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Studies on the active sites of *Bacillus cereus* sphingomyelinase substitution of some amino acids by site-directed mutagenesis

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Summary. Chemical modifications suggested that acidic amino acids such as aspartic and glutamic acids are involved in the active sites of *Bacillus cereus* sphingomyelinase. Among aspartic acid residues in the conserved regions of this enzyme, Asp-126, Asp-156, Asp-233 and Asp-295 were converted to glycine by site-directed mutagenesis. According to prediction on structural similarity to pancreatic DNase I, His-151 and His-296 were also converted to alanine. The Asp and His mutants, D126G, D156G, D233G, D295G, H151A and H296A, were produced in *Bacillus brevis* 47, a protein-hyperproducing strain. The catalytic activities of D295G, H151A and H296A were completely abolished, and sphingomyelin-hydrolyzing activity of D126G or D156G was reduced by more than 50%. The activity of D126G toward *p*-NPPC was comparable to that of the wild-type, while D156G catalyzed the hydrolysis of HNP and *p*-NPPC more efficiently than the wild-type. Hemolytic activities of the mutants were parallel to their sphingomyelin-hydrolyzing activities.

Keywords: Amino acids – *Bacillus cereus* – Sphingomyelinase – Pancreatic DNase I – Replacement of alanine – Replacement of histidine

Introduction

Sphingomyelinase is a specific phospholipase C which hydrolyzes sphingomyelin to produce ceramide and phosphorylcholine (Ikezawa et al., 1978). We have already isolated *Bacillus cereus* sphingomyelinase (Tomita et al., 1982), cloned its gene and deduced its amino acid sequence from the nucleotide sequence of the gene (Yamada et al., 1988). Apparently, the gene of this enzyme encodes the sequence of 333 amino acid residues. Also, *B. cereus* sphingomyelinase has been shown to be secreted into the culture fluid as a mature form. Since N-terminal signal peptide of the enzyme consists of 27 amino acid residues, the mature form of enzyme is composed of 306 amino acid residues, having the molecular weight of 34 Kd (Yamada et al., 1988). In

addition to these findings, we established a hyperproduction system of the *B. cereus* enzyme by use of *Bacillus brevis* 47 (Tamura et al., 1992).

B. cereus sphingomyelinase has unique enzymatic properties, accompanied by hemolytic activity toward mammalian erythrocytes (Ikezawa et al., 1980; Tomita et al., 1983). In the presence of Ca^{2+} or Mn^{2+}, the enzyme is able to adsorb specifically onto the erythrocyte membrane, while Mg^{2+}, Co^{2+} and Mn^{2+} accelerate hemolytic activity of the enzyme with the enhanced breakdown of sphingomyelin (Tomita et al., 1983; Ikezawa et al., 1986). Therefore, various functional domains such as adsorptive, catalytic and metal ion-binding sites might well exist in the molecule of this enzyme.

Chemical modifications suggested that the acidic amino acids such as aspartic and glutamic acids are involved in the catalytic and adsorptive activities of the enzyme (Tomita et al., 1993). In order to elucidate the role of aspartic acid residues in the enzymatic and hemolytic functions, Asp-126, Asp-156, Asp-233 and Asp-295 in the conserved regions of the enzyme were converted to glycine by site-directed mutagenesis. On the one hand, two histidines corresponding to those in the active site of pancreatic DNase I, were converted to alanine, according to the prediction on structural similarity between *B. cereus* sphingomyelinase and DNase I. In the present study, four Asp and two His mutants, D126G, D156G, D233G, D295G, H151A and H296A were produced in *Bacillus brevis* 47, a protein-hyperproducing strain, and determined for their catalytic and hemolytic activities.

Materials and methods

Plasmid pNUSM, an expression vector of *B. cereus* sphingomyelinase, was constructed by use of pNU211, an expression vector of *B. brevis*, as described previously (Tamura et al., 1992). Single amino acid mutations (Asp → Gly, His → Ala) were introduced into the sphingomyelinase gene by PCR-overlap extension procedures (Maruta et al., 1991) to amplify sequences from the full length of the gene in pUC119. The final PCR products were purified and cut by EcoRI and XbaI, and ligated into pBS(+) plasmid DNA. The 1.2 kb mutant sphingomyelinase gene thus cloned were introduced into pNU211 at EcoRI and XbaI sites as described previously (Tamura et al., 1992). Transformants carrying the mutant genes were isolated after screening. Thus *B. brevis* 47 transformed by wild and mutant pNUSMs were cultured in PX medium supplemented with 10 μg/ml erythromycin at 37°C for 3–4 days. After removal of the cells by centrifugation at 1000 × g, the resulting supernatants were subjected to the assays of hydrolyzing activities and to further purification of wild and mutant enzymes.

The hydrolyzing activities of the expressed sphingomyelinases toward 2-hexadecanoylamino-4-nitrophenylphosphorylcholine (HNP) or bovine brain sphingomyelin was determined as reported previously (Tomita et al., 1982). Lysophosphatidylcholine (lysoPC)-hydrolyzing activity was determined by the method of Saito et al. (1991) with slight modifications, using 4 mM 1-palmitoyl-*sn*-glycero-3-phosphorylcholine as the substrate. The activity toward *p*-nitrophenylphosphorylcholine (*p*-NPPC) was determined according to the method of Kurioka and Matsuda (1976).

Purification of wild and mutant sphingomyelinases from the culture medium of *B. brevis* was carried out according to the modified method of Ikezawa et al. (1978), by precipitation with $(NH_4)_2SO_4$ at 80% saturation, gel filtration on Sephadex G-75, DEAE-Toyopearl column chromatography and re-gel filtration on Sephadex G-75. Hemolytic activity of purified enzymes was determined by measuring hot-cold hemolysis of sheep erythrocytes, according to the modified method of Tomita et al. (1983).

Results

Ten Asp residues proved to be conserved in the amino acid sequence of sphingomyelinase from *B. cereus*, when compared with those from *Staphylococcus aureus* (Projan et al., 1987) and *Listeria interrogans* (Segers et al., 1990). Among these residues, Asp-126, Asp-156, Asp-233 and Asp-295 were selectively converted to glycine by site-directed mutagenesis. Figure 1 shows the secreted sphingomyelinase activity toward sphingomyelin or HNP, observed after 4 days' cultivation. As shown in Fig. 1A, sphingomyelin-hydrolyzing activity was reduced in all the mutants, as compared with wild sphingomyelinase. Especially in the culture broth of D295G mutant, sphingomyelin-hydrolyzing activity was completely abolished, although 34 Kd protein corresponding to sphingomyelinase was produced in the yield one-third as much as the wild enzyme (data not shown). Although sphingomyelin-hydrolyzing activity was exceedingly low in the culture broth of D126G, the amount of 34 Kd protein produced in D126G culture was almost comparable to that in the culture of wild enzyme (data not shown). As shown in Fig. 1B, it should be noted that D156G mutant expressed higher HNP-hydrolyzing activity than the wild enzyme.

Both Asp-126 and Asp-156 were located in the highly conserved region around a sole disulfide bond between Cys-123 and Cys-159 in the molecule of sphingomyelinase. Therefore, we purified the D126G, D156G and the wild-type enzymes from the culture broths of *B. brevis* 47, in a purity not less than 90%. Then, we determined their hydrolyzing activities toward four substrates such as sphingomyelin, HNP, lysoPC and *p*-NPPC (Fig. 2). Sphingomyelin-hydrolyzing activity of D126G or D156G became much lower than 50% that of wild enzyme (Fig. 2A), while the activity of D126G toward *p*-NPPC was comparable to that of the wild type (Fig. 2D). Interestingly, D156G catalyzed the hydrolysis of hydrophilic substrates such as HNP and *p*-NPPC at much higher rate than the wild enzyme (Fig. 2B and 2D). LysoPC-hydrolyzing

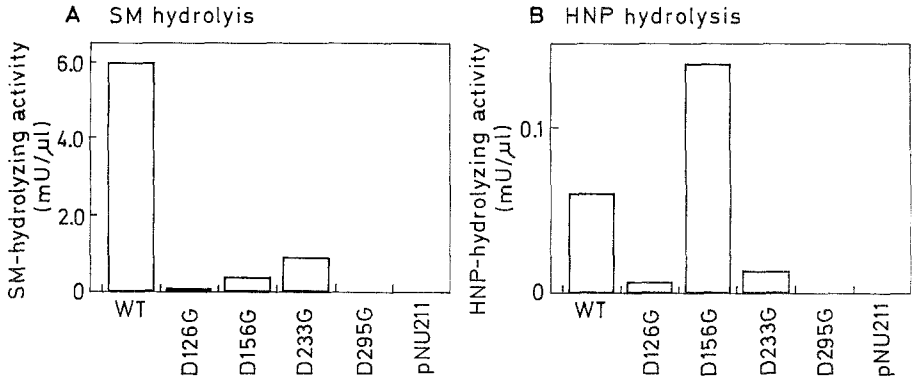

Fig. 1. Hydrolytic activities toward sphingomyelin (*SM*) (**A**) and HNP (**B**) secreted into the culture broths by the transformants carrying the mutant sphingomyelinase gene. After 4 days cultivation, cells were pelleted by centrifugation and the activities in the supernatants were determined, and expressed as mU/μl culture broth. The values were the average of duplicates

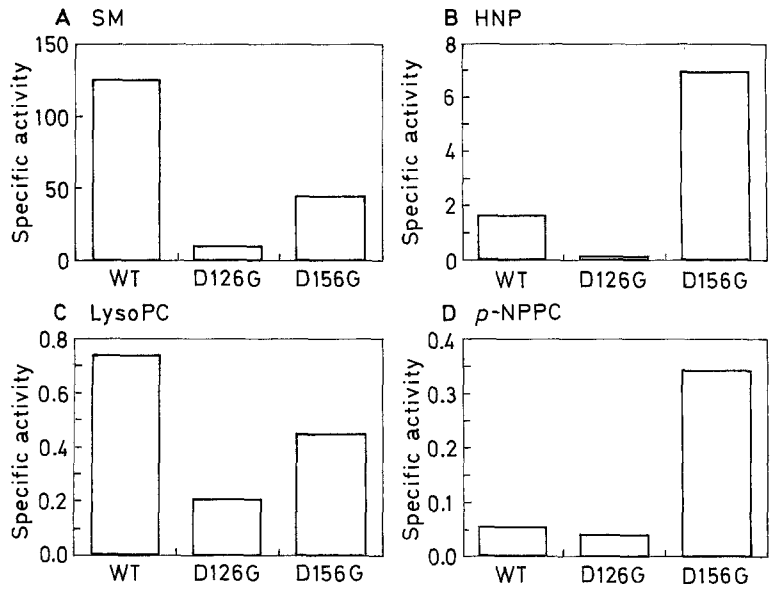

Fig. 2. The hydrolytic activities of the purified mutant (D126G and D156G) and the wild-type sphingomyelinases toward 4 different substrates, **A** sphingomyelin, **B** HNP, **C** lysoPC and **D** *p*-NPPC. Purified enzymes were determined for their activities toward 4 different substrates. Specific activity of sphingomyelinase for each substrate was expressed in term of the activity that degrades 1 μmole substrate/min/mg protein at 37°C. Values were the averages of duplicates

activity of D126G or D156G was lower than that of wild enzyme (Fig. 2C). Hemolytic activity of the purified D126G or D156G proved to be lowered in parallel with sphingomyelin-hydrolyzing activity, when compared with wild enzyme (data not shown).

Our recent studies revealed that there was at least 20% homology with two homologous regions between the amino acid sequences of *B. cereus* sphingomyelinase and bovine pancreatic DNase I. On the basis of sequence homology to DNase I with concomitant structural similarity, we replaced His-151 or His-296 of sphingomyelinase with alanine, respectively. The resulting mutants, H151A and H296A, proved to be completely inactive toward sphingomyelin.

Discussion

In the present study, four Asp and two His mutants of *B. cereus* sphingomyelinase were constructed and expressed, using a protein-hyperproducing strain, *B. brevis* 47. All the mutants partially or completely lost sphingomyelin-hydrolyzing activity, suggesting involvement of these Asp and His residues in the catalytic function or the recognition of substrate. Especially, the enzyme activity of H151A, D295G or H296A was completely abolished by the replacement. Both sphingomyelinase and DNase I share the highly homologous COOH-terminals containing the sequence SDHYPVE, in

which both Asp-295 and His-296 were involved. Since these two enzymes are known to be the typical phosphodiesterases which catalyze the hydrolysis of C-O-P-O-C bonds, these Asp and His residues must be directly responsible for the catalytic function of sphingomyelinase. Also, replacement of Asp-126 or Asp 156 with glycine resulted in the decrease in the activities toward amphiphilic substrates such as sphingomyelin and lysoPC, but was without serious effect on, or rather stimulative for, the activities toward hydrophilic substrates such as HNP and *p*NPPC. Thus these two Asp residues might well be related to the recognition site for the structure of substrate. Considering these two Asp residues to be proximate to the disulfide bond Cys-123-Cys-159, some conformational change might be brought about in the secondary structure of sphingomyelinase by replacement of aspartic acid with glycine. Probably, removal of carboxymethyl side chain of aspartic acid by this replacement, inevitably increased flexibility of the partial structure around disulfide bridge and enabled the hydrophilic substrates to approach the catalytic site more easily.

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