Bicyclo[3.2.1]octa-8-one. The synthesis of this ketone follows that of the bicyclo[3.3.1]nona-9-one. The starting enamine was prepared from cyclopentanone and morpholine. ^{1}H NMR ($CDCl_3$) (ppm); 1.5 (m, 10H), 2.2 (m, 2H): IR (CCl_4) (cm^{-1}); 3000 (m), 2940 (s), 2860 (s), 1710 (s), 1630 (m), 1408 (m), 1353 (m), 1233 (m), 1195 (m), 1129 (s), 1105 (m), 960 (m), 800 (m).

Acknowledgement. This work was supported by the Basic Science Research Institute (1987), Ministry of Education, Republic of Korea.

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Catalytic Properties of Phospholipase D using Phosphatidic Acid as an Activator

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The effects of phosphatidic acid(PA) on the activity of phospholipase D were examined in detail. The enzyme activity was examined in the liposome system containing phosphatidylcholine and PA, which was suspended in a desired buffer solution by ultrasonication. The substrate of large unilamella vesicle (LUV) state by ultrasonication was more effective on the enzyme activity than that of multilamella vesicle(MLV) by water-bath type sonication. The most effective molar ratio of PC-PA liposome for enzyme activity was found to be 1:0.7. The other optimum conditions were found 5 mM Ca^{2+} ion, pH 6.6, and incubation temperature of 27 °C. K_m and V_{max} values were estimated to be 1.43 mM and 0.8 nmole/min/μg protein respectively. These properties in a PC-PA liposome system were compared with those in a PC-SDS mixed micelle system. The effects of other phospholipids and organic phosphates on the enzyme activity were also examined.

Introduction

Phospholipase D (phosphatidylcholine phosphatido hydrolase, EC.3.1.4.4) catalyzes the hydrolytic cleavage of the terminal phosphate diester bond of glycerophosphatides containing choline, ethanolamine, serine or glycerol, resulting in the formation of phosphatidic acid. The enzyme also mediates transphosphatidylation by which the phosphatidyl group of phosphatidylcholine is transferred to alcohols. Phospholipase D occurs widely in the tissues of higher plants and especially in those of the genus Brassica.[1,2] In mammlian tissues, phospholipase D is enriched in the microsomal fraction of brain and lung tissues.[3,4] Taki *et al.* detected the enzyme activity in rat liver.[5] It appears to exist in plant cells in an insoluble form associated with plastids[6] but a soluble form can readily be demonstrated in cabbage[1], carrot[2], and cotton seed.[7] The enzyme in dry peanut shows the properties similar to those of the cabbage enzyme.[8]

The activities of the soluble enzyme of carrot and cabbage are stimulated by the addition of various organic solvents such as linear aliphatic ethers especially diethylether, ketones and esters,[2] except the phospholipase D of cotton seed which is not activated by diethylether.[6] Besides showing this solvent activation, the soluble enzyme from cabbage was stimulated by acidic phospholipid, phosphatidylinositol.[9] These observations on the stimulatory effects of solvents and surface active lipids suggest that the rates of enzyme hydrolysis are largely dependent on the physical states of the lipid

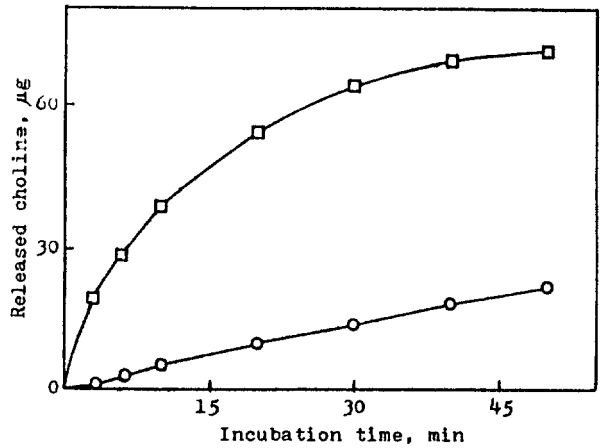

Figure 1. The effect of the dispersion state of the substrate on the activity of phospholipase D. Large unilamella vesicle (LUV) state (□-□); Multilamella vesicle (MLV) state (○-○).

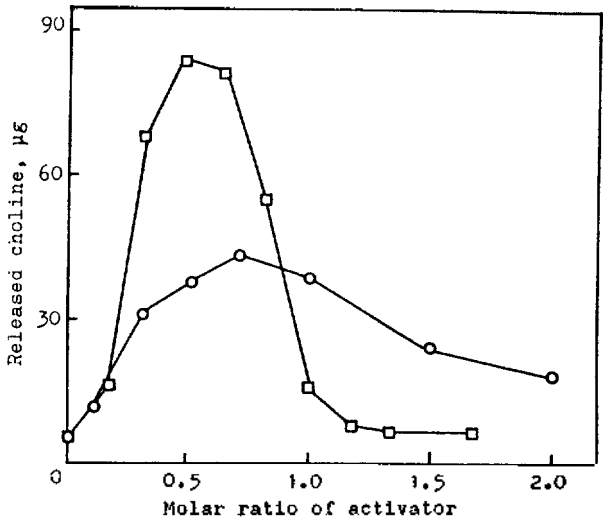

Figure 2. The effects of PA and SDS molar ratios to PC on the activity of phospholipase D using PA and SDS as activators. PC-PA liposome system (○-○); PC-SDS mixed micelle system (□-□).

pholipase D was determined in a liposomal state using PA as an activator. The substrate of large unilamella vesicle (LUV) obtained by ultrasonication was more effective toward the enzyme activity than that of multilamella vesicle (MLV) obtained by water bath type sonication as shown in Figure 1. This shows that the enzyme activity depends largely on the dispersion state of the substrates. When the effects of sonication time on the enzyme activity were examined, the maximum activity of the enzyme could be obtained from the substrate ultrasonicated for about 60 seconds at the maximum power of MSE ultrasonic disintegrator.

In order to see the effect of PA on the activity of phospholipase D, the concentration of PA in a reaction mixture varied at a fixed concentration of PC. The enzyme activity was maximized at a molar ratio of PC:PA of 1:0.7 in the PC-PA liposome system (Figure 2). At the optimum molar ratio the enzyme activity was 0.9 nmole/min/μg protein and the activity of Ca ion only system was 0.18 nmole/min/μg protein. The enzyme activity of PA liposome was 5 times greater than that of the Ca ion only system. When the concentration

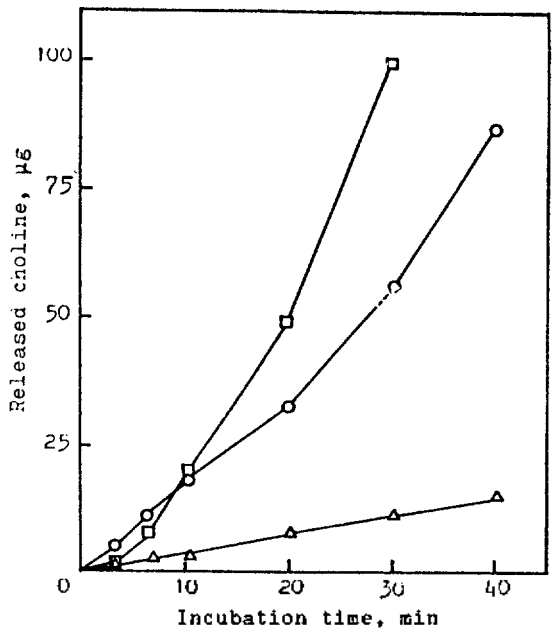

Figure 3. The effect of the incubation time on the enzyme activity of phospholipase D. PC-PA liposome system (○-○); PC-SDS mixed micelle system (□-□); Ca ion only system (△-△).

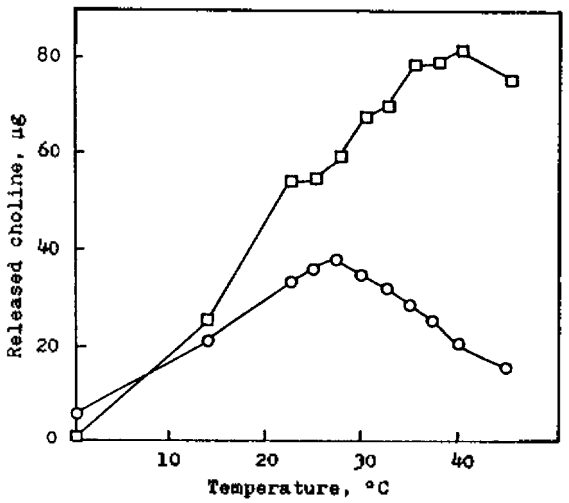

Figure 4. The effect of the incubation temperature on the activity of phospholipase D. PC-PA liposome system(○-○);PC-SDS mixed micelle system (□-□).

of SDS in a PC-SDS mixed micelle system varied at a fixed concentration of PC, the enzyme activity was maximized at a molar ratio of PC-SDS of 1:0.5. The molar ratio of activator (PA) to substrate (PC) in the PC-PA liposome was different from that of the PC-SDS mixed micelle. PA activated the enzyme over the broad molar ratios, but SDS activated the enzyme only on the basis of narrow molar ratios (Figure 2).

Effects of Incubation Time and Temperature in PC: PA Liposome System. When the effects of the incubation time on the enzyme activity were examined, the enzyme activity showed a linear increase until 20 minutes (Figure 3). But after 20 minutes the slope of the curve was upturned. When the effect of the incubation time was examined in a PC-SDS mixed micelle, the enzyme activity showed the concave

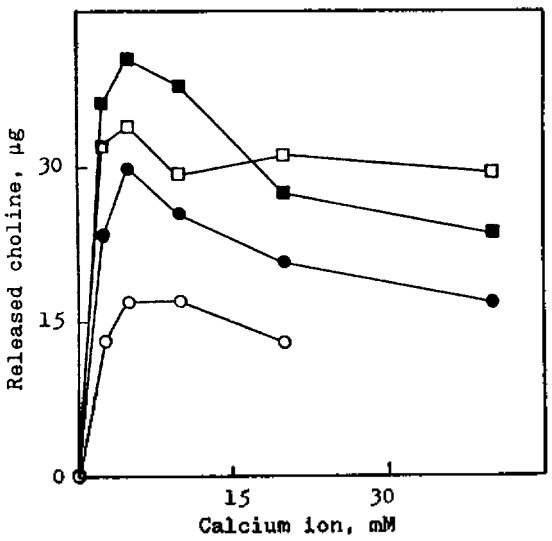

Figure 5. The effect of Ca ion concentration on the activity of phospholipase D. phosphatidylcholine 1 mM (2.5 μmole) (○-○); 2 mM (5 μmole) (●-●); 4 mM (10 μmole) (□-□); 8 mM (20 μmole) (■-■).

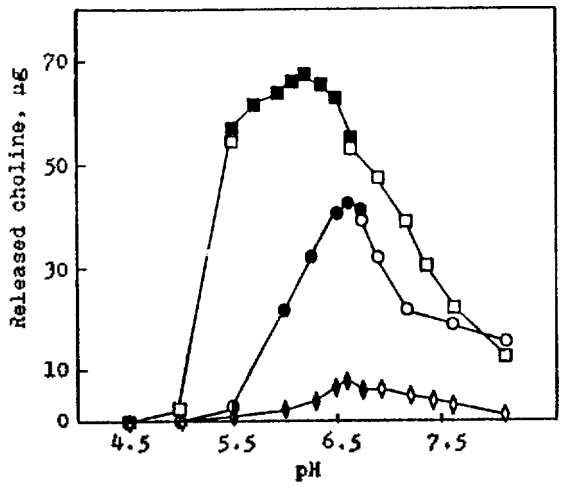

Figure 6. The effect of pH on the activity of phospholipase D. For the pH range of 4.5 to 5.6 the buffer solutions were 10 mM sodium acetate-acetic acid; for the pH range of 6.6 to 8.0 the buffer solutions were 10 mM HEPES. PC-PA liposome system (○-○); PC-SDS mixed micelle system (□-□); Ca ion only system (◇-◇).

curve until 10 minutes. From 10 minutes to 20 minutes the curve was almost linear. But after 20 minutes the slope of the curve was upturned like the PC-PA liposome. In case of Ca ion only system the enzyme activity showed a linear curve until 40 minutes but the activity was very low. One of the noticeable result was that the enzyme activity of PA system in an initial condition until 10 minutes of incubation time was greater than that of SDS system. When the effects of incubation temperature on the enzyme activity were examined, optimum temperature was obtained at 27 °C as shown in Figure 4. In the PC-SDS mixed micelle system, the optimum temperature was 40 °C. The differences of these properties were very interesting in view of that the activity of phospholipase D was largely dependent on the physical state of substrate.

Effect of Ca^{2+} Ion. When the effects of Ca ion concentration on the enzyme activity were investigated in various

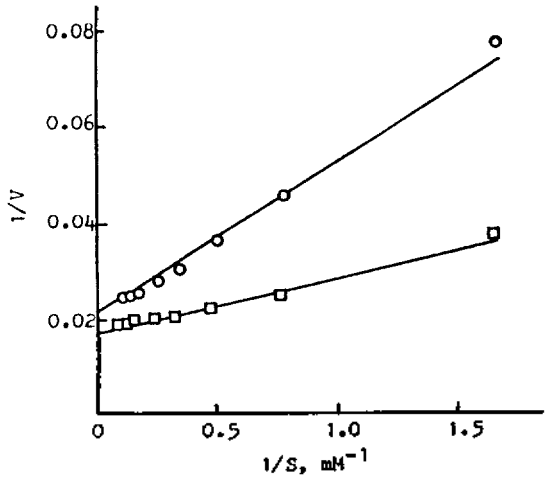

Figure 7. Lineweaver-Burk plot of the substrate dependence of phospholipase D activity. PC-PA liposome system (○-○); PC-SDS mixed micelle system (□-□).

substrate concentrations at a fixed molar ratio of PC:PA of 1:0.7, the optimum concentration of Ca ion was 5 mM in all cases, as shown in Figure 5. Therefore, it suggests that the optimum concentration of Ca ion is not dependent on the amount of substrate. In the PC-SDS mixed micelle system the optimum concentration of Ca ion was always 10 mM as previously determined.[14] Required concentration of Ca ion in the PC-PA liposome was smaller than that in the PC-SDS mixed micelle.

Other General Properties of PC:PA System. In order to obtain the optimum pH value, acetate-MES-HEPES buffer solutions were used. The reaction displayed an apparent optimum activity at pH 6.6, as shown in Figure 6. This optimum pH in PA liposome system was similar to that of Ca ion only system but differed from that of SDS system which showed an optimum at pH 6.2. When the effect of the protein amount was examined, the enzyme activity was almost proportional to the protein amount. When the effects of the substrate concentrations on the enzyme activity were examined, the enzyme activity at the PC:PA molar ratio of 1:0.7 showed a typical Michaelis Menten curve. From the Linewever-Burk plot (Figure 7), the K_m value of 1.43 mM and V_{max} of 0.80 nmole/min/μg protein were calculated. In SDS system K_m value was 0.67 mM and V_{max} was 1.20 nmole/min/μg protein. K_m value of PC-PA liposome was about 2 times greater than that of PC-SDS mixed micelle.

From these results, we established the following standard assay conditions: The most effective molar ratio of PC:PA was 1:0.7; Ca ion concentration was 5 mM; the optimum pH was 6.6 in 10 mM MES buffer; the optimum temperature was 27 °C; the concentration of substrate (PC) was 4 mM (10 μmoles); the sonication time was 60 seconds at the maximum power; incubation time was 10 minutes; the amount of the protein was 50 μg per tube.

Effect of Substrate Dispersion. The substrate of LUV was more effective toward the enzyme activity than that of MLV (Figure 1). The optimum pH (6.6) and the optimum temperature (27 °C) of PC-PA liposome were different from those (pH 6.20, 40 °C) of PC-SDS mixed micelle (Figure 4 and 6). These results suggest that of activity of phospholipase D is subtly dependent on the physical state of substrate.

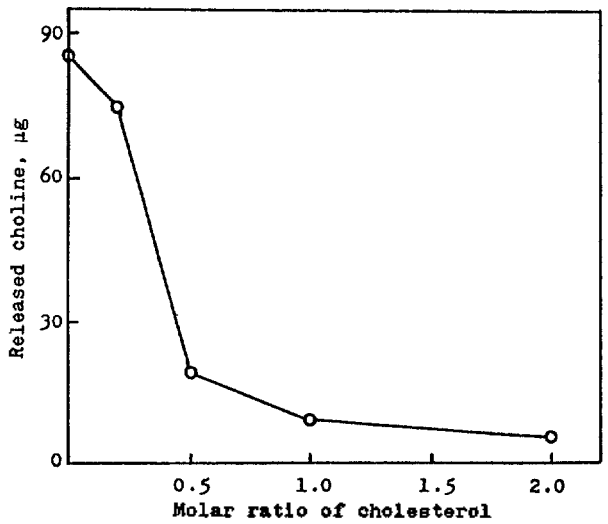

Figure 8. The effect of cholesterol on the activity of phospholipase D in the PC-PA liposome system.

In order to get a better dispersion of the substrate, triton X-100 (0.05%) was added to the liposome system containing PC and PA. But the triton X-100 was quite inhibitory, although triton X-100 activated the Ca ion only system to a smaller extent. When PA and SDS were added simultaneously to the reaction mixture, the enzyme activity of the system until 20 minutes of the incubation time was higher than those of PA system nor SDS system. But after about 20 minutes the enzyme activity of the system became lower than that of SDS system. These observations also support that the enzyme activity was largely dependent on the physical state of the substrate.

Effects of Lipids and Organic Phosphates. In order to investigate the effects of other lipids on the enzyme activity in the PC-PA liposome system, cholesterol and phosphatidylserine were added. Cholesterol was quite inhibitory as shown in Figure 8 and phosphatidylserine was also quite inhibitory. Other organic phosphates such as 2,3-diphosphoglycerate, O-phosphoethanolamine, acetyl phosphate, and inositolhexaphosphate did not activate the enzyme, except phosphoenolpyruvate which activated phospholipase D to a smaller extent.

Discussion

The present investigation has shown the general properties of phospholipase D in a heterogeneous system containing at least four components, *i.e.*, enzyme, substrate phosphatidylcholine, activator phosphatidic acid, and Ca ion. The most effective molar ratio was obtained with a phosphatidylcholine:phosphatidic acid of 1:0.7 and other conditions obtained were: The optimum concentration of Ca ion was 5 mM; the optimum pH was 6.6 in MES buffer; the optimum temperature was 27 °C; K_m value was 1.43 mM; V_{max} was 0.8 nmole/min/μg protein. In comparison with the PA liposome system, the PC-SDS mixed micelle system was studied and found following conditions: The most effective molar ratio of phosphatidylcholin: dodecylsulfate was 1:0.5; the optimum concentration of Ca ion was 10 mM; the optimum pH was 6.2 in MES buffer: the optimum temperature was 40 °C; K_m value was 0.67 mM; V_{max} was 1.2 nmole/min/μg protein. These results showed that the general catalytic properties of phospholipase D were quite different with the kinds of activators used, such as phosphatidic acid and dodecylsulfate.

In the time dependence of the enzyme activity, the curve was a concave pattern (Figure 3). This result was consistent with the fact known in the properties of phospholipase D by Dawson and Hemington.[20] This phenomenon seems to be the stimulating effect of the end product phosphatidic acid, which is a key intermediate in the phospholipid metabolism. The occurrence of turbid suspensions, observed after the addition of Ca ion, showed a formation of aggregates of "supersubstrate" molecules which might serve as an adequate molecules for enzyme binding. This supersubstrate, a macromolecular complex made of phosphatidylcholine, phosphatidic acid, and Ca ion, apparently binds the enzyme to bring its active sites to the vicinity of the reacting bonds in the molecules of the supersubstrate. It seems that an adsorption of the enzyme molecules onto the surface of the supersubstrate is followed by some sort of size transformation into a catalytically more active enzyme.

The lipids such as cholesterol and phosphatidylserine did not activate the enzyme. The addition of cholesterol or phosphatidylserine to the PC-PA liposome system (1:0.7) inhibited the enzyme activity (Figure 8). As the molar ratio of cholesterol or phosphatidylserine to phophatidylcholine increased, the enzyme activity were inhibited further. From the result, it can be speculated that lipid compositions of membranes may affect the enzyme activity by the lipid-lipid or lipid-protein interactions and alternatively the enzyme may control the lipid composition of membrane by modifying the polar head groups of phospholipids bound to the biological membranes while retaining the original lipid backbone. When we simultaneously used SDS and PA as activators in a reaction mixture, the enzyme activity of that system was higher than those of PA system or SDS system until 20 minutes of the incubation time. But the initial velocity of PA liposome system was faster than SDS mixed micelle system.

In present study PA, which is a key intermediate in phospholipid metabolism, could be a potent endogeneous activator on phospholipase D. The activity of phospholipase D was not dependent simply on the physical state of the substrate but activated only by a specific activator such as phosphatidic acid. Since the activity of phospholipase D and most of lipid metabolizing enzymes are affected by membrane environments such as phospholipid compositions, the studies of lipid effects on the lipid metabolizing enzymes are indispensible to elucidate the structure, stability, and function of biological membranes and lipoproteins.

Acknowledgement. This work was supported by a grant from the Korea Science and Engineering Foundation to E.K.

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Irreversible Thermoinactivation Mechanisms of Subtilisin Carlsberg

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In order to find the rational methods for improving the thermal stability of subtilisin Carlsberg, the mechanisms of irreversible thermoinactivation of the enzyme were studied at 90 °C. At pH 4, the main process was hydrolysis of peptide bond. This process followed first order kinetics, yielding a rate constant of $1.26 \times 10^{-1} h^{-1}$. Hydrolysis of peptide bond of PMS-subtilisin occurred at various sites, which produced new distinct fragements of molecular weights of 27.2 KD, 25.9 KD, 25.0 KD, 22.3 KD, 19.0 KD, 17.6 KD, 16.5 KD, 15.7 KD, 15.0 KD, 13.7 KD, and 12.7 KD. Most of the new fragments originated from the acidic hydrolysis at the C-side of aspartic acid residues. However 25.0 KD, 15.7 KD, and 13.7 KD which could not be removed in purification steps stemmed from the autolytic cleavage of subtilisin. The minor process at pH 4 was deamidation at asparagine and/or glutamine residues and some extend of aggregation was also observed. However, the aggregation was main process at pH 7 with a first order kinetic constant of 16 h^{-1}. At pH 9, the main process seemed to be combination of deamidation and cleavage of peptide bond.

Introduction

Inactivation of enzymes by high temperature can be classified into two types of mechanisms. One is the reversible thermoinactivations of enzymes caused by temperature-induced conformational changes in protein structures.[1] These phenomena have been extensively studied with various proteins and their mechanisms are relatively well understood.[2] On the other hand, irreversible thermoinactivations of enzymes caused by a combinations of aggregations, incorrect structure formations, or destruction of primary structure have remained somewhat mysterious, primarily because of severe conceptual and experimental problems encounted in their investigations.[3-5] However, recently, Klibanov group have partly succeeded in elucidating the detailed mechanisms of irreversible thermoinactivation of some well known enzyme such as lysozyme[6] and ribonuclease.[7] The processes leading to thermoinactivations were found to be deamination of Asn/Glu residues, hydrolysis of peptide bond at Asp residues, destruction of disulfide bonds, and formations of incorrectly folded and kinetically trapped structures. These findings provide the basis of rational reasonings for improving thermostabilities of enzymes.[8,9]

In the present work, we examined the thermoinactivation mechanisms of subtilisin Carlsberg in order to search for strategies to improve thermostability of the enzyme. Subtilisin is an extracellular serine protease (also known as alkaline endopeptidase) produced by *Bacillus subtilis*. Today this enzyme is one of the most important microbial protease economically because it is the predominant enzyme used in detergents. In contrast to other members of the serine protease family such as chymotrypsin and trypsin, subtilisin differs from them completely in sequence and tertiary structure but have an essentially identical arrangement of amino acid residues at the active site for its function.[10] This enzyme consists of a single polypeptide chain with 274 amino acids, corresponding to molecular weight of 27,277 daltons.[11] The crystal structures have been determined to 2.5 Å resolution[12]