Phytochemistry, 1971, Vol. 10, pp. 765 to 770. Pergamon Press. Printed in England.

CHANGES IN THE LEVEL OF ACID PHOSPHATASES IN *AVENA* LEAVES IN RESPONSE TO CELLULAR INJURY

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(*Received* 14 *April* 1970)

Abstract—In illuminated *Avena* mid-leaf blades, the activity of various phosphatases increased with time. Overall activity increased by about 30 per cent during an incubation period of 8 hr. The activity was resolved into five peaks by molecular sieve chromatography. One of the peaks was purified and proved to be homogeneous upon chromatography on DEAE cellulose and upon polyacrylamide gel electrophoresis. The enzyme was characterized as a non-specific acid phosphatase splitting a wide range of substrates. The phosphatase exhibited regular Michaelis kinetics and was competitively inhibited by inorganic phosphate. Each tested substrate was a competitive inhibitor of the other. The molecular weight of the enzyme was found to be around 26,000.

INTRODUCTION

AN INCREASE in the activity of a number of hydrolytic enzymes, including ribonuclease (RNase), phosphodiesterase (PDase), phosphatase and peptidase, has been reported to occur in excised leaf tissues.[1–13] It is tempting to attribute a role to the increased activities of hydrolytic enzymes in the regulation of senescence speeded up by leaf excision.[3,8,9] However, the prerequisite of a deeper understanding of the biochemical mechanism and role of increase in hydrolase levels in excised leaves is a characterization of the enzymes involved which has not been done in previous studies. Such an analysis is being carried out currently in our laboratory. We already reported on the behaviour and properties of various nucleases in excised *Avena* leaves.[10,14,15] The present paper deals with the phosphatases. It is shown that the *Avena* leaf contains several acid phosphatases differing in molecular size. The amount of all these enzymes increased moderately, and to about the same extent upon leaf excision. A detailed characterization of one of the phosphatases is given.

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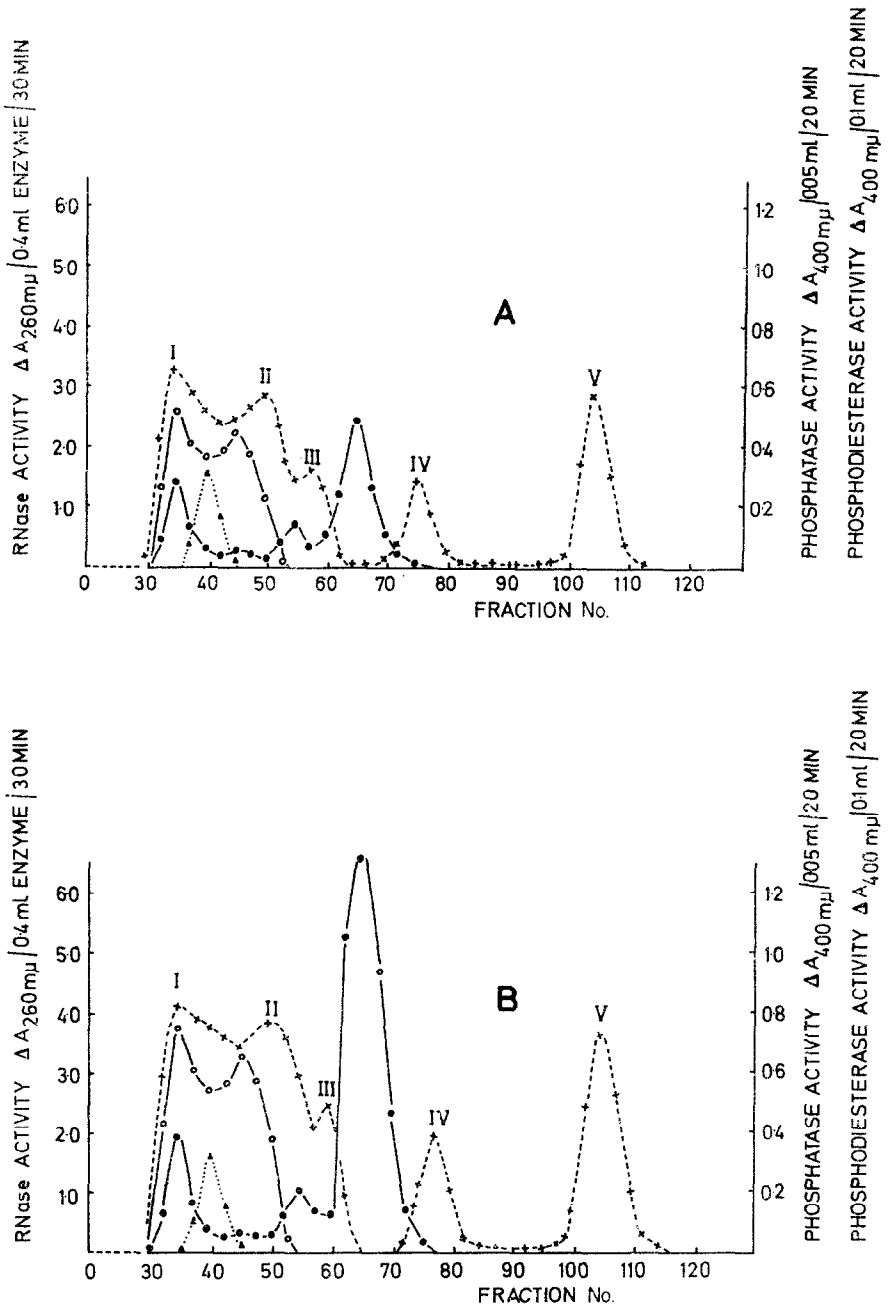

FIG. 1. CHROMATOGRAPHY ON SEPHADEX G-75 COLUMNS (2·3 × 100 cm) OF ENZYME PROTEINS EXTRACTED FROM INTACT (A) AND EXCISED (B) *Avena* LEAF TISSUES, RESPECTIVELY.

Proteins were precipitated with $(NH_4)_2SO_4$ (80 per cent saturation) from 10,000 *g* supernatants of cell-free extracts corresponding to 10 g of leaf tissue and placed on the columns equilibrated with 0·01 M Tris-HCl buffer, pH 7·5. Proteins were eluted with the same buffer and the fractions (3·0 ml) were assayed for RNase (●——●), acid phosphodiesterase (○——○), alkaline phosphodiesterase (▲.....▲) and acid phosphatase (×------×) activities.

RESULTS

Changes in Enzyme Profiles upon Leaf Excision

First leaves of 10-day old *Avena* seedlings were excised, the leaf tips and bases were removed to give possibly more homogeneous material and the remaining middle portion of the leaf blades were placed in Petri dishes on moist filter paper. After incubation for 8 hr at 8000 lx, proteins were extracted from both excised and control leaves, precipitated with ammonium sulphate and chromatographed on Sephadex G-75 columns as described in the Experimental. The enzyme profiles obtained are shown in Fig. 1. It may be seen that the major change upon leaf excision occured in the RNase (fraction Nos. 60–75) identified as a relative purine specific soluble RNase.[14] Some increase was experienced in the smaller RNase peaks as well. The alkaline phosphodiesterase activity, however, remained unaltered. Both peaks of acid phosphodiesterase activity increased moderately. The phosphatase activity was resolved into five peaks apparently representing different molecular sizes. All phosphatase peaks increased by about 30–40 per cent upon leaf excision. It is apparent from Fig. 1 that the phosphatase corresponding to peak No. V is not contaminated with other enzymes. Furthermore, the enzyme activity belonging to this peak proved to be homogeneous upon rechromatography on a DEAE-cellulose column (Fig. 2) and upon

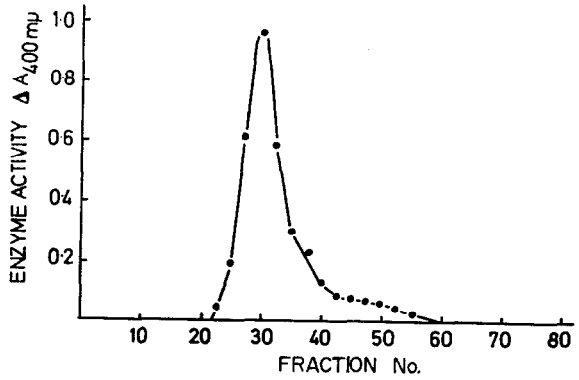

FIG. 2. CHROMATOGRAPHY ON A DEAE CELLULOSE COLUMN (0·8 × 26 cm) OF THE PHOSPHATASE CORRESPONDING TO PEAK NO. V OBTAINED UPON GEL-FILTRATION ON SEPHADEX G-75 OF $(NH_4)_2SO_4$ PRECIPITABLE PROTEINS OF CELL-FREE EXTRACTS FROM *Avena* LEAVES.

Pooled fractions from the Sephadex column (Nos. 102–110, Fig. 1) were loaded on the DEAE cellulose column equilibrated with 0·01 M Tris-HCl buffer, pH 7·5, and eluted with 300 ml of a linear gradient of NaCl (0·0–0·4 M) in 0·01 M Tris-HCl buffer, pH 7·5.

polyacrylamide gel electrophoresis. Therefore, the enzyme in peak V (Fig. 1) was characterized in a greater detail.

Substrate Specificity

The substrate specificity of the enzyme was tested on a wide range of phosphate esters. The results are summarized in Table 1. It may be seen that the enzyme has a very broad range of substrate specificity. In addition to phosphate monoesters, ATP and inorganic pyrophosphate also proved to be substrates of the enzyme which can thus be classified as a non-specific phosphatase. The various fractions belonging to phosphatase peak No. V (fractions 100–110, Fig. 1A) were assayed separately for their substrate specificity using

three different substrates (pNPP, glucose-6-P, ATP). The relative substrate specificity of all fractions was found to be the same. This demonstrates the homogeneity of the enzyme preparation.

TABLE 1. SUBSTRATE SPECIFICITY OF THE PHOSPHATASE ISOLATED FROM *Avena* LEAF TISSUES

Substrate	Relative enzyme activity (%)	Substrate	Relative enzyme activity (%)
pNPP	100·0	Fructose-6-P	15·0
ATP	58·6	Riboflavine-5-P	15·0
PP inorganic	54·1	AMP	11·2
GTP	48·9	Fructose-1-P	9·8
ADP	45·1	CMP	3·7
Thiamine-PP	41·3	Glucose-1-P	2·2
GDP	31·6	Glucose-6-P	2·0
UMP	26·6	GMP	2·0
NADP	22·5	Mannose-6-P	0·0
Fructose-1,6-P	15·8		

The standard assay based on the liberation of inorganic phosphorus was used.

pH-Optimum

The pH-dependence of enzyme activity was determined by using three different substrates (pNPP, β-glycerophosphate and ATP). The pH-optimum of the enzyme was found to be around 5·0 for each substrate.

Kinetic Studies

As shown by using pNPP, β-glycerophosphate and glucose-6-phosphate as substrates, the enzyme activity follows regular Michaelis–Menten kinetics. The Michaelis constants calculated from Lineweaver–Burk plots are 0·7 mM, 2·2 mM and 1·4 mM for pNPP, β-glycerophosphate and glucose-6-phosphate, respectively. Inorganic phosphate was shown to inhibit the enzyme competitively. Kinetic experiments indicated that two substrates added simultaneously to the enzyme apparently compete for the same binding site.

Molecular Weight

The results of a gel-filtration experiment which consisted of the chromatography of three marker proteins of known molecular weights plus the *Avena* phosphatase on a Sephadex G-100 column showed that the molecular weight of the latter was around 26,000.

DISCUSSION

The present paper provides strong evidence to show that the 'general' activation (and/or synthesis) of hydrolytic enzymes observed in excised leaf tissues[1–13] is a process which exhibits a fair degree of specificity in the sense that the level of different enzymes is altered to *different and characteristic extents.* The overall 'nuclease response' of the excised tissues obviously consists of several components which are not affected by leaf excision to the same extent. The great increase in 'ribonuclease activity' in the excised *Avena* leaf is mainly due to the increase in the level of a relative purine specific, soluble RNase.[14] Under they same conditions the amount of another nuclease, an alkaline phosphodiesterase which

splits both DNA and some RNA species,[15] is not altered at all. The level of some other enzymes involved in the breakdown of nucleic acids is increased in excised leaves to a moderate extent (30–100 per cent). The following enzymes belong to this category: an acid phosphodiesterase,[15] a minor endonuclease (which, according to unpublished results, exhibits a relative specificity different from that of the major RNase of the *Avena* leaf) and the acid phosphatases described in the present paper.

A number of acid phosphatases are present in the *Avena* leaf. Gel filtration resolved the phosphatase activity into five peaks. It seems significant that all these enzymes (or groups of enzymes) were affected by leaf excision to about the same extent. If we accept the general opinion that the acid phosphatases of plants are contained in special cell particles analogous to the lysosomes of animal cells[16–19] (cf. however Corbett and Price)[20] our observations seem to be in line with the conclusions of Balz[3] that the overall increase in hydrolytic enzyme activity in detached leaves might be due to an increase in the number of lysosomes. This would also imply that the *Avena* leaf lysosomes contain a number of different phosphatases. An alternative explanation for the increase in phosphatase level in excised *Avena* leaf fragments would be the activation of latent phosphatases by proteolytic or other unknown effects.[21] Latent phosphatase activity which can be activated by detergents has been described from plant tissues.[22,23] However, we observed only a slight increase in phosphatase activity upon treating the *Avena* leaf tissue extracts with Triton X-100.

In the present paper detailed data on the properties of one of the *Avena* leaf phosphatases are presented. The enzyme was characterized as an acid phosphatase with broad substrate specificity. This phosphatase, in contrast to true phosphomonoesterases, also hydrolyses pyrophosphate bonds. It is difficult to compare the substrate range of the *Avena* leaf phosphatase with that of other known plant phosphatases[24–29] since the various authors used different substrates in their substrate specificity tests. Still, some common features and some characteristic differences in the properties of the acid phosphatases of higher plant tissues appear to emerge from a comparison of the data available. Thus, it appears that the majority of plant phosphatases which possess a wide substrate specificity, including the *Avena* phosphatase, exhibit the highest (or almost the highest) activities with pNPP as substrate.[22,24,27,28] The K_m-values reported for some of the purified plant enzymes and determined for different substrates are in the range $1{\cdot}8–3{\cdot}5 \times 10^{-4}$ M.[24,29] Inhibition by fluoride and inorganic phosphorus appears to be a general property of the plant phosphatases[27–29] and this property is shared by the *Avena* enzyme. All acid phosphatases from plants investigated in detail, including the *Avena* enzyme, follow regular Michaelis–Menten kinetics.[24,29] No indication of the presence of subunits and of subunit interactions was

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obtained. To our knowledge, the present paper is the first to establish the approximate molecular weight of an acid phosphatase from plants. The molecular weight, 26,000, is one of the lowest described for an enzyme so far. This observation also suggests that the molecule consists of one polypeptide chain only. However, it must be stressed that, according to the gel filtration pattern, acid phosphatases of much higher molecular size are present in the *Avena* leaf. As shown by preliminary investigations, the other properties of these phosphatases are similar to those of the phosphatase characterized in detail. They are also non specific acid phosphatases.

EXPERIMENTAL

Plant Material

Avena sativa cv. Alaska seedlings were grown under controlled conditions (12 hr darkness, 12 hr light, 6000 lx, day temp. 28°, night temp. 22°) in sand culture. The first seedling leaves of 10- to 11-day old plants were used for the experiments.

Determination of Enzyme Activities

RNase, DNase, PDase and phosphatase assays were carried out as described previously.[10,14] In addition to the use of *p*-nitrophenyl phosphate (pNPP) as substrate,[10] phosphatase activity was also measured by the determination of inorganic phosphorus liberated.[30]

Enzyme Purification

Avena leaf tissues were extracted in 0·05 M Tris-HCl buffer, pH 7·5, at a ratio of 1:3. The homogenate was passed through 4 layers of cheese-cloth and centrifuged at 100,000 *g* for 1 hr in the Spinco L-50 preparative ultracentrifuge. Solid $(NH_4)_2SO_4$ was added to the supernate to 80% saturation; the precipitate was spun down at 10,000 *g* for 10 min and dissolved in 3 ml of 0·01 M Tris-HCl buffer, pH 7·5. The solution was applied to a 2·3 × 100 cm Sephadex G-75 column equilibrated with the same buffer. All fractions were assayed for RNase, DNase, PDase and phosphatase activities. Typical elution profiles are shown in Fig. 1. Fraction Nos. 102–110, representing a phosphatase peak (No. V), were pooled and used for further assays as a partially purified preparation.

DEAE-Cellulose Chromatography

This was carried out by eluting with a 0–0·4 M NaCl gradient the proteins adsorbed on a 0·8 cm × 50 cm DEAE-cellulose column equilibrated with 0·01 M Tris buffer, pH 7·5.

Polyacrylamide Gel Electrophoresis

Disc electrophoresis was carried out on 7·5% gels as described by Davis.[31] The gels were incubated with a reaction mixture containing 0·2 M sodium acetate buffer, pH 5·0, 40 mg lead acetate, 20 ml 3% sodium glycerophosphate in a final volume of 200 ml. Incubation was carried out at room temperature for 4 hr. Control gels were incubated in standard incubation mixtures containing 0·01 M NaF.

Determination of Molecular Weight

Gel-filtration on a Sephadex G-100 column was used for the determination of molecular weight as described by Bagi and Farkas.[32]

Estimation of Protein Content

Protein content was determined by the method of Lowry *et al.*[33] using bovine serum albumin as standard.

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