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Characterization of phospholipase A_2 from the pyloric ceca of two species of starfish, *Coscinasterias acutispina* and *Plazaster borealis*

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Abstract

Phospholipase A (PLA) activities in the pyloric ceca and viscera from seven species of marine invertebrates (four starfish, one sea urchin, and two shellfish) were determined. Relatively high PLA specific activities were found in the pyloric ceca of two species of starfish (*Coscinasterias acutispina* and *Plazaster borealis*). Phospholipase A_2s (PLA_2s) were partially purified from the pyloric ceca of the starfish, *C. acutispina* PLA_2 (C-PLA_2) and *P. borealis* PLA_2 (P-PLA_2). The C-PLA_2 and P-PLA_2 mainly released oleic acid from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine. Temperature optima of the C-PLA_2 and P-PLA_2 were at around 60 °C and 50 °C, respectively, and pH optima of the C-PLA_2 and P-PLA_2 were both at around pH 10.0. The activities of the C-PLA_2 and P-PLA_2 were enhanced by sodium deoxycholate and 1 mM or higher concentration of Ca^{2+}. The C-PLA_2 and P-PLA_2 did not show the fatty acid specificity for hydrolysis of phosphatidylcholine. Unlike porcine pancreatic PLA_2, the C-PLA_2 and P-PLA_2 hydrolyzed phosphatidylcholine more effectively than phosphatidylethanolamine.

Keywords: Phospholipase A_2; *Coscinasterias acutispina*; *Plazaster borealis*; Starfish; Marine invertebrate; Pyloric cecum; Polar group specificity

1. 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. PLA_2 is widely distributed in tissues of various organisms and is classified into extracellular and intracellular types. Extracellular PLA_2 is abundant in mammalian pancreas and snake venom, and these enzymatic and structural characteristics have been well studied (Arni & Ward, 1996; Dennis, 1983).

On the other hand, the PLA_2s from mammalian pancreas and snake venom have been used as diagnostic biochemical reagents. Stoll (1996) employed snake venom PLA_2 to analyze the positional distribution of fatty acids in glycerophospholipids from guinea pig and pig cardiac membranes. Mine (1997) examined structural and functional changes of hen egg-yolk low density lipoproteins (LDL) as a result of modifying its phospholipids using porcine pancreatic PLA_2. Additionally, commercial PLA_2, mainly produced from porcine pancreas, is used for industrial processes in the food industry. Soy lysophosphatidylcholine (lysoPC), which is an excellent emulsifier for food, is prepared by porcine pancreatic PLA_2-catalyzed hydrolysis of soy phosphatidylcholine (PC) (Aoi, 1990). The emulsion with soy lysoPC is stable under various conditions at high temperature, acidic solution and/or high salt concentration. Soy lysoPC is also a good solubilizer, and the interaction between soy lysoPC and protein is very strong. Dahlke, Buchold, Munch, and Paulitz (1995) reported

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that PLA_2 is suitable for the enzymatic degumming of edible oils. The enzymatic degumming of crude edible oils reduces the amounts of acids, bases and wastes during the refining processes. It also allows the extraction of PC and lysoPC as valuable by-products for the fortification of other foods. Recently, it was shown that LDL, modified by PLA_2, was removed from the circulation to the liver more rapidly than unmodified LDL (Labeque, Mullon, Ferreira, Lees, & Langer, 1993). Based on this finding, a novel therapy for hypercholesterolemia has recently been developed, that utilizes immobilized PLA_2 contained in an extracorporeal shunt.

In contrast, few studies exist on the enzymology and application of PLA_2 from marine invertebrates. In the previous study, we isolated PLA_2 from the pyloric ceca of starfish (*Asterina pectinifera*), and compared its enzymatic properties with those of porcine pancreatic PLA_2 (Kishimura & Hayashi, 1999b). The specific activity of the *A. pectinifera* PLA_2 for PC was about 30 times higher than that of the commercially available PLA_2 from porcine pancreas (Sigma). In addition, the *A. pectinifera* PLA_2 hydrolyzed PC more effectively than phosphatidylethanolamine (PE), like snake venom PLA_2 (Ibrahim, Sanders, & Thompson, 1964) but not porcine pancreatic PLA_2.

In this study, we partially purified PLA_2s from the pyloric ceca of the starfish (*Coscinasterias acutispina* and *Plazaster borealis*) and examined the characteristics of these enzymes.

2. Materials and methods

2.1. Materials

The starfish (*C. acutispina*) was caught off Uozu, Toyama Prefecture, Japan, and was stored at −20 °C until used. The starfish (*P. borealis*, *Solaster borealis*, and *Aphelasterias japonica*), sea urchin (*Strongylocentrotus franciscanus*), shellfish (*Neptunea arthritica* and *Patinopecten yessoensis*) and common squid (*Todarodes pacificus*) were caught off Hakodate, Hokkaido Prefecture, Japan and were stored at −20 °C until used. Porcine pancreatic PLA_2 was purchased from Sigma (St. Louis, MO, USA) and Amano Pharmaceutical Co. (Nagoya, Japan). Egg-yolk 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).

2.2. Preparation of crude enzyme solution

Crude enzyme solution was prepared from the pyloric ceca and viscera of the invertebrates by the same method as described by Kishimura and Hayashi (1999a). The pyloric ceca and viscera were homogenized in 4 volumes of chloroform–methanol (2:1, v/v) for 10 min, and the homogenates were filtered in vacuo on ADVANTEC No. 2 filter paper. Similarly, the residues were homogenized in 2 volumes of chloroform–methanol (2:1, v/v) and 1.3 volumes of acetone for 10 min, and then the residues were air-dried at room temperature. PLA was extracted by stirring the defatted powder for 3 h at 5 °C in 50 volumes of 50 mM Tris–HCl buffer at pH 8.0. The extracts were centrifuged at 10,000*g* for 10 min, and then the supernatants were concentrated by lyophilization into crude enzyme solution.

2.3. Purification of starfish PLA_2

The crude enzyme solutions were applied on a column (3.9×44 cm) of Sephacryl S-200 pre-equilibrated with 50 mM Tris–HCl buffer (pH 8.0), and PLA_2 was eluted with the same buffer. Main active fractions were concentrated by lyophilization and dialyzed against 10 mM Tris–HCl buffer (pH 8.0). The dialyzates were applied on a column (1.1×18 cm) of DEAE-cellulose, pre-equilibrated with 10 mM Tris–HCl buffer (pH 8.0). PLA_2 was eluted with a linear gradient of NaCl, from 0 to 0.5 M in 10 mM Tris–HCl buffer (pH 8.0), and main active fractions were obtained. The fractions were dialyzed against 10 mM Tris–HCl buffer (pH 8.0) and the dialyzates were concentrated by lyophilization. The concentrates were applied on a column (3.9×64 cm) of Sephadex G-50 pre-equilibrated with 10 mM Tris–HCl buffer (pH 8.0) and PLA_2 was eluted with the same buffer. Consequently, main active fractions were obtained. The final enzyme preparations were purified 63-fold (C-PLA_2) and 12-fold (P-PLA_2) from the crude enzyme solutions in yields of 14% and 5%, respectively. The C-PLA_2 and P-PLA_2 included small amounts of several proteins, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

2.4. Lipid extraction and analysis

The extraction of tissue lipids and lipid analyses 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 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.

2.5. PLA_2 activity assay

PLA_2 activity was measured as described by Kishimura and Hayashi (1999b). One unit of enzyme

activity was defined as 1 μg of PC hydrolyzed per min.

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

2.6. Protein determination

Protein concentration was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin fraction V as a standard protein.

3. Results

3.1. PLA activity in viscera of several marine invertebrates

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

3.2. Properties of C. acutispina and P. borealis partially purified PLA_2s

Because relatively high specific activities were detected in the pyloric ceca of the starfish, *C. acutispina* and *P. borealis*, PLA_2s were partially purified from the crude enzymes of the *C. acutispina* (C-PLA_2) and *P. borealis* (P-PLA_2). The positional specificities of the C-PLA_2 and P-PLA_2 were examined using POPC. The C-PLA_2 and P-PLA_2 mainly released oleic acid from POPC similar to the porcine pancreatic PLA_2 (Table 2). Fig. 1 shows the pH-dependences of the C-PLA_2 and P-PLA_2. Both C-PLA_2 and P-PLA_2 hydrolyzed egg-yolk PC effectively at alkaline pH with an optimum activity at about pH 10.0 (Fig. 1). Fig. 2 shows the temperature-dependences of the C-PLA_2 and P-PLA_2. The optimum temperatures of the C-PLA_2 and P-PLA_2 were at about 60 and 50 °C, respectively (Fig. 2). Fig. 3 shows the effects of $CaCl_2$ on the C-PLA_2 and P-PLA_2 activities. The C-PLA_2 and P-PLA_2 were activated by Ca^{2+} at 1 mM or higher. The fatty acid specificities of the C-PLA_2 and P-PLA_2 were examined using egg yolk PC as a substrate. The compositions of fatty acids released from the substrate by the C-PLA_2 and P-PLA_2 were similar to that released by the porcine pancreatic PLA_2 (Table 3). The polar-group specificities of the C-PLA_2 and P-PLA_2

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

	Organ	Activity (U[a]/g powder)	Specific activity (U/mg)
Starfish			
C. acutispina	Pyloric cecum	5400	17
P. borealis	Pyloric cecum	2000	17
S. borealis	Pyloric cecum	1900	14
A. japonica	Pyloric cecum	210	1.8
Sea urchin			
S. franciscanus	Viscera	120	2.0
Shellfish			
N. arthritica	Viscera	110	1.9
P. yessoensis	Hepatopancreas	70	0.6
Starfish			
A. pectinifera[b]	Pyloric cecum	540,000	1400
S. paxillatus[b]	Pyloric cecum	1000	12
D. nippon[b]	Pyloric cecum	70	0.5
A. amurensis[b]	Pyloric cecum	27	0.5

[a] One unit (U) of activity was determined as 1 μg of phosphatidylcholine hydrolyzed per minute.
[b] Kishimura and Hayashi (1999a).

Table 2
Composition of fatty acids released from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine by phospholipase A_2 from the pyloric ceca of starfish (*C. acutispina* and *P. borealis*) (%)

Fatty acid	*C. acutispina*	*P. borealis*	Porcine enzyme[a]
16:0	16.0	15.3	15.4
18:1n-9	84.0	84.7	84.6

[a] Phospholipase A_2 from porcine pancreas (Amano Pharmaceutical Co.).

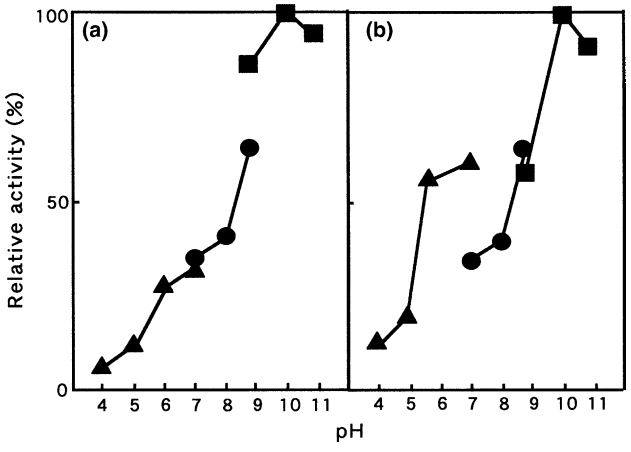

Fig. 1. Effects of pH on the activities of phospholipase A_2s of the starfish (*C. acutispina* and *P. borealis*). The activities were determined in 50 mM buffer solutions [acetic acid–sodium acetate from pH 4.0 to 7.0 (▲), Tris–HCl from pH 7.0 to 9.0 (●), and glycine–NaOH from pH 9.0 to 11.0 (■)] at 37 °C for 10 min. (a) *C. acutispina* phospholipase A_2, (b) *P. borealis* phospholipase A_2.

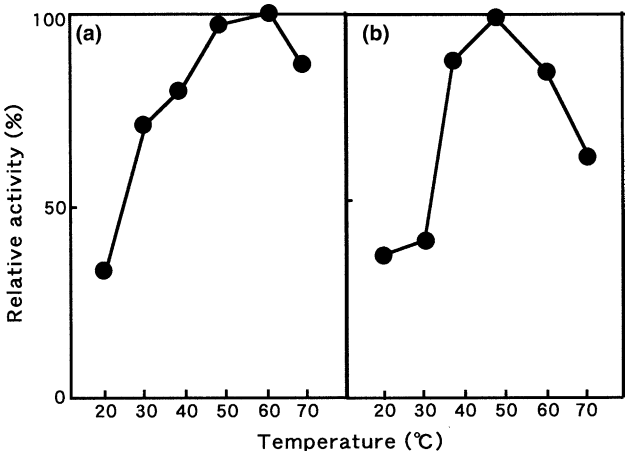

Fig. 2. Effects of temperature on the activities of phospholipase A_2s of the starfish (*C. acutispina* and *P. borealis*). The activities were determined in 50 mM Tris–HCl buffer (pH 8.5) at various temperatures for 10 min. (a) *C. acutispina* phospholipase A_2, (b) *P. borealis* phospholipase A_2.

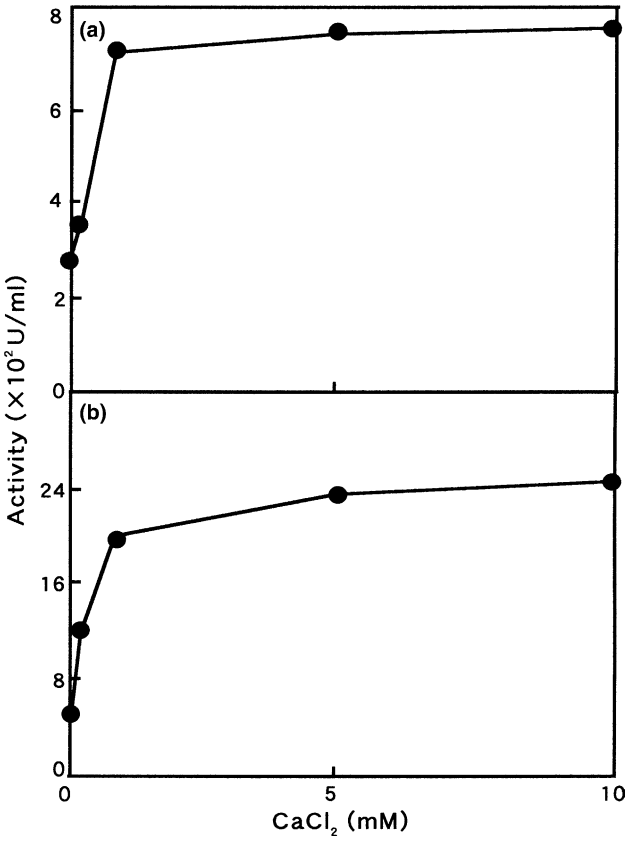

Fig. 3. Effects of Ca^{2+} on activity of phospholipase A_2s of the starfish (*C. acutispina* and *P. borealis*). The activities were determined in 50 mM Tris–HCl buffer (pH 8.5) at various concentrations of $CaCl_2$ at 37 °C for 10 min. (a) *C. acutispina* phospholipase A_2, (b) *P. borealis* phospholipase A_2.

were examined using squid PC and PE. The C-PLA_2 and P-PLA_2 hydrolyzed PC more effectively than PE (Fig. 4).

Table 3
Composition of fatty acids released from squid egg-yolk phosphatidylcholine by phospholipase A_2 from the pyloric ceca of starfish (*C. acutispina* and *P. borealis*)(%)

Fatty acid	*C. acutispina*	*P. borealis*	Porcine enzyme[a]
16:0	2.3	2.8	2.9
18:0	0.7	1.1	1.0
18:1n-9	59.0	55.9	57.6
18:1n-7	2.0	1.9	1.8
18:2n-6	24.9	26.9	24.2
20:4n-6	5.5	6.6	5.6
22:6n-3	1.5	1.1	1.6
Others[b]	4.1	3.7	5.3

[a] Phospholipase A_2 from porcine pancreas (Amano Pharmaceutical Co.).
[b] Consisted of minor (less than 1.0%) and unknown compounds.

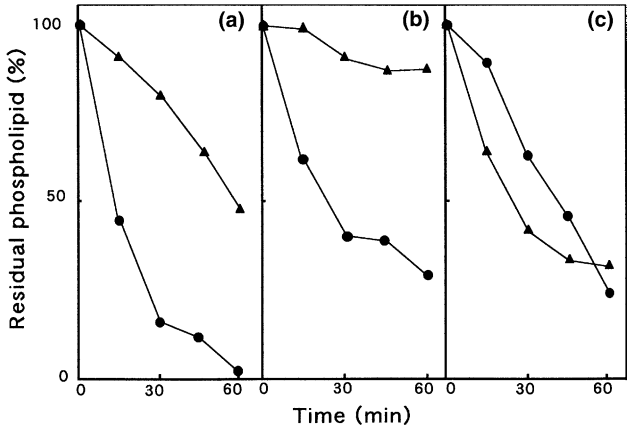

Fig. 4. Time-course of phosphatidylcholine and phosphatidylethanolamine hydrolyses by phospholipase A_2s of the starfish (*C. acutispina* and *P. borealis*). Hydrolysis of squid phosphatidylcholine (●) and phosphatidylethanolamine (▲) was carried out in 50 mM Tris–HCl buffer (pH 8.5) at 37 °C for various periods: (a) *C. acutispina* phospholipase A_2; (b) *P. borealis* phospholipase A_2; (c) porcine pancreatic phospholipase A_2 (Amano Pharmaceutical Co.).

4. Discussion

The PLA activities in the pyloric ceca and viscera from seven species of marine invertebrates were compared with those in the pyloric ceca from four species of starfish previously reported (Kishimura & Hayashi, 1999a). As shown in Table 1, the PLA specific activity in *A. pectinifera* was extremely high. Relatively high PLA specific activities were detected in *C. acutispina*, *P. borealis*, *S. borealis*, and *Solaster paxillatus*. Low PLA activities were detected in *A. japonica*, *Distolasterias nippon*, *Asterias amurensis*, *S. franciscanus*, *N. arthritica*, and *P. yessoensis*.

PLA_2s from the pyloric ceca of *C. acutispina* (C-PLA_2) and *P. borealis* (P-PLA_2) were partially purified. The C-PLA_2 and P-PLA_2 released mainly oleic acid from POPC. The C-PLA_2 and P-PLA_2 had an optimum alkaline pH of about 10.0, and was activated by Ca^{2+} at 1 mM or higher. These properties of the C-PLA_2 and

P-PLA_2 were similar to those of the mammalian pancreatic PLA_2s (Arni & Ward, 1996; Dennis, 1983) and other starfish PLA_2s (Kishimura & Hayashi, 1998; Kishimura & Hayashi, 1999b). Furthermore, the C-PLA_2 and P-PLA_2 hydrolyzed the fatty acid ester bond exclusively at the glycerol-*sn*-2 position of PC, regardless of the chain length and degree of unsaturation, similarly to the porcine pancreatic PLA_2 (De Haas, Postema, Nieuwenhuizen, & Van Deenen, 1968) and other starfish PLA_2s (Kishimura & Hayashi, 1998, 1999b). However, the optimum temperatures of the C-PLA_2 (about 60 °C) and P-PLA_2 (about 50 °C) in this study were higher than that of *S. paxillatus* PLA_2 (about 40 °C) (Kishimura & Hayashi, 1998).

Although the mammalian pancreatic PLA_2s hydrolyzed PC almost as effectively as 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_2s hydrolyzed PE more effectively than PC (Chang, Kudo, Tomita, & Inoue, 1987; Hara et al., 1989; Mizushima et al., 1989). In this study, the C-PLA_2 and P-PLA_2 hydrolyzed PC more effectively than PE, similarly to the snake venom PLA_2s. Kuipers et al. (1989) reported that the 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 (*Crotalus atrox*) venom PLA_2, and enhanced the catalytic activity on zwitterionic substrates. Therefore, possibly the primary structure of the C-PLA_2 and P-PLA_2 differed from that of the porcine pancreatic PLA_2 at the region corresponding to the pancreatic loop.

In conclusion, the C-PLA_2 and P-PLA_2 hydrolyzed PC more effectively than PE, unlike porcine pancreatic PLA_2, which hydrolyzed PC almost equally as well as PE. In addition, the specific activities of the C-PLA_2 (1066 U/mg) and P-PLA_2 (230 U/mg) for PC were similar to that of commercially available PLA_2 (440 U/mg) from porcine pancreas (Amano Pharmaceutical Co.). These results suggest that the pyloric ceca of the starfish (*C. acutispina* and *P. borealis*) would be a potential source of PLA_2.

References

Aoi, N. (1990). Soy lysolecithin. *Journal Japan Oil Chemist's. Society, 39*, 10–15.

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

Chang, H. W., Kudo, I., Tomita, M., & Inoue, K. (1987). Purification and characterization of extracellular phospholipase A_2 from peritoneal cavity of caseinate-treated rat. *Journal of Biochemistry, 102*, 147–154.

Dahlke, K., Buchold, H., Munch, E.-M., & Paulitz, B. (1995). First experiences with enzymatic oil refining. *Information, 6*, 1284–1291.

De Haas, G. H., Postema, N. M., Nieuwenhuizen, W., & Van Deenen, L. L. M. (1968). Purification and properties of phospholipase A from porcine pancreas. *Biochimica et Biophysica Acta, 159*, 103–117.

Dennis, E. A. (1983). *Phospholipases. The enzymes*. (pp. 307–353). New York: Academic Press.

Hara, S., Kudo, I., Chang, H. W., Matsuta, K., Miyamoto, T., & Inoue, K. (1989). Purification and characterization of extracellular phospholipase A_2 from human synovial fluid in rheumatoid arthritis. *Journal of Biochemistry, 105*, 395–399.

Hara, S., Chang, H. W., Horigome, K., Kudo, I., & 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., & Kishimura, H. (1996). Preparation and purification of DHA-enriched triacylglycerols from fish oils by column chromatography. *Fisheries Science, 62*, 842–843.

Ibrahim, S. A., Sanders, H., & Thompson, R. H. (1964). The action of phospholipase A on purified phospholipids, plasma and tissue preparations. *Biochemical Journal, 93*, 588–594.

Kishimura, H., & 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., & Hayashi, K. (1999a). Phospholipase A activity in the pyloric ceca of starfish. *Nippon Suisan Gakkaishi, 65*, 110–111.

Kishimura, H., & Hayashi, K. (1999b). Isolation and characteristics of phospholipase A_2 from the pyloric ceca of the starfish (*Asterina pectinifera*). *Comparative Biochemistry and Physiology, 124B*, 483–488.

Kuipers, O. P., Thunnissen, M. M. G. M., De Geus, P., Dijkstra, B. W., Drenth, J., Verheij, H. M., et al. (1989). Enhanced activity and altered specificity of phospholipase A_2 by deletion of a surface loop. *Science, 244*, 82–85.

Labeque, R., Mullon, C. J. P., Ferreira, J. P. M., Lees, R. S., & Langer, R. (1993). Enzymatic modification of plasma low density lipoproteins in rabbits: A potential treatment for hypercholesterolemia. *Proceeding of the National Academy of Sciences of the United States of America, 90*, 3476–3480.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry, 193*, 265–273.

Mine, Y. (1997). Structural and functional changes of hen's egg yolk low-density lipoproteins with phospholipase A_2. *Journal of Agricultural and Food Chemistry, 45*, 4558–4563.

Mizushima, H., Kudo, I., Horigome, K., Murakami, K., Hayakawa, M., Kim, D. K., et al. (1989). Purification of rabbit platelet secretory phospholipase A_2 and its characteristics. *Journal of Biochemistry, 105*, 520–525.

Stoll, U. (1996). The analysis of phospholipids from cardiac membranes by phospholipase A_2. *Fett/Lipid, 98*, 26–30.