Mutation in aspartic acid residues modifies catalytic and haemolytic activities of *Bacillus cereus* sphingomyelinase

Hiro-omi TAMURA,* Kaori TAMEISHI,* Atsuko YAMADA,* Masahiro TOMITA,† Yo MATSUO, ‡Ken NISHIKAWA‡ and Hiroh IKEZAWA*§

*Department of Microbial Chemistry, Faculty of Pharmaceutical Sciences, Nagoya City University, Nagoya, Aichi 467, †Faculty of Engineering, Mie University, Tsu, Mie 514, and ‡Protein Engineering Research Institute, Suita, Osaka 565, Japan

Four aspartic acid residues (Asp^{126}, Asp^{156}, Asp^{233} and Asp^{295}) of *Bacillus cereus* sphingomyelinase (SMase) in the conservative regions were changed to glycine by *in vitro* mutagenesis, and the mutant SMases [D126G ($Asp^{126} \rightarrow$ Gly etc.), D156G, D233G and D295G] were produced in *Bacillus brevis* 47, a protein-producing strain. The sphingomyelin (SM)-hydrolysing activity of D295G was completely abolished and those of D126G and D156G were reduced by more than 80%, whereas that of D233G was not so profoundly affected. Two mutant enzymes (D126G and D156G) were purified and characterized further. The hydrolytic activities of D126G and D156G toward four phosphocholine-containing substrates with different hydrophobicities, SM, 2-hexadecanoylamino-4-nitrophenylphosphocholine(HNP), lysophosphatidylcholine (lysoPC) and *p*-nitrophenylphosphocholine (*p*-NPPC), were compared with those of the wild-type. The activity of D126G toward water-soluble *p*-NPPC was comparable with that of the wild-type. On the other hand, D156G catalysed the hydrolysis of hydrophilic substrates such as HNP and *p*-NPPC more efficiently (> 4-fold) than the wild-type. These results suggested that Asp^{126} and Asp^{156}, located in the highly conserved region, may well be involved in a substrate recognition process rather than catalytic action. Haemolytic activities of the mutant enzymes were found to be parallel with their SM-hydrolysing activities. Two regions, including the C-terminal region containing Asp^{295}, were found to show considerable sequence identity with the corresponding regions of bovine pancreatic DNase I. Structural predictions indicated structural similarity between SMase and DNase I. An evolutionary relationship based on the catalytic function was suggested between the structures of these two phosphodiesterases.

INTRODUCTION

Sphingomyelinase (sphingomyelin cholinephosphohydrolase, EC 3.1.4.12) is an enzyme which hydrolyses sphingomyelin to ceramide and phosphocholine [1]. We have purified a bacterial sphingomyelinase (SMase) from *Bacillus cereus* to a homogeneous state, cloned the gene and sequenced it [1–3]. We also established a mass-production system of the protein using a protein-hyperproducing strain, *Bacillus brevis* 47 [4].

The enzyme, with a molecular mass of 34 kDa, exhibited unique enzymic and haemolytic activities. The enzyme can adsorb specifically on to the erythrocyte membranes and selectively hydrolyse sphingomyelin in the membrane of intact erythrocytes. Ca^{2+} and Mn^{2+} enhanced the adsorption of the enzyme to the erythrocyte membrane, whereas Mg^{2+} accelerated haemolytic activity together with the breakdown of sphingomyelin [5–8]. Various functional domains seem to exist in the enzyme, such as adsorptive, catalytic and metal-ion-binding sites. Analyses of the secondary structure of the enzyme based on the amino acid sequence and CD suggested that its enzyme molecule was enriched in turn or loop structure, but had relatively lower content of α-helix [9]. Chemical modifications of the enzyme suggested that the acidic amino acid residues such as Asp and Glu are involved in the catalytic and adsorptive activities of the enzyme [1,10].

To elucidate the role(s) of the aspartic acid residues in the enzymic and haemolytic functions, we constructed and expressed four mutant SMases with an Asp → Gly substitution in the conserved regions by site-directed *in vitro* mutagenesis and characterized their catalytic and haemolytic activities.

MATERIALS AND METHODS

Materials

B. brevis 47 was grown and transformed as described in [4]. PX medium used for culture of transformants consisted of 2% polypeptone S, 0.5% yeast extract, 1% glucose and 0.01% uracil. Plasmids pBS(+) and pUC119 were purchased from Stratagene and TAKARA (Kyoto, Japan) respectively. Plasmid pNUSM, an expression vector of SMase, was constructed as described previously [4]. Plasmid pUCSM used for *in vitro* mutagenesis was constructed by introducing a 1.2 kb *Eco*RI–*Xba*I DNA fragment of pNUSM, encoding the entire SMase gene, into pUC119 at the multicloning site. Oligonucleotides were synthesized by a DNA synthesizer (model 391; ABI, Chiba, Japan). The enzymes used for DNA manipulation were purchased from TAKARA. Reagents for PCR and DNA sequencing were purchased from ABI.

In vitro mutagenesis of SMase gene

Mutations were introduced into *B. cereus* SMase gene using PCR-overlap extension method [11] to amplify sequences from

Abbreviations: lysoPC, lysophosphatidylcholine; HNP, 2-hexadecanoylamino-4-nitrophenylphosphocholine; *p*-NPPC, *p*-nitrophenylphosphocholine; PI-PLC, phosphatidylinositol-specific phospholipase C; SM, sphingomyelin; SMase, sphingomyelinase; D126G, $Asp^{126} \rightarrow$ Gly mutation (etc.).

§ To whom correspondence should be sent.

the full-length SMase gene in pUC119. Briefly, each mutant construct was composed of two PCR fragments, joined at the site of the mutation, and together encompassing the 1.2 kb complete SMase coding sequence. Primers for the C-terminal fragment consisted of a primer containing the desired mutation as its central codon and M13-RV primer. Similarly, the N-terminal fragment was flanked by M13-M3 primer and a primer reverse complement of the mutant primer for the C-terminal fragment. The pairs of mutant primers used were as follows (mutation sites to change Asp to Gly were underlined); forward 5′-GGATGC-GGGCCAGGTAATTTATCGAA-3′ and reverse 5′-CGATAA-ATTACCTGGCCCCGAT-3′ for D126G; forward 5′-GCAGG-CTGAAGGTAGTATGTGCGG-3′ and reverse 5′-CGCACAT-ACTACCTTCAGCCTGCA-3′ for D156G; forward 5′-CAGC-GACTTGGGGTGCAACGACAA-3′ and reverse 5′-TTGTCG-TTGCACCCCAAGTCGCTG-3′ for D233G; forward 5′-GAT-TACTCTGGTGATTATCCAGT-3′ and reverse 5′-ACTGGA-TAATCACCAGAGTAATC-3′ for D295G. The N-terminal and C-terminal fragments were purified and used as a template for the PCR to amplify the 1.2 kb entire mutant SMase gene using M3 and RV primers. The final PCR fragments were purified and cut out by *Eco*RI and *Xba*I, and ligated into pBS(+) plasmid DNA. The cloned 1.2 kb DNA fragment of each mutant was sequenced to prove that only the mutation expected had occurred.

Expression of mutant SMases by *Bacillus brevis 47*

The cloned 1.2 kb mutated SMase genes were ligated into pNU211 at *Eco*RI and *Xba*I sites as described [4]. Transformants carrying the mutated SMase gene were screened and isolated. The transformants were cultured in PX medium supplemented with 100 μg/ml erythromycin at 37 °C. After cultivation for 3–4 days, the cells were pelleted by centrifugation at 1000 *g* and the supernatant was used for assay of hydrolysing activities and for purification.

Assays of hydrolysing activities of SMase

The hydrolysing activity of 2-hexadecanoylamino-4-nitrophenylphosphocholine (HNP) or bovine brain sphingomyelin (SM) of the expressed SMases was determined by the method of Gal et al. [12] or Ikezawa et al. [2] respectively. Lysophosphatidylcholine (lysoPC)-hydrolysing activity was determined by the method of Saito et al. [13] with slight modification. The mixture containing 4 mM 1-palmitoyl-*sn*-glycero-3-phosphocholine, 40 mM borate buffer, pH 7.5, 4 mM $MgCl_2$, and 0.02% BSA was incubated with enzyme at 37 °C for 20 min. The reaction was terminated by adding 0.1 ml of 5% BSA, followed by 0.4 ml of 10% perchloric acid on ice. After centrifugation, phosphorus in the supernatant was determined as reported previously [14,15]. The hydrolysis of *p*-NPPC was determined by the method of Kurioka and Matsuda [16]. The reaction mixture (0.5 ml), composed of 20 mM *p*-NPPC, 0.25 M Tris/HCl, pH 7.2, 20 mM $MgCl_2$, 36% sorbitol and enzyme was incubated at 37 °C for 30 min. The reaction was stopped by adding 0.5 ml of 25 mM EDTA and the absorbance at 410 nm was measured. One unit of the activity was defined as the activity that catalysed the hydrolysis of 1 μmol of substrate/min at 37 °C for all the substrates.

Purification of mutant SMases

Purification of SMase from the culture medium was performed by the modified method of Ikezawa et al. [2]. Originally, *B. brevis* 47 does not produce any detectable phospholipases C. Therefore, CM-Sephadex column chromatography was omitted from the original procedure for *B. cereus* which produces three phospholipases C, i.e., phosphatidylinositol-specific phospholipase C (PI-PLC), phosphatidylcholine-specific phospholipase C (PC-PLC) and SMase. Briefly, the culture broth was precipitated with $(NH_2)_4SO_4$ at 80% saturation, followed by gel filtration on Sephadex G-75 and ion- exchange column chromatography on a DEAE-Toyopearl column. Finally, rechromatography on Sephadex G-75 was carried out. The purity of the final preparation was determined by scanning the gel stained with Coomassie Blue after SDS/PAGE.

Determination of haemolytic activity of mutant SMases

Haemolytic activity secreted by the transformants was measured by blood-agar plates as follows; Colonies of *B. brevis* 47 harbouring mutant SMase genes, the wild-type one or the vector were streaked on T2ura plates containing 5%(v/v) fresh sheep erythrocytes. Cells were grown for 18 h at 37 °C, and haemolytic zones appeared after additional incubation of 24 h at 4 °C. Haemolytic activity ('hot–cold') of purified enzymes was determined by incubating 1% (v/v) sheep erythrocytes in 40 mM borate buffer (pH 7.5)/0.75%(w/v) NaCl/4mM $MgCl_2$/0.02% BSA with the enzyme in a volume of 200 μl at 37 °C for 5 min, and then at 4 °C for 30 min. The percentage haemolysis was calculated from the A_{545}. The activity showing 50% hemolysis was defined as one HD_{50} unit.

Other procedures

Analysis of the secondary structures was performed by the method of Nishikawa and Noguchi [17]. The '3D–1D' compatibility test was performed by the methods of Nishikawa and Matsuo [18] and Matsuo and Nishikawa [19,20]. Sequencing was performed by the dideoxynucleotide chain-termination method [21] using dye–dideoxynucleotides and appropriate primers, with an automated sequencer (ABI, model 373A). SDS/PAGE was performed by the method of Laemmli [22].

RESULTS

Construction and expression of Asp mutants of *B. cereus* SMase

Since chemical modification of *B. cereus* SMase by Woodward's reagent K reduced the catalytic and adsorptive activities [10], acidic amino acids such as aspartic and/or glutamic acids have been supposed to be involved in the action of the enzyme. As a first step to elucidate the roles of these amino acids in the enzymic functions, we decided to change Asp residues to a neutral amino acid, glycine, by site-directed mutagenesis.

Out of ten conserved Asp residues among SMases of three different bacterial species, we have selected four residues (Asp^{126}, Asp^{156}, Asp^{233} and Asp^{295}; numbered from the N-terminal amino acid of the mature enzyme) in the well-conserved regions of SMases as shown in Figure 1. By PCR-directed *in vitro* mutagenesis we constructed four mutant SMase genes and expressed them using *B. brevis* 47 as described previously [4]. Table 1 shows the SM- and HNP-hydrolysing activities secreted into the broths by the transformants during 4 days' cultivation. All the SM-hydrolysing activities of the mutants were reduced; especially, there was no detectable activity at all in the culture broth of the D295G ($Asp^{295} \rightarrow Gly$) transformant. Remarkably, the D156G-transformant expressed higher hydrolytic activity against HNP than the wild-type transformant, whereas the others exhibited activities more or less comparable with their SM-hydrolysing activities. As shown in Figure 2, analysis by

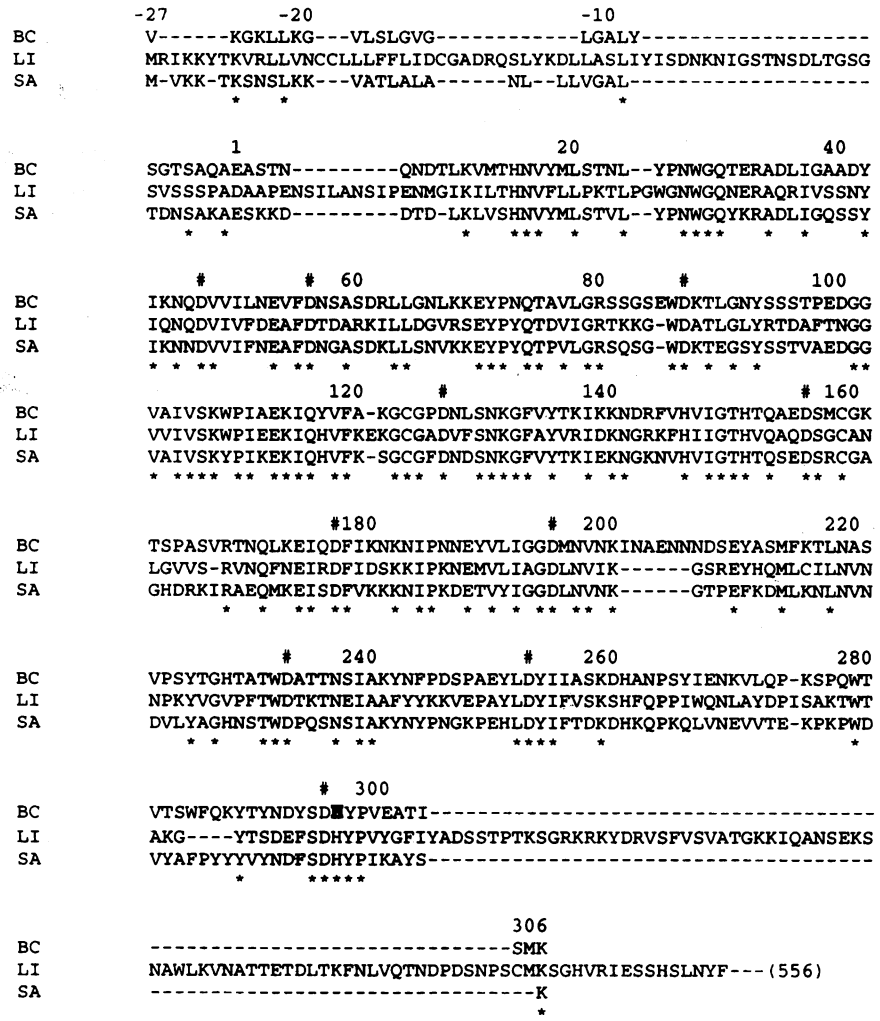

Figure 1 Comparison of amino acid sequences of SMases of three species

Amino acid sequences of SMases of *B. cereus* [3], *Leptospira interrogans* [34] and *Staphylococcus aureus* [35] were aligned by GENETYX-Mac software. Residues are numbered by taking the N-terminal residue of mature *B. cereus* SMase as 1 [3]. Identical residues are marked with an asterisk, and the conserved Asp residues are marked by #. Strokes indicate gaps introduced into the sequences for the alignment purpose. The outlined His^{296} in the sequence of *B. cereus* SMase is corrected for Asp^{296} reported previously [3], because of a sequencing error.

Table 1 HNP- and SM-hydrolytic activities secreted into the culture broths by the transformants carrying the mutant SMase gene

After 4 days cultivation, cells were pelleted by centrifugation and the activities in the supernatants were determined as described in the Materials and methods section. The values are averages for duplicate assays. The deviations were less than 5% of the each value.

	HNP-hydrolysing activity		SM-hydrolysing activity	
Transformant	(munits/μl of broth)	(%)	(munits/μl of broth)	(%)
Wild-type	0.060	100	6.00	100
D126G	0.007	11.7	0.09	1.6
D156G	0.138	230	0.39	6.3
D233G	0.014	23.3	0.89	14.8
D295G	0	0	0	0
pNU211	0	0	0	0

SDS/PAGE of the culture broths revealed that the amounts of SMases produced varied with the transformants; the amounts of SMases produced by the D233G and D295G transformants were approximately one-third that by the wild-type, estimated from the band intensities obtained by scanning the gel. This low production may be responsible for the low activity in the broth of the D233G transformant. However, this was not the case for the D295G SMase, since no activity was detected, even in the assay using a five-times-greater amount of the culture broth of the D295G-transformant (result not shown).

Characterization of the purified D126G and D156G mutant SMases

Mutation at Asp^{126} or Asp^{156} to Gly of *B. cereus* SMase reduced the SM-hydrolysing activity of the enzyme as described above, and these residues were located in the highly conserved region surrounded by the two cysteine residues (Cys^{123} and Cys^{159}) as shown in Figure 1. Previously, we have demonstrated that the disulphide bond formed by these two cysteine residues was essential for the catalytic activity [10]. So we decided to purify and characterize these two mutant SMases further.

We purified the D126G and D156G to a nearly homogeneous state ($\geq 90\%$) as well as the wild-type, and determined their hydrolytic activities toward four phosphocholine-containing sub-

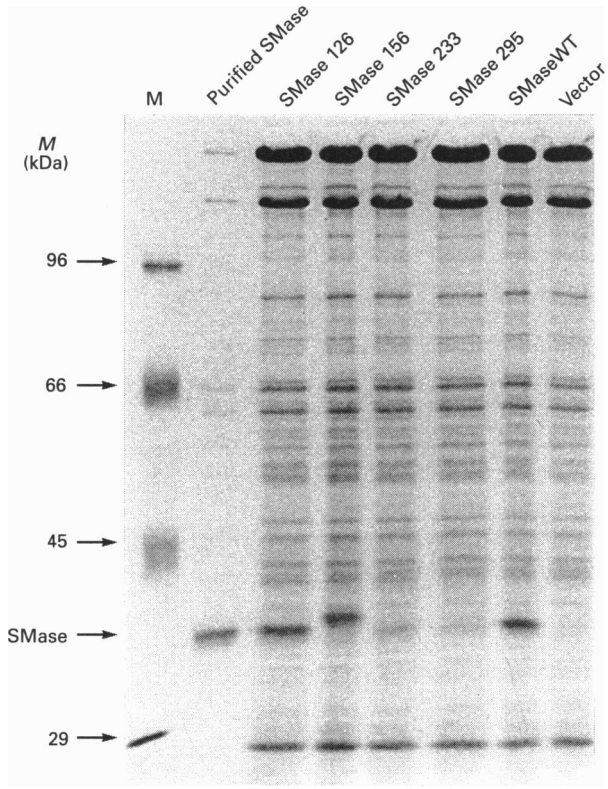

Figure 2 SDS/PAGE of the culture broths of the transformants

An aliquot (10 μl) of each culture broth was analysed. As a control, the purified enzyme (2 μg) from *B. cereus* was electrophoresed and indicated by '*SMase*'. M, molecular-mass (*M*) markers. Arrows indicate the positions of marker proteins with their sizes in kDa.

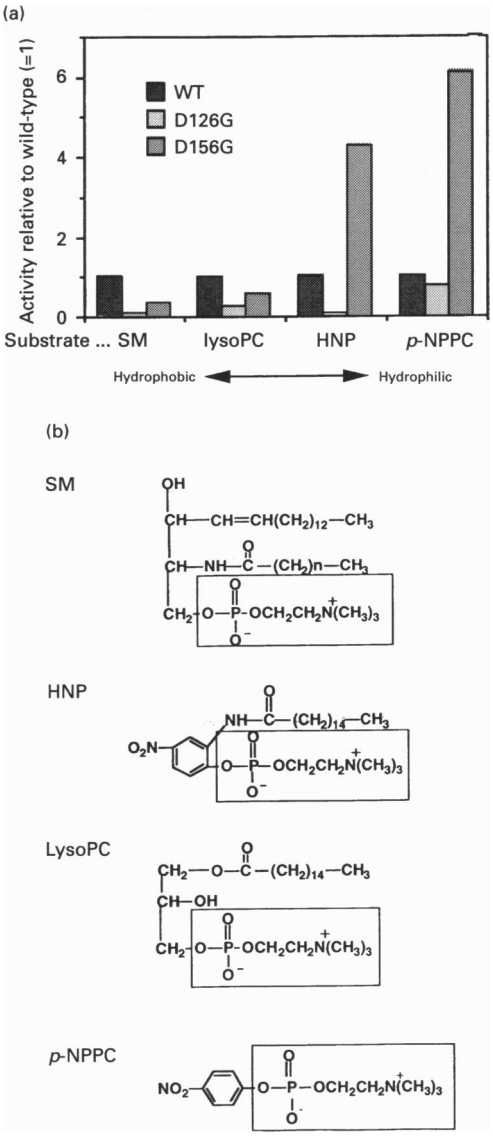

Figure 3 Changes in the relative activities of the mutant SMases (D126G and D156G) compared with the wild-type SMase with respect to four different substrates

(**a**) Relative activities for four phosphocholine-containing substrates (see **b**) of the mutant enzymes were calculated by taking the activity of the wild-type as unity. The order of the hydrophobicity was SM > lysoPC > HNP > *p*-NPPC as shown under the abscissa with the double-headed arrow. (**b**) Structures of the four substrates. The common phosphocholine moiety is boxed.

strates with different hydrophobicity (SM, HNP, lysoPC and *p*-NPPC; see Figure 3), in comparison with the purified, wild-type enzyme (Table 2). The preference of the wild-type enzyme toward these substrates were previously determined as SM > lysoPC $\simeq$ HNP > *p*-NPPC [5]. D126G exhibited the reduced activities toward all of these substrates, whereas D156G catalysed hydrolysis of the water-soluble substrates (HNP and *p*-NPPC) more effectively than the wild-type (Table 2 and Figure 3). Especially for *p*-NPPC, the relative activity of D156G to wild-type enzyme, 6.14, was 18 times as much as that for SM, 0.35 (Figure 3). Reduction of the activities toward lysoPC and *p*-NPPC of D126G was moderate while the activities toward SM and HNP were less than 10% of the wild-type activities.

The hydrolytic activity of the wild-type SMase is activated by Mg^{2+} and inhibited by Ca^{2+} as described in [7]. The effects of these bivalent cations on the hydrolytic activities of the purified D126G and D156G SMases were examined and found to be essentially the same as those on the wild-type (results not shown).

Haemolytic activity of the Asp mutant SMases

It has been shown that the hot–cold haemolysis of bovine erythrocytes induced by *B. cereus* SMase is closely related to the breakdown of SM on the erythrocytes by the enzyme [6]. To elucidate further the relationship between haemolysis and SM hydrolysis of erythrocytes, the haemolytic activity of the cells secreting mutant enzymes with reduced SM-hydrolysing activity were determined by blood-agar plate. As shown in Figure 4(a), all the transformants secreting mutant SMases displayed the lowered or no haemolytic activities toward sheep erythrocytes. Especially cells producing the D295G SMase were completely inactive.

The haemolytic activities of the purified mutant SMases (D126G and D156G) were also analysed quantitatively as described in the Materials and methods section (Figure 4b). The haemolytic unit (HD_{50}) was calculated as follows; 588 HD_{50}/mg protein for the wild-type and 233 HD_{50}/mg of protein for the D156G mutant. The activity of the D126G was too low to estimate the amount for 50% haemolysis. The ratio of the haemolytic activities of D156G to wild-type (233/588 = 0.40)

Table 2 Hydrolytic activities of the purified D126G, D156G and wild-type SMases toward four different substrates, SM, HNP, lysoPC and *p*-NPPC

The enzyme activities toward four different substrates were determined as described in the Materials and methods section. Values are averages for duplicate assays. The deviations were less than 5% of the each value.

		Activity [units (μmol of substrate hydrolysed/min)]			
Enzyme	Substrate ...	SM	HNP	lysoPC	*p*-NPPC
Wild-type		125	1.61	0.738	0.056
D126G		9.8	0.12	0.201	0.043
D156G		44.0	6.96	0.448	0.346

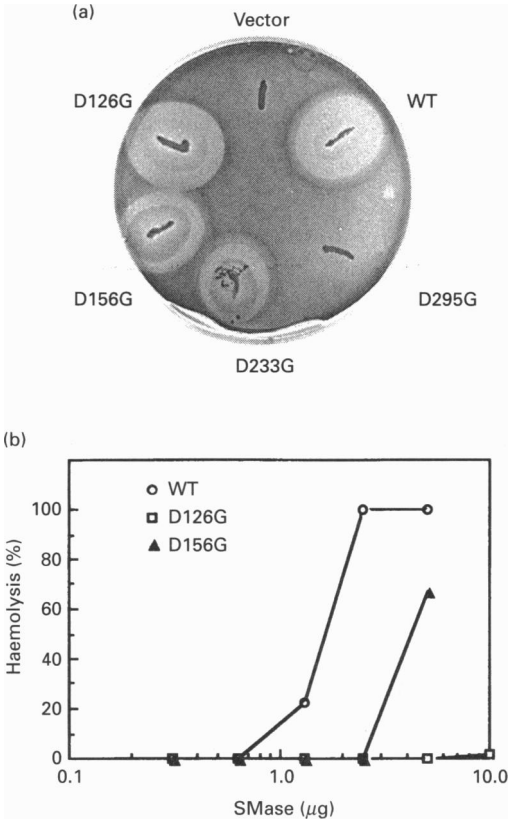

Figure 4 Haemolytic activities of the mutant SMases

(**a**) Haemolytic activities of the mutant SMases were measured by streaking the transformants on an agar plate containing 5% sheep erythrocytes. Haemolytic zones were revealed after incubation at 37 °C for 2 days and the plates then cooled at 4 °C overnight. (**b**) Haemolytic activities of the purified mutant SMases were quantitatively determined as described in the Materials and methods section. The values are the averages for duplicate assays. From the dose giving 50% haemolysis, the HD_{50} was calculated. Abbreviation: WT, wild-type.

was comparable with that for the SM-hydrolysing activities (44/125 = 0.35 obtained from Table 2).

Sequence similarity of *B. cereus* SMase to bovine pancreatic DNase I

Recent sequence analysis of cDNA for *B. cereus* SMase revealed that Asp^{296} in the amino acid sequence of this enzyme [3] should be corrected to His^{296}, as shown in Figures 1 and 5. With this corrected sequence, a 'homology' search revealed that there were at least two regions of sequence identity between *B. cereus* SMase and bovine DNase I, as shown in Figure 5(a). The one was an 11-amino-acid stretch (from Tyr^{293} to Ile^{303}) of *B. cereus* SMase containing Asp^{295} residue. This region shows sequence identity with the C-terminal region of bovine pancreatic DNase I (from Ile^{246} to Leu^{256}, numbered as in [23]). The other was a longer stretch, from Ile^{43} to Asn^{73}, of SMase showing sequence identity with the region from Val^{27b} to Asn^{58} of DNase I. More than half the identical residues in the two regions of identity were also identical among three bacterial SMases (Figure 5a). Although the overall sequence similarity was not so high (~ 20%, calculated by GENETYX-Mac software), several identical residues have been demonstrated to be involved in the catalytic centre, as shown in Figure 5(b). Analysis by the method of Nishikawa and Noguchi [17] predicted the significant similarity of the secondary structures of the two proteins over the entire sequences (Figure 6).

3D-1D compatibility analysis of *B. cereus* SMase

The SMase sequence was compared with 325 known structures taken from the Protein Data Bank (PDB [26]) using the 3D–1D compatibility method of Nishikawa and Matsuo [18–20]. Of the structures, the bovine pancreatic DNase I structure (PDB code, 1ATND) showed the best compatibility score ($S_{tot.}$, −2.56), as shown in Table 3. This suggests a structural similarity between SMase and DNase I.

DISCUSSION

We constructed and expressed four mutants with substitution at an aspartic acid in the well-conserved regions to glycine (D126G, D156G, D233G and D295G) and found that all mutants exhibited reduced or no SM-hydrolysing and haemolytic activities (Table 1 and Figure 4a).

Mutations at Asp^{126} and Asp^{156} in the stretch of the most conserved region among SMases of three different bacterial species impaired profoundly the SM-hydrolysing activity of *B. cereus* SMase. We have already demonstrated that disruption of the disulfide bridge between Cys^{123} and Cys^{159} inactivated the catalytic activity of the enzyme [10]. These results indicated that this conserved region plays a critical role(s) in the hydrolysis of SM.

The substitution of Asp^{126} with Gly more profoundly reduced the activities toward SM and HNP than those toward lysoPC and *p*-NPPC (Table 2 and Figure 3). The presence or absence of *N*-acyl moiety of the substrates might be a determinant for the difference in the activities of D126G. The mutation Asp^{156} → Gly

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(a)
(1)  SMase     43 IKNQDVVILNEVFDNS--ASDRLLGNLKKEYPN 73
                  ... *.*...** *.    * ..**. *... **
     DNaseI   27b VRRYDIVLIQEVRDSHLVAVGKLLDYLNQDDPN 58

(2)  SMase    293 YSDHYPVEATISMK 306(COOH)
                  .*******.*.
     DNaseI   246 ISDHYPVEVTLT   257(COOH)
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(b)
SMase  1 EASTNQNDTL KVMTHNVYML STNLYPNWGQ TERADLIGAA DYI-KNQDVV ILNEVFD--N
                  * *    *          *       * *       *    * *    ** *
DNaseI 1           L KIAAFNIRTF GESKMSN--- ---ATLASYI VRIVRRYDIV LIQEVRDSHL
                                                    (1)------------------

   S  58 SASDRLLGNL RKEYPNQTAV LGRSSGSEWD KTLGNYSSST PEDGGVAIVI KWPIAEKIQY
          *   **  *     ** *      *        **   *                *       **
   D  43 VAVGKLLDYL NQDDPN-TYH YVVS------ EPLG-RNSYK ERYLFLFRPN KVSVLDTYQY
         ----------------->

   S 118 -VFAKGCGPD NLSNKGFVYT KIKKNDRFVH VIGTHLQAED SMCGKTSPAS VRTNQLKEIQ
               ** *   *    *     *        *      *   *     * *     * *
   D  95 DDGCESCGND SFSREPAV-- -VKFSSHSTK VKEFAIVALH S---APSDAV AEINSLYDVY


   S 177 DFIKNKNIPN NEYVLIGGDM NVNKINAENN NDSEYASMFK TLNASVPSYT GHTATWDATT
             *   *    *   **       **                 * *           * *
   D 149 LDVQQKWHLN --DVMLMGD- ----FNADCS YVTSSQWSSI RLRTSSTFQW LIPDSADTTA


   S 237 NSIAKYNFPD SPAEYLDYII ASKDHANPSY IENKVLQPKS PQWTVTSWFQ KYTYNDYSDH
          *           * *          * *                                ***
   D 202 TSTNCAYDRI VVAGSLLQSS VVPGSAAP-- ---------- FDFQAAYGLS NEMALAISDH
                                                                  (2)----

   S 297 YPVEATISMK
         **** *
   D 250 YPVEVTLT
         ------->
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Figure 5 Sequence identity between *B. cereus* SMase and bovine pancreatic DNase I

(**a**) Two highly similar regions are shown. Identical residues are marked by an asterisk, and conserved mutations are marked with a dot. Strokes indicate a gap introduced into the sequence for alignment purpose. Conserved amino acids among SMases of three species are underlined. The numbering is taken from [3] and [23]. (B) Alignment of SMase and DNase I along the entire sequences. Identical residues are indicated by an asterisk. Among identical residues, residues considered to be involved in the active centre of DNase I are underlined. Broken lines under the sequences indicate two highly similar regions as shown in (**a**). Numbering is taken from [3] and [23].

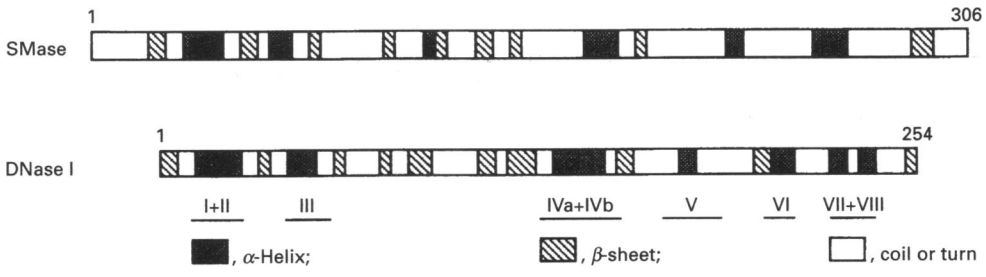

Figure 6 Similarity of the predicted secondary structures of SMase and DNase I

The structural segments of both enzymes were schematically represented according to a prediction based on the method of Nishikawa and Noguchi [17]. Stippled and hatched boxes represent α-helix and β-sheet respectively. Open boxes indicate coil or turn structures. Bars with roman numbers denote the numbers and regions of the α-helixes that were predicted by X-ray crystallography [23].

Table 3 Compatibility of the *B. cereus* sphingomyelinase sequence with known structures

The *B. cereus* SMase sequence was compared with 325 known structures from the Protein Data Bank [26], using the 3D–1D compatibility method developed by Nishikawa and Matsuo [18–20]. The structures were sorted in order of their compatibility scores, $S_{tot.}$. The best 20 structures are listed. The auxiliary scores, $S^{tot.}_{res.}$, which assess the compatibility per residue, are also shown. The detail of the method for calculating the scores, $S_{tot.}$ and $S^{tot.}_{res.}$ were described by Matsuo and Nishikawa [22].

Rank	Structure	PDB*	% id†	$S_{tot.}$	$S^{tot.}_{res.}$
1	Deoxyribonuclease	1ATND	9.7	−2.56	−2.40
2	Bean-pod-mottle-virus capsid protein	1BMV2	9.1	−2.27	−1.06
3	Xylose isomerase	4XIS	6.9	−2.27	−1.08
4	Phthalate dioxygenase reductase	2PIA	8.2	−2.25	−1.12
5	Ovalbumin	1OVAA	9.6	−2.03	−0.74
6	DG3PH‡	1GD1O	11.0	−2.03	−1.13
7	RuBisCO§	5RUBA	9.1	−2.01	−0.80
8	Aldose reductase	1ADS	7.2	−2.00	−0.99
9	Triosephosphate isomerase	5TIMA	11.7	−1.90	−1.63
10	Narbonin	1NAR	10.1	−1.89	−1.02
11	recA protein	2REB	6.1	−1.83	−0.88
12	Methylamine dehydrogenase HC	2BBKH	6.4	−1.83	−0.51
13	Acid α-amylase	2AAA	7.8	−1.78	−0.65
14	Endothiapepsin	2ER7E	9.3	−1.75	−0.53
15	Nitrogenase iron protein	1NIPA	8.2	−1.72	−0.88
16	3-Isopropylmalate dehydrogenase	1IPD	11.0	−1.70	−0.55
17	Tomato-bushy-stunt-virus coat protein	2TBVC	9.3	−1.70	−0.54
18	L-Arabinose-binding protein	8ABP	8.5	−1.69	−0.80
19	Dihydrolipoamide dehydrogenase	3LADA	9.8	−1.64	−0.43
20	Galactose oxidase	1GOF	8.7	−1.60	−0.42

* Protein Data Bank code.
† Percentage sequence similarity to the SMase sequence.
‡ DG3PH, d-glyceraldehyde-3-phosphate dehydrogenase.
§ RuBisCO, ribulose bisphosphate carboxylase.
HC, heavy chain.

resulted in more-than-4-fold enhancement in the activity toward the water-soluble synthetic substrates, HNP and *p*-NPPC, whereas reduction was observed in the SM- and lysoPC-hydrolysis, as shown in Figure 3. These results suggested that Asp^{126} and Asp^{156} of SMase might be involved in the substrate-recognition process rather than in the active centre. Further analyses using substrates with different structures will enable one to clarify this point.

Since there were no significant differences in the effects of Mg^{2+} and Ca^{2+} cations on the hydrolytic activities of the mutants (D126G and D156G) from that of the wild-type (results not shown), both Asp^{126} and Asp^{156} of SMase seemed not to be involved in the metal-binding sites.

The hot–cold haemolytic activities displayed by the mutant enzymes were almost parallel with their SM-hydrolysing activities (Figure 4), showing that hydrolysis of SM in the erythrocyte membrane is one of the rate-limiting steps of haemolysis as previously suggested by Tomita et al. [6]. To clarify the mechanism of haemolysis in detail, the adsorptive activity toward erythrocytes of the mutant enzymes should be further determined.

Mutation at Asp^{295} completely disrupted the hydrolytic and haemolytic activities of *B. cereus* SMase (Table 1 and Figure 4). This strongly suggests that Asp^{295} is one of the target residues modified by Woodward's reagent K [10]. The region containing Asp^{295} was found to have identity with the C-terminal region of bovine DNase I, as shown in Figure 5(a). Since the residues Asp^{248} and His^{249} of DNase I in this region of identity have been identified to be involved in the catalytic centre by X-ray-crystallographic analysis [23–25], the sequence Asp^{295}-His^{296} of SMase corresponding to Asp^{248}-His^{249} of DNase I may well be involved in the active centre of SMase. Lack of the hydrolytic activity of D295G supported this hypothesis.

Another region of identity between the two enzymes was located close to the N-termini of both enzymes. More than half the identical residues in this region were conserved among SMases of three species (Figure 5a). Therefore this region might also play (an) important role(s) in the hydrolysis of SM. The acidic amino acid residues in this region of identity (Asp^{47}, Glu^{53} and Asp^{56}) of SMase might be responsible for the inactivation by Woodward's reagent K.

Comparative analyses of the secondary and tertiary structures between the two enzymes predicted a structural similarity between them (Figure 6 and Table 3). The method used to predict the secondary structures seemed to be reliable, since the prediction for DNase I was consistent with the results obtained from the X-ray-crystallographic analysis, as shown in Figure 6. The 3D-1D compatibility test recently developed by Matsuo and Nishikawa [18–20] is a method for evaluation of the compatibility of a sequence with a 3D structure. Using this method, one can identify the most likely structure of a protein from a library of known structures. By this test the structure of DNase I was the most compatible with that of SMase among 325 known structures (Table 3). Both enzymes catalyse the hydrolysis of phosphodiester bonds, and several functionally important residues are conserved (Figure 5b). All these results suggested a distant evolutionary relationship between SMase and DNase I, although they are different in substrate specificity and obtained from phylogenically distant sources.

Clustering of PC-PLC and SMase genes in the *B. cereus* genome suggests that these two genes diversed from an ancestor

gene, although no apparent sequence identity was observed [3,27,28]. A possible, tempting scenario explaining the structural similarity between SMase and DNase I is that there is a protogene encoding a proto-enzyme which catalysed the hydrolysis of phosphodiester bond, and the two genes for SMase and DNase I had been evolved from that protogene. Volbeda et al. also reported the structural similarity between the enzymes related to hydrolysis of phospholipids and nucleic acids: the PC-PLC of *B. cereus* and the P1 nuclease of *Penicillium citrinum* [29]. Several reports demonstrated that the distant relationship between enzymes had been clarified after the determination of their structures [29–33]. Further structural and functional analysis of SMase would answer this question.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan. We are grateful to Dr. R. Taguchi and Ms. T. Kobayashi for their critical comments.

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Received 3 January 1994/7 March 1995; accepted 15 March 1995