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MEMBRANE-BOUND NUCLEOLYTIC ACTIVITY OF CORN ROOT CELLS

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Abstract– The plasma membrane and mitochondrial fractions isolated from a homogenate of maize roots by differential centrifugation and discontinuous sucrose density gradient centrifugation were characterized by means of marker enzymes. Nuclease activity was noted in both fractions. The enzyme from the plasma membrane fraction was isolated and identified as nuclease I.

INTRODUCTION

The occurrence of deoxyribonuclease activity in the plasma membranes of maize roots has been reported [1]. The fact that the estimation of enzyme activity was made using whole, unhomogenized maize roots immersed in a medium containing the substrate showed that the enzyme was released from the root cells into the medium. Wilson [2–5] found on differential centrifugation of a root homogenate in sucrose that about 75% of the nucleolytic activity precipitated in the 10000 *g* fraction ('mitochondria') and about 14% precipitated in the 104000 *g* fraction ('microsomes') [2].

The plasma membrane and mitochondria of corn root cells have been isolated by differential centrifugation followed by discontinuous sucrose density gradient centrifugation [6]. The 13000 *g* (mitochondria) and 13000 *g* to 80000 *g* (plasma membrane) pellets from the same homogenate were centrifuged in non-linear sucrose density gradients [6]. The isolated fractions were characterized on the basis of the following criteria: (a) increase of K^+-stimulated, Mg^{2+}-dependent ATPase activity at pH 6.5 in the plasma membrane as compared with mitochondrial ATPase activity at pH 6.5; (b) lower cytochrome *c* oxidase activity of the plasma membrane as compared with that of mitochondria. More recently, Gallagher and Leonard [7] have pointed out that the plasma membrane ATPase activity can be distinguished by its sensitivity to vanadate and its insensitivity to molybdate or azide.

In the present study the plasma membrane- and mitochondria-rich fractions were isolated from maize roots and characterized using the above criteria. Particular attention was paid to the occurrence of nucleolytic activity in these two fractions.

RESULTS

To obtain the characteristics of isolated fractions of the plasma membrane and mitochondria, and to calculate the degree of their mutual cross-contamination, the activity of ATPase at pH 6.5, the effect of inhibitors of ATPase, as well as the activity of cytochrome *c* oxidase, were estimated (Table 1). In addition, the alkaline phosphodiesterase activity in these two fractions was also measured. The data presented in Table 1 show that at pH 6.5, the ATPase activity of the plasma membrane fraction was about twice as high as that of the mitochondrial fraction. At pH 6.5, the ATPase activity of the plasma membrane fraction was inhibited 37% by 225 μM vanadate and 7% by 0.1 mM azide while 0.1 mM molybdate was without effect. The pH 6.5-ATPase of mitochondria was sensitive to vanadate (17% inhibition). The latter effect was presumably due to contamination of the mitochondrial fraction with plasma membrane ATPase activity.

The activity of the mitochondrial marker enzyme, cytochrome *c* oxidase, in the plasma membrane fraction constituted 46% of the activity found in the mitochondrial fraction. This showed that the plasma membrane fraction was contaminated with mitochondria. The results presented in Table 1 show that the plasma membrane and mitochondrial fractions were free from the alkaline phosphodiesterase activity present in the homogenate. There was no exonucleolytic activity towards thymidine-5′-*p*-nitrophenyl-P. It was assumed that in the fractions

Table 1. Activities of the marker enzymes in the plasma membrane and mitochondria from corn root cells

Fraction	Cytochrome *c* oxidase (μmol/mg/min)	Alkaline phosphodiesterase (units/mg)	K^+-stimulated ATPase (pH 6.5)			
			Control (μmol Pi/mg/hr)	Vanadate	Molybdate	Azide
				Inhibition (%)		
Plasma membrane	1.6	0	1.9	37	2	7
Mitochondria	3.5	0	0.8	17	15	25

investigated, contamination by soluble cytosolic proteins did not occur. The DNAase activity of various fractions of corn cells is presented in Table 2. It is apparent that the DNAase activity of the plasma membrane and that of mitochondria accounted for 4.5 and 43.3%, respectively, of the overall DNAase activity found in the homogenate. Since the DNAase activity measured for a given fraction in consecutive experiments scarcely varied, if at all, it may be suggested that DNAase activity is bound to the particles and is not derived from the soluble fraction. The total DNAase activity of the mitochondria was found to be ten times higher than that of the plasma membrane, while the specific activity of the enzyme was highest in the latter fraction. The data (Table 2) reveal that the specific activity of DNAase of the plasma membrane was very much higher than that of the homogenate and supernatant after centrifugation at 80000 *g*. These results suggest, therefore, that DNAase activity is not a result of adsorption on the membranes of the soluble fraction.

As can be seen from Table 3, the specific activity of nuclease in the plasma membrane fraction was higher than that of the mitochondrial fraction.

The ratio of DNAase to RNAase activity, estimated at pH 6.2, in the homogenate was 0.95 and that associated with the membranes was 0.62 (Table 3). This again suggests that nuclease activity is not the result of enzyme adsorption on the membrane in the course of homogenization.

Plasma membrane nuclease revealed two peaks of enzyme activity dependent on pH: at pH 5.5 and pH 7.4 (not shown). The question arises whether two peaks of nucleolytic activity are related to the same enzyme or to two different ones. The enzymes were extracted from the plasma membranes using 0.5 M potassium chloride. Isolation of the enzymes was carried out on a Sephadex G-75 column and by chromatofocusing. The results are presented in Table 4. The nucleolytic activity from the Sephadex G-75 column was applied to a gel PBE 94 column from which two peaks of enzyme activity were eluted: the first was eluted at pH 6.5 and the second at pH 5.2. The properties of the enzyme eluted from the second peak were studied. As shown in Table 4, the nucleolytic activity towards RNA increased six-fold in the course of the isolation procedure. The enzyme hydrolysed single-stranded DNA 1.5 times faster than native double-stranded DNA. The effect of pH on nuclease activity was studied, and a pH optimum of 6.2 for enzymatic hydrolysis of both the denatured DNA as well as RNA was determined (not shown).

Treatment of the products of plasma membrane nuclease digestion of RNA and denatured DNA with either snake venom phosphodiesterase or spleen phosphodiesterase resulted in more extensive degradation in the case of venom phosphodiesterase treatment. This indicates that the plasma membrane nuclease degrades DNA and RNA chains to 3'-OH and 5'-phosphate terminated fragments.

The endonucleolytic action of the enzyme is indicated by the fact that the nuclease did not show exonucleolytic activity against thymidine-5'-*p*-nitrophenyl phosphate (Table 1). In Table 5 the relative activity of nuclease towards various nucleic acids and polynucleotides, measured by formation of acid-soluble products, is shown. Nuclease hydrolysed preferentially *Escherichia coli* rRNA and highly polymerized wheat germ RNA. The commonly used substrate of maize nuclease I—soluble *t*RNA from yeast - was hydrolysed more slowly. The enzyme hydrolysed single-stranded DNA from calf thymus, synthetic double-stranded complex poly(A)-poly(U), single-stranded complex poly(G)-(U) and native double-stranded DNA. Of a number of synthetic ribohomopolymers tested, poly(U) and then poly(A) and poly(C) were most rapidly hydrolysed by nuclease, whereas poly(G) was slightly hydrolysed.

Table 2. Distribution of DNAase activity in various fractions from corn root cells. The activity was measured with [2-^{14}C-*thymine*]DNA [15]

Fraction	Total protein (mg)	Specific activity (cpm × 10^{-3}/ mg protein)	Total activity (%)
Homogenate	16	1.42	100
1000 *g* precipitate	1.3	5.65	32.3
13000 *g* precipitate 'mitochondria'	4.5	2.19	43.3
Discontinuous sucrose gradient 'plasma membrane'	0.35	2.91	4.5
80000 *g* supernatant solution	8.2	0.54	19.4

Table 3. Nucleolytic activity of homogenate, plasma membrane and mitochondria of corn root cells, measured at pH 6.2. Nucleolytic activities were determined spectrophotometrically [13, 14]

Fraction	Specific activity (units/mg protein)			DNAase:RNAase ratio
	Denatured DNA	Native DNA	RNA	
Homogenate	18.0	17.2	18.1	0.95
Plasma membrane	70.0	38.0	61.0	0.62
Mitochondria	43.0	19.0	23.0	0.82

Table 4. Isolation of nuclease I from plasma membrane of corn roots

Fraction	Fraction No.	Total protein (mg)	Specific activity (units/mg protein) RNAase	DNAase: Single-stranded DNA	DNAase: Double-stranded DNA
Extract of plasma membrane		10.2	36.2	42.0	23.0
Sephadex G-75	3–5	4.0	66.0	32.0	18.0
Gel PBE 94					
Peak I	17–20	1.5	13.6	10.6	7.5
Peak II	45–46	1.1	221.8	158.1	92.7

Table 5. Specificity of nuclease I isolated from plasma membrane of corn roots

Substrate	Specific activity (units/mg protein)
E. coli rRNA	306
Wheat germ RNA	303
Yeast *t*RNA	221
Calf thymus single-stranded DNA	158
Poly(U)	145
Complex poly(A)-poly(U)	109
Complex poly(G):(U)	98
Calf thymus double-stranded DNA	92
Poly(A)	56
Poly(C)	52
Poly(dC)	50
Poly(G)	5

Deoxyribohomopolymer such as poly(dC) was also digested. It may be concluded, therefore, that the investigated nuclease may digest almost all tested nucleic acids and synthetic polymers of nucleotides.

DISCUSSION

According to Wilson [4], nuclease I is located in the nucleus, mitochondria, spherosomes, endoplasmic reticulum and vacuoles of corn root cells. Moreover, the occurrence of nucleolytic activity associated (at pH 6.5) with ribosomes, isolated from corn root cells, has been described by Hsiao [8]. The observation by Chang and Bandurski [1] that deoxyribonuclease activity might be located in plasma membranes of corn root cells is supported by the results obtained in the present work.

The data presented in this paper indicate that nuclease is bound to the plasma membrane *in vivo*, and is not associated with this membrane as a result of its adsorption onto the membranes during homogenization. Thus, the enzyme cannot be removed from the plasma membrane fraction by repeated differential centrifugation or by sedimentation through the sucrose gradient used in this work. The DNAase activity associated with the plasma membranes constitutes 4.5% of the total DNAase activity of the homogenate under the experimental conditions described. This value was repeatable for successive preparations. The ratio of DNAase to RNAase activity in the homogenate was 0.95 whereas that associated with the membrane was 0.62. The alkaline phosphodiesterase activity which occurs in soluble form in the homogenate was not found in the isolated plasma membrane fraction. The specific activity of nuclease in the plasma membrane fraction was higher than that of the homogenate.

The results of the present study indicate that the enzyme responsible for nucleolytic activity and isolated from the plasma membrane of corn roots is nuclease I. The following data on the characteristics of the isolated enzyme support this fact: optimum enzyme activity at pH 6.2, lack of specificity towards the sugar residue in the hydrolysed compound, preference of enzyme for single-stranded DNA, products of enzyme activity are characteristic of nuclease I, and finally, the endonucleolytic mode of action of the enzyme. The foregoing features of the enzyme are consonant with those described by Wilson for nuclease I isolated from maize root [2]. Wilson isolated nuclease I from particles of a corn root homogenate and pointed out that the fraction precipitated during centrifugation of corn roots homogenates at 10000 *g* is the best material for isolation of nuclease I [2–4]. However, the observed variability of nucleolytic activity distribution within the subcellular and soluble fractions is attributable to the media used for homogenization of corn roots [2].

The contamination of the plasma membrane fraction with mitochondria had to be clarified in the present investigation since nucleolytic activity, which is known to occur in mitochondria [2], has been found in both fractions. According to Leonard and VanDerWoude, the centrifugation of maize root homogenates at 13000 *g* isolates the mitochondrial fraction in 90% yield [6]. However, there is a certain cross-contamination between the mitochondrial and plasma membrane fractions [6]. Cytochrome *c* oxidase activity in the plasma membrane fraction reached about 7.3% of that found in the mitochondrial fraction [6]. In the present study, cytochrome *c* oxidase activity in the plasma membrane fraction reached about 45% of that found in the mitochondrial fraction. The identification of the isolated fractions in the present investigation was done mainly by means of measurement of vanadate-sensitive ATPase activity in the plasma membrane fraction as well as by the occurrence of a high level of cytochrome *c* oxidase activity in the mitochondrial fraction. The nuclease activity found in the plasma

membrane cannot originate only from contamination by mitochondria since the specific activity of nuclease was higher in the former fraction than in the latter. Nucleolytic enzymes have so far been investigated in higher plants mainly in cell organelles in which synthesis and processing of DNA and RNA occur, i.e. nuclei, mitochondria or plastids. Recently, however, some authors have suggested that nucleolytic enzymes can be bound to membranes [2]. The latter localization of nucleases might be of physiological importance for storage of the enzyme and its release at the appropriate time.

EXPERIMENTAL

Plants. Corn seeds (*Zea mays* L. var. 72) were supplied by the Plant Breeding Station (Kobierzyce, Poland). The seeds were soaked for 12 hr, then germinated for 48 hr in the dark at 26° and the roots harvested.

Reagents. Highly polymerized wheat germ RNA was obtained from Calbiochem. Calf thymus DNA type I, *t*RNA from yeast type X, sucrose grade II, ATP, Poly(G):(U) (polyguanylic : uridilic acid) and thymidine 5'-*p*-nitrophenyl phosphate were purchased from Sigma. *r*RNA from *E. coli* was purchased from BDH Chemicals. Poly(A), Poly(U), Poly(C), Poly(G), Poly(dC), Poly(G) and Poly(A : Poly U) (polyadenylate : polyuridylate) were purchased from Miles. Snake venom phosphodiesterase and spleen phosphodiesterase were products of Worthington Biochem. Gel PBE^{TM}94 for chromatofocusing and Sephadex G-75 were purchased from Pharmacia Fine Chemicals. Polybuffer TM 74 was a gift from Pharmacia. Cytochrome *c* from porcine heart and vanadium pentoxide were from PPH (Poland). [2-^{14}C-*Thymine*]DNA from *E. coli* (1.21 MBq/mg) was obtained from the Institute for Research, Production and Application of Radioisotopes, Prague, Czechoslovakia.

Isolation of mitochondria and plasma membrane. The roots were rinsed and then homogenized with 0.25 M sucrose/0.001 M Tris–MES buffer, pH 7.2, in a mortar. The homogenate was filtered through cheesecloth and centrifuged at 1000 *g* for 10 min and at 13000 *g* for 20 min. The resulting supernatant was collected and centrifuged at 80000 *g* for 30 min. Isolation of the plasma membrane and mitochondria by means of a non-linear sucrose gradient was performed according to the method of ref. [6]. The pellets from the 13000 *g* and 80000 *g* centrifugations were suspended in 0.25 M sucrose/0.001 M Tris–MES buffer, pH 7.2, and layered onto 36 ml gradients consisting of 28 ml 45% and 8 ml 34% sucrose in 0.001 M Tris–MES, pH 7.2. The gradients were centrifuged for 2 hr at 82500 *g* in a Beckman SW-27 rotor. The plasma membrane or mitochondria were collected from the interface and used for further expts.

Activity of the marker enzymes. ATPase activity was measured according to the method of ref. [9]. The incubation mixture contained 3 mM ATP–Tris form, 3 mM $MgSO_4$, 30 mM Tris–MES (pH 6.5), 50 mM KCl and the membrane or mitochondria preparation in a final vol. of 1 ml. The final concns of inhibitors in the incubation mixture were as follows: 225 μM vanadate, 0.1 mM molybdate, 0.1 mM azide. The Pi released during incubation was estimated according to the method described in ref. [10].

Cytochrome *c* oxidase activity was assayed as described in ref. [11] by measuring the oxidation of reduced cytochrome *c* at 550 nm.

Alkaline phosphodiesterase assay was performed according to the method described in ref. [12]. Activity against thymidine-5'-*p*-nitrophosphate was assayed by incubation of the substrate (5 mM) in 0.1 ml 0.1 M Tris–HCl buffer, pH 7.5, with the appropriate amount of enzyme preparation at 37°. One unit of alkaline phosphodiesterase activity is defined as the amount of enzyme which liberates 1 μmol *p*-nitrophenol from thymidine 5'-*p*-nitrophosphate in 15 min under these conditions.

Nucleolytic activity determination. RNAase and DNAase activities were determined according to the methods described in refs. [13, 14]. The absorbance of the acid-soluble nucleotides released from the nucleic acids by the enzymes were measured at 260 nm. For either enzyme, one unit of activity is defined as the amount of enzyme which, under the condition of assay after 1 hr incubation, leads to an increase by 0.1 at ΔE = 260 nm. Sp. act. is expressed in units/mg protein.

Hydrolysis of [2-^{14}C-*thymine*]DNA from *E. coli* was examined as described in ref. [15]. The reaction mixture contained 22 μmol acetate buffer (pH 6.2), enzyme preparation and radioactive DNA (15000 cpm). After incubation at 37°, the mixture was cooled and 10 μg of calf thymus DNA was added. 100 μl of this mixture was applied to a Whatman glass microfibre paper (GF-C filter), dried and acid-insoluble DNA precipitated by immersing the filter in 15% trichloroacetic acid at 4°. The filters were washed twice with EtOH and once with Et_2O and counted in a liquid scintillation counter using PPO–POPOP–toluene scintillation liquid.

Hydrolysis of ribo- and deoxyribohomopolymers and their complexes was carried out according to the method of ref. [16]. The following modification was introduced: the reaction was terminated by addition of 12% $HClO_4$ in 20 mM lanthanum acetate. The termini produced by scission of DNA and RNA by corn root plasma membrane nuclease were analysed according to the method described in ref. [17].

Protein was determined by the methods of refs. [18, 19].

Extraction of proteins. The plasma membranes were incubated in 0.5 M KCl/0.05 M Tris–HCl, pH 7.4, for 18 hr at 4°. The plasma membranes were removed by centrifugation at 80000 *g* and the supernatant was dialysed against Tris–HCl buffer, pH 7.4.

Sephadex G-75 chromatography. The proteins extracted from the plasma membrane were applied onto a Sephadex G-75 column (1.5 × 12 cm) which had been previously equilibrated with 0.05 M Tris–HCl buffer, pH 7.4. Elution of proteins was carried out using the same buffer. 2 ml fractions were collected and their ΔE (280 nm) was measured. The most active fractions in the activity peak (Nos 3–5) were pooled and used for the next step of the enzyme isolation procedure.

Separation of proteins by chromatofocusing. The fractions containing nuclease activity were applied onto a column (1 × 9 cm) of PBE 94 gel (anion exchanger, Pharmacia), equilibrated with 0.025 M imidazole buffer, pH 7.5. Separation of the proteins was carried out in a gradient produced by 0.025 M starting buffer: imidazole–HCl, pH 7.5, and elution buffer 0.0075 mmol/pH unit/ml: polybuffer 74 + HCl, pH 4.0. These two buffers were gradually mixed in the mixing chamber of a gradient maker (MSE Cat. No. 36657). Fractions of 2 ml were collected and the activities of RNAase and DNAase estimated. Proteins were determined by measuring the absorption at ΔE (280 nm) and by the methods described in ref. [19].

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