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Molecular forms of phosphatase and ribonuclease in phosphate deficient and N,N-dimethylmorpholinium chloride treated *Spirodela oligorrhiza (Lemnaceae)*

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Abstract

Soluble, membrane bound, and extracellular phosphatases (EC 3.1.3.2 and 3.1.3.1) of control, N,N-dimethylmorpholinium chloride (DMMC) treated, and phosphate deficient (—P) axenic *Spirodela oligorrhiza* plants were analysed by Sephadex G-150 gel filtration. Soluble, acid enzymes of control plants were separated into two molecular forms with apparent MW ≧ 400 000 and 85 000. Phosphatase with MW 34 000 replaced the latter isoenzyme in the presence of DMMC. Two alkaline enzymes with apparent MW 210 000 and 36 000 were detected in —P plants.

Triton X-100 solubilized a number of acid and alkaline phosphatases from membrane material. DMMC caused the appearance of two membrane bound enzymes (MW 48 000 and 14 000) which were not detected in the control. Senescing control and DMMC treated plants released an acid phosphatase (MW 48 000; pH optimum 5.2) into the nutrient medium. —P plants released, in addition ,an alkaline phosphatase (MW 170,000; pH optimum 7.8-8.2). Ribonucleases (EC 2.7.7.17.) with apparent MW 31 000 and 28 000 daltons were induced by DMMC and —P, respectively.

INTRODUCTION

Unspecific acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) is ubiquitous in plants. The enzyme is heterogenous upon chromatography and electrophoresis (Bailey et al. 1976; Foster and Weber 1973; Kubicz et al. 1972; Lorens-Kubis et al. 1975; Rychter et al. 1972; Murray and Collier 1977; Papageorgakopoulou and Georgatsos 1977). Total activity

Abbreviation used: DMMC, N,N-dimethylmorpholinium chloride; NPP, p-nitrophenylphosphate; —P, phosphate deficiency; M, nutrient medium; MB and S, membrane bound and soluble enzymes; Ac, acid; Alk, alkaline.

of this enzyme markedly increases in plants grown in conditions of mineral deficiency (Hewitt 1958; Hewitt and Tatham 1960). Some micronutrients, *e.g.* zinc, may decrease the phosphatase activity in plants (Cox and Thurman 1978).

Soluble phosphatase of *Spirodela oligorrhiza* (Kurz) Hegelmaier, a small aquatic plant of the family *Lemnaceae* (McClure and Alston 1966), has pH optimum 5.7-6.0 (Bieleski 1974) and it exists in two molecular forms (Knypl 1976). In a response to phosphate deficiency two isoenzymes of alkaline phosphatase (EC 3.1.3.1) are synthesized (Reid and Bieleski 1970a; Bieleski 1974). These derepressible enzymes have a pH optimum at 7.5, and their relative activity prevails manyfold over the activity of a constitutive acid enzyme. The alkaline enzyme is not released into the nutrient medium (Bieleski 1974). Nevertheless, around 40% of the alkaline phosphatase activity appears to be available to phosphate esters in the external medium, *i.e.* the enzymes seem to be localised externally on the outer surface of the cells (Bieleski 1974), possibly in the plasmalemma (Knypl and Sobolewska 1978). There are evidences that both acid and alkaline phosphatase activity is released by the plants to the external medium in some "special" conditions (Knypl 1976b).

It has recently been reported that N,N-dimethylmorpholinium chloride (DMMC), a plant growth retardant (Knypl et al. 1976), increased total phosphatase activity in *Spirodela*, and that phosphate deficiency caused the release of some phosphatase activity into the nutrient medium (Knypl 1977). A considerable phosphatase activity was also detected in tissue debris remaining upon the extraction of the soluble enzymes; this residual activity was particularly marked in senescing and –P plants (Knypl and Kabzińska 1977). This fact seems to indicate that in *Spirodela* a proportion of phosphatase activity is associated with the cell membranes (Knypl and Sobolewska 1978), as it is in other plants (Hirai and Asahi 1973; Williams and Juo 1976). The aim of this study was to analyse multiple forms of soluble, membrane bound and extracellular phosphatases in the control, DMMC treated and phosphate deficient *Spirodela* cultures. Since –P and treatments with DMMC induce high RNase activity (Reid and Bieleski 1970a; Knypl 1976b; Knypl and Kabzińska 1977), analyses of this enzyme were also performed.

MATERIAL AND METHODS

Plants

Axenic *Spirodela oligorrhiza* (Kurz) Hegelm. was grown according to Bollard (1966) under continuous illumination (1.5 Klx) produced by fluorescent tubes (Flora LF40W, Unitra-Polam) at 25°C in Roux

bottles containing 200 ml of nutrient media. The control nutrient medium (C) contained 1.0% glucose, macro- and microelements, and 5 mM ammonium sulphate as sole N source (K n y p l 1976a). This medium was supplemented with 0.4 mM of filter sterilized DMMC (DMMC-treated plants) or deprived of orthophosphate (—P plants). In the latter case K_2HPO_4 (1 mM) was replaced by K_2SO_4. The nutrient media were buffered with solid sterile $CaCO_3$ added after autoclaving (B o l l a r d 1966), then inoculated with *ca.* 70 fronds (control series) or 200-400 fronds (DMMC and —P series). For analyses of extracellular phosphatase in the —P plants, the plants were grown in 100 ml conical flasks containing 30 ml of the —P nutrient medium.

Doubling time for the control plants was 44h. DMMC and —P decreased the growth rate of the plants, producing typical morphological symptoms that already have been described (K n y p l et al. 1976; R e i d and B i e l e s k i 1970b).

Extraction of enzymes

All extractions were performed at 0-4°C. 0.05 M Tris-HCl buffer, pH 7.4 (as measured at 20°C) was used, if not otherwise stated.

Soluble phosphatase. Plants were washed with distilled water, blotted dry, frozen in liquid nitrogen and ground in a mortar and pestle with hydrated Polyclar AT (2 g PVP per g fr wt of tissue) and Tris-HCl buffer (4 ml per g fr wt). Tissue debris remaining after centrifugation at 3500 g for 20 min was extracted twice with this buffer (2 ml per g original fr wt; each extraction time 20 min). Pooled supernatant fractions were passed through 4 layers of Miracloth, then centrifuged at 15 000 g for 20 min and concentrated twice by passing through dry Sephadex G-25 in a bench centrifuge (800 g for 10 min; *cf.* Kohl 1969). The resulting extract contained crude soluble enzyme.

Membrane bound phosphatase. Tissue debris remaining after extraction of soluble fraction was gently stirred with 0.05 M Tris-HCl buffer, pH 7.4, supplemented with 0.5% Triton X-100 (*cf.* Williams and Juo 1976) for 30 min and cleared by centrifugation. The pellet was again agitated with the same buffered solution of the detergent, and centrifuged. Combined supernatants (3 ml per g original tissue fr wt) were precipitated with 70% acetone (—21°C, 30 min) and solubilized in 0.05 M Tris-HCl buffer without Triton X-100. Insoluble material was discarded and the supernatant fraction concentrated twice with dry Sephadex G-25. The final extract contained "membrane bound" phosphatase activity. The term "membrane bound" means that the enzyme activity could not be solubilized with the buffer alone. None tests have been performed to determine the *in situ* localization of this enzyme activity.

Extracellular phosphatase. $CaCO_3$ from nutrient media remaining after culture the plants was discarded by sedimentation. Chilled acetone (−21°C) was added to a final concentration of 70%, and the precipitate formed during standing for 30 min at −21°C sedimented at 3500 g (10 min). The pellet was extracted twice with Tris-HCl buffer (1 ml per 10 ml of original nutrient medium) by continuous shaking for 30 min, and insoluble salts discarded by centrifugation. The supernatant fraction was concentrated by means of dry Sephadex G-25 as described.

Soluble, membrane bound and extracellular phosphatases were extracted from the same culture of a given type (C, DMMC or −P) with some exceptions. MB phosphatase activity in the control 12-day old plants was too low to be analysed by gel chromatography, and this enzyme fraction was extracted from 2 days older culture. For the same reason the extracellular phosphatase was extracted from a nutrient medium of senescent 25-day old control plants.

Gel chromatography

2.6 × 90 cm bed of Sephadex G-150 (column K-26/100, Pharmacia AG, Uppsala, Sweden) was equilibrated with 0.05 M Tris-HCl buffer, pH 7.4, and calibrated with protein standards of known MW: cytochrome C, MW 12 400 (Polfa); RNase A, MW 13 700 (Koch-Light Laboratories, Ltd.); horseradish peroxidase, MW 40 000 (Koch-Light Laboratories, Ltd.); ovoalbumin, MW 45 000 (Serva Feinbiochemica); human serum γ-globulin, fraction II, MW 160 000 (Calbiochem); ferritin, MW 480 000 (Serva Feinbiochemica); and dextran blue 2000, MW 2 000 000 (Pharmacia AG). Enzymes applied to the column in a volume of 2 to 5 ml were eluted with downward flow of 0.05 M Tris-HCl buffer, pH 7.4. 5 ml fractions were collected each 15 min.

Ten gramms of DEAE-Sephadex A-25 which had been swollen in 0.05 M Tris-HCl buffer, pH 7.4, was poured into a column and pressed to form a bed of 2.6 × 10 cm. Combined eluates from Sephadex G-150 chromatography, comprising peaks of phosphatase and/or RNase activity, were applied to the column (usually in a volume of 30 ml), washed with Tris-HCl buffer, and eluted with a linear gradient of 0-0.4 M NaCl in the same buffer. 5 ml fractions were collected.

Chromatography was performed at 4-8°C.

Enzyme assays

Phosphatase and ribonuclease activities were assayed essentially as described by Knypl (1977).

RNase (EC 2.7.7.17). 0.1 ml of highly polymerized yeast RNA was mixed with 0.1 ml of 0.1 M citrate buffer (pH 6.0), and 0.1 ml of eluate from a column. Reaction was allowed to proceed for 60 min at 30°C, then stopped by adding 3.0 ml of magnesium-lanthanum precipitating reagent (Ambellan and Hollander 1966). Absorbance at 260 nm was read, and values of A 260 nm corrected for 11-fold dilution with the precipitating reagent and for t_o blanks into which enzyme was added after the precipitating reagent. RNase activity is expressed as $\triangle A_{260}$ nm (0.1 ml eluate)$^{-1}$ per equivalent of g fr wt applied to the column of Sephadex G-150.

Phosphatase (EC 3.1.3.1. and 3.1.3.2). 8 mM *p*-nitrophenylphosphate in glycine-maleate-Tris-citrate buffer (Bieleski 1974; Knypl and Sobolewska 1978) was used as a substrate for phosphatase assays at pH 5, 6 and 7.5 (as measured in a whole assay mixture at 30°C). The assay mixture contained 0.1, 0.2 or 0.5 ml eluate in a final volume of 1.5 ml. The reaction was allowed to proceed for 20 min at 30°C, and stopped by adding 1.0 ml of 0.3 N NaOH. Absorbance at 404 nm was read and corrected for t_o blanks into which NaOH solution was added before the eluate. The phosphatase activity, unless otherwise stated, is expressed as $\triangle A_{404}$ nm (0.5 ml eluate)$^{-1}$ per an equivalent of g fr wt of tissue applied to a column, or per such a volume of nutrient medium which was equivalent to 1 g of floating plants at the very time of analyses. Phosphatase activity in crude nutrient media is expressed as $\triangle A_{404}$ nm (0.2 ml medium)$^{-1}$. Unit (U) of phosphatase activity in crude extracts is defined as the amount of enzyme which releases one μmole of *p*-nitrophenol min^{-1} g^{-1} fr wt.

K_m of extracellular phosphatases towards NPP was estimated according to Eisenthal and Cornish-Bowden (1974).

Highly polymerized yeast RNA and NPP were purchased from POCh (Gliwice) and E. Merck (Darmstadt), respectively. DMMC was kindly synthesized by Dr. Małgorzata Oświęcimska (The Technical University, Wrocław).

RESULTS

Soluble phosphatase and RNase

Two molecular forms of soluble phosphatase were detected in extracts of control plants which had been taken for analyses at the end of a log phase of growth. One of these enzymes, subsequently denoted CS-I, was eluted in a void volume of the Sephadex G-150 column. Its MW is equivalent to or higher than 400 000, since exclusion limit for

this type of Sephadex is 400 000. Second molecular form of the enzyme, CS-II, was eluted as a broad peak (Fig. 1). Its apparent MW is around 85 000. CS-I and CS-II had a pH optimum at 5.8-6.0.

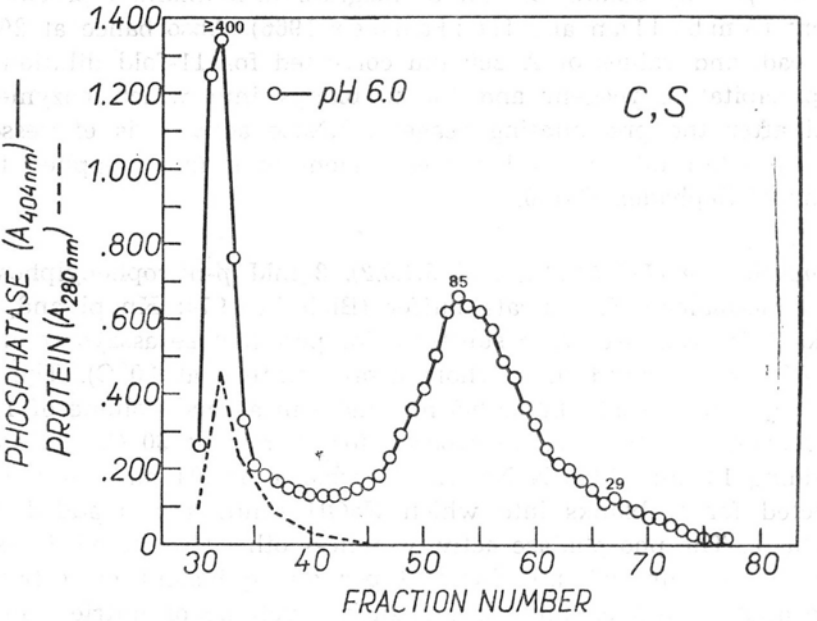

Fig. 1. Elution profile of soluble phosphatases of control plants. Phosphatase was extracted from 12-day old plants (1.2 U g^{-1} fr wt); 80% of applied activity recovered from a columns of Sephadex G-150. Enzyme activity is expressed as ΔA_{404} nm (0.5 ml eluate)$^{-1}$ (20 min)$^{-1}$ per equivalent of 1 g fr wt. Numbers above peaks of enzyme activity in this Fig. and all others mean MW $\times 10^{-3}$.

Pooled fractions containing CS-I and CS-II were rechromatographed on DEAE-Sephadex A-25. CS-I proved to be heterogenous (Fig. 2a). Around a one third of the total enzyme activity was eluted with the washing buffer. The K_m of this fraction towards NPP as a substrate is 0.25 mM. The remaining part of enzyme activity was recovered upon elution with a NaCl gradient (peak of enzyme activity at 0.14 M NaCl, Fig. 2). Discrete shoulders suggest that a number of isoenzymes are present in that portion of CS-I which binds to the anion exchange gel.

Almost total CS-II was eluted with washing buffer (Fig. 2b). However, a minor proportion of enzyme activity was bound to DEAE-Sephadex and eluted with NaCl. This small peak appeared again when the first peak of phosphatase activity was pooled and rechromatographed after one day of storage at 0°C. Thus, the enzyme present in this small peak is obviously formed upon the storage of the extracts. This minor fraction is "more acid" than the other one, as values of an equation: activity at pH 6 to activity at pH 7.5, were 1.20 and 1.70 for the fractions eluting with washing buffer and buffered NaCl solution, respectively.

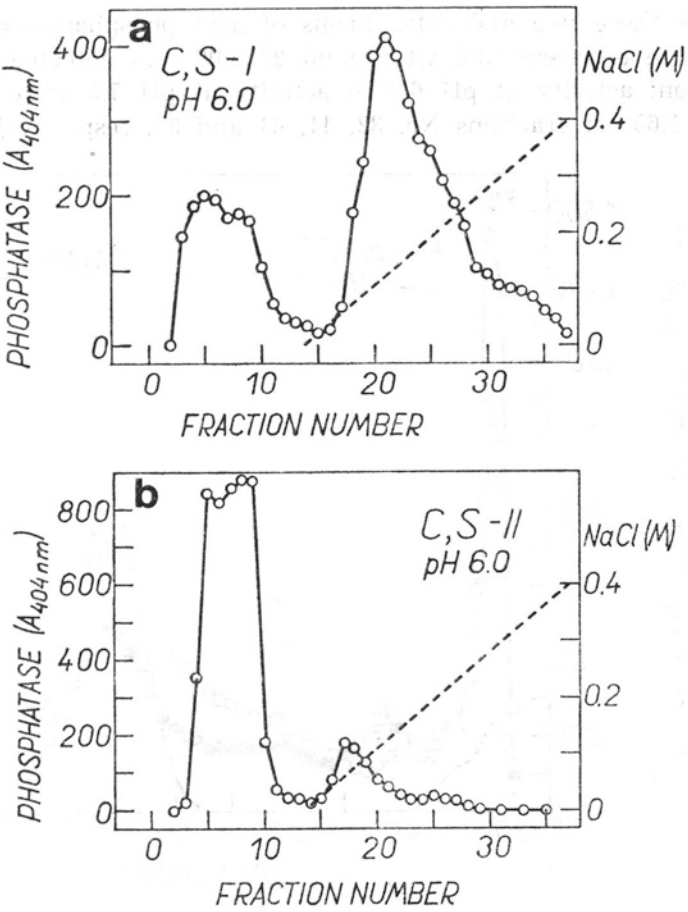

Fig. 2. Fractionation of CS-I and CS-II isoenzymes on DEAE-Sephadex A-25. Source of CS-I and CS-II: fractions No. 30-40 and 47-61 of Fig. 1, respectively· 90% of applied activity was recovered.

Ribonuclease was present in extracts prepared from the control plants (K n y p l 1977) but its activity was too low to be detected in eluates from the column of Sephadex G-150.

The optimum of phosphatase activity in crude extracts of the DMMC treated plants was at pH 6.0-6.3, whereas in the control maximum activity was at pH 5.8-6.0. There was present a high mol wt phosphatase DMMC,S-I. The presence of isoenzyme corresponding to CS-II was manifested by a very minor peak (Fig. 3, tubes No. 51-52). CS-II was replaced by a very remarkable isoenzyme denoted DMMC,S-II with a molecular weight of around 34 000. Peak of activity of this enzyme was constantly saddle-shaped as if it comprised two components with apparent MW of *ca.* 32 000 (alkaline) and 36 000 (acid). Phosphatase DMMC,S-II was eluted together with a high RNase activity (MW 31 000; pH optimum 5.2).

Besides these two molecular forms of acid phosphatase an alkaline enzyme with apparent mol wt around 220 000 was detected. Values of an equation: activity at pH 6.0 to activity at pH 7.5 were 1.59, 0.66, 2.15, and 1.65 for fractions No. 32, 41, 61 and 66, respectively (Fig. 3).

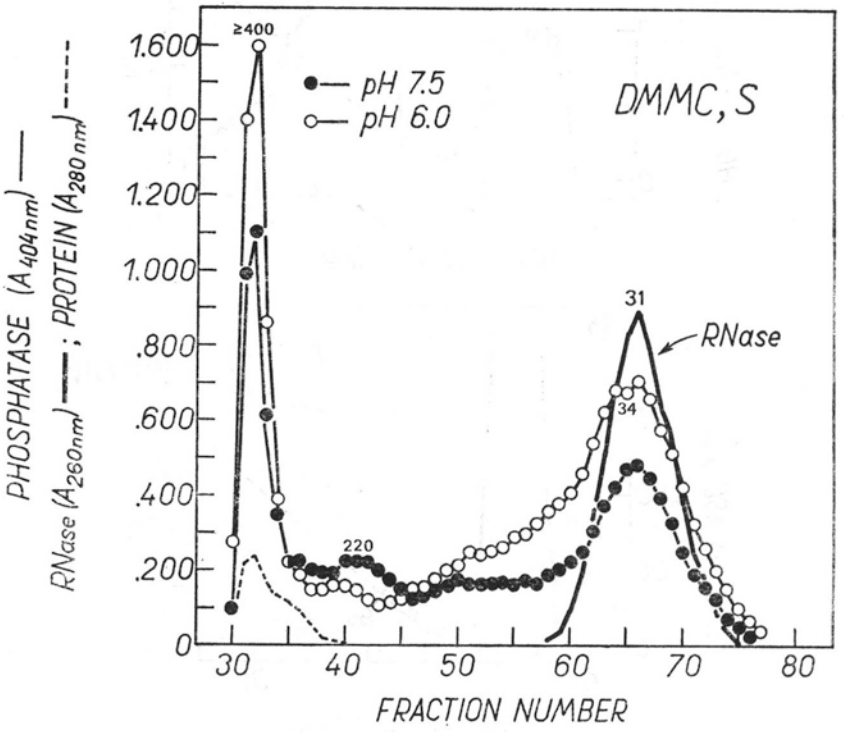

Fig. 3. Elution profile of soluble phosphatase and RNase of DMMC treated plants. The plants were grown in 0.4 mM DMMC for 9 days.

Elution profile of DMMC,S-I from DEAE-Sephadex was similar to that of CS-I (data not shown). Also elution profile of DMMC, S-II was similar to that of CS-II except that a third minor peak of enzyme activity eluting with 0.16-0.17 M NaCl appeared (Fig. 4).

DMMC-treated plants showed 20- to 50-fold higher RNase activity in comparison with the control plants (K n y p l 1976b, 1977). All the DMMC enhanced RNase activity was bound to DEAE-Sephadex and major portion of the activity eluted with 0.12 M NaCl (Fig. 4). Chromatography on DEAE-Sephadex A-25 can, thus, be used for separating RNase activity from phosphatase activity in extracts of soluble enzymes of the DMMC treated *Spirodela oligorrhiza*.

Crude extracts of soluble phosphatase from *Spirodela* which had been grown for 17 days in —P nutrient medium showed 6.00, 4.14, and 4.08 U g^{-1} fr wt at pH 7.5, 6.0 and 5.0, respectively. Chromatography on Sephadex G-150 revealed the presence of three phosphatase isoenzymes,

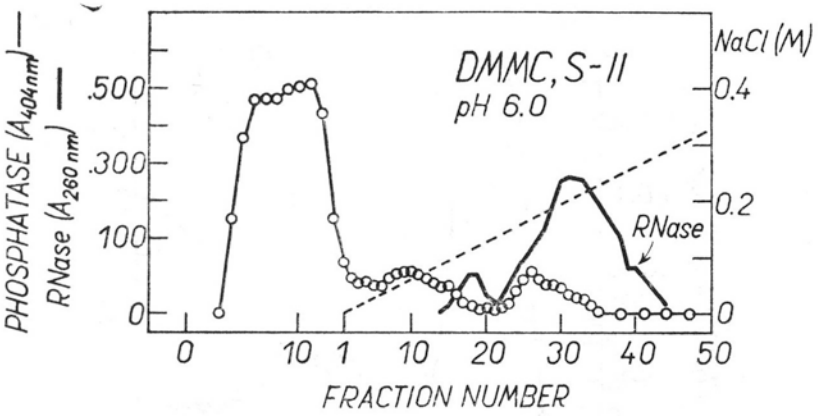

Fig. 4. Fractionation of DMMC,S-II isoenzyme on DEAE-Sephadex A-25. 90% of applied activity (Fig. 3, fractions No. 61-71) was recovered.

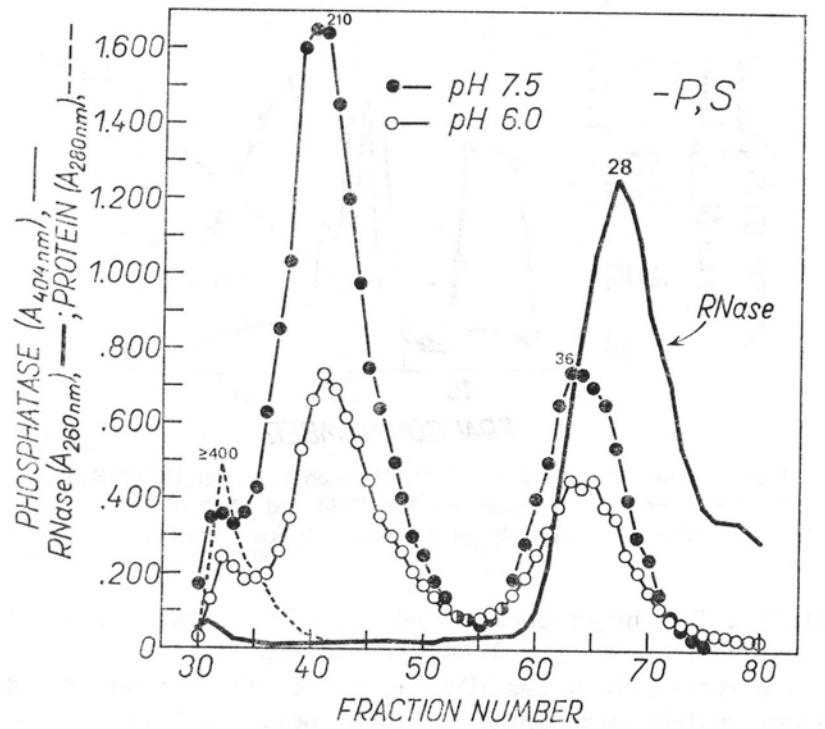

Fig. 5. Elution profile of soluble phosphatases and RNase of —P plants. Plants were grown for 17 days on —P nutrient medium yielding 3.5 fr wt per bottle; total soluble enzyme activity: 6.00 and 4.10 U g^{-1} fr wt at pH 7.5 and pH 6.0, respectively. 102% of applied activity was recovered from a column of Sephadex G-150. Enzyme activity in this Figure, in contrast to all others, is expressed per 0.1 ml eluate. RNase activity: ΔA_{260} nm h^{-1} (0.1 ml eluate)$^{-1}$.

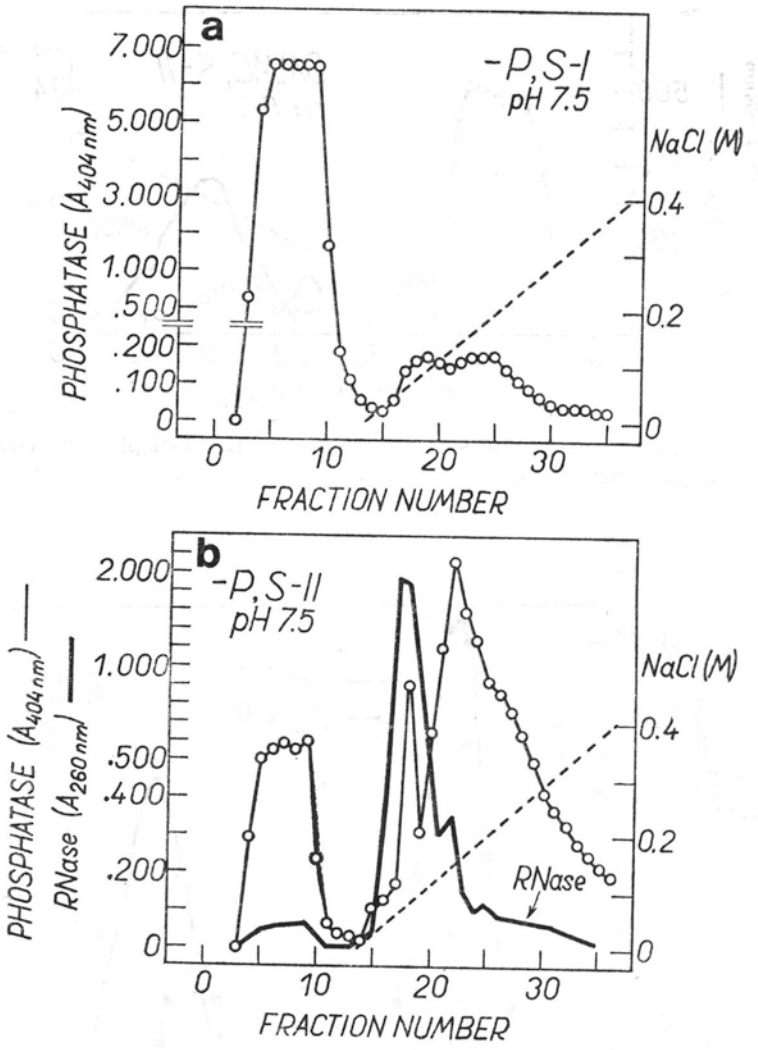

Fig. 6. Fractionation of —PS-1 and —PS-II isoenzymes on DEAE-Sephadex A-25. Source of —PS-I and —PS-II: fractions No. 36-48 and 67-72 of Fig. 5, respectively. 104% and 96% of applied phosphatase activity recovered in the eluates.

all alkaline. Two major ones, —PS-I and —PS-II, are characterized by mol wt around 210 000 and 36 000, respectively. Third relatively minor peak of enzyme activity has MW equal to or higher than 400 000 (Fig. 5). RNase activity was eluted in three peaks. Mol wt of the main RNase fraction was estimated for 28 000 daltons (Fig. 5, fraction No. 67).

The main —P soluble phosphatases eluted from a column of Sephadex G-150 differ in respect to activity at pH 7.5 and pH 6.0. Values of an equation: activity at pH 7.5 to activity at pH 6.0 were 1.67, 0.65, and

1.33 for fractions No. 40 (—PS-I), 55, and 65 (—PS-II), respectively (Fig. 5).

Almost total phosphatase —PS-I was not bound to DEAE-Sephadex A-25, but a minor fraction representing ca. 5% of total recovered activity was eluted with 0.14 and 0.18 M NaCl (Fig. 6a). This minor fraction of enzyme activity appeared again when the first peak of phosphatase activity was pooled and rechromatographed. It is, thus, formed upon storage of the eluates.

Low molecular weight phosphatase —PS-II was heterogenous. Of three distinct fractions, first one was eluted with washing buffer whereas the others were eluted with 0.08 M and 0.14 M NaCl. RNase activity was eluted mainly with 0.08 M NaCl. Nevertheless, minor peaks of RNase activity were associated with each phosphatase peak (Fig. 6b).

Membrane bound phosphatase

Activity of phosphatase extracted with 0.5% Triton X-100 from control plants was too low to be analysed by gel chromatography. Since the activity of membrane bound phosphatase increases in senescing plants (Knypl and Kabzińska 1977; Knypl and Sobolewska 1978) older cultures with few yellowing fronds per bottle were taken for analyses. Crude extract hydrolysed NPP most actively at pH 6.0. However, chromatography on Sephadex G-150 revealed the

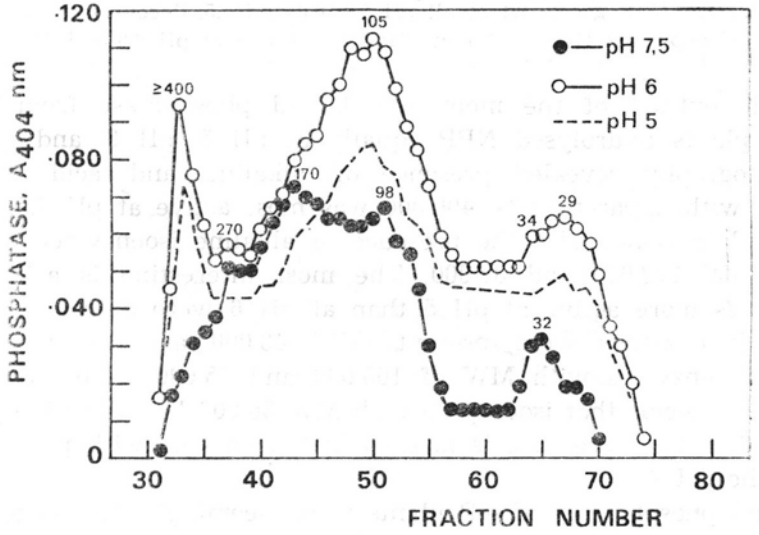

Fig. 7. Elution profile of membrane bound phosphatase of control *Spirodela*. Enzyme was extracted from 2-week old plants. Activity in crude extract: 0.4 U g^{-1} fr wt (pH 6.0). Recovery from the column of Sephadex G-150: 65%.

presence of multiple forms of phosphatase in the membrane bound fraction. When the eluates were tested at pH 6.0, four molecular forms were detected with MW ≧ 400 000, 270 000, 105 000 and 29 000; the presence of minor fractions with MW of 34 000 and 85 000 was manifested by shoulders (Fig. 7). When the eluates were tested at pH 7.5, isoenzymes with MW of 270 000, 170 000, 98 000 and 32 000 were detected. No phosphatase with optimum activity at pH 5.0-5.2 was detected.

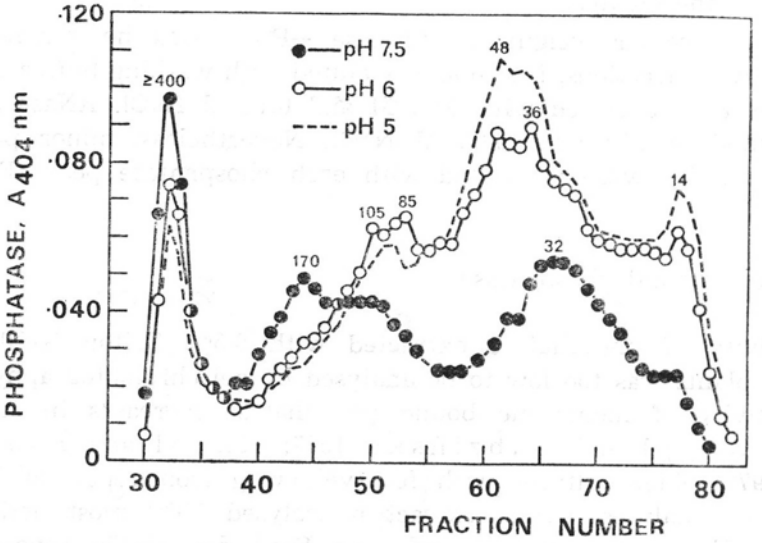

Fig. 8. Elution profile of membrane bound phosphatases of DMMC treated plants. The plants were grown in 0.4 mM DMMC for 9 days. Phosphatase activity in crude extract: 0.45 U g^{-1} fr wt at all pH 5.0, 6.0 and 7.5. Recovery from a column of Sephadex G-150: 38% at pH 7.5, and 56% at pH 6.0 and 5.0.

Crude extract of the membrane bound phosphatase from DMMC treated plants hydrolysed NPP equally at pH 5, pH 6 and pH 7.5. Chromatography revealed presence of alkaline and acid enzymes. Enzyme with apparent MW 400 000 was most active at pH 7.5. Assays at this pH revealed also the presence of alkaline isoenzymes with apparent MW 170 000 and 32 000. The most interesting is a fact that isoenzymes more active at pH 5 than at pH 6 were detected; at least two such enzymes were present (MW 48 000 and 14 000; Fig. 8). Only two enzymes with MW of 105 000 and 85 000 were most active at pH 6. It seems that isoenzyme with MW 36 000 has also pH optimum around 6, but its presence is masked by an enzyme with pH optimum below the pH 6.

Bound phosphatases of —P plants were, seemingly, the same as the soluble ones (Fig. 9). Activity of these alkaline phosphatases was, however, very high in comparison with the control, and they might mask minor quantities of constitutive membrane bound enzymes.

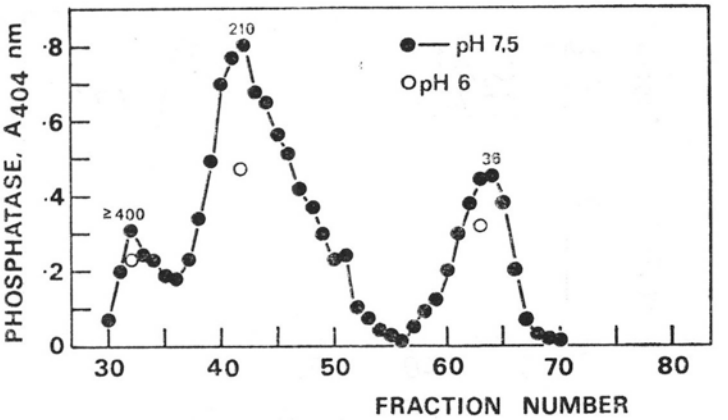

Fig. 9. Elution profile of membrane bound phosphatases of —P plants. 17-day old —P plants were used for extraction of MB enzymes. In contrast to Fig. 6 and Fig. 7, the enzyme was not precipitated with acetone before application to a column of Sephadex G-150. Enzyme activity is expressed per. 0.25 ml of eluate; recovery: 250% or original enzyme activity as tested in crude extract.

Extracellular phosphatase

In nutrient medium of senescing control plants phosphatase activity with optimum at pH 5.2 was detected (Fig. 10). At the very time of analyses (25- and 35-days old cultures) there was neither orthophosphate or ammonium in the nutrient media. Plants showed typical symptoms of advanced nitrogen starvation (F e r g u s o n and B o l l a r d 1969). Gel chromatography revealed the presence of extracellular phosphatase with MW 48 000 (Fig. 11). Similar enzyme was detected in nutrient medium

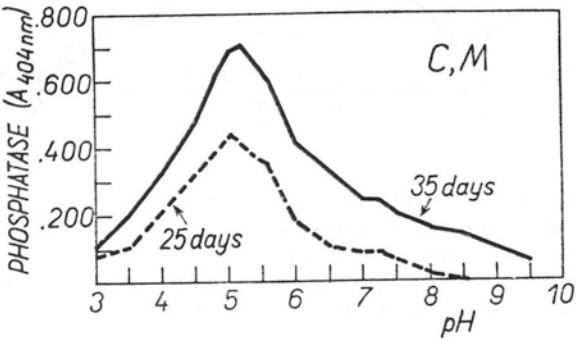

Fig. 10. Effect of pH on extracellular phosphatase activity in nutrient media of control plants. 0.2 ml aliquots of nutrient media were tested for phosphatase activity at different pH. Enzyme activity expressed as ΔA_{404} nm (0.2 ml medium)$^{-1}$ (20 min)$^{-1}$. At the very time of analyses there was 8.5 g fr wt of tissue and 180 ml medium in the case of 25-day old culture, and 13.9 g fr wt and 165 ml medium in the case of 35-days old culture.

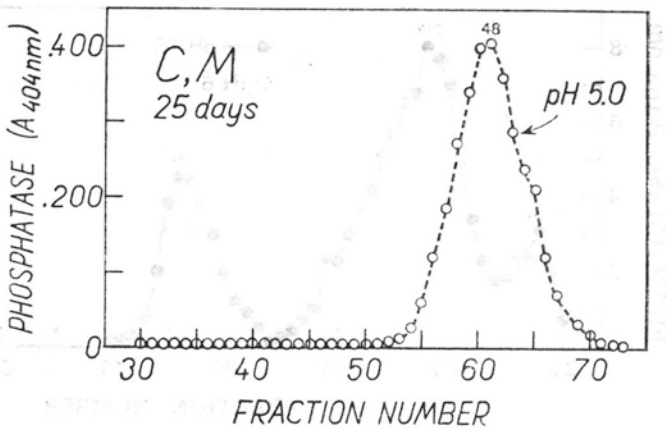

Fig. 11. Elution profile of extracellular phosphatase extracted from nutrient medium of 25-day old control *Spirodela*. Enzyme activity: ΔA_{404} nm (0.5 ml eluate)$^{-1}$ (20 min)$^{-1}$. Equivalent of 21 ml original medium, corresponding to 1 g of floating plants, was applied to a column of Sephadex G-150. Recovery: 72% of applied enzyme activity.

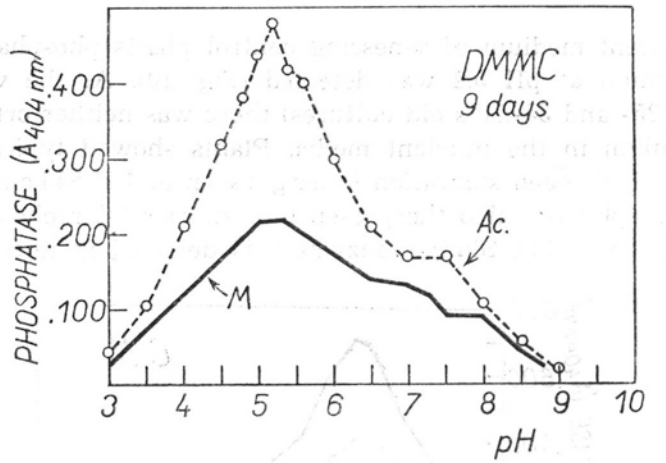

Fig. 12. Effect of pH on activity of extracellular phosphatase in nutrient medium of DMMC treated plants. 0.1 ml aliquots of crude medium (M) of *Spirodela* grown for 9 days in the presence of 0.4 mM DMMC, or 0.2 ml aliquots of purified enzyme (Ac.) were tested. At the very time of analyses there were 5 g fr wt of tissue and 180 ml of nutrient medium.

of the DMMC treated plants. Both crude medium and purified enzyme hydrolyzed NPP most effectively at pH 5.2 (Fig. 12).

Extracellular phosphatase was not detected in nutrient media of *Spirodela* in a log phase of growth. Bacteriological tests confirmed sterility of the cultures (cf. K n y p l and S o b o l e w s k a 1978).

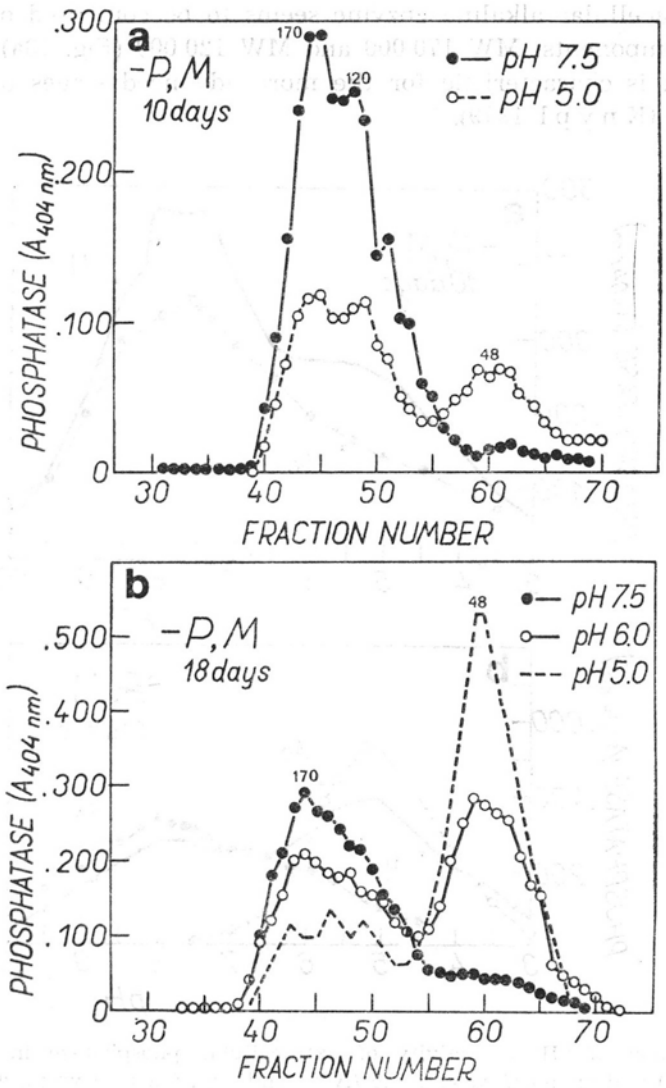

Fig. 13. Elution profile of extracellular phosphatase extracted from nutrient media of 10- and 18-day old phosphate deficient *Spirodela*. 10-day old plants were dark green, none of the fronds was yellow; yield of fr wt was 750 mg per 100 ml flask containing 30 ml of nutrient medium. 18-day old plants: young fronds were dark green whereas the old ones turned yellow, roots elongated to *ca.* 12 mm, much anthocyanins accumulated on the downward side of fronds. Yield was 800 mg fr wt per 100 ml flask. Recovery from the column of Sephadex G-150: 86—96% of applied enzyme activity.

Two phosphatase fractions were present in nutrient media of —P plants (Fig. 13): an alkaline one (MW 170 000) and an acid one (MW 48 000). In younger cultures the activity of an alkaline enzyme prevailed over that of the acid one, whereas in older cultures the reverse was

true. Extracellular alkaline enzyme seems to be composed of two overlapping components: MW 170 000 and MW 120 000 (Fig. 13a). The lower component is characteristic for the more advanced stages of phosphate deficiency (K n y p l 1979).

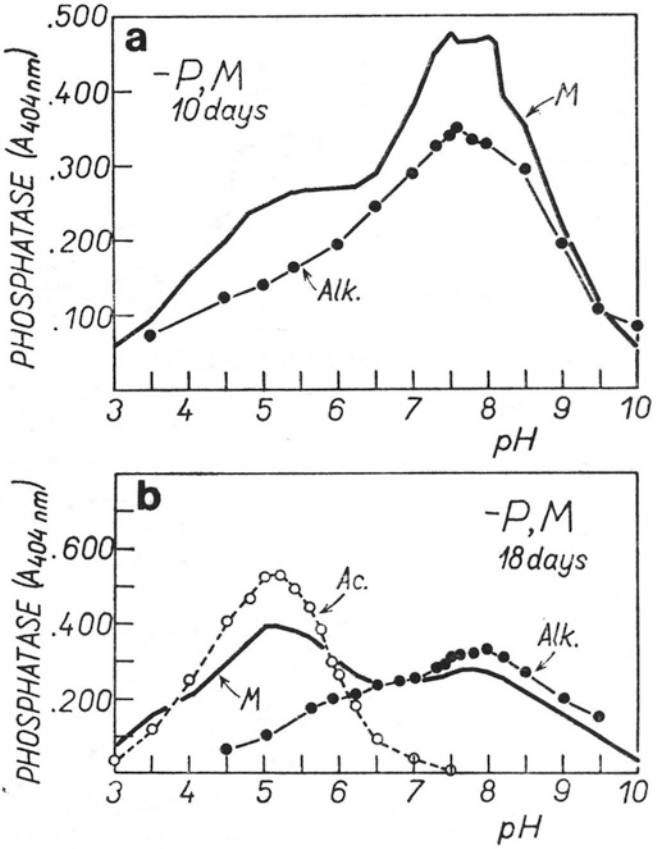

Fig. 14. Effect of pH on activity of extracellular phosphatase in —P nutrient medium (M) and on purified alkaline (Alk.) and acid (Ac.) enzymes. 0.2 ml aliquots of crude media or pooled fractions containing alkaline phosphatase (Fig. 13, fractions No. 42-43) or acid phosphatase (Fig. 13, fractions No. 59-60) were assayed. pH of crude media were 7.5 and 7.9 in the case of 10- and 18-day old cultures, respectively.

There were two pH optima of hydrolysis of NPP by crude —P nutrient media: at pH 5.2 and at pH 8. The curves representing hydrolysing activity of unpurified media towards NPP as a substrate seem to be simply a result of summed activity of alkaline and acid extracellular enzymes (Fig. 14a). It is of interest that activity of the acid enzyme is sharply dependent on pH. On the contrary, alkaline enzyme is active in a much broader range of pH. This enzyme at pH 6 and pH 9 showed

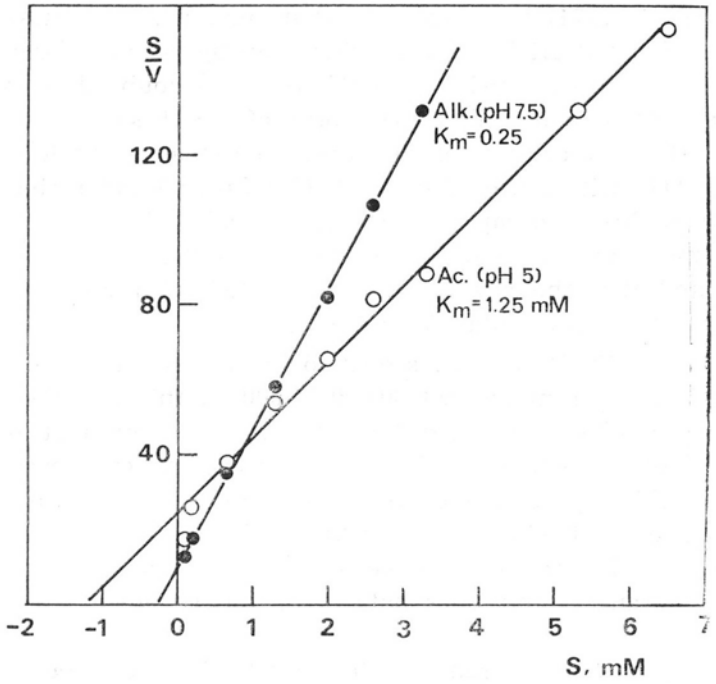

Fig. 15. Determination of K_m values for alkaline (pH 7.5) and acid (pH 5.0) extracellular phosphatases according to Woolf. Source of enzymes: collected fractions No. 43-48 (Alk) and No. 57-63 (Ac) from Fig. 14, 18-day old —P plants.

around 60% of activity in comparison with 100% at the optimum pH 8 (Fig. 14b).

The values of K_m for acid and alkaline extracellular enzymes were estimated for 1.25 mM and 0.25 mM, respectively, when NPP was used as a substrate (Fig. 15).

Senescent control, —P, or DMMC treated plants produced neither extracellular RNase or phosphodiesterase.

DISCUSSION

This study has shown that soluble phosphatases of control and —P *Spirodela oligorrhiza* differ both in pH optima and apparent molecular weights. The values of mol wt were estimated on a basis of 2-4 separate chromatographic runs. Nevertheless, higher plan phosphatases are glycoproteins (*cf.* K r u z e k and M o r a w i e c k a 1978). The same is true for the enzymes of *Spirodela;* for example, the phosphatase DMMC, S-II contains around 40% of sugar (K n y p l 1978), and for that reason true values of MW may differ from the values reported in this paper.

Bieleski (1974) has suggested that high mol wt phosphatase in —P plants (denoted HI-P) may represent an aggregated form of a low mol wt isoenzyme (denoted LO-P). There is no doubt that constitutive phosphatase CS-I is an oligomeric form of CS-II since both enzymes are eluted from a column of Sephadex G-150 as a single peak (MW around 100 000) with buffered 0.4 M NaCl (Knypl 1978). Heterogeneity of CS-I upon chromatography on DEAE-Sephadex (Fig. 2a) may indicate that this molecular form of the enzyme partially dissociates to CS-II *in vitro*, and that the enzyme activity eluted with washing buffer is identical with the molecular form CS-II.

Phosphatase CS-II in turn seems to be a product of association of subunits with apparent mol wt 30 000-34 000. If this was the case, then phosphatase DMMC,S-II could be regarded as a product of *in vivo* dissociation of the enzyme CS-II. There are some evidences that dissociation of this type occurs during a course of senescence of this plant (Knypl and Sobolewska 1978). Peak of enzyme activity corresponding to this range of molecular weight was, in fact, detected among membrane bound phosphatases of the senescent control plants (Fig. 7).

DMMC produced a remarkable modification of the pattern of membrane bound phosphatases. The most interesting seems to be a fact that in the presence of DMMC phosphatases with pH optimum in a range of pH 5.0-5.2 appeared (Figs 7 and 8). One of these enzymes (MW 48 000) may be identical with the extracellular acid enzyme. Acid phosphatases have been found to be associated with membranes of chloroplasts, mitochondria and microsomes (Młodzianowski 1972; Hirai and Asahi 1973; Nakano and Asahi 1972), and in *Spirodela* around 4% of constitutive acid phosphatase is accessible to external phosphate esters (Bieleski 1974). DMMC at a concentration of 0.4 mM as applied in this study inhibited growth and chlorophyll accumulation. It could, thus, induce some biochemical events which occur in naturally senescing plants. Marked increase of RNase activity supports a view that this growth retardant at high concentrations might be a factor inducing senescence (*cf.* Hodge and Sacher 1975). The synthesis of RNase in —P plants can also be regarded as a symptom of senescence (*cf.* De Leo and Sacher 1970; Hanson et al. 1965; Udvardy and Farkas 1972), or as a symptom of stress conditions (*cf.* Blekhman 1977).

Since extracellular acid phosphatase found in nutrient media of senescent control and DMMC treated plants differs from soluble enzymes in pH optimum and molecular weight, it seems to be excreted by living plants. If it was a product of cell lysis, then other enzymes should be detected in the media of both DMMC treated and —P plants. Proper analyses revealed, that this was not the case.

Extracellular alkaline phosphatase released by —P plants is possibly identical with the membrane bound alkaline enzyme with MW 170 000 detected in the control and DMMC-treated plants (Figs. 7 and 8), whereas the acid extracellular enzyme seems to be identical with the MB enzyme (MW 48 000) detected in the DMMC treated plants (Fig. 8).

It is of interest that —P plants released, initially, alkaline extracellular phosphatase. This enzyme showed around 5 times higher affinity towards NPP than the acid phosphatase (Fig. 15), and in the presence of both enzymes external phosphate esters could be hydrolyzed in a very broad range of pH (Fig. 14). The release of an alkaline phosphatase followed by an acid enzyme into the surrounding medium can be regarded as a symptom of adaptation of *Spirodela* to phosphate deficiency. The enzymes, in natural environment, can possibly release orthophosphate from P-esters otherwise unavailable to this plant (Bieleski 1974; Knypl 1978b).

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Molekularne formy fosfatazy i rybonukleazy w Spirodela oligorrhiza (Lemnaceae) hodowanej wobec chlorku N,N-dwumetylomorfoliniowego lub przy braku ortofosforanu

Streszczenie

Techniką chromatografii żelowej (Sephadex G-150) analizowano fosfatazy (EC 3.1.3.2 oraz 3.1.3.1) rozpuszczalne, związane z membranami i ekstracelularne rzęsy wodnej [*Spirodela oligorrhiza* (Kurz) Hegelm.], hodowanej wobec chlorku N,N-dwumetylomorfoliniowego (DMMC) lub przy braku ortofosforanu (—P). Rozpuszczalny enzym roślin kontrolnych rozdzielono na dwie frakcje, CS-I i CS-II, charakteryzujące się c.mol. ok. 400 000 i 85 000; obie molekularne formy enzymu były optymalnie aktywne przy pH 6.0. DMMC spowodował zanik frakcji CS-II i pojawienie się enzymu o c.mol. ok. 34 000. Brak fosforanu powoduje pojawienie się adaptacyjnych enzymów alkalicznych (c.mol. 210 000 i 36 000; optimum pH 7.5).

W roślinach kontrolnych występuje kilka form fosfataz związanych z membranami, przy czym są to enzymy zarówno kwaśne jak i alkaliczne. Pod wpływem DMMC pojawiają się dwie formy molekularne fosfatazy optymalnie aktywne przy

pH 5 (c.mol. 48 000 i 14 000), nie wykryte w materiale kontrolnym. Fosfatazy związane z membranami roślin —P są identyczne z fosfatazami rozpuszczalnymi.

Starzejące się rośliny kontrolne oraz rośliny hodowane wobec DMMC wydzielają fosfatazę kwaśną (optimum pH 5.2; c.mol. 48 000) do pożywki. Rośliny —P oprócz wymienionej fosfatazy kwaśnej wydzielają również fosfatazę alkaliczną (optimum pH 7.8-8.2; c.mol. 170 000). K_m extracelularnej fosfatazy kwaśnej i alkalicznej wynosi 1.25 mM i 0.25 mM NPP.

DMMC i brak fosforanu powodują silny wzrost aktywności RNazy (EC 2.7.7.17) o c.mol. 31 000 i 28 000.

Sugeruje się, iż spektrum fosfataz w rzęsie wodnej zależy od wieku roślin oraz od dostępności makro- i mikroelementów.