Bull. Fish. Sci. Hokkaido Univ.
55(1), 11-16, 2004.

Partial purification and characteristics of phospholipase A_2 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 A_2 was partialy 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 Ca^{2+} at 1 mM or higher. The enzyme had no fatty acid specificity. Partialy purified *P. borealis* phospholipase A_2 hydrolyzed phosphatidylcholine more effectively than phosphatidylethanolamine similar to other starfish phospholipase A_2.

Key words: Marine invertebrate, Phosphatidylcholine, Phospholipase A_2, *Plazaster borealis*, Polar group specificity, Pyloric cecum, Starfish

Introduction

Phospholipase A_2 (PLA_2) (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. PLA_2 is widely distributed in the tissues of various organisms and is classified into extracellular and intracellular types (Dennis, 1997). Extracellular PLA_2 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 PLA_2 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 PLA_2 (Kishimura and Hayashi, 1998; Kishimura and Hayashi, 1999a; Kishimura and Hayashi, 1999b). The starfish PLA_2 exhibited similar enzymatic properties to mammalian pancreatic PLA_2 in terms of Ca^{2+} requirement, fatty acid specificity and optimum pH. However, the specific activity of *A. pectinifera* PLA_2 was markedly higher than that of commercially available PLA_2 from porcine pancreas. In addition, *A. pectinifera* PLA_2 showed distinct substrate specificity hydrolyzing phosphatidylcholine more efficiently than phosphatidylethanolamine, whereas the porcine PLA_2 hydrolyzed them almost equally. Recently, we have reported the complete amino acid sequence of *A. pectinifera* PLA_2 and its structural characteristics, compared with the porcine pancreatic PLA_2 (Kishimura et al., 2000a; Kishimura et al., 2000b). *A. pectinifera* PLA_2 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 PLA_2. The amino acid sequences of the putative active site and Ca^{2+}-binding loop of *A. pectinifera* PLA_2 showed a moderately high homology to those of porcine pancreatic PLA_2. However, in the alignment of the amino acid sequences of *A. pectinifera* PLA_2 and porcine pancreatic PLA_2, 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* PLA_2, and determined some biochemical properties of the recombinant PLA_2 (Kishimura et al., 2001). Although N-terminal Ser in the native *A. pectinifera* PLA_2 was replaced by Ala in the recombinant PLA_2, the recombinant PLA_2 showed essentially the same properties as those of the native PLA_2 with respect to specific activity, substrate specificity, optimum pH and temperature, and Ca^{2+} requirement.

As noted above, PLA_2 from the pyloric ceca of the starfish *A. pectinifera* has functional and structural

Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan
(e-mail: kishi@fish.hokudai.ac.jp)
(北海道大学大学院水産科学研究科生物資源化学講座)

properties different from those of mammalian pancreatic PLA_2. In this study, to clarify whether the properties of *A. pectinifera* PLA_2 are common among asteroids, we determined PLA activities in pyloric ceca and viscera from six species of marine invertebrates and partially purified PLA_2 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 PLA_2 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 PLA_2

PLA_2 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.

PLA_2 activity assay

PLA_2 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 PLA_2

Because a relatively high activity was detected in the pyloric ceca of *P. borealis*, PLA_2 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* PLA_2 was examined using POPC. The enzyme mainly released oleic acid from POPC similar to the porcine pancreatic PLA_2 (Table 3). Figure 1 shows the pH and temperature dependence of the *P. borealis* PLA_2. 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 $CaCl_2$ on *P. borealis* PLA_2 activities. The enzyme was activated by Ca^{2+} at 1 mM or higher. The fatty acid specificity of the *P. borealis* PLA_2 was examined using egg yolk PC as a substrate. The composition of fatty acids released from the substrate by the *P. borealis* PLA_2 was similar to that released by the porcine pancreatic PLA_2 (Table 4). The polar group specificity of the *P. borealis* PLA_2 was examined using squid PC and PE. The *P. borealis* PLA_2 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			
Plazaster borealis	Pyloric cecum	2,000	17
Solaster borealis	Pyloric cecum	1,900	14
Aphelasterias japonica	Pyloric cecum	210	1.8
Sea urchin			
Strongylocentrotus franciscanus	Viscera	120	2.0
Shellfish			
Neptunea arthritica	Viscera	110	1.9
Patinopecten yessoensis	Hepatopancreas	70	0.6
Starfish			
*Asterina pectinifera**2	Pyloric cecum	540,000	1,400
*Coscinasterias acutispina**3	Pyloric cecum	5,400	17
*Solaster paxillatus**2	Pyloric cecum	1,000	12
*Distolasterias nippon**2	Pyloric cecum	70	0.5
*Asterias amurensis**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 A_2 of *P. borealis*

Purification step	Protein (mg)	Total activity ($\times 10^3$ 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 hydrovyzed per minute.
DEAE: diethylaminoethyl

Table 3. Composition of fatty acids released from POPC by the partially purified phospholipase A_2 of *P. borealis*

(wt%)*1

Patty acid	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 A_2 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*.

PLA_2 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 Ca^{2+} at 1 mM or higher. These properties of the enzyme were similar to those of the mammalian pancreatic PLA_2 (Dennis, 1983; Arni and Ward, 1996) and

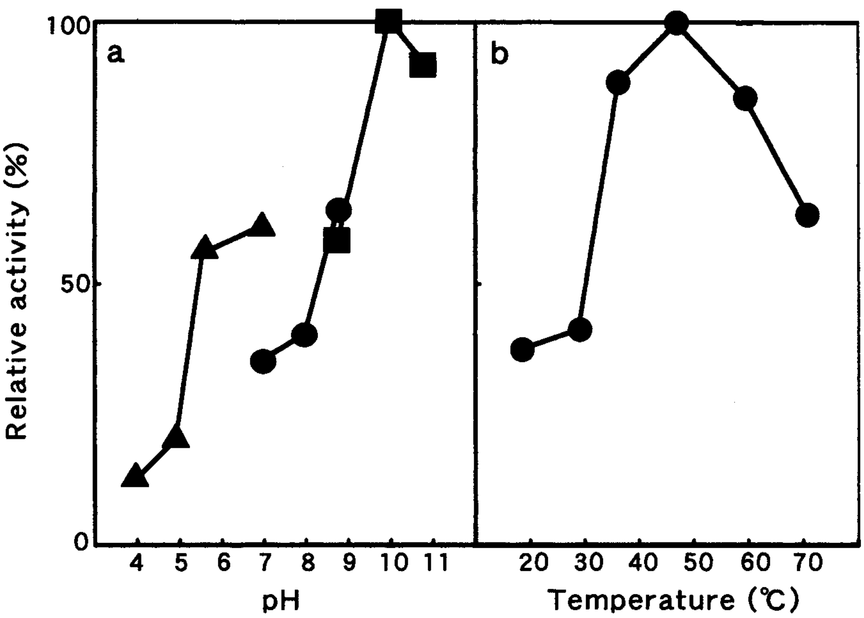

Fig. 1. Effects of pH and temperature on the activity of phospholipase A_2 of *P. borealis.* a : Reaction mixture containing 21 μg of starfish phospholipase A_2, 100 μg of egg yolk PC, 2.7 mM sodium deoxycholate, and 5 mM $CaCl_2$ in a total volume of 130 μl was incubated at 37°C for 10 min. The buffers 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 μg of starfish phospholipase A_2, 100 μg of egg yolk PC, 2.7 mM sodium deoxycholate, 5 mM $CaCl_2$, and 50 mM Tris-HCl (pH 8.5) in a total volume of 130 μl was incubated at various temperatures for 10 min.

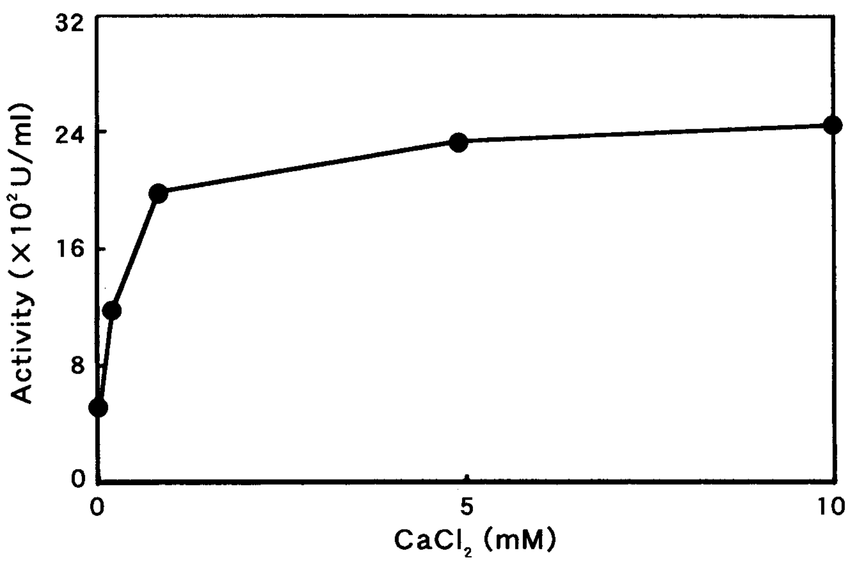

Fig. 2. Effects of Ca^{2+} on activity of phospholipase A_2 of *P. borealis.* Reaction mixture containing 21 μg starfish phospholipase A_2, 100 μg egg yolk PC, 2.7 mM sodium deoxycholate, 50 mM Tris-HCl buffer (pH 8.5), and various concentrations of $CaCl_2$ in a total volume of 130 μl was incubated at 37°C for 10 min.

other starfish PLA_2 (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 PLA_2 (De Haas et al., 1968) and other starfish PLA_2 (Kishimura and Hayashi, 1998; Kishimura and Hayashi, 1999b; Koyama et al., 2001). However, the optimum temperature (about 50°C) of the *P. borealis* PLA_2 in this study was higher than that of *S. paxillatus* PLA_2 (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 A_2 of *P. borearis* (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 A_2 from porcine pancreas (Amano Pharmaceutical Co.).
*3Consisted of minor (less than 1.0%) and unknown compounds.

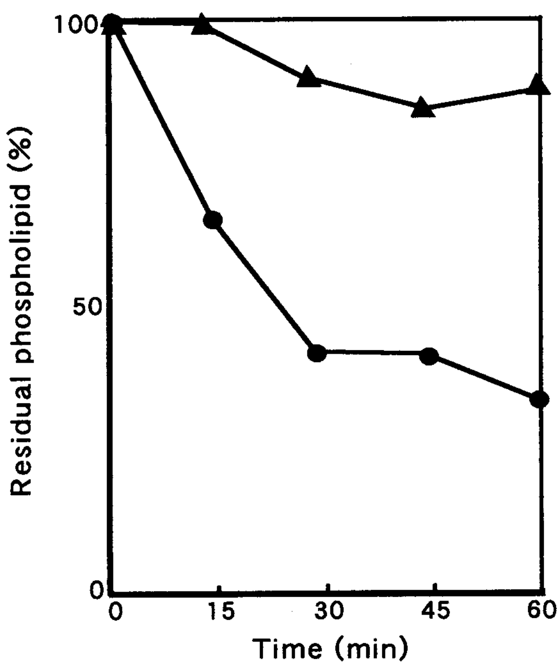

Fig. 3. Time course of phosphatidylcholine and phosphatidylethanolamine hydrolyses by phospholipase A_2 of *P. borealis*. Reaction mixture containing 21 μg starfish phospholipase A_2, 100 μg PC (●) or PE (▲) prepared from squid mantle muscle, 2.7 mM sodium deoxycholate, 5 mM $CaCl_2$, and 50 mM Tris-HCl buffer (pH 8.5) in a total volume of 130 μl was incubated at 37°C for various periods.

C. acutispina PLA_2 (about 60°C) (Koyama et al., 2001).

Although the mammalian pancreatic PLA_2 hydrolyzed PC almost equally to PE (De Haas et al., 1968; Hara et al., 1991), the snake venom PLA_2 hydrolyzed PC more effectively than PE (Ibrahim et al., 1964). Moreover mammalian nonpancreatic extracellular PLA_2 hydrolyzed PE more effectively than PC (Chang et al., 1987; Hara et al., 1989; Mizushima et al., 1989). In this study, *P. borealis* PLA_2 hydrolyzed PC more effectively than PE similar to the snake venom PLA_2. Kuipers et al. (1989) reported that a recombinant porcine pancreatic PLA_2 mutant with a deletion of the pancreatic loop at positions 62-66 provided an intermediate conformation between the wild type porcine PLA_2 and the snake venom PLA_2, and enhanced the catalytic activity on zwitterionic substrates. In the alignment of the amino acid sequences of *A. pectinifera* PLA_2 and the porcine pancreatic PLA_2, 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* PLA_2 purified in this study also differed from that of the mammalian pancreatic PLA_2 at the region corresponding to the pancreatic loop.

References

Arni, R.K. and Ward, R.J. (1996) Phospholipase A_2—a structural review. *Toxicon*, **34**, 827-841.

Chang, H.W., Kudo, I., Tomita, M. and Inoue, K. (1987) Purification and characterization of extracellular phospholipase A_2 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 A_2 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 A_2 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 A_2. *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 A_2-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 A_2 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 A_2 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 A_2 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 A_2. *Comp. Biochem. Physiol.*, **128B**, 565-573.

Koyama, N., Kishimura, H., Hayashi, K. and Fujita, D. (2001) Partial purification and properties of phospholipase A_2 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 A_2 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 A_2 and its characteristics. *J. Biochem.*, **105**, 520-525.