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PHOSPHOLIPASE A ACTIVITY IN COMMERCIAL NUCLEASES

IMPLICATIONS FOR MEMBRANE VESICLE ISOLATION

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Phospholipase A activity was detected in commercial DNAases I and II and in RNAase preparations. The amount of phospholipase correlates inversely with the degree of nuclease purification. The assessment of the level of phospholipase in commercial nucleases is important in cases where enzymatic properties other than those of DNAases and RNAases are to be investigated and when these preparations are to be used in the isolation of biological membranes.

In the procedures for fractionation of cellular membranes from Gram-negative bacteria, small amounts of DNAase and RNAase are usually added for cell disruption and the isolation of outer and inner membrane vesicles [1–5]. Our recent work on isolated membrane fractions from *Escherichia coli* and *Salmonella anatum*, however, indicated that the addition of commercial nuclease preparations introduced phospholipase activities that initially interfered with the interpretation of some of our data [5]. Similar observations were made by Thiel and Astrachan [6] who had studied the phospholipase activity in phage lysates; they reported that the commercial DNAase preparation used in their studies contained phospholipase. We were concerned with the isolation of vesicles of the outer and inner cell membranes and with the identification of markers for membrane fractions of intermediate sucrose density. One marker was found to be the membrane-bound phospholipase A which appears to be active at these sites [5]. We used a DNAase I which had been purified by Lipson and Stuehr (unpublished data) by a modification of the procedures of Kunitz [7] and Price et al. [8]. In this procedure, the commercial enzyme preparation was treated with phenylmethylsulfonyl fluoride and was subjected to chromatography on DEAE-Sephadex A-25 in 50 mM Tris-HCl, 10 mM Ca^{2+} buffer at pH 7.2; the concentrated DNAase $I_{(A)}$ had a specific activity of about 3000 Kunitz units (Lipson, K., personal communication). The enzyme was essentially phospholipase free.

The amount of phospholipase A activity present in commercial nucleases was assessed by measuring the release of free fatty acids from a ^{14}C-labeled glycerophospholipid substrate. Preparations of the substrate followed essentially the procedure outlined by Doi and Nojima [9]. 2 μCi [^{14}C]palmitic acid (16 : 0) and 2 μCi [^{14}C]oleic acid (18 : 1) (both from New England Nuclear) were dried under N_2 and added to 2 mg/ml fatty acid-free bovine serum albumin (Sigma) in double-distilled H_2O. 1 ml of a mid-log phase *E. coli* B suspension and 8 ml nutrient broth were incubated on a shaker for 3 h at 37°C with the ^{14}C-labeled fatty acid mixture. The culture was harvested by centrifugation, followed by two washes in nutrient broth (containing 2% bovine serum albumin). The pellet, suspended in 0.15 M NaCl, was autoclaved for 30 min at 120°C. Cells were washed twice in sterile saline solution (containing 2% bovine serum al-

bumin) and resuspended in a total volume of 1 ml saline solution without bovine serum albumin. 0.1-ml aliquots were frozen at −70°C. Total ^{14}C activity was about $4–5 \cdot 10^5$ cpm/ml. Phospholipase A activity was assayed by a slight modification of the procedures given by Folch et al. [10] and Scott et al. [4] (see legend to Table I). A mixture of chloroform/methanol (2 : 1, v/v) was used to stop the incubation process and to extract fatty acids and lysoglycerophospholipids (extraction overnight, room temperature). Chloroform and 50 mM KCl were added to the methanol mixture, and the lipid-containing chloroform phase was dried under N_2. The enzyme digests yielded free fatty acids, lysoglycerophospholipids and acylglycerols; they were separated by thin-layer chromatography (TLC) on silica gel H plates (Analabs, Inc.) in a solvent system of petroleum ether/diethyl ether/glacial acetic acid (80 : 20 : 1.1, v/v) at 4°C [11]. Unlabeled oleic acid (Analabs), palmitic acid (Sigma), phosphatidylethanolamine (Sigma) and lysophosphatidylethanolamine (Sigma) were run as controls on the same TLC plates. Chromatograms were stained with iodine vapor. R_f sections of 1.2 cm length covering the entire distance from origin to solvent front were scraped off and counted in a liquid scintillation counter (Beckman LS-233). The amount of lysoglycerophospholipids as well as that of mono- and diacylglycerols liberated by nucleases from the substrate was very small if no phospholipase A_2 (Sigma) was added (i.e., only 1.2–1.5%). However, almost all of the tested commercial nucleases exhibited substantial amounts of phospholipase A activity (Table I). The amount of free fatty acids released from the substrate by 1 mg of the nuclease ranged from 10.3 to 30.3%. Our data show that the degree of DNAase I purification correlates inversely with the amount of phospholipase present in these preparations whereas no significant corre-

TABLE I

PHOSPHOLIPID HYDROLYSIS BY COMMERCIAL NUCLEASES

Nucleases: 1 mg in 200 µl of buffer (M9 medium [12]); incubation with ^{14}C-fatty acid-labeled *E. coli* in a mixture of 100 mM Tris-HCl, pH 7.5, and 200 mM $CaCl_2$ for 2 h at 37°C.

Nuclease	Source	% of free fatty acids released from substrate (±S.E.)
DNAase I (DN-100)	1305 Kunitz U/mg protein (Sigma)	30.3±4.3
DNAase I (DP)	Purified, 1400–1800 Kunitz U/mg dry wt. (Worthington)	27.8±4.8
DNAase I (grade 1)	Lyophilized, 1684 Kunitz U/mg (Boehringer, Mannheim)	20.9±1.4
DNAase I (DN-EP)	Electrophoresis purified, 2770 Kunitz U/mg protein (Sigma)	10.3±0.5
DNAase $I_{(A)}$	Purified from Sigma DNAase I by DEAE-Sephadex A-25 chromatography, approx. 3000 Kunitz U/mg dry wt.	3.9±2.8
DNAase II (Type V)	800 Kunitz U/mg protein (Sigma)	20.2±8.3
DNAase II (B-grade)	Lyophilized, 240 Kunitz U/mg material (Calbiochem)	14.3±1.7
RNAase A (Type IIA)	64 Kunitz U/mg (Sigma)	27.7±3.0 [a] 3.8±1.5 [b]
RNAase A (R)	Twice crystallized, 2650 Kunitz U/mg dry wt. (Worthington)	30.3±3.3 [a] 4.9±2.9 [b]
Phospholipase A_2	10 µg (from bee venom, Sigma), 1300 Kunitz U/mg protein	89.2±3.5
Sterile double-distilled H_2O	Control	2.6±1.2

[a] Sample boiled for 1 min on water bath.
[b] Sample boiled for 6 min on water bath.

lation could be deduced from the activities of the two DNAase II preparations tested. Boiling RNAase A for 6 min also destroyed practically all phospholipase A activity. In control experiments, 10 μg of phospholipase A_2 (Sigma) hydrolyzed 89.2% of the substrate into free fatty acids.

This study will help to assess the level of phospholipase activities in commercial nucleases which should be especially important in cases where degradative properties other than those of DNAase and RNAase are to be investigated.

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