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Bacillus cereus sphingomyelinase recognizes ganglioside GM3

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ABSTRACT

Sphingomyelinase (SMase) from *Bacillus cereus* (*Bc*-SMase) hydrolyzes sphingomyelin (SM) to phosphocholine and ceramide in a divalent metal ion-dependent manner, and is a virulence factor for septicemia. *Bc*-SMase has three characteristic sites, viz., the central site (catalytic site), side-edge site (membrane binding site), and β-hairpin region (membrane binding site). Here, we show that the β-hairpin directly binds to gangliosides, especially NeuAcα2-3Galβ1-4Glcβ1-1ceramide (GM3) through a carbohydrate moiety. Neuraminidase inhibited the binding of *Bc*-SMase to mouse peritoneal macrophages in a dose-dependent manner. SPR analysis revealed that the binding response of *Bc*-SMase to liposomes containing GM3 was about 15-fold higher than that to liposomes lacking GM3. Moreover, experiments with site-directed mutants indicated that Trp-284 and Phe-285 in the β-hairpin play an important role in the interaction with GM3. The binding of W284A and F285A mutant enzymes to mouse macrophages decreased markedly in comparison to the binding by wild-type enzymes. Therefore, we conclude that GM3 is the primary cellular receptor for *Bc*-SMase, and that the β-hairpin region is the tethering region for gangliosides.

1. Introduction

Sphingomyelinase (SMase), which catalyzes the hydrolysis of sphingomyelin (SM) to produce phosphocholine and ceramide, is widely distributed throughout eukaryotes and prokaryotes. Eukaryotic SMases have been classified according to their pH preference as acid [1], alkaline [2], and neutral [3,4] SMases. In prokaryotes, some broad-specificity phosphatidylcholine (PC) phospholipase C enzymes possess SM-hydrolyzing activity [5,6], but a number of pathogenic bacteria, such as *Staphylococcus aureus* (β-hemolysin [7,8]), *Bacillus cereus* [9], *Leptospira interrogans* [10], and *Listeria ivanovii* [11,12], produce SM-specific phospholipases. These bacterial SMases share sequence homology with eukaryotic SMases and have a similar catalytic mechanism and overall structure [13,14]. Bacterial SMase has been confirmed to be a member of the DNase 1-like folding superfamily [15–17], and the amino acid residues in the putative active site of bacterial SMase were found to be geometrically identical to the corresponding amino acid residues of enzymes in the DNase 1-like folding superfamily.

Bc-SMase belongs to a group of bacterial extracellular enzymes; it exhibits potent hemolytic activity, and is a virulence factor for septicemia caused by *B. cereus*. Crystal analysis showed that *Bc*-SMase possesses 3 characteristic sites, viz., the central site, side edge site, and β-hairpin site. We also showed that the metal ion(s) in the central region plays an important role in the enzymatic activity of *Bc*-SMase [18]. Several researchers reported that Glu-53, His-151, Asp-195, Asn-197, and His-296 at the central site participate in the hydrolysis of substrates [18–21]. Recently, we provided evidence that the metal ion of the side-edge site of *Bc*-SMase is involved in the binding of membranes [22].

However, little detail is known about the mechanism of membrane binding by the β-hairpin, which is conserved in bacterial SMases, but not in mammalian SMases (Fig. 1). It is known that various bacterial toxins binds to cell membranes through specific recognition of the polysaccharide moiety of glycosphingolipids [23–26]; thus, in this study, we investigated the relationship between the β-hairpin region and binding to gangliosides.

2. Materials and methods

2.1. Drugs

NeuAcα2-3Galβ1-4Glcβ1-1ceramide (GM3) was obtained commercially (Calbiochem, USA). Neuraminidase from *Clostridium perfringens* and a ganglioside mixture from bovine brain were

Abbreviations: *B. cereus*, *Bacillus cereus*; SMase, sphingomyelinase; *Bc*-SMase, *Bacillus cereus* sphingomyelinase; GM3, NeuAcα2-3Galβ1-4Glcβ1-1ceramide; SPR, surface plasmon resonance.

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Fig. 1. Amino acid sequence alignment of sphingomyelinases (SMases) from various species. Amino acid sequences of SMases from *B. cereus* (*Bc*-SMase), *S. aureus* (*Sa*-SMase), and *L. ivanovii* (*Li*-SMase), human neutral SMase (hnSMase), and human neutral SMase2 (hnSMase2) were aligned using the program T-Coffee [31]. Amino acid residues conserved between bacterial SMases and mammalian SMases are indicated by boxes. Red characters indicate the amino acid residues participating in forming the β-hairpin region. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

obtained commercially (Sigma–Aldrich, USA). All other chemicals were of analytical grade.

2.2. Preparation of Bc-SMase and variants

Bc-SMase was overexpressed in *Bacillus subtilis* ISW1214 transformed with the plasmid vector pHY300PLK, which contains cDNA of *Bc*-SMase cloned from *B. cereus* IAM1029. Expression and purification of the recombinant *Bc*-SMase and variant enzymes were performed as described previously [18].

2.3. Cy3-coupled enzyme

Bc-SMase (3 mg/mL) was labeled with a Cy3-labeling kit (GE healthcare, USA) following the manufacturer's protocol. Hemolysis in sheep erythrocytes treated with Cy3-coupled *Bc*-SMase (Cy3-*Bc*-SMase) was the same as that of cells treated with the non-labeled enzyme.

2.4. Glycoarray assay

Glycoarray plates (Sumitomo Bakelite, Japan) were incubated with 100 μg Cy3-*Bc*-SMase in PBS containing 1 mM $CaCl_2$ for 15 min; this was followed by washing the plate with PBS. Bound Cy3-*Bc*-SMase was measured with an Affymetrix 428 array scanner (Affymetrix, USA).

2.5. Site-directed mutagenesis

A transformer site-directed mutagenesis kit (BD Biosciences, USA) was used along with the primers W284A: 5′-TGGACTGTTACATCAGCGTTCCAAAAATAT-3′, and F285A: 5′-ACTGTTACATCATGGGCGCAAAAATATACG-3′, to prepare a modified plasmid. The genetic sequence of *Bc*-SMase in each plasmid was confirmed with an ABI3500 PRISM™ genetic analyzer (Life technologies, USA).

2.6. Mice

Six- to eight-week-old male wild-type ICR mice (Nihon SLC, Japan) were used. Experimental protocols were approved by the Institute Animal Care and Use Committee at Tokushima Bunri University. Mice were housed in plastic cages under controlled environmental conditions (temperature 22 °C, humidity 55%). Food and water were freely available.

2.7. Culture of macrophages

Mouse macrophages were isolated from cells in peritoneal exudates using 2 mL of phenol red-free RPMI1640 medium (Wako Pure Chemical Industries, Japan), supplemented with 5% fetal bovine serum (FBS; Biowest, USA). After centrifugation at 170×*g* for 10 min at 4 °C, the cell pellet was resuspended in phenol red-free RPMI1640 medium supplemented with 5% FBS. Adherent macrophage monolayers were obtained by plating the cells in 96- or 48-well plastic trays (Falcon, USA).

2.8. Binding of Bc-SMase to mouse macrophages

Mouse macrophages were seeded on a poly-L-lysine glass-bottom dish (MatTek, USA). Cy3-*Bc*-SMase was added at room temperature for various times, and the cells were fixed with 2% paraformaldehyde in PBS, then washed 3 times with PBS. The fluorescence level of Cy3-*Bc*-SMase in each cell was analyzed by fluorescent microscopy (BIOREVO BZ-9000; Keyence, Japan) using the microscope-associated analysis software package (BZ-H2A).

2.9. Preparation of GM3-liposomes

Phosphatidylcholine from egg yolk (Nacalai, Japan), sphingomyelin from bovine brain (Nacalai, Japan), and GM3 (Calbiochem, USA) were mixed in a 1:1:0.1 M-ratio, and was dried from chloroform/methanol (1:2 v/v) under nitrogen gas, resuspended in HBS-N (0.01 M HEPES pH 7.4, 0.15 M NaCl; GE healthcare, USA), and sonicated in a water bath for 5 min to ensure that the lipid was fully hydrated. The liposome suspension was passed 21 times (0.5 mL at a time) through a Liposofast (Avestin, USA) liposome extruder with 100-nm pore size polycarbonate membranes.

2.10. Bc-SMase membrane-binding assay

A membrane-binding assay was performed using surface plasmon resonance (SPR) analysis employing a Biacore3000 system (GE healthcare, USA) and the associated analysis software package (BIAevaluation; GE healthcare, USA). *Bc*-SMase and variants at concentrations of 100, 50, 10, 5, and 0 nM were applied to GM3-liposomes or liposomes without GM3-coated L1 sensor chips at a flow rate of 10 μL/min in running buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 1 mM $CaCl_2$) at 25 °C. Dissociation was monitored for at least 150 s in a constant flow of enzyme-free running buffer.

2.11. Statistical analysis

All data presented are expressed as the mean ± SEM. Comparisons of mean values among experimental groups were made using Student's *t*-test. $P < 0.01$ was considered statistically significant.

3. Results

3.1. Bc-SMase binds directly to GM3

We investigated the interaction between *Bc*-SMase and various gangliosides using an *in vitro* glycoarray system (Fig. 2A). The array plate was incubated with 100 μg/mL Cy3-*Bc*-SMase at 37 °C for 15 min, and the fluorescence signal of *Bc*-SMase was significantly localized to the spot corresponding to the carbohydrate chain of GM3 (Fig. 2A). The enzyme was also bound to gangliosides, such as GD3 and GD1a (Fig. 2A). Binding of *Bc*-SMase to GD3 and GD1a was weaker than that to GM3 (Fig. 2A).

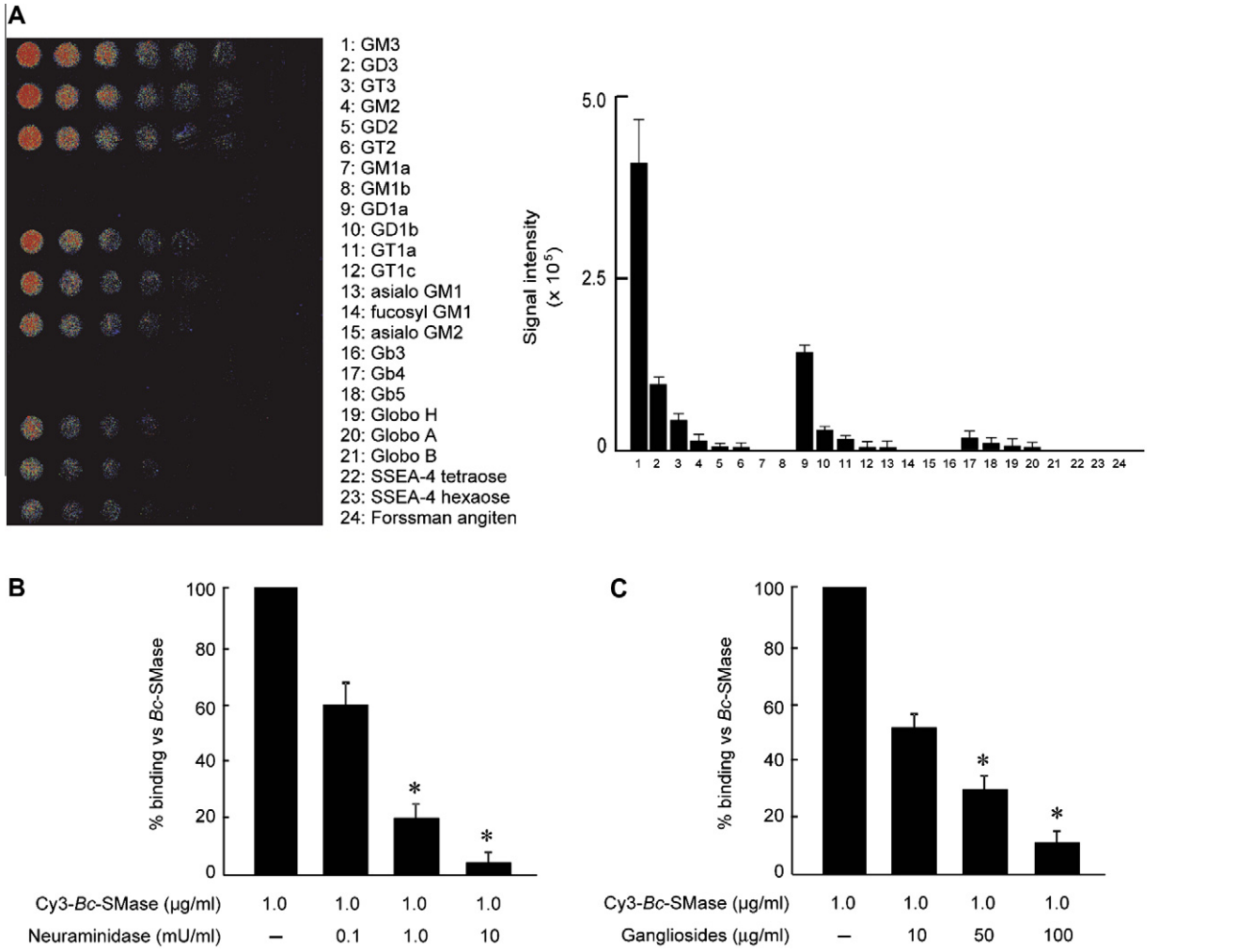

Fig. 2. Relationship between Bc-SMase and gangliosides. (A) Glycoarray plates were incubated with 100 μg/mL of Cy3-*Bc*-SMase for 15 min. Fluorescence intensity of the enzyme on the plate was measured using a fluorescence scanner. Values represent the mean ± SD ($n = 3$). (B) Mouse peritoneal macrophages were treated with various concentrations of neuraminidase at 37 °C for 60 min, then the cells were incubated with 1.0 μg/mL Cy3-*Bc*-SMase for 10 min. (C) Mouse peritoneal macrophages were incubated with 1.0 μg/mL Cy3-*Bc*-SMase and with a gangliosides mixture from bovine brain for 10 min. The treated cells were fixed in 4% paraformaldehyde and analyzed using fluorescent microscopy. The fluorescence intensity in the visual fields was measured as described under Section 2. The binding of *Bc*-SMase to the intact cells was set as the maximal response (100%) against which all other results were compared. Values represent the mean ± SD; $n = 3$; $^*P < 0.01$, compared with the binding of *Bc*-SMase in untreated cells.

3.2. Gangliosides mediate the binding of Bc-SMase

Bc-SMase induced attenuation of membrane fluidity and impaired phagocytosis in mouse peritoneal macrophages [27]. To investigate the effect of gangliosides and sialic acid on the binding of *Bc*-SMase to mouse peritoneal macrophages, cells were treated with neuramidase, which catalyzes the hydrolysis of terminal sialic acid residues, at 37 °C for 60 min. After this pretreatment, the cells were incubated with 1.0 μg/mL *Bc*-SMase. Treatment with neuraminidase inhibited the binding of Cy3-*Bc*-SMase to the cells in a dose-dependent manner (Fig. 2B). In addition, the binding of *Bc*-SMase to cells was dose-dependently inhibited by treatment with a gangliosides mixture form bovine brain (Fig. 2C).

3.3. Role of the β-hairpin on binding of Bc-SMase to mouse peritoneal macrophages

We previously reported that replacement of Trp-284 and Phe-285, which are located at the apex of the β-hairpin of *Bc*-SMase (Fig. 3A, *red*), with alanine, W284A, and F285A, reduced binding of *Bc*-SMase to SM-liposomes, and caused disruption of SM-liposomes and sheep erythrocytes [18]. Here, we investigated the binding of W284A and F285A mutant *Bc*-SMases to mouse macrophages. Fig. 3B shows that binding of W284A and F285A mutant enzymes to the cells was significantly decreased compared with that of wild-type enzymes.

3.4. Surface plasmon resonance analysis

We analyzed the affinity of these variants for GM3-liposomes by SPR. Fig. 4 shows both the liposome association, represented by an increase in resonance units, and dissociation, represented by a decrease in resonance units. Wild-type enzyme rapidly associated with GM3-liposomes and slowly dissociated from them, and the resonance units of wild-type enzyme binding to GM3-liposomes increased dose-dependently (Fig. 4A). The steady-state binding parameters were determined using BIAevaluation software, and the K_D value for the wild-type was estimated to be 25 nM. The binding curves for the *Bc*-SMase variants to GM3-liposomes (Fig. 4B and C) showed estimated K_D values of 3220 nM and 2850 nM for W284A and F285A, respectively. The K_D values of W284A and F285A were more than 100-fold that of the wild-type

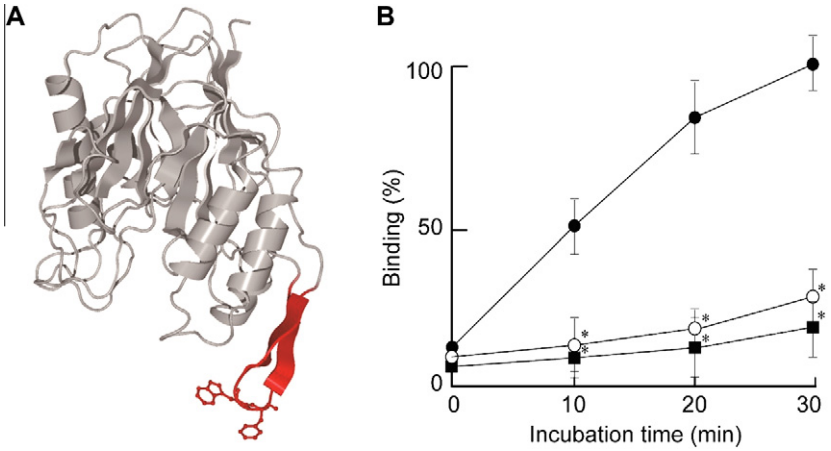

Fig. 3. Binding assay of *Bc*-SMase β-hairpin mutants. (A) The structure of *Bc*-SMase is shown using a ribbon representation. β-hairpin, Trp-284, and Phe-285 are colored *red*. (B) Mouse peritoneal macrophages were incubated with 1.0 μg/mL Cy3-*Bc*-SMase (black circle), Cy3-W284A (white circle), or Cy3-F285A (black square) for the indicated periods. Cells were fixed in 4% paraformaldehyde and analyzed using fluorescent microscopy. The fluorescence intensity in the visual fields was measured as described under Section 2. The binding of *Bc*-SMase to the intact cells at 30 min was set as the maximal response (100%) against which all other results were compared. Values represent the mean ± SD; $n = 3$; $^{*}P < 0.01$, compared with the binding of *Bc*-SMase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

enzyme, suggesting that the affinity of W284A and F285A for GM3-liposomes was markedly reduced compared to that of the wild-type enzyme. In addition, the binding of wild-type enzyme, W284A, and F285A to liposomes in the absence of GM3 showed estimated K_D values of 325 nM, 4620 nM, and 3650 nM, respectively (Fig. 4D–F). Therefore, it appears that *Bc*-SMase strongly interacts with GM3 from phospholipids such as SM and PC.

4. Discussion

Bc-SMase catalyzes hydrolysis of SM to form ceramide in cell membranes, and is used as a tool in various studies, such as investigation of apoptosis or of internalization of viruses [28–30]. Despite the recent identification of the residues critical for the enzymatic activity of *Bc*-SMase [18–20], the binding mechanism of this enzyme is still not completely understood. The present study has demonstrated that the β-hairpin in *Bc*-SMase plays an important role in tethering to GM3 on membranes. Our mutagenesis study has now identified two specific amino acid residues, Trp-284 and Phe-285, which play a critical role in the binding of *Bc*-SMase to a carbohydrate moiety of GM3. We measured the binding capacity of *Bc*-SMase to various gangliosides and found that the binding activity of *Bc*-SMase to gangliosides was in the order of GM3 >> GD1a > GD3 (Fig. 2A and B). In addition, neuraminidase

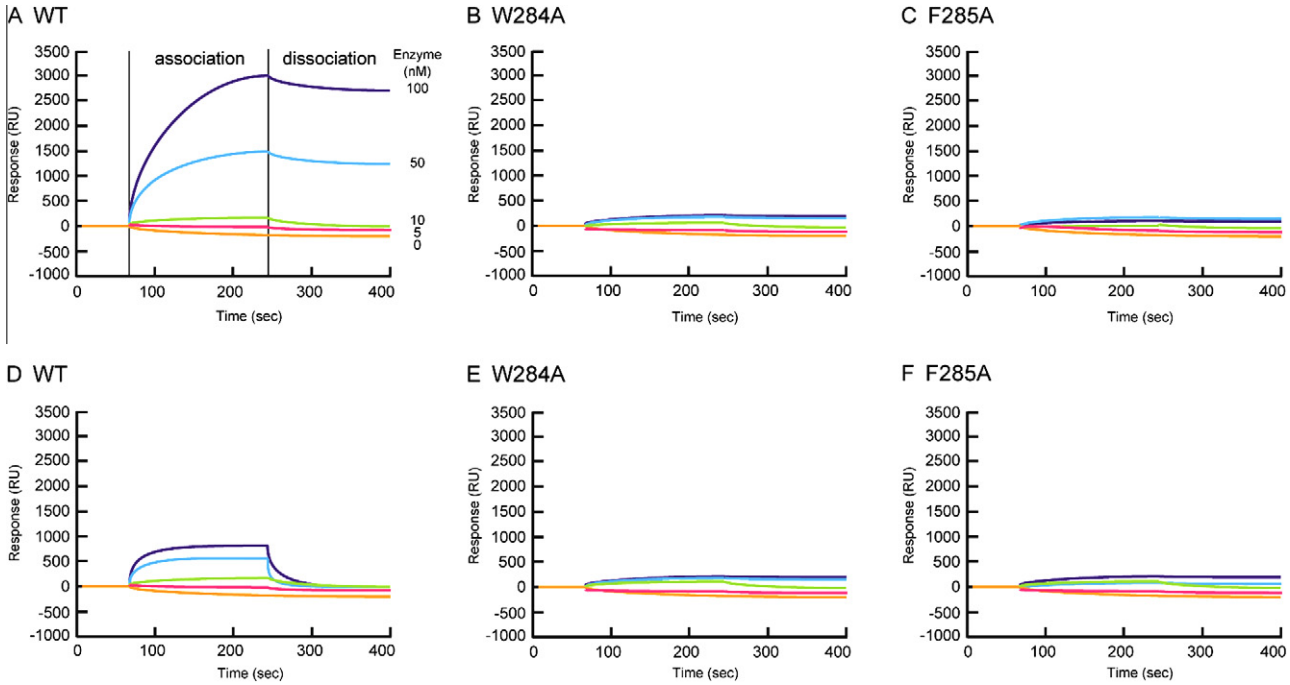

Fig. 4. Surface plasmon resonance analysis of binding of variants to GM3-liposomes. GM3-liposomes (A–C) or liposomes without GM3 (C–E) were immobilized on L1 sensor chips. Binding of the wild-type (A and C), W284A (B and D), and F285A (C and E) mutant *Bc*-SMases to each type of liposome was measured using a Biacore 3000. *Blue, light blue, green, pink, and red* lines indicate 100, 50, 10, 5, 0 nM concentrations of the variants, respectively. Association and dissociation represent the buffer in the presence or absence of these enzymes, respectively. A representative result from 1 of 3 experiments is shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

dose-dependently inhibited the binding of *Bc*-SMase to mouse peritoneal macrophages. These results suggest that the sialic acid of GM3 is required for effective binding to *Bc*-SMase.

Bc-SMase has an aromatic-residue rich β-hairpin (Trp-279 to Tyr-290), which is conserved in hemolytic SMase from *S. aureus* and from *L. ivanovii* [12,18]. This region is not found in human SMase, and in other structurally characterized members of the DNase 1-like folding superfamily. Replacement of Trp-284 and Phe-285 with alanine in the β-hairpin, viz., the W284A and F285A mutants, reduced binding to mouse macrophages in the early stage (Fig. 3). SPR analysis confirmed the marked reduction in the binding of GM3-liposomes binding to these mutants compared with the wild-type enzyme (Fig. 4A–C). In addition, binding of *Bc*-SMase to GM3-liposomes was about 15-fold higher than that to liposomes lacking GM3. Therefore, it appears that *Bc*-SMase initially recognizes GM3 rather than phospholipids such as SM and PC.

We reported that *Bc*-SMase has another membrane-binding region, which is the solvent-exposed loop and side-edge metal-binding site (Asn-92 to Asp-100, side-edge site) [18]. This suggests that the β-hairpin region and the side-edge site is involved in the early and the late stage of membrane-binding by the enzyme, respectively.

In conclusion, we have here revealed that Trp-284 and Phe-285, located at the apex of the β-hairpin in *Bc*-SMase, plays an important role in the binding of the enzyme to the sialic acid moiety of GM3 or GM3-like structures on cell membranes, and that the β-hairpin region participates in the pathogenic activity of SMase-secreting bacteria. Thus, gangliosides, especially GM3, can be a potential target for the development of drugs against *B. cereus*.

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