Chromogenic Assay for the Activity of Sphingomyelinase from Bacillus cereus and Its Application to the Enzymatic Hydrolysis of Lysophospholipids

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Received July 26, 2004; accepted August 25, 2004; published online August 26, 2004

We developed a convenient chromogenic assay method for the activity of sphingomyelinase (SMase) from Bacillus cereus. SMase reaction was quenched by Zn\(^{2+}\), and the released phosphocholine was converted into a chromogenic dye by the action of alkaline phosphatase. After that, the released phosphocholine was measured by titration with base at an appropriate constant pH value (pH-stat method). Assays using radiolabeled or unlabeled substrate generally requires physical separation of the product from substrate, except for the pH-stat method. This separation is usually accomplished by liquid-liquid extraction that can effectively separates the lipophilic substrate and hydrophilic product into the organic and aqueous phases, respectively. Furthermore, the quantification of inorganic phosphate requires pretreatment of wet-ashing. The pH-stat method, which is used for unlabeled SM as a substrate, can provide a continuous monitoring assay, generating the time course of the reaction, but it can not measure simultaneously many samples. These methods are therefore not suitable for the measurement of a large number of test samples.

Previously, a chromogenic assay for the activity of phosphatidylcholine-specific PLC from Bacillus cereus has been reported. That was based on the conversion of phosphocholine and hydrophilic product into the chromogen. This method never requires the separation of the product from the substrate, but it is not applicable to SMase, since this enzyme reaction could not be quenched with Tris.

In this study, we tried to construct a facile and convenient assay method for the activity of SMase from Bacillus cereus that was based on the conversion of phosphocholine into a chromogenic dye. In this assay, ZnCl\(_2\) was used for quenching of SMase reaction, and the assay was performed using 96-well microtiter plates to test a large number of samples in a short period of time.
known to hydrolyze lyso platelet-activating factor (lysoPAF, alkyl subclass of lysoPC), but it is uncertain whether bacterial SMase can hydrolyze this lipid. Since the present assay method is applicable not only to the hydrolysis of SM but also to those for lysoPC and lysoPAF, the kinetics of the hydrolysis of these substrates by *B. cereus* SMase were examined.

**MATERIALS AND METHODS**

**Materials**  SM from bovine brain (S7004), alkaline phosphatase from bovine intestinal mucosa (P6774), and peroxidase from horseradish (P8250) were obtained from Sigma. Choline oxidase from *Alcaligenes* sp. and MgCl$_2$·6H$_2$O were purchased from Wako Pure Chemicals (Japan). LysoPCs having different chain lengths were from Avanti Polar Lipids, and 1-O-hexadecyl-sn-glycero-3-phosphocholine (C$_{16}$lysoPAF) was from Cayman. Other chemicals were of the highest purity commercially available.

SMase from *Bacillus cereus* was purchased from Higeta Shouyu (Japan). The preparation was dissolved in 2 M urea, and the solution was dialyzed against 0.2 M NaCl and centrifuged. Concentration of the SMase solution obtained from the supernatant was determined spectrophotometrically based on its molar absorption coefficient of $5.82 \times 10^4$ M$^{-1}$ cm$^{-1}$ at 280 nm, which had been calculated on the basis of the Tyr and Trp contents by their respective molar absorption coefficients of $1.4 \times 10^4$ and $5.5 \times 10^3$ M$^{-1}$ cm$^{-1}$ at 280 nm. The enzyme solution containing 2 mM Triton X-100 was stored at 4°C as a SMase stock solution.

**Measurement of SMase Activity**  Enzymatic hydrolysis was started by the addition of 5 μl of the SMase stock solution to 495 μl of the substrate solution containing certain respective concentrations of Mg$^{2+}$, substrate, and buffer component. Final concentrations of Mg$^{2+}$ and N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) were 10 and 50 mM, respectively. After incubation at 37°C and pH 7.5 for an appropriate period of time, 0.5 ml of a stop solution containing 10 mM ZnCl$_2$, 30 units/ml alkaline phosphatase, and 0.1 mM Tris–HCl at pH 8.0 was added to the reaction mixture so as to quench the SMase action, and the mixture was incubated at 37°C for 1 h to produce choline from phosphocholine by phosphatase reaction. Then 2.0 ml of chromogenic substrate solution containing 0.5 unit/ml choline oxidase, 0.5 unit/ml peroxidase, 1 mM 4-aminoantipyrine, 2 mM phenol, 15 mM Triton X-100, 7.5 mM EDTA, and 0.1 mM Tris–HCl at pH 8.0 was added, and the mixture was incubated at 37°C for 1 h so as to produce the chromogenic dye from choline. Then, the absorbance at 492 nm with a microplate reader. The absorbance was read at 492 nm with a microplate reader.

The calibration curve was constructed according to the same protocol as described above, except that various concentrations of phosphocholine were subjected to the assay reaction.

**RESULTS AND DISCUSSION**

As shown in Fig. 1, the assay method for the activity of *B. cereus* SMase described here is based on the estimation of the phosphocholine produced from the substrate by the SMase action, by the coupling system of alkaline phosphatase and choline oxidase/peroxidase. In this assay, choline generated from phosphocholine by the action of alkaline phosphatase induced the formation of a red dye by the actions of choline oxidase and peroxidase. Therefore, this assay depends on whether the SMase activity remains selectively inhibited during the chromogenic reaction (phosphatase, choline oxidase, and peroxidase reactions).

Previously, a similar assay method has been reported for phosphatidylcholine-specific PLC (PC-PLC). In that case, the activity of PC-PLC was quenched with Tris. However, SMase activity could not be quenched with Tris. Initially, we expected that SMase reaction could be arrested by the addition of EDTA, since the SMase was known to be a Mg$^{2+}$-dependent enzyme. However, the alkaline phosphatase reaction was also inhibited by the presence of EDTA, since this...
enzyme is a Zn$^{2+}$-dependent one. Therefore, we tried to use Zn$^{2+}$ to quench the SMase activity, since the enzyme activity of B. cereus SMase is known to be inhibited strongly by Zn$^{2+}$. However, Zn$^{2+}$ was found to interfere with the choline oxidase/peroxidase reaction. Therefore, the chromogenic reaction was divided into two separate parts, the phosphatase reaction and the choline oxidase/peroxidase reaction, and the latter reaction was performed in the presence of EDTA to trap the Zn$^{2+}$ and Mg$^{2+}$. In the latter reaction, furthermore, Triton X-100 was added to the reaction mixture, in order to remove the turbidity generated from ceramide which had been produced by the SMase reaction. This turbidity disturbed the measurement of absorbance but did not disturb the SMase reaction, because the linear time course of the reaction was obtained.

Until now, SMase activity toward unlabeled SM has been measured by quantification of the inorganic phosphate obtained by wet-ashing of the phosphocholine which had been extracted from the reaction mixture. Figure 2 compares the results of the chromogenic assay for B. cereus SMase activity with those of the inorganic phosphate quantification assay. The calibration curves for both assay methods showed nearly identical sensitivities (Fig. 2A). The limits of detection for both assay methods were about 10 μM phosphocholine in the reaction mixture of SMase. Similar time courses of reactions were obtained by both methods for the SMase-catalyzed hydrolysis of mixed micellar SM with Triton X-100 (the molar ratio of 1 : 10) (Fig. 2B). However, the chromogenic assay was thought to be more convenient than the inorganic phosphate quantification assay, since the latter method requires the physical separation of the product generated from the substrate and the wet-ashing of the product.

Since the chromogenic assay does not require the physical separation and wet-ashing, it can be performed using microtiter plates to test a large number of the test samples. Figure 3 shows the calibration curve obtained by the microtiter plate assay. The limit of detection for this assay method was about 10 μM phosphocholine in the reaction mixture of SMase. Figure 4A shows the time courses of the enzymatic hydrolysis of mixed micellar SM with Triton X-100 (1 : 10), in the microplate assay using various amounts of SMase. The time course curves were linear with time, and the slopes of the straight lines were taken as the velocities of the enzyme reaction in nmol/min (Fig. 4A). Figure 4B shows the velocities of the enzyme reaction plotted as a function of the amounts of SMase. The velocities were directly proportional to the amounts of enzyme in their range examined.

To determine the kinetic parameters for the hydrolysis of SM and lysoPC catalyzed by B. cereus SMase, the products of SMase reaction at various concentrations of the substrates were measured for 10 min at 2 min intervals, and the initial velocities were determined by a regression analysis of the time course of the enzyme reactions. Figures 5A and B show the Lineweaver-Burk plot of the data on the hydrolysis of mixed micellar SM with Triton X-100 (1 : 10) and that of mixed micellar C<sub>16</sub>lysoPC with varying molar ratios of Triton X-100, respectively. The kinetic parameters were determined according to the Michaelis–Menten equation by a non-linear regression analysis and summarized in Table 1.

In these experiments, the initial velocity could not be measured at a substrate concentration below 0.1 mM, because the time course of the SMase reaction did not show a straight line owing to the detection limit of this method. Therefore, it was difficult to obtain the accurate values of $K_m$ when the
lower, respectively, than the corresponding values for the SMase reaction and used test tubes instead of microtiter plates. The SMase reaction without changing the total volume of chromogenic assay using 96-Well Microtiter Plates different types of substrates obtained from the chromogenic assay using 96-Well Microtiter Plates (A) Time courses of the enzyme reaction. The enzymatic hydrolysis of mixed micellar SM with Triton X-100 (1:10) was followed with time at 37 °C with various amounts of B. cereus SMase in the presence of 4 mM Mg$^{2+}$ and 33 mM TES (pH 7.5) at ionic strength 0.2. The amounts of SMase added were 0.35 ( ), 0.29 ( ), 0.23 ( ), 0.18 ( ), 0.11 ( ), and 0.06 ( ) ng. Values represent the means±S.D. of three experiments. (B) The slopes obtained from the straight lines in (A) were plotted as a function of the amount of SMase. Values represent the means±S.E.M. from regression analysis of the time course of the enzyme reactions (n=7).

Fig. 5. Lineweaver–Burk Plots of the Data on the Hydrolysis of Two Different Types of Substrates Obtained from the Chromogenic Assay Using 96-Well Microtiter Plates (A) The SMase-catalyzed hydrolysis of mixed micellar SM with Triton X-100 (1:10) in the presence of 4 mM Mg$^{2+}$ and 33 mM TES (pH 7.5) at ionic strength 0.2 and 37 °C. (B) The enzymatic hydrolysis of mixed micellar C$_{16}$lysoPC with several molar ratios of Triton X-100. The molar ratios of C$_{16}$lysoPC: Triton X-100 were 1:10 ( ), 1:6.2 ( ), 1:3.2 ( ), and 1:0 ( ). Values represent the means±S.E.M. from regression analysis of the time course of the enzyme reactions (n=5).

values were less than 0.1 mM. If we increased the volume of SMase reaction without changing the total volume of chromogenic reaction and used test tubes instead of microtiter plates, more accurate $K_m$ values could be determined.

As shown in Table 1, in comparison of the kinetic parameters for SM with those for C$_{16}$lysoPC in the presence of the same molar ratio of Triton X-100 to the substrate (1:10), the $k_{cat}$ and $K_m$ values for SM were 300-fold higher and 17-fold lower, respectively, than the corresponding values for C$_{16}$lysoPC. This result indicates that higher activity of SMase toward SM is ascribable to the higher catalytic center activity ($k_{cat}$) rather than the binding capability for the substrate (1/$K_m$). As also shown in Table 1, the $k_{cat}$ and $K_m$ values of SMase toward C$_{16}$lysoPC decreased and increased, respectively, with increase in the molar ratio of Triton X-100 to C$_{16}$lysoPC. Similar observations have been reported for phospholipid-degrading enzymes such as phospholipase A$_2$ and phospholipid-biosynthetic enzymes such as phosphatidylinositol-4-kinase. These phenomena can be interpreted in terms of “surface dilution”.

Sawai et al. reported that lysoP AF was a good substrate for nSMase 1, but it was still unclear whether lysoP AF could be a substrate for bacterial SMase. Moreover, the effect of the chain length of its related substrate, lysoPC on the SMase activity remains to be clarified. The chromogenic assay method was therefore applied to the studies on lysoP AF and lysoPCs having various chain-lengths.

As shown in Table 1, the $k_{cat}$ and $K_m$ values toward micellar C$_{16}$lysoP AF were much lower and higher than the corresponding values for SM, and the catalytic efficiency ($k_{cat}/K_m$) toward micellar C$_{16}$lysoP AF was 2000-fold lower than the corresponding value for SM. Recently, the enzyme activities of nSMase 1 and nSMase 2 toward several phospholipids have been reported. These two nSMases hydrolyzed SM more predominantly than lysoP AF in the presence of Triton X-100 and phosphatidylserine. We found that the hydrolysis of lysoP AF catalyzed by B. cereus SMase was restrained to the same extent as that of lysoPC in the presence of Triton X-100 (data not shown). These observations suggest that the substrate specificity of B. cereus SMase toward lysoP AF and SM is similar to that for nSMases.

As also shown in Table 1, the $k_{cat}$ values toward lysoPCs having different chain lengths (n=14 to 20) were nearly identical to each other, whereas the $K_m$ values showed a little tendency to increase above C$_{18}$ and the lowest value was obtained for C$_{16}$lysoPC. These results suggest that the chain length in these substrate species slightly influences the affinity of enzyme to the substrate but not the catalytic center activity.

In the present study, we developed an assay method for the activity of B. cereus SMase which was applicable to the studies on the phosphocholine-containing phospholipid substrates and was convenient for testing a large number of samples. However, enlarging the volume of SMase reaction was found to be required to obtain the accurate values of $K_m$ when the values were lower than 0.1 mM. Nevertheless, our present assay method for the SMase activity would be useful especially in screening the activating or inhibiting reagents.
toward this enzyme.

Acknowledgments  This work was supported in part by Grants-in-Aid for the Encouragement of Young Scientists (No. 14771297 to S. F.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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