IN VIVO ACTIVATION BY POLYMYXIN B OF PHOSPHOLIPASE FROM *PSEUDOMONAS AERUGINOSA* AND *ESCHERICHIA COLI*

Sir:

As reported previously, we observed a marked degradation of phospholipid in *Pseudomonas aeruginosa* AMS 6073 induced by polymyxin B(PLB).[1] We wish to report in this communication that this degradation of phospholipid is due to the *in vivo* activation of phospholipase which liberates fatty acids from the substrate.

The activity of the phospholipase was assayed by the formation of either free fatty acids and fatty acid methyl esters or lysophosphatidylethanolamine from the substrate, phosphatidylethanolamine. The control enzyme from the non-treated cells of *P. aeruginosa* AMS 6073 was prepared as follows.[2] The cells were grown in a nutrient broth (100 ml) at 30°C with shaking. When the optical density (O.D.) of the culture at 660 nm reached 0.55~0.60, the cells were harvested by centrifugation and washed with cold saline. The washed cells were suspended in 2 ml of 5 mM Tris-HCl buffer (pH 7.0) and sonicated at 10 kHz for 2 minutes at 0°C. The sonicate was centrifuged twice at 8,500×*g* at 0°C for 15 minutes. The supernatant was used as the crude enzyme preparation. The enzyme from the PLB-treated cells was prepared similarly except that 5 μg/ml of PLB was added when the O.D. at 660 nm of the culture reached 0.3 and the culture was continued at 30°C with shaking for an additional 60 minutes. The O.D. at 660 nm at the time of harvest was approximately 0.42~0.45. The crude enzyme was prepared from the PLB-treated cells as above. The substrate, ^{14}C-phosphatidylethanolamine [^{14}C-PE, specific radioactivity = 703 c.p.m./mμ mole, exclusively labeled at fatty acid moieties (Personal communication of Dr. S. NOJIMA of the University of Tokyo)] was prepared according to the procedure of DOI *et al.*[3] from cells of *Escherichia coli* W2252 grown in the presence of (1-^{14}C) acetate. The standard reaction mixture contained 0.5 ml of the enzyme solution, 0.15 ml of 1 mM $CaCl_2$, 0.5 ml of 0.1 M Tris-HCl buffer (pH 8.0), 0.25 ml of 800 μM ^{14}C-PE and 1.5 ml of methanol, in a final volume of 3 ml. Incubation was carried out at 37°C with shaking for 30 minutes. After the incubation, the mixture was immediately chilled and 1.5 ml of methanol and 6.0 ml of chloroform were added. The lipid materials in the reaction mixture were extracted by the modified procedure of BLIGH and DYER.[4] Chloroform in the extract was evaporated to dryness. The resulting residue was dissolved in a small volume of chloroform - methanol (2: 1, v/v) and spotted onto a pre-coated thin-layer plate (Silica gel 60, Merck: Code No. 5724). The plate was developed with a solvent system consisted of chloroform - methanol - water (65: 25: 4, v/v). After the autoradiography, areas corresponding to phosphatidylethanolamine (Rf 0.54), lysophosphatidylethanolamine (Rf 0.23), free fatty acids (Rf 0.87) and fatty acid methyl esters (Rf 0.92) were scraped off from the thin-layer plate. Their radioactivities were counted by a liquid scintillation spectrometer (Packard Tri-Carb 3385) using a toluene scintillation fluid.

As shown in Table 1, the enzyme preparation from the PLB-treated cells of *P. aeruginosa*

Table 1. Activation of ^{14}C-PE degradation by PLB-treated enzyme preparation

Enzyme source (mg protein/ml)			Substrate	Reaction product		Reaction product/ Total (%)	Relative activity (%)/mg protein
			PE (D.P.M.)	LPE* (D.P.M.)	FA + FAME** (D.P.M.)		
P. aeruginosa	None-treated	(13.7)	15,400	250	540	4.9	0.36
	PLB-treated 5 μg/ml	(10.8)	15,040	230	1,430	9.9	0.92
E. coli	None-treated	(7.7)	13,000	1,620	1,820	21.0	2.73
	PLB-treated 3 μg/ml	(6.5)	11,100	2,510	2,970	33.0	5.08
	5 μg/ml	(6.1)	8,570	4,090	4,840	51.0	8.36

* Lysophosphatidylethanolamine.

** Fatty acids and fatty acid methyl esters.

caused a marked degradation of ^{14}C-PE, and approximately 10% of exogeneously added substrate was degraded to free fatty acids and fatty acid methyl esters. On the other hand, no such marked degradation was observed with the control enzyme. Although data are not shown, a similar activation of phospholipase was observed with the cells which were incubated with PLB (5 μg/ml) only for 5 minutes.

Essentially the same results were obtained with *Escherichia coli* W2252 (Table 1). The crude enzymes were prepared from the non-treated and PLB (3 and 5 μg/ml)-treated cells of *E. coli* in the same way as those of *P. aeruginosa*. The detergent-resistant phospholipase A activity was measured by the same method as described by NOJIMA *et al.*[2] It is known that the enzyme was slightly activated by the sonication process[5]. In keeping with this, the control enzyme from *E. coli* showed a higher activity than that of *P. aeruginosa*. However, the enzyme fraction prepared from the PLB-treated *E. coli* cells contained a much higher phospholipase A activity than the control enzyme.

The activation of phospholipase occurred only under *in vivo* conditions, since there was no marked effect on the activity of the control enzymes when incubated with various concentrations of PLB *in vitro*. The mechanism of the activation of phospholipase by PLB is now under investigation.

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References

1) KUSANO, T.; K. IZAKI & H. TAKAHASHI: Degradation of phospholipid in *Pseudomonas aeruginosa* induced by polymyxin B. J. Antibiotics 28: 689~695, 1975
2) DOI, O.; M. OHKI & S. NOJIMA: Two kinds of phospholipase A and lysophospholipase in *Escherichia coli*. Biochim. Biophys. Acta 260: 244~258, 1972
3) DOI, O. & S. NOJIMA: Phospholipase C from *Pseudomonas fluorescens*. Biochim. Biophys. Acta 248: 234~244, 1971
4) BLIGH, E. G. & W. J. DYER: A rapid method of total lipid extraction and purification. Canad. J. Biochem. Physiol. 37: 911~917, 1959
5) DOI, O. & S. NOJIMA: (in Japanese) Kagaku-to-Seibutsu 11: 178~190, 1973