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Determination of covalently bound *myo*-inositol in bovine erythrocyte acetylcholinesterase and porcine kidney alkaline phosphatase

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Bovine erythrocyte acetylcholinesterase and porcine kidney alkaline phosphatase were purified to a homogeneous state. By using gas chromatography-mass spectrometry, we demonstrated the presence of covalently bound *myo*-inositol in these purified enzymes. The quantitative data suggest that one molecule of *myo*-inositol is bound to each subunit of these enzyme proteins. The covalently bound inositol was removed from these enzyme molecules by deamination with nitrous acid, suggesting the possibility that *myo*-inositol is directly bound to amino sugar.

Alkaline phosphatase; Acetylcholinesterase; Phosphatidylinositol-specific phospholipase C; Phosphatidylinositol anchor

1. INTRODUCTION

Slein and Logan [1,2] first reported the release of alkaline phosphatase from slices of animal tissues by DEAE-cellulose eluate of *Bacillus cereus* culture filtrate. After a decade, alkaline phosphatase-releasing activity of phosphatidylinositol (PI)-specific phospholipase C was confirmed by us, using purified enzyme of *B. cereus* [3]. Using immunochemical methods, we further demonstrated that the activity inducing alkaline phosphatase release in vitro and in vivo is identical with PI-hydrolyzing enzyme activity [4]. Thereafter, alkaline phosphatase [5–7] and other membrane-bound enzymes such as 5′-nucleotidase [5,6], acetylcholinesterase [8,9], alkaline phosphodiesterase I [10,11], and trehalase [12] have been reported to be released from plasma membranes and microsomes of animal tissues and erythrocytes by the action of PI-specific phospholipase C of *Clostridium novyi, Staphylococcus aureus* and *Bacillus thuringiensis*. From these studies, the presence of so-called 'PI-anchoring enzymes' has been established. Recently, Ferguson et al. [13] reported the existence of PI-glycan covalently bound to a variant surface glycoprotein of *Trypanosoma brucei* and confirmed the structure of PI-glycan. Futerman et al. [14] also reported that acetylcholinesterase of *Torpedo* electric organ contains *myo*-inositol by using gas chromatography-mass spectrometry (GC-MS). In this report, we confirmed the presence of covalently bound *myo*-inositol in the purified preparation of bovine erythrocyte acetylcholinesterase and porcine kidney alkaline phosphatase by GC-MS.

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2. MATERIALS AND METHODS

The adsorbents such as Sepharose-4B and DEAE-Toyopearl were purchased from Pharmacia

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and Toyo-Soda, respectively. All other chemicals used were of analytical reagent grade unless otherwise stated.

2.1. *Preparation and assay of PI-specific phospholipase C*

As described in a foregoing report [15], purified PI-specific phospholipase C was obtained from the culture broth of *Bacillus thuringiensis* IAM 12077 in a homogeneous state, as indicated by polyacrylamide gel electrophoresis. Assay of PI-specific phospholipase C was performed as reported [16] with PI as a substrate.

2.2. *Solubilization and purification of acetylcholinesterase by PI-specific phospholipase C*

20 ml of 10% bovine erythrocyte suspension were incubated with 1 unit of PI-specific phospholipase C at 37°C for 60 min with gentle shaking. The reaction mixture was centrifuged at $2000 \times g$ for 5 min and then $30000 \times g$ for 20 min. The resulting supernatant was concentrated by PT-10000 membrane (Millipore) and then purified to a homogeneous state with affinity chromatography as reported previously [16]. Acetylcholinesterase activity was determined by the method of Ellman et al. [17]. To 0.1 ml aliquots of enzyme solution were added 0.8 ml of 100 mM sodium phosphate buffer (pH 7.5), 50 μl of DTNB, and 50 μl of acetylthiocholine. The reaction was followed spectrophotometrically at 412 nm at 37°C.

2.3. *Solubilization and purification of alkaline phosphatase*

Alkaline phosphatase was solubilized from porcine kidney slices by PI-specific phospholipase C and purified to a homogeneous state with Con A-Sepharose, arsenate-binding Sepharose 4B, and Toyopearl HW-55F and DEAE-Toyopearl. Alkaline phosphatase activity was determined with *p*-nitrophenyl phosphate as a substrate according to Engstrom [18].

2.4. *Gas chromatography-mass spectrometry*

GC-MS was performed using Jeol DX300 mass spectrometry fitted with a JMA-3500 mass data analysis system. Both sugar and inositol derivatives were separated on a glass column (2 m 2 mm) packed with 3% silicone SE30 on a Uniport HP 60/80 mesh. A temperature program of 170 – 250°C at 4°C/min was employed for analysis. The MS fragments of *myo*-inositol hexatrimethylsilyl ether at *m/z* 612, 507, 432 and 305 were used in these measurements.

2.5. *Protein determination*

Protein was determined according to Lowry et al. [19] with bovine serum albumin as a standard.

3. RESULTS AND DISCUSSION

3.1. *Determination of* myo-*inositol in the purified preparations of acetylcholinesterase and alkaline phosphatase*

Purified alkaline phosphatase did not adsorb to the phenyl-Sepharose CL-4B or butyl-Toyopearl column as purified acetylcholinesterase [20]. This indicated that these purified enzymes had already lost the diglyceride moiety from PI by the action of PI-specific phospholipase C and that these purified enzymes are completely lipid-free.

Purified acetylcholinesterase (100 μg) were hydrolyzed in sealed glass ampoules with 6 N HCl at 110°C for 18 h. After hydrolysis, the samples were taken to dryness at 37°C under N_2 stream and then by a centrifugation-evaporation system, thereafter placed in vacuo with a rotary pump so as to ensure thorough drying. To the dried samples 10 μl of *N*-trimethylsilylimidazole, 2 μl of trimethylchlorosilane and 10 μl of *N,O*-bis(trimethylsilyl)trifluoroacetamide were added. After mixing vigorously and standing for 60 min, 10 μl samples were subjected to GC-MS analysis. Standard *myo*-inositol hexatrimethylsilyl ether was eluted at a retention time of 12 min 48 s in the GC spectrum and exhibited the typical mass spectrum pattern at *m/z* 612, 507, 432 and 305. As shown in fig.1, we detected the typical peak of TMS derivatives from *myo*-inositol in the sample at the same retention time as the standard. By using a mass chromatogram, we calculated the amount of *myo*-inositol in purified acetylcholinesterase. Our results indicate that 100 μg purified acetylcholinesterase contain more than 100 ng *myo*-inositol.

100 μg purified porcine kidney alkaline phosphatase were hydrolyzed in sealed glass ampoules with 6 N HCl at 110°C for 18 h in the presence of 400 ng *scyllo*-inositol (400 ng) as an in-

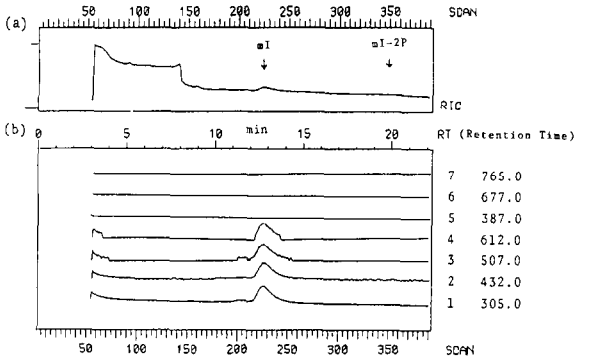

Fig.1. Analysis of hydrolyzed bovine erythrocyte acetylcholinesterase. Purified bovine acetylcholinesterase (100 μg) was heated with 6 N HCl at 110°C for 18 h. TMS derivatives of the hydrolysate were analyzed by GC-MS as described in the text. (a) Total ion chromatogram, (b) mass chromatogram. TMS derivatives of standards: mI, *myo*-inositol; sI, *scyllo*-inositol; mI-2P, *myo*-inositol 2-phosphate.

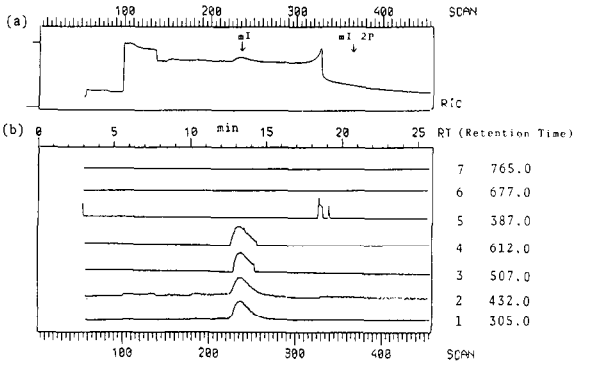

Fig.3. Analysis of *myo*-inositol released after deamination of acetylcholinesterase. Purified acetylcholinesterase was deaminated by nitrous acid. TMS derivatives of the released molecules were analyzed by GC-MS as described in the text. (a) Total ion chromatogram, (b) mass chromatogram. Abbreviations as for fig.1.

ternal standard. As shown in fig.2, typical peaks of *myo*- and *scyllo*-inositol were detected on the gas chromatogram, corresponding to retention times of their standard peaks, 9 min 51 s and 8 min 47 s, respectively. From the mass chromatogram data of *m/z* 507 and 305, the content of *myo*-inositol per mol alkaline phosphatase was estimated as 1.4 mol. This indicated that 0.7 molecules of *myo*-

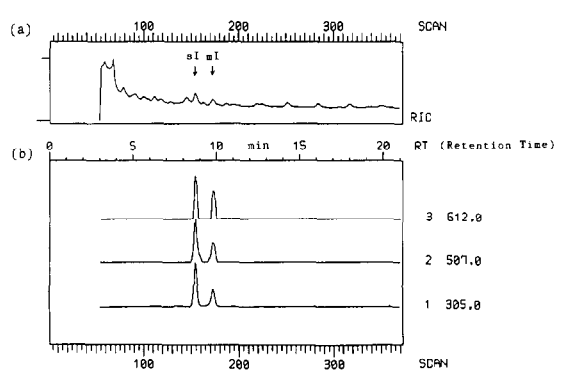

Fig.2. Analysis of hydrolyzed porcine kidney alkaline phosphatase by GC-MS. Purified porcine kidney alkaline phosphatase (100 μg) and *scyllo*-inositol (400 ng, internal standard) were heated with 6 N HCl at 110°C for 18 h. TMS derivatives of the hydrolysate were analyzed by GC-MS as described. (a) Total ion chromatogram, (b) mass chromatogram. Abbreviations as for fig.1.

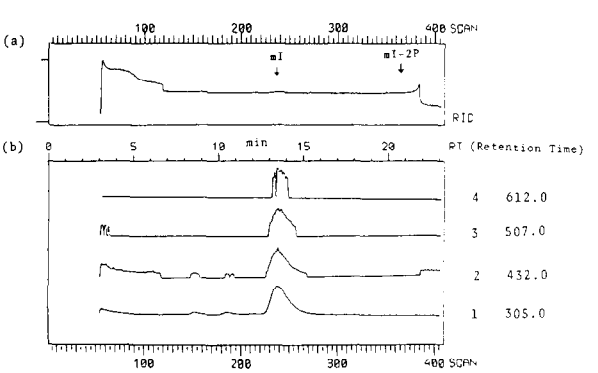

Fig.4. Analysis of *myo*-inositol released after deamination of alkaline phosphatase. Purified alkaline phosphatase was deaminated by nitrous acid. TMS derivatives of the released molecules were analyzed by GC-MS as described in the text. (a) Total ion chromatogram, (b) mass chromatogram. Abbreviations as for fig.1.

inositol is contained in each subunit of alkaline phosphatase.

3.2. *Deamination of acetylcholinesterase and alkaline phosphatase*

Purified acetylcholinesterase and alkaline phosphatase were incubated with 0.2 M sodium nitrite at 25°C for 5 h. Then the reaction mixture was desalted on a Dowex X-8(H^+) column. Breakthrough fractions were dried under N_2 stream and thoroughly dried by vacuum evaporator. As shown in figs 3 and 4, only a TMS derivative of *myo*-inositol was detected at the retention time of 13 min 35 s and TMS derivatives of *myo*-inositol 1- or 2-phosphates (*m/z* 387, 677 and 765) were not, as in figs 1 and 2. These data indicate that the phosphate residue might be hydrolyzed after solubilization of these enzymes from plasma membranes during purification, and that a *myo*-inositol molecule is directly bound to an amino sugar such as glucosamine in both enzymes [13,21].

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