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5'-NUCLEOTIDASE AND GLUCOSE-6-PHOSPHATASE IN A PURIFIED CELL-WALL FRACTION FROM *PHASEOLUS VULGARIS*

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Abstract—A purified cell-wall fraction was isolated from a homogenate of cotyledon tissue from *Phaseolus vulgaris*. The fraction was enriched in cellulose and had a phospholipid content equivalent to about 1 per cent of the level in the homogenate. Measurements of 5'-nucleotidase (5'-ribonucleotide phosphohydrolase EC 3.1.3.5) and of glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) activities showed enrichments in the fraction of from 11- to 14-fold and 2- to 5-fold respectively, relative to homogenate, on a specific activity basis. Levels of the enzyme activities in the cell-wall preparation ranged from 7 to 8 per cent of homogenate activity for 5'-nucleotidase and 2 to 3 per cent for glucose-6-phosphatase. The data clearly indicate that these activities are associated with the cell wall, but do not preclude the possibility that the enzymes, rather than being part of the protein normally present in the cell wall, are bound to membrane which in turn is imbedded in the wall.

INTRODUCTION

A NUMBER of enzymes have been reported as being present in the cell wall of a variety of tissues. For example, Kivilaan *et al.* were able to identify ATP ase (ATP phosphohydrolase, E.C. 3.6.1.3) invertase (β-D-fructofuranoside fructohydrolase, E.C. 3.2.1.26), inorganic pyrophosphatase (pyrophosphate phosphohydrolase, E.C. 3.6.1.1) and uridine diphosphate glucose pyrophosphorylase (UTP: α-D-glucose-1-phosphate uridylyl transferase, E.C. 2.7.7.9) as well as a nonspecific phosphatase activity responding to α-glycerophosphate as a substrate in a cell-wall fraction isolated from maize coleoptiles.[1] In addition, for roots of both barley seedlings[2] and maize,[3] the major proportion of cellular ascorbate oxidase activity (L-ascorbate: oxygen oxidoreductase, E.C. 1.10.3.3) has been found in the cell wall and ATPase has been detected in an isolated cell-wall fraction from oat root.[4] Peroxidase (donor: hydrogen-peroxide oxidoreductase, E.C. 1.11.1.7) has been reported as being present in the cell wall of pea roots by Siegel[5] and more recently Jong has demonstrated by enzyme histochemistry that this enzyme is primarily associated with the wall in mature onion cells.[6]

One of the earliest procedures used for isolating cell wall involved extraction in hot ethanol in order to obtain an alcohol-insoluble polysaccharide residue, a technique that was employed primarily for analysis of wall constituents.[7-9] However, various other methods

[1] A. KIVILAAN, T. C. BEAMAN and R. S. BANDURSKI, *Plant Physiol.* **36**, 605 (1961).
[2] S. I. HONDA, *Plant Physiol.* **30**, 174 (1955).
[3] D. MERTZ, *Am. J. Botany* **48**, 405 (1961).
[4] J. FISHER and T. K. HODGES, *Plant Physiol.* **44**, 385 (1969).
[5] S. M. SIEGEL, *Physiol. Plantarum* **8**, 20 (1955).
[6] D. W. JONG, *J. Histochem. Cytochem.* **15**, 335 (1967).
[7] M. A. JERMYN and F. A. ISHERWOOD, *Biochem. J.* **64**, 123 (1956).
[8] J. CRONSHAW, A. MYERS and R. D. PRESTON, *Biochim. Biophys. Acta* **27**, 89 (1958).
[9] R. C. BEAN and L. ORDIN, *Anal. Biochem.* **2**, 544 (1961).

utilizing homogenization have also been developed. For example, Kivilaan *et al.* obtained a purified preparation of wall from corn coleoptiles by homogenizing in glycerol and subsequently filtering through glass beads.[10] Homogenization, followed by various schemes of centrifugation, has also been used.[9] In this study, a purified cell-wall fraction was isolated from cotyledon tissue of *Phaseolus vulgaris* by low-speed centrifugation of a homogenate and ultimately centrifugation through a sucrose barrier. Levels of phospholipid in the preparation were determined in order to get some indication of the extent of cytoplasmic contamination. Evidence is presented which clearly demonstrates that glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, E.C. 3.1.3.9) and 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, E C. 3.1.3.5) activities are associated with the purified wall fraction.

RESULTS AND DISCUSSION

A two-step homogenization procedure was used in the preparation of the isolated cell-wall fraction in order to minimize cytoplasmic contamination. The tissue was initially ground with a mortar and pestle and the suspension filtered through cheese-cloth. The debris retained on the cheese-cloth was then rehomogenized with an Omnimixer and it was this suspension that was fractionated. It is likely that the majority of the cells were broken open by the initial treatment with a mortar and pestle, thus allowing much of the cytoplasm to escape. Consequently, the debris from this homogenization should have been somewhat enriched in cell wall fragments. The second homogenization further helped to ensure that all cells were broken and that as much as possible of the cytoplasm was released.

When wet mounts of the purified cell-wall fraction were examined qualitatively for the presence of cellulose by staining with I-KI and H_2SO_4, the cell-wall fragments turned dark blue. The preparation was contaminated with starch granules and these also turned blue in the presence of this stain, but could be easily distinguished from cell-wall fragments on the basis of their differing morphologies. It is apparent, however, from a consideration of levels of cellulose per mg of protein that there was an enrichment of cellulose of from 3 to 8 times relative to homogenate in the purified cell-wall fraction (Table 1). Thus even though starch contamination was still prevalent, the enrichment of cellulose in the isolated wall fraction indicated that a significant purification of cell wall away from cytoplasmic protein was achieved during the isolation procedure.

Glucose-6-phosphatase and 5′-nucleotidase activities were found to be enriched in the purified cell-wall fraction by from 2- to 5-fold and 11 to 14 respectively, relative to homogenate, on a specific activity basis (Table 1). The wall fraction also showed an enrichment in ATPase activity (Table 1) and this is in agreement with a previous report by Fisher and Hodges of ATP hydrolyzing activity in a cell-wall fraction isolated from oat root.[4] Since the nature of the two-step homogenization procedure used to prepare the purified wall fraction did not permit preservation of a sample of original homogenate, a reference homogenate was prepared by treatment of cotyledons from the same batch of seedlings with the Omnimixer. This homogenate was taken to be representative of that from which the cell-wall fraction was derived and was used for all homogenate analyses. Recovery values with respect to the reference homogenate ranged from 75 to 110 per cent for 5′-nucleotidase, 67–90 per cent for glucose-6-phosphatase and 84–109 per cent for ATPase. Levels of enzyme activities in the fraction were found to be about 7–8 per cent of that in the homogenate for 5′-nucleotidase, 2–3 per cent for glucose-6-phosphatase and 5–10 per cent for ATPase (Table 2).

[10] A. KIVILAAN, T. C. BEAMAN and R. S. BANDURSKI, *Nature* **184**, B.A. 81 (1959).

TABLE 1. COMPARATIVE CHEMICAL AND ENZYMATIC DATA FOR HOMOGENATE AND ISOLATED CELL WALL FROM COTYLEDON TISSUE OF *Phaseolus vulgaris*

	Expt.	Homogenate	Cell-wall preparation
5′-Nucleotidase	A	4·8	52·4
	B	6·8	93·2
	C	8·0	116·0
Glucose-6-phosphatase	A	35·6	76·0
	B	26·4	116·0
	C	38·8	194·0
ATPase	A	2·5	33·5
	B	3·5	53·6
	C	2·1	42·4
Cellulose/mg protein	A	82·8	230
	B	61·3	462
	C	122·0	315

Enzyme activities are expressed as μg P/mg protein/hr for 5′-nucleotidase and glucose-6-phosphatase, and as mg P/mg protein/hr for ATPase. Cellulose levels are expressed as mg glucose/mg protein.

TABLE 2. LEVELS OF ENZYME ACTIVITIES IN A CELL-WALL FRACTION ISOLATED FROM COTYLEDON TISSUE OF *Phaseolus vulgaris*

	Expt.	Percentage of homogenate activity
5′-Nucleotidase	A	7·5
	B	7·1
	C	8·5
Glucose-6-phosphatase	A	1·5
	B	2·3
	C	3·0
ATPase	A	6·5
	B	5·4
	C	9·9

As suggested by Hubscher and West,[11] EDTA and KF were routinely present in the assay mixture for glucose-6-phosphatase at a final concentration of 4 mM and in a few instances were also included at the same concentration as additional components in the assay mixture for 5′-nucleotidase when activities of homogenates were being measured. Acid phosphatase and alkaline phosphatase (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.1 and E.C. 3.1.3.2 respectively) have been found to be almost completely inhibited by EDTA and KF in cotyledon tissue of *Phaseolus vulgaris*.[12] Thus, the inclusion of these two components in the glucose-6-phosphatase assay mixture on a routine basis helped to ensure that glucose-6-phosphate was not being hydrolyzed by nonspecific phosphatases. Furthermore, the addition of EDTA and KF to the 5′-nucleotidase assay did not cause any inhibition of enzyme

[11] G. HUBSCHER and G. R. WEST, *Nature* **205**, 799 (1965).
[12] J. E. THOMPSON, *Can. J. Biochem.* **47**, 685 (1969).

activity. In fact, in some instances there was a stimulation in the presence of the inhibitors. This would seem to indicate that adenosine-5′-monophosphoric acid as well is not being acted upon by nonspecific phosphatases. It would seem likely therefore that very little if any of either of these two enzyme activities found in the cell wall is due to the action of acid or alkaline phosphatase.

The phospholipid P content of the isolated cell-wall fraction was found to be about 1 per cent of that in the homogenate (0·9, 1·0 and 1·2 per cent from experiments A, B and C respectively; see Tables 1 and 2). This low level indicated that the preparation was relatively free of membranous contamination but these figures cannot be interpreted as absolute values because they are dependent upon the proportion of total cell wall isolated. It can be assumed, however, that a fraction sedimented from a reference homogentate by centrifugation at 2500 *g* for 10 min would contain all of the cell-wall fragments, in addition to some cytoplasmic components. A comparison of the glucose-6-phosphatase and 5′-nucleotidase levels in such a fraction, expressed as percentage of reference homogenate activity, with those in the isolated cell-wall fraction expressed on the same basis, indicated that from 10 to 25 per cent of the total cell wall was present in the isolated fraction. Thus by applying this approximation as a correction factor to the values for phospholipid given above it becomes apparent that the realistic level of phospholipid contamination in the isolated cell-wall fraction probably ranged from 4 to 10 per cent of that in the homogenate. It is quite well documented that portions of cytoplasmic membranes are imbedded in plant cell walls, particularly in the regions of plasmadesmata.[13] Moreover, it has been demonstrated for mammalian tissues that 5′-nucleotidase is localized on the plasma membrane[14–16] and glucose-6-phosphatase on endoplasmic reticulum.[17] Thus the possibility that the enzymes detected in the cell-wall fraction are bound to membranes imbedded in the cell wall, rather than being simply part of the protein complement of the wall, still remains tenable.

EXPERIMENTAL

Materials

Amylopsin was obtained from Difco Laboratories Ltd., diastase from Parke Davis and Co., glucostat reagent from Worthington Biochemical Corp., and glucose-6-phosphate, adenosine-5′-monophosphoric acid and DL-α-tocopherol from Sigma Chemical Co. All other chemicals and solvents were Fisher reagent grade.

Cell-Wall Isolation

Seeds of *Phaseolus vulgaris*, variety Kinghorn, were germinated and grown in vermiculite in the dark at 28° and the cotyledons harvested after 4 days. For preparation of a purified cell-wall fraction, the cotyledons were first cut up finely with scissors. All subsequent operations were carried out at 5°. An initial homogenization consisted of grinding 20 g of tissue with a mortar and pestle in 60 ml of precooled 50 mM $NaHCO_3$, pH 7·5. This suspension was filtered through six layers of cheese-cloth and the filtrate discarded. The debris was immediately resuspended in 60 ml of $NaHCO_3$ and macerated in a blade-type homogenizer (Sorvall Omnimixer) at maximum speed for three periods of 20 sec, each separated by 30-sec intervals for cooling. An initial centrifugation of this suspension at 2500 *g* for 10 min yielded a double-layered pellet consisting of a tightly packed bottom portion, primarily starch, and a more loosely packed upper portion. The supernatant was removed and the upper portion of the pellet resuspended in the original volume of $NaHCO_3$. This suspension was again centrifuged as before, and the upper portion of the pellet resuspended in one-half of the original volume of $NaHCO_3$. These procedures of resuspension and centrifugation were repeated until a homogeneous pellet with no apparent trace of the original bottom portion was obtained. This final pellet was then

[13] W. G. WHALEY, H. H. MOLLENHAUER and J. H. LEECH, *Am. J. Botany* **47**, 401 (1960).
[14] A. B. NOVIKOFF and E. ESSNER, *Fed. Proc.* **21**, 1130 (1962).
[15] R. COLEMAN and J. B. FINEAN, *Biochim. Biophys. Acta* **125**, 197 (1966).
[16] R. COLEMAN, R. H. MICHELL, J. B. FINEAN and J. N. HAWTHORNE, *Biochim. Biophys. Acta* **135**, 573 (1967).
[17] S. GOLDFISCHER, E. ESSNER and A. B. NOVIKOFF, *J. Histochem. Cytochem.* **12**, 72 (1964).

subjected to centrifugation through a layer of sucrose of density 1·22 in a Spinco 25·1 swinging-bucket rotor. For this purpose, the pellet was resuspended in 35 ml of $NaHCO_3$ and, for each centrifuge tube, 10 ml of this suspension were layered on 20 ml of sucrose, density 1·22. After centrifugation for 30 min at 58,750 g a faint layer was present at the interface and there was a pellet at the bottom of the tube. The bottom pellet was the purified cell-wall fraction.

The reference homogenate was prepared by suspending 10 g of cotyledons from the same batch of seedlings used for the cell-wall preparation in 30 ml of $NaHCO_3$ solution and homogenizing with the Sorvall Omnimixer for six periods of 20 sec with 30-sec intervals between each treatment for cooling.

Enzymatic and Chemical Determinations

All tissue fractions and aliquots of homogenates were stored at −10° until required. Glucose-6-phosphatase and 5′-nucleotidase were assayed according to the methods of Hubscher and West[11] and Michell and Hawthorne[18] respectively. ATPase was measured as described by Fisher and Hodges;[4] no stimulating ions were present in the assay mixture. Assays for 5′-nucleotidase were always carried out on the day following the preparation and, for glucose-6-phosphatase and ATPase, within 4 days of the date of preparation. These storage conditions were found to have no noticeable effect on the enzyme activities of the fractions. Protein was routinely determined by the method of Lowry *et al.*[19]

For phospholipid determinations, lipids were extracted as described by Dallner *et al.*[20] 2-ml aliquots of the homogenate and cell-wall suspension were each extracted with 12 ml of $CHCl_3$–ethanol (1:1 on a volume basis), containing DL-α-tocopherol at a concentration of 10 mg/ml, for 16 hr in N_2. The extraction mixture was then filtered and aliquots (0·4 ml for homogenate and 1·0 ml for the cell-wall fraction) were placed in digestion flasks and evaporated to dryness at 100°. Digestions and determinations of P were carried out as described by King.[21] P levels of undigested samples were subtracted.

The qualitative tests for cellulose were performed on the isolated cell-wall fraction by applying a solution of I_2–KI together with sulphuric acid (H_2SO_4) to wet mounts of the preparation in the manner described by Jensen.[22] For quantitative determinations of cellulose, levels of glucose were measured subsequent to acid hydrolysis. Prior to hydrolysis, however, starch was made soluble by treatment with diastase and amylopsin according to the assay procedure of Bernfeld.[23] The reaction mixture consisted of 0·5 ml of sample suspended in 1·5 ml of 0·02 M phosphate buffer, pH 6·0, and to this were added 100 mg each of diastase and amylopsin together with two drops of toluene to inhibit bacterial growth. The samples were incubated for 20 hr at 20° and then centrifuged at 2000 *g* for 15 min. The pellets were washed several times in distilled water by centrifugation and finally subjected to hydrolysis in H_2SO_4 as described by Adams.[24] The hydrolysate was neutralized (NaOH) and glucose levels measured using a prepared glucostat reagent according to the method of Dahlqvist.[25]

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