Changes in Enzymatic and Membrane-Adsorbing Activities of Sphingomyelinase from *Bacillus cereus* by Modification with a Polyethylene Glycol Derivative

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Sphingomyelinase (SMPLC) from *Bacillus cereus* was modified with a polyethylene glycol (PEG) derivative, methoxypolyethylene glycol–succinimidyl succinate (ss-PEG). The molecular weight of the ss-PEG-modified SMPLC was calculated to be approx. 150 kDa by gel-filtration whereas that of the native enzyme, was 25 kDa. By this modification, the enzyme increased its thermostability and retained its hydrolytic activity toward 2-(*N*-hexadecanoylamino)-4-nitrophenylphosphocholine (HNP) and sphingomyelin (SM) in the mixed micelles with the surfactants such as Triton X-100 and sodium deoxycholate (SDC). However, the activity toward liposomal SM was significantly decreased, and all the enzyme activities toward bovine erythrocytes, including membraneous SM-hydrolyzing and hemolytic activities as well as the enzyme adsorption onto the erythrocyte membranes, were completely lost.

Keywords sphingomyelinase; *Bacillus cereus*; polyethylene glycol; chemical modification

Recently, modification by use of polyethylene glycol (PEG) derivatives has been shown to induce some favorable changes in the properties of proteins or enzymes, *e.g.* reduction of their antigenicity, enhancement of biological activities *in vivo* by retardation of their decay in the blood stream, and stimulation of their enzyme activities in organic solvents.[1] In the previous study,[2] we have presented the results of modification of phospholipase D by a *N*-hydroxysuccinimide–activated PEG, *i.e.* methoxypolyethylene glycol–succinimidyl succinate (ss-PEG). This PEG derivative can combine with free amino groups in the molecules of proteins at neutral pH under mild conditions without causing marked inactivation.

For more than a decade, we have also developed the study on *Bacillus cereus* sphingomyelinase, a sphingomyelin-hydrolyzing phospholipase C (SMPLC, EC 3.1.4.12).[3–13] This enzyme was isolated from the culture broth of *B. cereus*[4] and its properties and biological activities were examined in detail.[3,5] Also, the primary and secondary structures of this enzyme were deduced from sequencing of the cloned complementary deoxyribonucleic and (cDNA)[10] and from computer analysis with circular dichroism (CD) spectra,[11] respectively. On the basis of amino acid sequence,[10] the SMPLC molecule contains 26 free amino groups ready for modification by ss-PEG, *i.e.* 25 lysyl ε-amino groups and a N-terminal α-amino group.

In the present study, we succeeded in the modification of *B. cereus* sphingomyelinase by ss-PEG, without an appreciable loss of enzyme activity toward micellar sphingomyelin (SM). The modified enzyme was also examined for its activity toward SM-liposomes and 2-(*N*-hexadecanoylamino)-4-nitrophenylphosphocholine (HNP), a water-soluble, synthetic substrate for SMPLC assay. Furthermore, the effects of modification on the hemolytic activity of SMPLC, as well as on the extent of its adsorption to bovine erythrocytes, were investigated in the presence of divalent metal ions such as Mg^{2+} and Ca^{2+}.

Materials and Methods

Chemicals SM and phosphatidylcholine (PC) were prepared from bovine brain and from egg yolk, respectively, and purified by silicic acid column chromatography.[14] Phosphatidylinositol (PI) was extracted from an autolysate of baker's yeast with chloroform–methanol (1 : 1, v/v) and purified by silicic acid column chromatography or by batch operation with diethylaminoethyl (DEAE)-cellulose.[14,15] A synthetic substrate for SMPLC, HNP, and the enzyme-modifying agent, ss-PEG (average molecular weight: 5000), were purchased from Sigma Chemical Co., Ltd. All other chemicals used were of analytical reagent grade unless otherwise stated.

Preparation of Phospholipases C According to the method reported previously,[4,12] a purified preparation of SMPLC was obtained from the culture broth of *Bacillus cereus* IAM1208 in a homogeneous state as indicated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE),[16] followed by silver staining.[17] From the same culture broth, phosphatidylcholine-hydrolyzing phospholipase C (PCPLC, EC 3.1.4.3) was partially purified by the method used in the foregoing study.[4] The purity of PCPLC preparation obtained was estimated to be approximately 50%, according to SDS–PAGE followed by Coomassie brilliant blue-staining.

Phosphatidylinositol-specific phospholipase C (PIPLC, EC 3.1.4.10) was purified from the culture broth of *Bacillus thuringiensis* IAM 12077 to a homogeneous state,[18] according to SDS–PAGE followed by Coomassie brilliant blue-staining. Final preparations of purified phospholipases C were dissolved in 5 mM *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (HEPES)–NaOH buffer (pH 7.5) containing 50% (v/v) glycerol.

Estimation of Protein Protein contents were determined according to the method of Lowry *et al.*[19] with bovine serum albumin as the standard, and the method of Bensadoun and Weinstein.[20]

Enzyme Assays The assay of SMPLC activity was carried out with mixed SM–Triton X-100 micelle as a substrate, as reported previously.[4,12] One unit of SMPLC was defined as the amount catalyzing the hydrolysis of 1 μmol SM per minute at pH 7.5 and 37 °C. The activities of PCPLC and PIPLC were determined as described in the foregoing reports.[8,21]

Modification of Phospholipases C with ss-PEG Modification of SMPLC was carried out by incubating the reaction mixture composed of 10 mg ss-PEG and 0.1 ml each of purified SMPLC (7.5 units) and 10 mM HEPES–NaOH buffer (pH 7.5) at 37 °C. The amount of ss-PEG added was approximately 70 times excess for free amino groups (α-amino and Lys ε-amino groups) of SMPLC. Except for the experiment following the activity change in the process of modification, incubation was continued for 2 h.

The progress of PCPLC modification was followed by incubating the reaction mixture composed of 10 mg ss-PEG and 0.1 ml each of purified PCPLC (6.5 units) and 10 mM HEPES–NaOH buffer (pH 7.5) at 37 °C for 2 h. The amount of ss-PEG added was approximately 100 times excess for free amino groups of PCPLC. Similarly, the process of PIPLC modification was followed by incubation of the reaction mixture containing 1 mg ss-PEG and 0.1 ml each of purified PIPLC (2.5 units) and 10 mM HEPES–NaOH buffer (pH 7.5) at 37 °C for 2 h. The amount of ss-PEG was approximately 10 times excess for free amino groups of PIPLC.

Gel Filtration of SMPLCs for Molecular Weight Determination Modified and unmodified SMPLCs were applied to a Sephadex G-100 column (12 mm × 400 mm), and eluted with 10 mM Tris–HCl (pH 7.5). Elution of the column was followed by absorbance at 280 nm. As the molecular-weight standards, marker proteins (Boehringer Mannheim) consisting of aldolase (158 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), chymo-

trypsinogen A (25 kDa) and cytochrome c (12.5 kDa) were used.

Thermostability of SMPLCs Unmodified and modified SMPLC solutions in 10 mM Tris–HCl (pH 8.0) containing 10 munits of the enzyme and 0.1% bovine serum albumin, were heated for 10 min at temperatures ranging from 35 °C to 95 °C, then cooled to room temperature and subjected to activity measurement.

Estimation of SMPLC Adsorption onto Bovine Erythrocytes, and Determination of the Hydrolytic and Hemolytic Actions of SMPLCs toward the Erythrocytes Bovine erythrocyte suspensions were prepared according to the method reported previously.[9,13] Fresh bovine blood samples were mixed well with an equal volume of Alsever's solution[22] and centrifuged at $600 \times g$ for 5 min, and the plasma and buffy coat were removed by aspiration.

Then the collected erythrocytes were washed three or four times with borate-buffered saline (pH 8.0), which was made isotonic by the addition of 0.75% NaCl to 0.05 M borate. The washed cells were suspended in the same buffered saline and then subjected to incubation with SMPLC as follows: With gentle shaking, 5% (hematocrit) suspensions of fresh bovine erythrocytes containing 1 mM $CaCl_2$, both 1 mM $CaCl_2$ and 1 mM $MgCl_2$, or 1 mM $MnCl_2$ were incubated at 37 °C with 15 munits of modified or unmodified SMPLC. After incubation for 5, 10 or 30 min, adsorption of SMPLC onto bovine erythrocyte membrane, breakdown of SM in the membrane and hemolysis of bovine erythrocytes were measured according to the method reported previously.[9,13]

For determination of SMPLC adsorption to the membrane, the reaction mixtures (1 ml) were centrifuged at $600 \times g$ for 5 min, after 10 min incubation. Then, the activity of SMPLC in the resulting supernatants (unadsorbed activity) was determined, and the amount of enzyme adsorbed onto the erythrocyte membrane, *i.e.*, adsorbed activity, was calculated by subtracting unadsorbed activity from the total activity, which was obtained by incubation of the reaction mixture without erythrocytes at 37 °C. For the purpose of the determination of SM degradation, the reaction mixtures (1 ml) were extracted with chloroform–methanol by the method of Bligh and Dyer,[23] after 30 min of incubation. Then the extracts were separated into SM and other phospholipids by Thincrod-S-II (crystal rods coated with silica gel) thin-layer chromatography (TLC) with chloroform–methanol–acetic acid–water (83 : 17 : 10 : 4, v/v/v/v) and quantitatively analyzed for the SM content by Iatroscan TH-10 TLC analyzer containing FID (frame ionization detector) of high sensitivity (Iatron Laboratories, Inc., Tokyo). For the measurement of hemolysis (so-called "hot–cold hemolysis"), the reaction mixtures (1 ml) were cooled in an ice bath for 10 min, after 10 min incubation. Then, the mixtures were centrifuged at $600 \times g$ for 5 min. From the resulting supernatants, 0.2 ml-aliquots were withdrawn, diluted with 0.8 ml of cold borate-buffered saline (pH 8.0), and determined for the extent of hemolysis of measuring absorbance at 550 nm.

Measurement of SMPLC Activity toward Bovine Erythrocyte Ghosts, Liposomes and Micelles of SM, and HNP Unsealed ghosts of bovine erythrocytes were prepared by the method of Dodge *et al.*[24] The reaction mixtures (1 ml) containing unsealed ghosts (0.267 mg protein), 1 mM $MgCl_2$ and 15 munits of modified or unmodified SMPLC in 0.25 M sucrose–20 mM Tris–HCl buffer (pH 7.5), were incubated at 37 °C for 20 min. After incubation, the reaction mixtures were extracted with chloroform–methanol by the method of Bligh and Dyer.[23] Then, the extracts were analyzed for the extent of SM degradation, as shown in the case of the extracts from bovine erythrocyte suspensions.

SM-liposomes such as large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV), were prepared according to the reverse-phase evaporation method of Szoka and Papahadjopoulous,[25] and the method of Tomita *et al.*,[7] respectively. The reaction mixtures (0.5 ml) containing liposomes (SM: 2 mM), 2 mM $MgCl_2$ and 30 munits of modified or unmodified SMPLC in 3 mM sucrose—10-fold diluted borate-buffered saline (pH 7.5), were incubated at 37 °C for 20 min. The reaction was terminated by the addition of 2.5 ml of chloroform–methanol–35% HCl (66 : 33 : 1, v/v/v). Then, the phosphate content in the water–methanol layer, equivalent to hydrolyzed SM, was determined.[26,27]

For determination of SMPLC activity toward SM/sodium deoxycholate (SDC) or SM/Triton X-100 micelles, the reaction mixtures (0.5 ml) containing 2 mM $MgCl_2$, 2 mM SM, 0.16% SDC (w/v) or 0.312% Triton X-100 (w/v), and 10 munits of modified or unmodified SMPLC in 40 mM borate–NaOH buffer (pH 7.5), were incubated at 37 °C for 20 min. Like the determination of enzyme activity toward liposomal SM, the reaction was terminated and the phosphate content in the water–methanol layer of the extracts was determined.

For determination of SMPLC activity toward HNP, the reaction mixtures containing 4 mM $MgCl_2$, 2 mM HNP and 60 munits of modified or unmodified SMPLC were incubated at 37 °C for 30 min. The reaction was terminated by the addition of 1 ml of 0.1 M glycine–NaOH buffer (pH 10.5). Then, the amount of liberated 2′-hydroxy-5′-nitrohexadecanolide was determined by the absorbance at 415 nm.[28]

Results

Comparison of Bacterial Phospholipase C Activities after ss-PEG Modification First of all, three kinds of bacterial phospholipases C having different substrate specificities, *i.e.* SMPLC (sphingomyelinase) from *B. cereus*, PCPLC from *B. cereus* and PIPLC from *B. thuringiensis*, were modified by ss-PEG and followed the change in enzyme activities during the course of modification, as shown in Fig. 1. Use of ss-PEG is of great advantage in that *N*-hydroxy-succinimide ester can specifically attack lysyl ε-amino groups as well as an α-amino group of N-terminal amino acid. As shown in Fig. 1a, it was surprising that SMPLC retained more than 90% of its original activity (specific activity: 179 munits/mg) toward SM/Triton X-100 mixed micelle, after 2 h incubation with ss-PEG. Even after 12 h incubation, 80% of the enzyme activity still remained. Usually, the reaction between phospholipases and ss-PEG must be completed within at least 30 min. Therefore, the ss-PEG-modified SMPLC obtained was extremely stable. In contrast with SMPLC, both PCPLC and PIPLC were sensitive to this modification, reducing their activities to 30% and 50% of the initial level, respectively, by treatment with ss-PEG for 2 h (Fig. 1b and c). Thus, in this study we decided to further study the properties of ss-PEG-modified

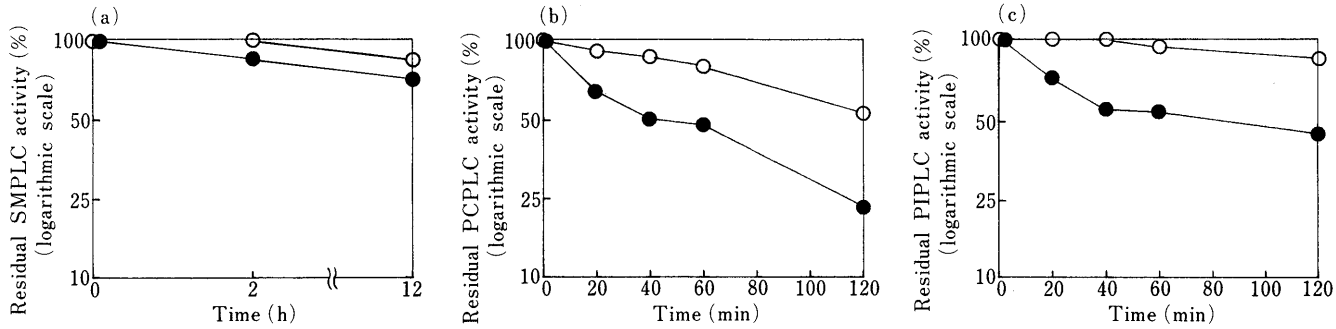

Fig. 1. The Activity Changes of Bacterial Phospholipases C during the Course of Modification with ss-PEG

To 100 μl aliquots of enzyme solutions containing 7.5 units of *B. cereus* SMPLC (a), 6.5 units of *B. cereus* PCPLC (b) or 2.5 units of *B. thuringiensis* PIPLC (c), was added 10 mg (a, b) or 1 mg (c) of ss-PEG, and the mixtures were incubated at 37 °C, as described in Materials and Methods. At the times indicated, residual activities of phospholipases C (closed circles) were determined. As control runs, residual activities of enzyme solutions which were incubated in the absence of ss-PEG (open circles), were also determined.

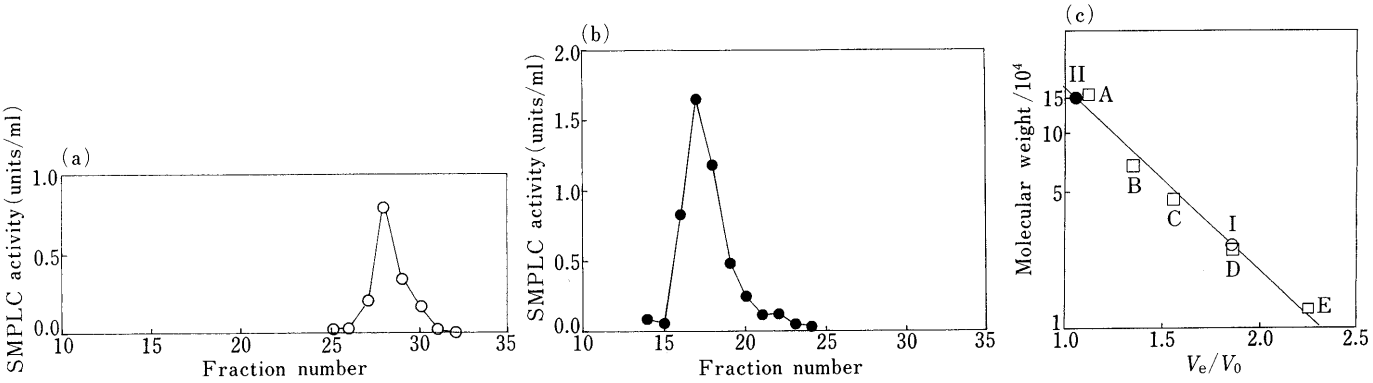

Fig. 2. Size-Exclusion Column Chromatography

Elution patterns of native SMPLC (a) and ss-PEG-modified SMPLC (b) were shown. The enzyme samples were applied to a Sephadex G-100 column (10 mm × 400 mm) and eluted with 10 mM Tris–HCl buffer (pH 7.5). Sixty-five drops (approx. 2 ml) of fractions were collected. Open and closed circles denote SM-hydrolyzing activity of native and modified SMPLC fractions. In (a), unreacted ss-PEG was detected around the fraction No. 35, according to A_{280}. (c) Calibration of molecular weights. Molecular weights of standard proteins: A, aldolase (158 kDa); B, bovine serum albumin (68 kDa); C, ovalbumin (50 kDa); D, chymotrypsinogen A (25 kDa); E, cytochrome c (12.5 kDa). I, native SMPLC; II, ss-PEG-modified SMPLC. Linearity of the calibration curve was assured by the method of least squares.

SMPLC, which were prepared by incubation of the enzyme with the modifying agent at 37 °C for 2 h.

Purification of Modified SMPLC In order to separate ss-PEG-modified SMPLC from both native SMPLC and unreacted ss-PEG, size-exclusion column chromatography was carried out with Sephadex G-100. The elution profile of SMPLC activity (Fig. 2a) was completely shifted to the region of much higher molecular weight by modification with ss-PEG (Fig. 2b). Substantially, there was no overlapping zone between the elution profiles of native and modified SMPLC activities. Therefore, complete modification of the enzyme was achieved, and the modified SMPLC proved to be free from the native enzyme and unreacted ss-PEG (molecular weight: approximately 5 kDa). Thus, the molecular weight of modified SMPLC must be more than 100 kDa whereas that of the native enzyme estimated was 25 kDa (the value from primary structure: 34 kDa), as shown in Fig. 2c. On the other hand, modified SMPLC migrated as a relatively broad band around 135 kDa, when applied to SDS–PAGE (data not shown).

Effect of Modification on Thermostability of SMPLC Figure 3 shows that SMPLC was significantly stabilized by modification with ss-PEG against heat denaturation. On heating at 65 °C, modified SMPLC still retained 60% of its initial activity, while the activity of native SMPLC was reduced to 30% of its original activity. The midpoint of heat inactivation (T_m) for modified SMPLC was approx. 70 °C whereas that for the native enzyme was 55 °C. In spite of the remarkable increase in thermostability, the range of pH optimal for SMPLC activity, pH 5—10, was not significantly altered by modification with ss-PEG (data not shown).

Effect of Modification on the Hydrolytic and Hemolytic Activities of SMPLC When bovine erythrocytes were incubated at 37 °C for 10 min with 15 munits of native SMPLC in the presence of both 1 mM Ca^{2+} and 1 mM Mg^{2+}, the cells became temperature-sensitive, resulting in 80—90% hemolysis by cold shock. The hemolysis was induced by the enzymatic breakdown of membrane SM, amounting to 80—90% of total SM. The same activity units of modified SMPLC, however, did not exert hemolytic action on bovine erythrocytes nor did they hydrolyze SM in the erythrocyte membrane at all, under the same condition. Also, in the

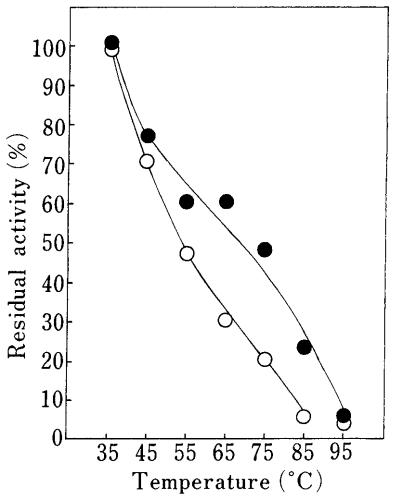

Fig. 3. Heat Stability

Enzyme solutions of native (○) and PEG-modified (●) SMPLC were allowed to stand for 10 min at varying temperatures (35 °C to 95 °C), then cooled to room temperature and the enzyme activity was determined, as shown in Materials and Methods.

presence of 1 mM Mn^{2+}, 10 munits of native SMPLC caused nearly complete hemolysis of bovine erythrocytes after incubation at 37 °C for 10—20 min, while the same units of modified SMPLC did not exhibit appreciable hemolytic activity toward the erythrocytes, as shown in Fig. 4. In the presence of 1 mM Ca^{2+} alone, 70—80% of native SMPLC activity was adsorbed to bovine erythrocyte membrane by incubation with the cells at 37 °C for 10 min, while no appreciable adsorption of modified SMPLC took place by similar incubation with the erythrocytes. Therefore, ss-PEG modification of SMPLC resulted in the disappearance of accessibility and hydrolytic activity toward bovine erythrocyte membrane, although the enzyme activity toward micellar SM was not significantly affected by modification (Table I).

Effect of Modification on the Activities toward Bovine Erythrocyte Ghosts, SM-Containing Liposomes and Micelles, and HNP Table I shows the effect of ss-PEG modification on catalytic activity of SMPLC toward membranous, liposomal and micellar SMs as well as a synthetic substrate, HNP. By this modification, the enzyme activity

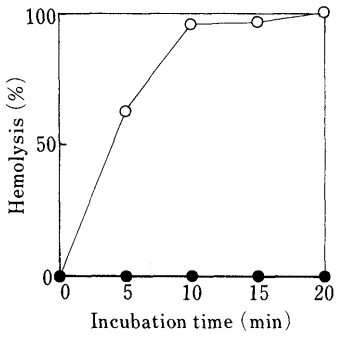

Fig. 4. Time Course of the Hemolysis of Bovine Erythrocytes Induced by Native and Modified SMPLC

The 5% bovine erythrocyte suspension in borate-buffered saline, pH 8.0 (1 ml) containing 1 mM Mn^{2+} was added to 10 munits of native (○) or PEG-modified (●) SMPLC, and each reaction mixture was incubated at 37 °C for 0, 5, 10, 15 or 20 min. Then, the mixtures were cooled in an ice bath for 5 min. After centrifugation, 0.1 ml of the supernatant was diluted with 0.9 ml of the cold buffer and determined for the extent of hemolysis by A_{550}.

TABLE I. Substrate Specificity of Native or Modified SMPLC

Natural and synthetic substrates	SMPLC activity (μmol/min·units)		Modified/native
	Native	Modified	
SM in various forms			
Erythrocyte ghosts	0.08	0	0
Large unilamellar liposomes	0.43	0.13	0.30
Small unilamellar liposomes	0.26	0.12	0.47
SM/SDC micelles	0.93	0.81	0.87
SM/Triton X-100 micelles	1[a]	1.2	1.2
Water-soluble substrate			
HNP	0.0065	0.0066	1.0

a) This should be normalized as 1, since the assay of SMPLC was carried out in the system of SM/Triton X-100 and all the SMPLC activities are calculated in terms of μmol/min·units.

toward membranous SM in bovine erythrocyte ghosts as well as the intact erythrocytes was completely lost. The activity toward liposomal SMs was reduced to one-third or half that of native SMPLC. On the other hand, modified SMPLC retained full activity toward micellar SMs and a water-soluble substrate, HNP. Therefore, SMPLC acquired selectivity toward amphiphilic or aqueous substrate as the result of modification, by virtue of the loss of accessibility to membranous SM.

Discussion

In the present study, the activity of sphingomyelinase from *Bacillus cereus* proved to be stable, even after the modifications of Lys residues in the SMPLC molecules by ss-PEG, a derivative of macromolecular PEG (molecular weight = approx. 5000), while PCPLC of *B. cereus* and PIPLC of *B. thuringiensis* were significantly inactivated by the same treatment.

Aurebekk and Little[29] reported that PC-hydrolytic activity was drastically decreased by the modification of Lys residues in the PCPLC of *B. cereus* with pyridoxal 5′-phosphate. Pyridoxal 5′-phosphate is known to be a chemical modifier which can specifically make a covalent bond with Lys residues through the formation of Schiff base. Therefore, this suggests that Lys residues in the PCPLC molecules may be involved in the catalytic center. In contrast, our preliminary results show that SMPLC activity was not affected after the modification of Lys residues by Bolton–Hunter reagent, 3-(*p*-hydroxyphenyl)-propionic acid *N*-hydroxysuccinimide ester,[30] or pyridoxal 5′-phosphate (Tomita *et al.* in preparation). The former reagent has the same functional group as ss-PEG used in the present study for the modification of the SMPLC molecules. Thus, Bolton–Hunter reagent can also specifically modify Lys residues under mild neutral pH regions. No information has been obtained on the functions of Lys residues in the molecules of PIPLC of *Bacillus* sp. Among three phospholipases C, Lys residues in the SMPLC molecules are certainly not involved in the catalytic center. Even though attachment of macromolecular PEG to the SMPLC molecules might distort the tertiary structure of the enzyme molecules and result in partial unfolding of the SMPLC molecules, ss-PEG-modified SMPLC retains full activity.

It is probable that a unique secondary structure of *B. cereus* SMPLC would contribute to the extreme stability of the SMPLC molecule after PEG modifications. In the foregoing study,[11] we reported that almost half of the total amino acid residues (306 residues) of SMPLC were involved in the loop or turn structure, while only 65 or 73 amino acid residues participate in α-helix or β-structure. Especially, analysis by CD spectra confirmed the result of the low content of α-helix predicted by the computer analysis method. Since a SMPLC molecule consists of a small amount of α-helix (0—5%) and is enriched in the loop or turn structure, this molecule must be very flexible. This flexibility of the SMPLC molecules may well keep the catalytic site intact, even after the attachment of macromolecular PEG.

Hough *et al.*[31] reported that PCPLC molecules from *B. cereus* contained 66% of α-helical structure in their secondary structure on the basis of the analysis of X-ray diffraction study. Therefore, the secondary structure of PCPLC seems to take more delicate configurations than that of SMPLC. In the present study, the destruction of the helical structure by ss-PEG probably resulted in the decrease of accessibility of PCPLC toward PC in the mixed micelles with the surfactants.

As shown in the foregoing reports,[3-6,9,12] the activity of *B. cereus* SMPLC is stimulated in the presence of Mg^{2+}, Mn^{2+} and Co^{2+}, while Ca^{2+}, Sr^{2+} and Zn^{2+} are inhibitory for the enzyme activity. The native enzyme catalyzes not only the hydrolysis of micellar or liposomal SM,[7,12] and a synthetic, water-soluble SM analog, HNP,[12] but also causes the breakdown of SM on the surface of mammalian erythrocytes in the presence of Mg^{2+} or Mn^{2+}, resulting in the hemolysis of these cells.[6,9] The ss-PEG-modified SMPLC, however, seems to acquire selectivity toward micellar and water-soluble substrates. Both HNP and mixed-micelle SM are hydrolyzed by the enzyme action of PEG-modified SMPLC almost at the same rate as that of native SMPLC. On the other hand, modification of SMPLC shows complete loss of accessibility toward biomembranes such as erythrocyte ghosts and intact erythrocytes. In the presence of Ca^{2+} alone, the native enzyme is significantly adsorbed to the surface of bovine erythrocytes, without causing the degradation of SM and hemolysis of the cells.[9] In other words, the native SMPLC forms a stable "enzyme–Ca^{2+}–substrate" complex on the surface of erythrocytes,

since the enzymatic breakdown of SM is completely inhibited by Ca^{2+}.[9] In the presence of both Mg^{2+} and Ca^{2+}, however, the enzyme is rapidly adsorbed to the bovine erythrocyte surface, then the activity once adsorbed is rapidly recovered in the supernatant as the breakdown of SM proceeds.[9] A similar phenomenon is observed in the presence of Mn^{2+} alone, although the process of enzyme adsorption is much slower than in the presence of both Mg^{2+} and Ca^{2+}.[6] Bovine erythrocytes attacked by SMPLC in the presence of Mg^{2+}, both Mg^{2+} and Ca^{2+}, or Mn^{2+}, causes hemolysis by cold shock.[6,9] However, ss-PEG-modified SMPLC is now shown to be completely inert to bovine erythrocytes; the modified enzyme does not exert hemolytic action on bovine erythrocytes by degradation of SM in the presence of Mg^{2+} and Ca^{2+} nor in the presence of Mn^{2+}. Also, the adsorptive property in the presence of Ca^{2+} is lost by modification of SMPLC with ss-PEG.

Interestingly, liposomal SMs were moderately hydrolyzed by PEG-modified SMPLC at the rate slower than the native enzyme. From the viewpoint of steric hindrance, it is reasonable that the large molecule of PEG-modified SMPLC is more accessible to small molecular substrates such as HNP and mixed-micellar or liposomal SM than SM in erythrocyte ghosts and intact erythrocyte membranes. Also, there is another possibility. SMPLC molecules modified by PEG lost their accessibility toward only erythrocyte membranes. Mixed micelles and liposomes are simply composed of SM and surfactant, or SM molecule alone. However, erythrocyte membranes are very condensed and are composed of many other constituents such as neutral lipids, glycolipids and glycoproteins besides phospholipids. Thus, it is possible that high surface pressure on the erythrocyte membranes decreased the accessibility of macromolecular PEG-modified SMPLC.

PEG-modified SMPLC was eluted at an extremely higher molecular range in Sephadex G-100 size exclusion chromatography (Fig. 2). From TNBS (trinitrobenzene sulfonic acid) titration, SMPLC modification was estimated to be approximately 60% by the method of Fields[32] (data not shown). Thus, from the results of gel filtration (Fig. 2) and the fact that SMPLC contains 26 free (lysyl or N-terminal) amino groups within a molecule according to its primary structure deduced from the sequence of cDNA,[10] the mean numbers of modified amino groups were nearly 15 per one SMPLC molecule. Since the molecular weight of ss-PEG was approx. 5000 on the average, the mean molecular weight of modified SMPLC might be calculated to be more than 100 kDa, being three fold (or more) that of native enzyme, 34 kDa. Although the preparation of native SMPLC was homogeneous, the SDS–PAGE profile of modified SMPLC became relatively broad (data not shown) owing to the heterogeneous size in the ss-PEG molecules, showing the heterogeneity of modified enzyme in the molecular mass.

The disappearance of SMPLC adsorption onto erythrocyte membranes seems not to be due to the blockage of the adsorptive site of this enzyme by ss-PEG, since chemical modification with two low-molecular weight NH_2-modifiers such as pyridoxal 5′-phosphate and Bolton–Hunter reagent, 3-(*p*-hydroxyphenyl) propionic acid *N*-succinimide ester did not show any appreciable effect on enzyme adsorption nor on hemolytic activity toward bovine erythrocytes (Tomita *et al.* in preparation). Probably, modification of free amino groups with ss-PEG inevitably induces alteration in the secondary or tertiary structures of SMPLC, *e.g.* the numbers of loops or β-turn structures, resulting in the repulsion of membranous SM against the modified enzyme.

Since these two amino group modifiers reduced its immunoreactivity to antiserum by enzyme-linked immunosorbent assay, immunoreactivity of the enzyme may be reduced by PEG-modification of SMPLC.

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