



HOKKAIDO UNIVERSITY

Title	Partial purification and characteristics of phospholipase A2 from pyloric ceca of starfish <i>Plazaster borealis</i>
Author(s)	KOYAMA, Noriki; KISHIMURA, Hideki; HAYASHI, Kenji
Citation	北海道大学水産科学研究彙報, 55(1), 11-16
Issue Date	2004-08
Doc URL	https://hdl.handle.net/2115/21989
Type	departmental bulletin paper
File Information	55(1)_P11-16.pdf



Partial purification and characteristics of phospholipase A₂ from pyloric ceca of starfish *Plazaster borealis*

Noriki KOYAMA, Hideki KISHIMURA and Kenji HAYASHI

(Received 18 January 2004, Accepted 14 May 2004)

Abstract

Phospholipase A activities in the pyloric ceca and viscera from six species of marine invertebrates were determined. A high activity of the enzyme was found in the pyloric ceca of the starfish *Plazaster borealis*. Phospholipase A₂ was partially purified from the pyloric ceca of the *P. borealis*. The enzyme was purified 12-fold from the crude enzyme solution with a yield of 5 %. The optimum pH and temperature of the enzyme were approximately 10.0 and 50°C, respectively, and the activity was enhanced by Ca²⁺ at 1 mM or higher. The enzyme had no fatty acid specificity. Partially purified *P. borealis* phospholipase A₂ hydrolyzed phosphatidylcholine more effectively than phosphatidylethanolamine similar to other starfish phospholipase A₂.

Key words: Marine invertebrate, Phosphatidylcholine, Phospholipase A₂, *Plazaster borealis*, Polar group specificity, Pyloric cecum, Starfish

Introduction

Phospholipase A₂ (PLA₂) (EC3.1.1.4) catalyzes the selective hydrolysis of the *sn*-2-acyl group in 1,2-diacyl-*sn*-glycero-3-phospholipids and the production of fatty acids and lysophospholipids. PLA₂ is widely distributed in the tissues of various organisms and is classified into extracellular and intracellular types (Dennis, 1997). Extracellular PLA₂ is abundant in mammalian pancreas and snake venom, and the enzymatic properties and amino acid sequences have been well characterized (Dennis, 1983; Arni and Ward, 1996).

On the other hand, there are few studies on PLA₂ present in digestive glands of marine invertebrates. Recently, we have found high phospholipase A (PLA) activities in crude enzyme fractions from the pyloric ceca of the starfish *Solaster paxillatus* and *Asterina pectinifera* and succeeded in isolating a PLA₂ (Kishimura and Hayashi, 1998; Kishimura and Hayashi, 1999a; Kishimura and Hayashi, 1999b). The starfish PLA₂ exhibited similar enzymatic properties to mammalian pancreatic PLA₂ in terms of Ca²⁺ requirement, fatty acid specificity and optimum pH. However, the specific activity of *A. pectinifera* PLA₂ was markedly higher than that of commercially available PLA₂ from porcine pancreas. In addition, *A. pectinifera* PLA₂ showed distinct substrate specificity hydrolyzing phosphatidylcholine more efficiently than phosphatidylethanolamine, whereas the porcine PLA₂ hydrolyzed them almost

equally. Recently, we have reported the complete amino acid sequence of *A. pectinifera* PLA₂ and its structural characteristics, compared with the porcine pancreatic PLA₂ (Kishimura et al., 2000a; Kishimura et al., 2000b). *A. pectinifera* PLA₂ consisted of 137 amino acids with a Mr 15,300 and contained 14 Cys residues at positions that have been shown to be involved in the formation of intramolecular disulfide bonds in the porcine pancreatic PLA₂. The amino acid sequences of the putative active site and Ca²⁺-binding loop of *A. pectinifera* PLA₂ showed a moderately high homology to those of porcine pancreatic PLA₂. However, in the alignment of the amino acid sequences of *A. pectinifera* PLA₂ and porcine pancreatic PLA₂, two amino acid deletions in the pancreatic loop region, and sixteen insertions and three deletions in the β -wing region were required to maximize the sequence homology. In the previous study, we constructed a bacterial expression system for *A. pectinifera* PLA₂, and determined some biochemical properties of the recombinant PLA₂ (Kishimura et al., 2001). Although N-terminal Ser in the native *A. pectinifera* PLA₂ was replaced by Ala in the recombinant PLA₂, the recombinant PLA₂ showed essentially the same properties as those of the native PLA₂ with respect to specific activity, substrate specificity, optimum pH and temperature, and Ca²⁺ requirement.

As noted above, PLA₂ from the pyloric ceca of the starfish *A. pectinifera* has functional and structural

properties different from those of mammalian pancreatic PLA₂. In this study, to clarify whether the properties of *A. pectinifera* PLA₂ are common among asteroids, we determined PLA activities in pyloric ceca and viscera from six species of marine invertebrates and partially purified PLA₂ from the pyloric ceca of the starfish *Plazaster borealis*, and examined the characteristics of this enzyme.

Materials and Methods

Materials

The starfish (*P. borealis*, *Solaster borealis* and *Aphelasterias japonica*), sea urchin (*Strongylocentrotus franciscanus*), shellfish (*Neptunea arthritica* and *Patinopecten yessoensis*) and squid (*Todarodes pacificus*) were caught off Hakodate, Hokkaido Prefecture, Japan and were stored at -20°C until use. Porcine pancreatic PLA₂ was purchased from Sigma (St. Louis, MO, USA) and Amano Pharmaceutical Co. (Nagoya, Japan). Egg yolk phosphatidylcholine (PC) was purchased from Wako Pure Chemicals (Osaka, Japan). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA).

Preparation of crude enzyme solution

The crude enzyme solution was prepared from the pyloric ceca and viscera of the invertebrates by the method of Kishimura and Hayashi (1999a).

Partial purification of starfish PLA₂

PLA₂ was partially purified from the crude enzyme extracted from the defatted powder of the pyloric ceca of *P. borealis* using sequential column chromatography: gel filtration on Sephacryl S-200, diethylaminoethyl (DEAE)-cellulose anion-exchange column chromatography, and gel filtration on Sephadex G-50 (Kishimura and Hayashi, 1999b).

Lipid extraction and analysis

The extraction of tissue lipids and lipid analysis by thin-layer chromatography (TLC), preparative TLC, TLC-frame ionization detection method (TLC/FID), and gas-liquid chromatography (GLC) were performed as described by Hayashi (1989) and Hayashi and Kishimura (1996).

PC and phosphatidylethanolamine (PE) were prepared from the total lipids of the squid mantle muscle using preparative TLC with chloroform-methanol-acetic acid-water (55:17:3:2, v/v/v/v) as a developing solvent.

PLA₂ activity assay

PLA₂ activity was measured as described by Kishimura and Hayashi (1999b). One unit of enzyme activity was defined as the micrograms of PC hydrolyzed per min.

Positional specificity, fatty acid specificity, and polar group specificity were analyzed by the method of Kishimura and Hayashi (1999b).

Protein determination

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin fraction V as a standard protein.

Results

PLA activity in viscera of several marine invertebrates

We compared PLA activities in the pyloric ceca and viscera from six species of marine invertebrates. As shown in Table 1, maximum PLA activity was detected in *P. borealis*, followed by *S. borealis*. Low PLA activities were detected in *S. franciscanus*, *N. arthritica* and *P. yessoensis*, and *A. japonica*.

Properties of *P. borealis* partially purified PLA₂

Because a relatively high activity was detected in the pyloric ceca of *P. borealis*, PLA₂ was partially purified from the crude enzyme extracted from the defatted powder of the pyloric ceca of the starfish (Table 2). The enzyme, which was purified 12-fold from the crude enzyme solution with a yield of 5 %, included several proteins as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The positional specificity of the partially purified *P. borealis* PLA₂ was examined using POPC. The enzyme mainly released oleic acid from POPC similar to the porcine pancreatic PLA₂ (Table 3). Figure 1 shows the pH and temperature dependence of the *P. borealis* PLA₂. The enzyme hydrolyzed egg yolk PC effectively at an alkaline pH with an optimum activity at about pH 10.0 (Fig. 1a), and an optimum temperature at about 50°C (Fig. 1b). Figure 2 shows the effects of CaCl₂ on *P. borealis* PLA₂ activities. The enzyme was activated by Ca²⁺ at 1 mM or higher. The fatty acid specificity of the *P. borealis* PLA₂ was examined using egg yolk PC as a substrate. The composition of fatty acids released from the substrate by the *P. borealis* PLA₂ was similar to that released by the porcine pancreatic PLA₂ (Table 4). The polar group specificity of the *P. borealis* PLA₂ was examined using squid PC and PE. The *P. borealis* PLA₂ hydrolyzed PC more effectively than PE (Fig. 3).

Table 1. Phospholipase A activities in pyloric ceca and viscera of several marine invertebrates

	Organ	Activity (U/g powder)* ¹	Specific activity (U/mg)
Starfish			
<i>Plazaster borealis</i>	Pyloric cecum	2,000	17
<i>Solaster borealis</i>	Pyloric cecum	1,900	14
<i>Aphelasterias japonica</i>	Pyloric cecum	210	1.8
Sea urchin			
<i>Strongylocentrotus franciscanus</i>	Viscera	120	2.0
Shellfish			
<i>Neptunea arthritica</i>	Viscera	110	1.9
<i>Patinopecten yessoensis</i>	Hepatopancreas	70	0.6
Starfish			
<i>Asterina pectinifera</i> * ²	Pyloric cecum	540,000	1,400
<i>Coscinasterias acutispina</i> * ³	Pyloric cecum	5,400	17
<i>Solaster paxillatus</i> * ²	Pyloric cecum	1,000	12
<i>Distolasterias nippon</i> * ²	Pyloric cecum	70	0.5
<i>Asterias amurensis</i> * ²	Pyloric cecum	27	0.5

*¹One unit (U) of activity was determined as the microgram of phosphatidylcholine hydrolyzed per minute.

*²Kishimura and Hayashi (1999a).

*³Koyama et al. (2001).

 Table 2. Purification of phospholipase A₂ of *P. borealis*

Purification step	Protein (mg)	Total activity (×10 ³ U)*	Specific activity (U/mg)	Purity (fold)	Yield (%)
Crude enzyme solution	23,500	438	19	1	100
Sephacryl S-200	4,400	78	18	1	18
DEAE-Cellulose	600	38	63	3	9
Sephadex G-50	103	24	230	12	5

*One unit of activity was defined as the microgram of phosphatidylcholine hydrolyzed per minute.

DEAE : diethylaminoethyl

 Table 3. Composition of fatty acids released from POPC by the partially purified phospholipase A₂ of *P. borealis*

Fatty acid	(wt%)* ¹	
	Starfish enzyme	Porcine enzyme* ²
16:0	15.3	15.4
18:1n-9	84.7	84.6

*¹Weight % relative to total fatty acid.

*²Phospholipase A₂ from porcine pancreas (Amano Pharmaceutical Co.)

Discussion

The PLA activities in the pyloric ceca and viscera from six species of marine invertebrates were compared

with those in the pyloric ceca from five species of starfish previously reported (Kishimura and Hayashi, 1999a; Koyama et al., 2001). As shown in Table 1, the PLA activity in *A. pectinifera* was extremely high. High PLA activities were detected in *P. borealis*, *Coscinasterias acutispina*, *S. borealis* and *Solaster paxillatus*. Low PLA activities were detected in *A. japonica*, *Distolasterias nippon* and *Asterias amurensis*, *S. franciscanus*, and *N. arthritica* and *P. yessoensis*.

PLA₂ from the pyloric ceca of *P. borealis* was partially purified. The partially purified enzyme released mainly oleic acid from POPC. The enzyme had an optimum alkaline pH of about 10.0, and was activated by Ca²⁺ at 1 mM or higher. These properties of the enzyme were similar to those of the mammalian pancreatic PLA₂ (Dennis, 1983; Arni and Ward, 1996) and

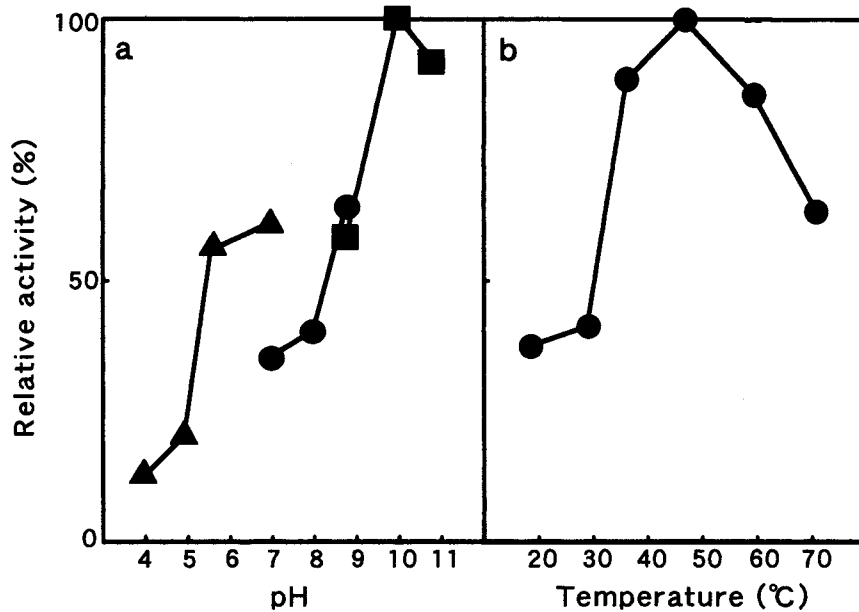


Fig. 1. Effects of pH and temperature on the activity of phospholipase A₂ of *P. borealis*. a: Reaction mixture containing 21 μ g of starfish phospholipase A₂, 100 μ g of egg yolk PC, 2.7 mM sodium deoxycholate, and 5 mM CaCl₂ in a total volume of 130 μ l was incubated at 37°C for 10 min. The buffers used were 50 mM acetic acid-sodium acetate from pH 4.0 to 7.0 (▲), 50 mM Tris-HCl from pH 7.0 to 9.0 (●), and 50 mM glycine-NaOH from pH 9.0 to 11.0 (■). b: Reaction mixture containing 21 μ g of starfish phospholipase A₂, 100 μ g of egg yolk PC, 2.7 mM sodium deoxycholate, 5 mM CaCl₂, and 50 mM Tris-HCl (pH 8.5) in a total volume of 130 μ l was incubated at various temperatures for 10 min.

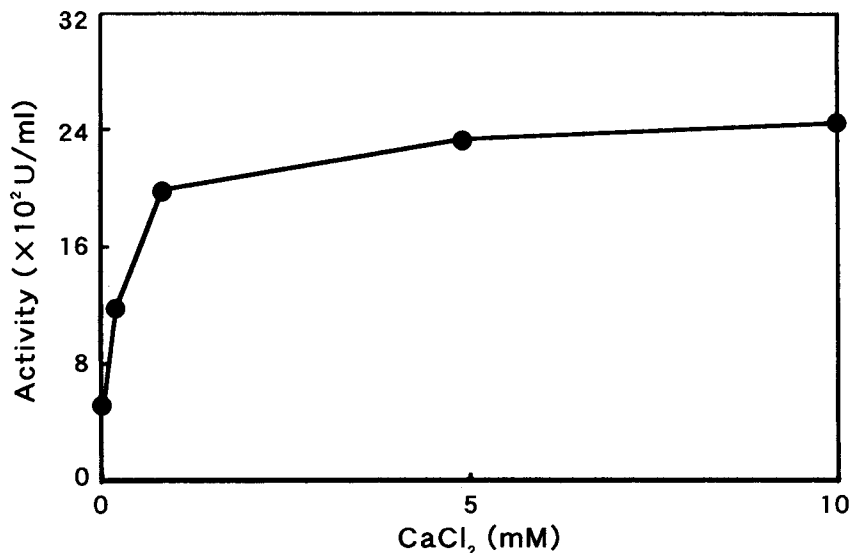


Fig. 2. Effects of Ca²⁺ on activity of phospholipase A₂ of *P. borealis*. Reaction mixture containing 21 μ g starfish phospholipase A₂, 100 μ g egg yolk PC, 2.7 mM sodium deoxycholate, 50 mM Tris-HCl buffer (pH 8.5), and various concentrations of CaCl₂ in a total volume of 130 μ l was incubated at 37°C for 10 min.

other starfish PLA₂ (Kishimura and Hayashi, 1998; Kishimura and Hayashi, 1999b; Koyama et al., 2001). Furthermore, the enzyme hydrolyzed fatty acid ester bond exclusively at the glycerol-*sn*-2 position of PC regardless of the chain length and degree of unsaturation, similar to the mammalian pancreatic PLA₂ (De

Haas et al., 1968) and other starfish PLA₂ (Kishimura and Hayashi, 1998; Kishimura and Hayashi, 1999b; Koyama et al., 2001). However, the optimum temperature (about 50°C) of the *P. borealis* PLA₂ in this study was higher than that of *S. paxillatus* PLA₂ (about 40°C) (Kishimura and Hayashi, 1998), but lower than that of

Table 4. Composition of fatty acids released from PC by the partially purified phospholipase A₂ of *P. borealis* (wt%)*¹

Fatty acid	Egg yolk PC	
	Starfish enzyme	Porcine enzyme* ²
16:0	2.8	2.9
18:0	1.1	1.0
18:1n-9	55.9	57.6
18:1n-7	1.9	1.8
18:2n-6	26.9	24.2
20:4n-6	6.6	5.6
22:6n-3	1.1	1.6
Others* ³	3.7	5.3

*¹Weight % relative to total fatty acid.

*²Phospholipase A₂ from porcine pancreas (Amano Pharmaceutical Co.).

*³Consisted of minor (less than 1.0%) and unknown compounds.

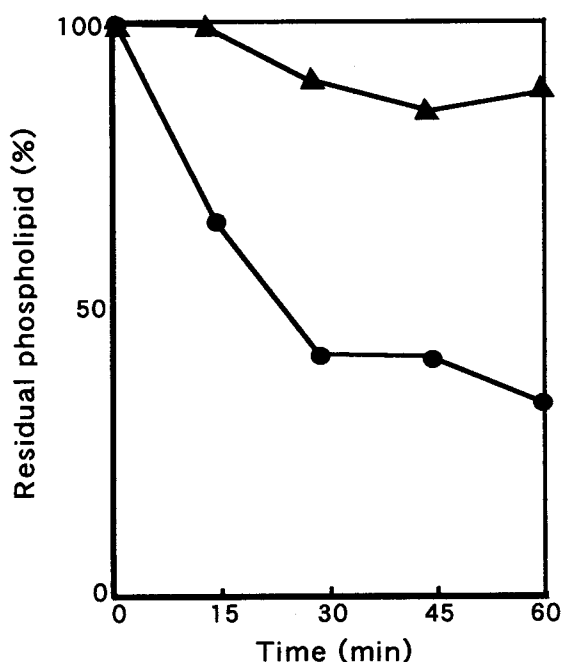


Fig. 3. Time course of phosphatidylcholine and phosphatidylethanolamine hydrolyses by phospholipase A₂ of *P. borealis*. Reaction mixture containing 21 μg starfish phospholipase A₂, 100 μg PC (●) or PE (▲) prepared from squid mantle muscle, 2.7 mM sodium deoxycholate, 5 mM CaCl₂, and 50 mM Tris-HCl buffer (pH 8.5) in a total volume of 130 μl was incubated at 37°C for various periods.

C. acutispina PLA₂ (about 60°C) (Koyama et al., 2001).

Although the mammalian pancreatic PLA₂ hydrolyzed PC almost equally to PE (De Haas et al., 1968; Hara et al., 1991), the snake venom PLA₂ hydrolyzed

PC more effectively than PE (Ibrahim et al., 1964). Moreover mammalian nonpancreatic extracellular PLA₂ hydrolyzed PE more effectively than PC (Chang et al., 1987; Hara et al., 1989; Mizushima et al., 1989). In this study, *P. borealis* PLA₂ hydrolyzed PC more effectively than PE similar to the snake venom PLA₂. Kuipers et al. (1989) reported that a recombinant porcine pancreatic PLA₂ mutant with a deletion of the pancreatic loop at positions 62–66 provided an intermediate conformation between the wild type porcine PLA₂ and the snake venom PLA₂, and enhanced the catalytic activity on zwitterionic substrates. In the alignment of the amino acid sequences of *A. pectinifera* PLA₂ and the porcine pancreatic PLA₂, two amino acid deletions in the pancreatic loop region were required to maximize the sequence homology (Kishimura et al., 2000a; Kishimura et al., 2000b). Therefore, possibly the primary structure of *P. borealis* PLA₂ purified in this study also differed from that of the mammalian pancreatic PLA₂ at the region corresponding to the pancreatic loop.

References

- Arni, R.K. and Ward, R.J. (1996) Phospholipase A₂—a structural review. *Toxicon*, **34**, 827–841.
- Chang, H.W., Kudo, I., Tomita, M. and Inoue, K. (1987) Purification and characterization of extracellular phospholipase A₂ from peritoneal cavity of caseinate-treated rat. *J. Biochem.*, **102**, 147–154.
- De Haas, G.H., Postema, N.M., Nieuwenhuizen, W. and van Deenen, L.L.M. (1968) Purification and properties of phospholipase A from porcine pancreas. *Biochim. Biophys. Acta*, **159**, 103–117.
- Dennis, E.A. (1997) The growing phospholipase A₂ superfamily of signal transduction enzymes. *Trends Biochem. Sci.*, **22**, 1–2.
- Dennis, E.A. (1983) Phospholipases. pp. 307–353. *The Enzymes*. 3rd ed. Vol. XVI. Academic Press, New York.
- Hara, S., Kudo, I., Chang, H.W., Matsuta, K., Miyamoto, T. and Inoue, K. (1989) Purification and characterization of extracellular phospholipase A₂ from human synovial fluid in rheumatoid arthritis. *J. Biochem.*, **105**, 395–399.
- Hara, S., Chang, H.W., Horigome, K., Kudo, I. and Inoue, K. (1991) Purification of mammalian nonpancreatic extracellular phospholipase A₂. *Methods Enzymol.*, **197**, 381–389.
- Hayashi, K. (1989) Occurrence of diacyl glyceryl ethers in liver lipids of gonatid squid *Gonatopsis borealis*. *Nippon Suisan Gakkaishi*, **55**, 1383–1387.
- Hayashi, K. and Kishimura, H. (1996) Preparation and purification of DHA-enriched triacylglycerols from fish oils by column chromatography. *Fisheries Sci.*, **62**, 842–843.
- Ibrahim, S.A., Sanders, H. and Thompson, R.H. (1964) The action of phospholipase A on purified phospholipids, plasma and tissue preparations. *Biochem. J.*, **93**, 588–594.

- Kishimura, H. and Hayashi, K. (1998) Purification and properties of phospholipase A₂-like enzyme from the pyloric ceca of the starfish *Solaster paxillatus*. *Nippon Suisan Gakkaishi*, **64**, 264-269.
- Kishimura, H. and Hayashi, K. (1999a) Phospholipase A activity in the pyloric ceca of starfish. *Nippon Suisan Gakkaishi*, **65**, 108-109.
- Kishimura, H. and Hayashi, K. (1999b) Isolation and characteristics of phospholipase A₂ from the pyloric ceca of the starfish *Asterina pectinifera*. *Comp. Biochem. Physiol.*, **124B**, 483-488.
- Kishimura, H., Ojima, T., Tanaka, H., Hayashi, K. and Nishita, K. (2000a) Amino acid sequence of phospholipase A₂ from the pyloric ceca of the starfish *Asterina pectinifera*. *Fisheries Sci.*, **66**, 104-109.
- Kishimura, H., Ojima, T., Hayashi, K. and Nishita, K. (2000b) cDNA cloning and sequencing of phospholipase A₂ from the pyloric ceca of the starfish *Asterina pectinifera*. *Comp. Biochem. Physiol.*, **126B**, 579-586.
- Kishimura, H., Ojima, T., Hayashi, K. and Nishita, K. (2001) Bacterial expression and characterization of starfish phospholipase A₂. *Comp. Biochem. Physiol.*, **128B**, 565-573.
- Koyama, N., Kishimura, H., Hayashi, K. and Fujita, D. (2001) Partial purification and properties of phospholipase A₂ from the pyloric ceca of starfish *Coscinasterias acutispina*. *Nippon Suisan Gakkaishi*, **67**, 728-734.
- Kuipers, O.P., Thunnissen, M.M.G.M., de Geus, P., Dijkstra, B.W., Drenth, J., Verheij, H.M. and de Haas, G.H. (1989) Enhanced activity and altered specificity of phospholipase A₂ by deletion of a surface loop. *Science*, **244**, 82-85.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265-273.
- Mizushima, H., Kudo, I., Horigome, K., Murakami, K., Hayakawa, M., Kim, D.K., Kondo, E., Tomita, M. and Inoue, K. (1989) Purification of rabbit platelet secretory phospholipase A₂ and its characteristics. *J. Biochem.*, **105**, 520-525.