5.5 and 3 mM disodium p-nitrophenylphosphate. The final volume was 1 ml. Reaction was started by adding a sample of vacuoles (or vacuole subfraction) containing 4-20 μg of protein. Samples were incubated at 30°C for up to 1 h and reaction was stopped by the addition of 2 ml of 10% [w/v] Na₂CO₃. The absorbance at 410 nm was measured and p-nitrophenol release was estimated by comparison with a standard curve of p-nitrophenol in 10% Na₂CO₃, measured at the same wavelength. For the experiment described in Table 1 (substrate specificity) the total volume was reduced to 0.3 ml, proportionally less vacuolar protein was used and substrate hydrolysis was measured by determining Pi, release as described for ATPase.

Phospholipids were extracted by dissolving 0.5 ml of sample in 12 ml of chloroform: methanol (2:1). The extracts were washed with 3 ml of 0.9% [w/v] NaCl, then with 2 ml of water and were evaporated to dryness in a stream of nitrogen. Combustion of lipid and estimation of Pi, were as described by Ames (1966). Protein was estimated by the method of Lowry et al. (1951) after the samples had been precipitated and washed with ice-cold 7% [w/v] TCA. BSA was used as a standard. Betanin was determined as previously described (Leigh et al. 1979).

**Chemicals.** Common laboratory reagents and chemicals not mentioned below were obtained from B.D.H. Chemicals Limited, Poole, Dorset, U.K. BSA was from Armour Pharmaceutical Com-