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EXTRACELLULAR PHOSPHOHYDROLASES FROM SUSPENSION CULTURES OF *NICOTIANA TABACUM*

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Abstract—Several phosphohydrolase activities were detected in the medium after growth of suspension cultures of tobacco cells. A rapid rate of hydrolysis was observed with DNA, RNA, 3′-nucleotides, phosphoric anhydrides, *p*-nitrophenyl phosphate, and synthetic phosphodiesters. A fractionation of some of these phosphohydrolase activities was achieved by gel chromatography.

INTRODUCTION

IT HAS been established that certain enzymes are released into the medium during the growth of cultured plant cells. These enzymes include peroxidase,[1,2] indoleacetic acid oxidase,[1] α-amylase,[3] and other glycosidases.[4,5] Reports of extracellular phosphohydrolases have also appeared. Straus and Campbell[1] detected an enzyme in tobacco medium that catalyzed the hydrolysis of *p*-nitrophenyl phosphate. Ueki and Sato[6] demonstrated that the synthesis of this enzyme is repressed by inorganic phosphate in the culture medium. These workers also observed that other phosphomonoesters and inorganic pyrophosphate are hydrolyzed by enzymes present in the tobacco culture medium. Enzymes that cleave RNA have been detected in tobacco medium by Yamaoka *et al.*[5] and in wild carrot medium by Wilson.[7] In this communication we report the presence of several phosphohydrolase activities in tobacco culture medium. Some of these enzyme activities are distinguishable from the acid phosphatases studied by Ueki and Sato.[6]

RESULTS

Phosphohydrolase activities of tobacco culture medium

A typical sample of B5 nutrient medium that has supported the growth of cultured tobacco cells for a period of 10 days contains approximately 200 μg of polysaccharide and 50 μg of protein per ml. Although the amount of protein released by the cells is quite small, substantial amounts of phosphohydrolase activities are found in this solution. The ability of dialyzed culture medium to catalyze the hydrolysis of several phosphomonoesters and

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[5] YAMAOKA, T., HAYASHI, T. and SATO, S. (1969) *J. Fac. Sci. Univ. Tokyo, Sect. III* **10**, 117.
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phosphoric anhydride compounds is shown in Table 1. The rate of hydrolysis of 3′-AMP was much greater than that of any other phosphomonoester tested. *p*-Nitrophenyl phosphate and the phosphoric anhydride compounds (PP_i, ADP, and ATP) were also hydrolyzed at substantial rates, but the other compounds tested were hydrolyzed slowly.

Enzymes present in dialyzed extracellular medium catalyzed the hydrolysis of DNA, RNA and model substrates for phosphodiesterases (Table 2). Nuclease activity for DNA was observed to be greater than that for RNA, and *bis-p*-nitrophenyl phosphate was the most effective synthetic phosphodiester.

TABLE 1. HYDROLYSIS OF PHOSPHOMONOESTERS AND PHOSPHORIC ANHYDRIDES BY TOBACCO CULTURE MEDIUM

Substrate	Activity (milliunits/ml)
p-Nitrophenyl phosphate	34·5
Adenosine 2′-monophosphate	2·25
Adenosine 3′-monophosphate	87·0
Adenosine 5′-monophosphate	1·80
Glucose-6-phosphate	2·30
α-Glycerophosphate	2·60
β-Glycerophosphate	5·20
Phytic acid	0·05
Inorganic pyrophosphate	21·0
ADP	24·0
ATP	20·3

TABLE 2. HYDROLYSIS OF PHOSPHODIESTERS BY TOBACCO CULTURE MEDIUM

Substrate	Activity (milliunits/ml)
DNA	93·4
RNA	67·6
bis-p-Nitrophenyl phosphate	113·2
p-Nitrophenyl-3′-thymidylate	27·3
p-Nitrophenyl-5′-thymidylate	33·0

Gel chromatography

Additional information on the nature of the extracellular phosphohydrolases was obtained by gel chromatography of concentrated extracellular medium on a column of Sephadex G-75. Phosphohydrolases were present in three fractions of the eluate. Fraction I contained material that eluted between 320 and 360 ml. This fraction, which represented the void volume of the column, contained a large amount of high MW polysaccharide. Fractions II and III contained material that eluted between 360 and 410 ml, and 410 and 480 ml, respectively. The enzyme activities that catalyzed the hydrolysis of DNA, RNA and 3′-AMP were eluted in a single peak (Fraction III) and none were found in Fractions I and II. Fraction III also cleaved 3′-UMP at 30% of the rate observed with 3′-AMP. The enzyme activity that catalyzed the hydrolysis of *p*-nitrophenyl phosphate was eluted in two separate peaks. The activity was almost evenly divided between Fractions I and II

(48% and 44%, respectively). The small amount of *p*-nitrophenyl phosphatase that was present in Fraction III (8%) represented some tailing of the peak in Fraction II. The results with the phosphohydrolases active on *bis*-*p*-nitrophenyl phosphate were similar to those observed for the *p*-nitrophenyl phosphatase, except that the activity was found primarily in Fraction I (80%). Fractions II and III contained 18% and 2% of the total, respectively.

DISCUSSION

The enzyme activities that catalyze the hydrolysis of polynucleotides and *bis*-*p*-nitrophenyl phosphate are present at high levels in tobacco culture medium. Gel chromatography produced an almost complete separation of the nuclease activities from the enzymes active on *bis*-*p*-nitrophenyl phosphate. The enzymes in tobacco culture medium that cleave this synthetic phosphodiester do not attack DNA or RNA under our conditions of assay. The hydrolytic activity for *bis*-*p*-nitrophenyl phosphate may reflect the presence in the tobacco medium of an enzyme similar to that observed by Suzuki and Sato[8] in the cell walls of cultured tobacco cells.

Our observations on phosphomonoesterase activities in tobacco culture medium resemble those of Sato and co-workers,[5,6] although they did not test the activity with adenine nucleotides. Adenosine 2′- and 5′-monophosphate are hydrolyzed very slowly by tobacco culture medium. The results of the Sephadex G-75 chromatogram reveal that the acid phosphatase that cleaves *p*-nitrophenyl phosphate at a high rate has little, if any, activity on 3′-AMP. The latter compound is hydrolyzed by a highly active 3′-nucleotidase that is distinguishable from the *p*-nitrophenyl phosphatase by gel chromatography.

Two peaks of enzyme activity for each of the *p*-nitrophenyl esters were observed on gel chromatography. These multiple components may reflect the presence of more than one enzyme capable of attacking each substrate. Alternatively, the enzyme found in Fraction I may be an aggregate (specific or nonspecific) of the enzyme found in Fraction II. In addition, each peak of enzyme activity may contain more than one enzyme capable of acting on the substrate. Additional purification of the enzyme activities may clarify the relationship of these multiple components.

Under our conditions phytic acid is hydrolyzed at a barely detectable rate. Ueki and Sato[6] also found this compound to be a poor substrate, but Olson[9] reported that tobacco culture medium catalyzed the hydrolysis of phytic acid at 55° in a solution buffered at pH 5·2 at a rate of 0·8 μmol/min/mg protein. We have also assayed for phytic acid degradation by tobacco medium under the conditions of temperature and pH employed by Olson; P_i was released at the rate of 0·05 μmol/min/mg protein. This value is only 2% of the rate of hydrolysis of *p*-nitrophenyl phosphate under these conditions. Differences in the cell lines or conditions of growth may be responsible for the variance of the observations of Olson with those of Sato's group and ourselves.

The enzymes in the extracellular medium that are active on 3′-nucleotides, RNA, and DNA were not separated by gel chromatography. Although other fractionation procedures may possibly separate these activities, multifunctional enzymes that cleave 3′-nucleotides, RNA and DNA have been purified from differentiated tissues of higher

[8] SUZUKI, T. and SATO, S. (1973) *Plant and Cell Physiol.* **14**, 585.
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plants.[10–14] The phosphohydrolase activities found in Fraction III of the Sephadex chromatogram may reflect the presence in tobacco culture medium of a nuclease of this type.

EXPERIMENTAL

Tobacco cell culture and harvest of extracellular medium. The XD line[26] of *Nicotiana tabacum* L. cv. Xanthi (obtained from Dr. Philip Filner of Michigan State Univ.) was cultured on B5 medium[4] at 26°. Suspension cultures (250 ml in 1-liter Delong flasks) were incubated on a rotary shaker for 8–10 days. Small portions of each culture were removed just before harvest and plated on B5 agar and Difco nutrient agar to demonstrate that the culture was free from microbial contamination. The remainder of each culture was filtered through Miracloth (Calbiochem) to remove the cells. The filtrate was clarified by centrifugation (12000 *g*, 15 min) and dialyzed at 4° against 0·05 M Na acetate buffer (pH 6). The dialyzed medium was stored on ice. Phosphohydrolase activities in each preparation were assayed within one week of harvest.

Analytical methods. Standard methods were used to determine protein[15] and carbohydrate.[16] Polynucleotides were determined by analysis of total phosphate,[17] and quantities were expressed in terms of μmol polynucleotide-phosphate.

Preparation of radioactive substrates. Radioactive T4 bacteriophage were prepared by infection of a culture of *Escherichia coli* B in glycerol medium;[18] 5 min after infection, uracil-[2-^{14}C] (0·05 mM, 2 Ci/mol) was added, and the culture was incubated at 37° until lysis occurred. The virus in the lysate were purified[19] and then extracted with pHOH[20] to yield a preparation of ^{14}C-labeled T4 DNA (sp. act., $2·7 \times 10^5$ cpm/μmol). ^{14}C-labeled ribosomal RNA (sp. act., $2·9 \times 10^5$ cpm/μmol) was prepared by the method of Kirby[21] from *E. coli* that had been grown in M9 medium[22] supplemented with adenine-[8-^{14}C] (0·2 mM, 0·5 Ci/mol).

Nuclease assays. The assay medium contained 20 nmol ^{14}C-labeled polynucleotide (RNA or heat-denatured DNA), 15 μmol Tris-acetate buffer (pH 5·8), 0·2 mg bovine serum albumin and enzyme in a final vol. of 0·3 ml. The reaction mixtures were incubated for 30 min at 37° C, chilled in an ice bath, and then treated with 0·2 ml of a soln containing 0·25 mg yeast RNA, 1 mg albumin and 0·5 ml of 10% trichloroacetic acid. The vessels were stored for 10 min at 0° and the ppt. removed by centrifugation. The supernatant was mixed with 0·2 ml of 2 N NH_3 and 5 ml of Bray's Solution[23] and then counted in a liquid scintillation spectrometer.

Assay of enzymes active on p-nitrophenyl esters. The assay medium contained 0·5 μmol substrate, 50 μmol Tris-acetate buffer (pH 6), 5 μmol $MgCl_2$, 0·1 mg albumin and enzyme in a total vol. of 1 ml. The vessels were incubated for 30 min at 37° and the reaction was terminated by the addition of 0·5 ml of 0·05 M EDTA in 0·3 M NaOH. The A at 400 nm was measured. An $a_{0.1M}$ of 12·0 was used to calculate the amount of *p*-nitrophenol formed during the reaction.[24]

Assay of other phosphohydrolases. The assay was performed by a modification of the method employed for *p*-nitrophenyl esterases. Substrate was present at a level of either 1 μmol (phytic acid) or 2·5 μmol (other compounds). The reaction was terminated by addition of 0·5 ml cold 15% trichloroacetic acid, and a portion of the mixture was taken for analysis of P_i by the method of Chen *et al.*[17] (acid-stable substrates) or of Dreisbach[25] (acid-labile substrates).

One unit of enzyme activity is defined as the amount that catalyzes the formation of 1 μmol of product per min at 37° under the conditions of the assay.

Gel chromatography. The dialyzed extracellular medium was concentrated 15× by dialysis against 30% (w/v) polyethylene glycol (Carbowax 6000) in 0·05 M Na acetate buffer (pH 6). A portion of this concentrate was applied to a column (3 × 105 cm) of Sephadex G-75 and eluted with 0·2 M NaCl in 0·02 M Na maleate buffer (pH 6·5).

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