Arch Pharm Res Vol 28, No 8, 923-929, 2005

Archives of Pharmacal Research
http://apr.psk.or.kr

Identification of Three Competitive Inhibitors for Membrane-Associated, Mg^{2+}-Dependent and Neutral 60 kDa Sphingomyelinase Activity

Seok Kyun Kim, Sang Mi Jung, Kyong Hoon Ahn, Hyung Jun Jeon, Dong Hun Lee, Kwang Mook Jung, Sung Yun Jung, and Dae Kyong Kim

Department of Environmental & Health Chemistry, College of Pharmacy, Chung-Ang University, Dongjak-Ku, Seoul 156-756, Korea

(Received April 7, 2005)

Methanol extracts of domestic plants of Korea were evaluated as a potential inhibitor of neutral pH optimum and membrane-associated 60 kDa sphingomyelinase (*N*-SMase) activity. In this study, we partially purified *N*-SMase from bovine brain membranes using ammonium sulfate. It was purified approximately 163-fold by the sequential use of DE52, Butyl-Toyopearl, DEAE-Cellulose, and Phenyl-5PW column chromatographies. The purified *N*-SMase activity was assayed in the presence of the plant extracts of three hundreds species. Based on the *in vitro* assay, three plant extracts significantly inhibited the *N*-SMase activity in a time- and concentration-dependent manner. To further examine the inhibitory pattern, a Dixon plot was constructed for each of the plant extracts. The extracts of *Abies nephrolepis*, *Acer tegmentosum*, and *Ginkgo biloba* revealed a competitive inhibition with the inhibition constant (*Ki*) of 11.9 μg/mL, 9.4 μg/mL, and 12.9 μg/mL, respectively. These extracts also inhibited in a dose-dependent manner the production of ceramide induced by serum deprivation in human neuroblastoma cell line SH-SY5Y.

Key words : Neutral sphingomyelinase, *Abies nephrolepis*, *Acer tegmentosum*, *Ginkgo biloba*, Sphingomyelinase inhibitor

INTRODUCTION

Sphingomyelin (SM) hydrolysis in mammalian cells occurs *via* the action of SMase; SM-specific forms of phospholipase C, which hydrolyzes the phosphodiester bond of SM, yielding ceramide and phosphocholine. Ceramide is now recognized as an important signaling mediator in cells. It has been proposed that ceramide, the backbone of various sphingolipids, may play a crucial role in cell responses such as cell differentiation (Okazaki *et al.*, 1990), cell cycle arrest (Jayadev *et al.*, 1995), cellular senescence (Venable *et al.*, 1995), and apoptosis (Obid *et al.*, 1993).

Many different forms of SMase have been identified in mammalians. Initially an acidic sphingomyelinase (A-SMase) was discovered and was found to have an optimum pH at 5.0 (Spence, 1993). Although A-SMase has been primarily found in the lysosomes, it has also been detected as a soluble form in cytosol and extracellular media (Quintern *et al.*, 1987). This enzyme has been cloned and is deficient in Neimann-Pick cells. It is likely to play a role in radiation-induced apoptosis (Haimovitz-Friedman *et al.*, 1994) and to be activated by Fas (Cifone *et al.*, 1994) and tumor necrosis factor-α (TNF-α) (Schutze *et al.*, 1992). A neutral pH-optimum and Mg^{2+}-dependent SMase (*N*-SMase) has also been described in various cells (Chatterjee, 1993). Most of the isoforms of this enzyme are associated with membranes. This family of SMase is known to be activated in response to TNF-α, Fas, Ara-C, and serum deprivation. Their activation appears to be closely related to growth suppression and apoptosis (Strum *et al.*, 1994; Jayadev *et al.*, 1995;Tepper *et al.*, 1995). Recently, a cytosolic Mg^{2+}-independent *N*-SMase was partially purified from HL-60 cells following treatment with vitamin D_3 (Okazaki *et al.*, 1994). In

Correspondence to: Dae Kyong Kim, Department of Environmental & Health Chemistry, College of Pharmacy, Chung-Ang University, Dongjak-Ku, Seoul 156-756, Korea
Tel: 82-2-820-5610, Fax: 82-2-825-7920
E-mail: proteinlab@hanmail.net

addition, a Zn^{2+}-dependent A-SMase was detected in sera from a variety of mammalian species (Spence *et al.*, 1989), recent studies have suggested that it may be localized in the lysosome (Schiessel *et al.*, 1996). Finally, an alkaline pH-optimum SMase has been described in intestinal cells (Nyberg *et al.*, 1996).

In particular, *N*-SMase is mainly found in the brain (Spence *et al.*, 1979) and activation of *N*-SMase may be a vital event in the onset of stroke and neurodegenerative diseases. Thus an inhibitor blocking the activation of *N*-SMase can provide alternate avenues for therapy of such diseases. It has been reported that ceramide, generated by the activation of *N*-SMase, could mediate hypoxic cell death in neuronal PC12 cells (Yoshimura *et al.*, 1998). In contrast, it is known that *N*-SMase is strongly expressed in the central nervous system (Spence *et al.*, 1978). The specific activity of *N*-SMase increases during the first two weeks of the neonatal period in the oncogenesis of a rat's brain, participating in the normal growth and maturation of the neural cells.

However, the role of *N*-SMase in these specific cellular reactions and diseases are not yet fully understood. Any direct link between the brain *N*-SMase and specific signaling systems have not yet been established. To establish a clear picture of the metabolic links, a *N*-SMase inhibitor is strongly required. One molecule, scyphostatin, has been described to exert inhibitory activity against *N*-SMase (Nara *et al.*, 1999; Bernardo *et al.*, 2000) and it has been used to associate *N*-SMase with the outgrowth process of hippocampal neurons in response to nerve growth factor (Brann *et al.*, 1999). Recently, GW4869 was also found as another inhibitor of *N*-SMase and it was characterized in MCF7 breast cancer cells treated with TNF-α (Luberto *et al.*, 2002).

In this study, we focused on the identification of novel inhibitors that specifically inhibits *N*-SMase. We turned to the extracts of domestic plants of Korea.. They were evaluated *via* an *in vitro* *N*-SMase assay system, using a single unilamellar vesicle of [*N*-methyl-^{14}C]SM as an exogenous substrate and a purified *N*-SMase from bovine brain as an enzyme source. Here, we show that the extracts of each stem bark of *Abies nephrolepis*, *Acer tegmentosum* and *Ginkgo biloba* inhibit *N*-SMase activity competitively. All the extracts also suppress ceramide production caused by serum deprivation in SH-SY5Y cells.

MATERIALS AND METHODS

Materials

Three hundred extracts of domestic plants of Korea were obtained from the Plant Extract Bank (Daejeon, Korea). Each of the samples were extracted three times using reflux in hot methanol and were then dissolved in dimethylsulfoxide (DMSO), in a concentration of 20 mg/mL. [*N*-methyl-^{14}C]SM ([*N*-methyl-^{14}C]SM, 47 mCi/mmol) and [^{3}H]palmitic acid (1.2 Ci/mmol), which were purchased from Amersham Pharmacia Biotech U.K. Ltd. (Buckinghamshire, England). DE52 anion exchange column, Butyl-Toyopearl hydrophobic column, DEAE-Cellulose anion exchange HPLC column and Phenyl-5PW hydrophobic HPLC column were purchased from Tosoh Co. (Tokyo, Japan). Bovine brain tissues were obtained from a local slaughterhouse in Seoul and kept at -70°C. All other chemicals (Sigma Chemical Co.) were of the highest, commercial available, purity.

Cell culture

SH-SY5Y human neuroblastoma cells (originally from ATCC) were maintained in DMEM with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified, CO_2-controlled (5%) incubator. Cells were detached from the plates using 0.25% trypsin containing 1 mM EDTA.

Assessment of *N*-SMase activity

The substrate, [*N*-methyl-^{14}C]SM (labeled with ^{14}C on the choline moiety), was dried under a nitrogen stream, resuspended in ethanol and sonicated with a cell disrupting sonicator to make a single unilamellar vesicle. The standard incubation system (100 μL), for assessment of *N*-SMase activity, contained 10 mM $MgSO_4$, 2.5 μM [*N*-methyl-^{14}C]SM (approximately 30,000 cpm), 2 mM sodium deoxycholate (SDC) and 100 mM Tris-HCl, pH 7.0. Reactions were carried out at 37°C for 10 min and stopped by adding 320 μL of chloroform/methanol (1:1, by volume) and 30 μL of 2N-HCl into the reaction mixture according to the Bligh & Dyer's method (Bligh and Dyer, 1959). After mixing vigorously, the mixtures were micro-centrifuged to separate the two phases. 200 μL of clear aqueous phase was put into 2.5 mL of scintillation solution (Insta gel-XF, Packard Instrument Co., Meriden, CT, U.S.A.) and tested for radioactivity in a Packard Tri-carb liquid β-scintillation counter. The active fractions were pooled for the next step.

Purification of the salt-extractable, Mg^{2+}-dependent and membrane-bound *N*-SMase from bovine brain

As described elsewhere (Jung *et al.*, 2000), a salt-extractable form of the membrane-bound *N*-SMase, was purified. First, the fresh bovine brain (2 kg) was kept at -70°C and homogenized using 5 volumes (of 10 liters) of homogenizing buffer V (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 3 mM $MgCl_2$, 50 mM KCl, and 10 mM 2-mercaptoethanol) containing a Polytron homogenizer

(Model PT-MR 6000, Kinematica, Switzerland). The homogenate was centrifuged at 10,000×g for 10 min in order to remove the cell debris and nuclei. The resulting supernatants were again centrifuged at 10,000×g at 4°C for 1 h. The resulting pellets were resuspended using 1.2 liters of buffer V and centrifuged at 40,000×g at 4°C for 1 h. The resulting 40,000×g pellets were again resuspended using 1.2 liters of buffer V, adjusted to 0.5 M $(NH_4)_2SO_4$, and stirred at 4°C for 1 h. It was followed by centrifugation at 40,000×g at 4°C for 1 h. The resulting supernatants, termed "ammonium sulfate extracts", were collected and used as the enzyme source for the purification of *N*-SMase. The ammonium sulfate extracts (1.2 liters) were applied to a DE-52 anion exchange column (bed volume of DE52 gel, 1.0 liters) pre-equilibrated with buffer D (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 10 mM 2-mercaptoethanol). The protein bound to the column was eluted at a flow rate of 20 mL/min using a stepwise application of buffer D containing 0.5 M $(NH_4)_2SO_4$ and 0.1% Triton X-100. An aliquot (10 μL) of each fraction (40 mL) was assayed for *N*-SMase activity. The active fractions were pooled and sonicated, at 4°C with a cell disruptor (Sonics & Materials Inc., Danbury, CT, U.S.A.), six times for 3 sec with 5 sec intervals at an output setting of amplitude 70% and again centrifuged at 100,000×g at 4°C for 1 h. An aliquot of stock solution of 4.0 M $(NH_4)_2SO_4$ was added to the resulting supernatant to adjust to 0.5 M $(NH_4)_2SO_4$. The sample as applied to a Butyl-Toyopearl hydrophobic column (bed volume of Butyl-Toyopearl gel, 150 mL) pre-equilibrated with buffer D containing 0.5 M $(NH_4)_2SO_4$. The protein bound to the column was eluted at a flow rate of 15 mL/min using a stepwise application of distilled water. An aliquot (10 μL) of each fraction (40 mL) was assayed for *N*-SMase activity. The active fractions were pooled and the sample was applied to a DEAE-Cellulose anion exchange HPLC column previously equilibrated with buffer D. The protein bound to the column was eluted at a flow rate of 5 mL/min with a 200 mL-linear gradient of buffer D, containing 0.5 M $(NH_4)_2SO_4$, and 0.1% Triton X-100 as an elution buffer. An aliquot (10 μL) of each fraction (5 mL) was assayed for *N*-SMase activity. The active fractions were pooled and applied to a Phenyl-5PW hydrophobic HPLC column (21.5 mm×15 cm, Tosoh Co., Tokyo, Japan), which was equilibrated with buffer D containing 0.5 M $(NH_4)_2SO_4$. The protein bound to the column was eluted at a flow rate of 5 mL/min with a 100 mL-gradient elution of distilled water. Finally, each sample of the active fractions were applied to a 10 % SDS-PAGE gel and visualized using a silver staining kit.

Inhibitory effect of the extracts on the purified *N*-SMase activity

An aliquot of the active fractions from the final step was preincubated with different concentrations of each of the plant extracts at 37°C for 10 min in the reaction buffer (100 mM Tris-HCl, pH 7.0, 10 mM $MgSO_4$, 2 mM sodium deoxycholate (SDC)). Substrate was added and the mixture was further incubated at 37°C. After 10 min, the reaction was stopped and assayed for the *N*-SMase activity as described in the previous section.

Assessment for ceramide production

SH-SY5Y cells (three 6-well vessels/condition) were washed once using PBS and then incubated in DMEM, supplemented with 10% FBS and 1 μCi/mL [^{3}H]palmitic acid. After 1 day, the cells were washed three times using 10 mL PBS. The indicated concentrations of the extracts were added and incubated in DMEM with 10% FBS or without the FBS for 15 h, respectively. The produced ceramide was separated on a TLC as follows. The lipids were first separated by using chloroform/methanol/acetic acid/water (170:9:10:1; vol/vol/vol/vol). The plates were dried out and then further separated by using chloroform/methanol/acetic acid/water (39:15:5.3:2.7; vol/vol/vol/vol) as the second solvent. Individual lipids were visualized using iodine vapor staining. The radioactive spots corresponding to ceramide were scrapped and determined by a β-liquid scintillation counter.

RESULTS

Preparation of *N*-SMase from bovine brain

To examine whether the plant extracts inhibited the *N*-SMase activity, we used the active fractions from the final purification step (Phenyl-5PW HPLC column). First, to prepare the enzyme source for the salt-extractable and membrane-bound *N*-SMase, the bovine brain tissues were homogenized and centrifuged as described in "Materials & Methods". The $(NH_4)_2SO_4$-extracts showed a specific activity of 140 pmol/min/mg. The extracts were loaded on a DE52 anion exchange column. A portion (~20%) passed through the anion exchange column, but the majority (~80%) bound to the column. Elution with buffer D, containing 0.5 M $(NH_4)_2SO_4$, and 0.1% Triton X-100, removed the bound protein. The yield was 35%. The turbid pool of DE52 gel was clarified by ultracentrifugation, and the majority (63%) of the remaining protein was recovered in 100,000×g supernatants with a 4.3-fold purification increase. Next, supernatants by ultracentrifugation were subjected to the Butyl-Toyopearl hydrophobic column. A portion (~15%) passed through the anion exchange column, but the majority (~85%) bound to the column. Elution resulted in a yield of 35%. The active fractions were pooled and subjected to a DEAE-Cellulose anionic exchange HPLC column. A portion (~10%) passed through the anion exchange column, but the majority

(~90%) bound to the column. Elution resulted in a yield of 66%. Then active fractions were pooled and applied to a Phenyl-5PW hydrophobic HPLC column. A portion (~5%) passed through the anion exchange column, but the majority (~95%) bound to the column. Elution resulted in a yield of 18%. These sequential procedures for brain *N*-SMase resulted in a 163-fold purification and excluded other isoforms of SMase. The supernatant was separated in 10% SDS-PAGE gel and visualized with a silver staining kit (Fig. 1).

Effect of the plant extracts on the *N*-SMase activity

The inhibitory effect of the natural plant extracts of *Abies nephrolepis*, *Acer tegmentosum*, and *Ginkgo biloba* on brain *N*-SMase activity was further characterized. Preincubation of the enzyme with 10 μg/mL, 25 μg/mL, and 50 μg/mL of each of the extracts took place for 10 min. It was followed by the addition of the substrate at 37°C. As shown in Fig. 2, the enzyme activity was inhibited in a concentration-dependent manner. It's activity was reduced to less than 40% at 50 μg/mL. All extracts showed a similair inhibitory effect. As shown in Fig. 3, the inhibitory effects of *Abies nephrolepis*, *Acer tegmentosum*, and *Ginkgo biloba* extracts on *N*-SMase activity were in a time-dependent manner. Furthermore, compared to the control, when incubated with 10, 25, and 50 μg/mL of the extracts for 60 min at 37°C, the inhibition of brain *N*-SMase was 57, 62, and 64%, respectively. *Abies nephrolepis*, *Acer tegmentosum*, and *Ginkgo biloba* extracts inhibited the activity in similar fashions.

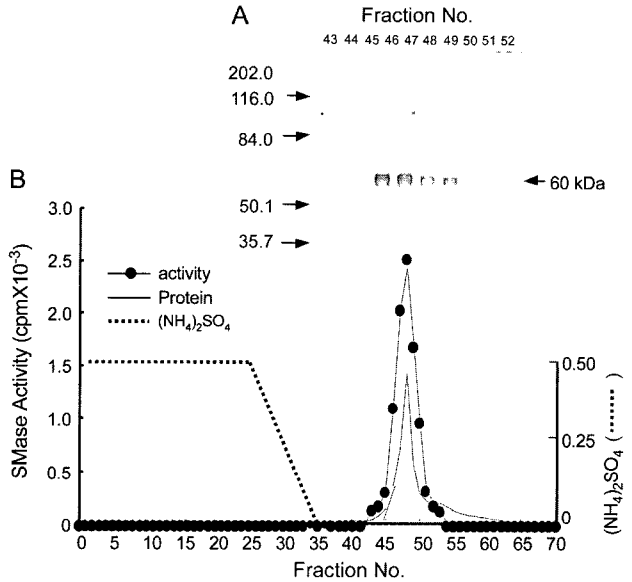

Fig. 1. Purification of a 60 kDa *N*-SMase from the salt-extractable bovine brain membranes. The 60 kDa *N*-SMase activity was purified as described in "Materials & Methods". Finally, the active pool of the DEAE-Cellulose anion exchange column was subjected to a phenyl-5PW hydrophobic column pre-equilibrated with 25 mM Tris (pH 7.0) buffer containing 1 mM EDTA, 0.5M $(NH_4)_2SO_4$ at a flow rate of 5 mL/min. The column was eluted at a flow rate of 5 mL/min with 100 mL of the same buffer in a linear gradient of 0.5~0.0 M $(NH_4)_2SO_4$. An aliquot (10 μL) of each fraction was assayed for *N*-SMase activity and an aliquot (5 μL) of each fraction was applied to the SDS-PAGE and visualized using a silver staining kit.

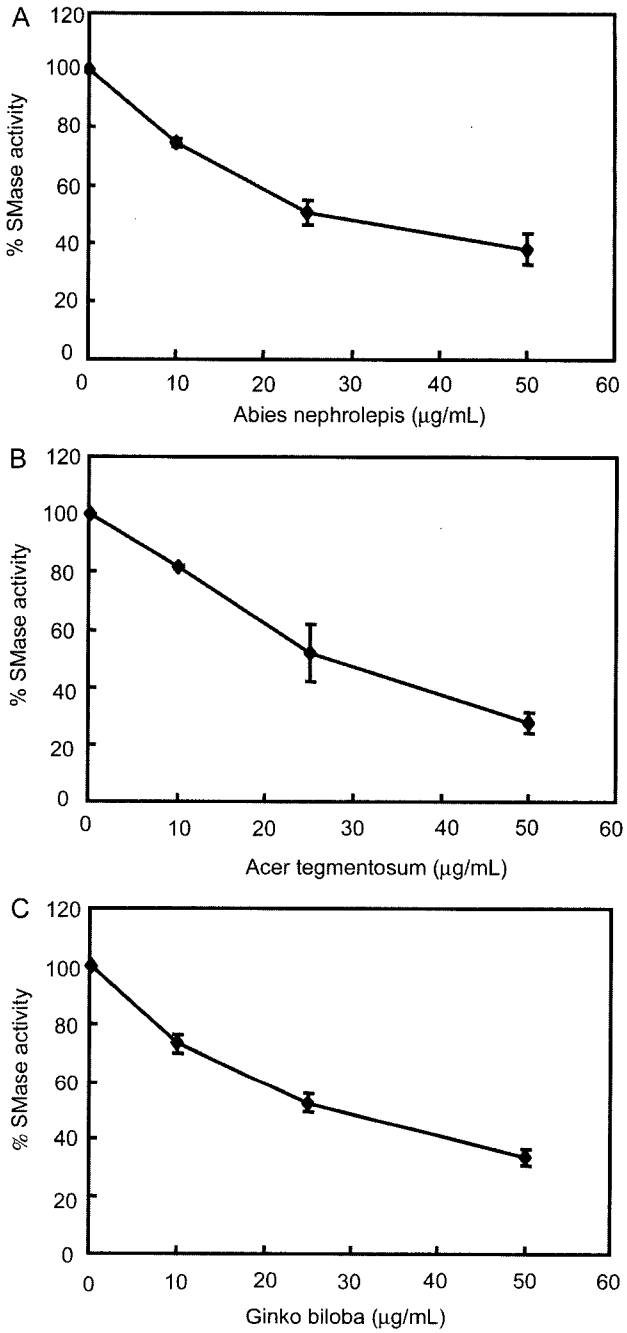

Fig. 2. Dose-dependent inhibition of natural plant extracts on the purified *N*-SMase activity. The *N*-SMase activity was assayed in the presence of increasing concentrations of inhibitors as described in "Materials & Methods". Inhibitors were preincubated with the *N*-SMase activity (5,000 cpm/10 μL aliquot) for 10 min at 37°C. Each data point represents the mean±S.E.M. of three independent experiments.

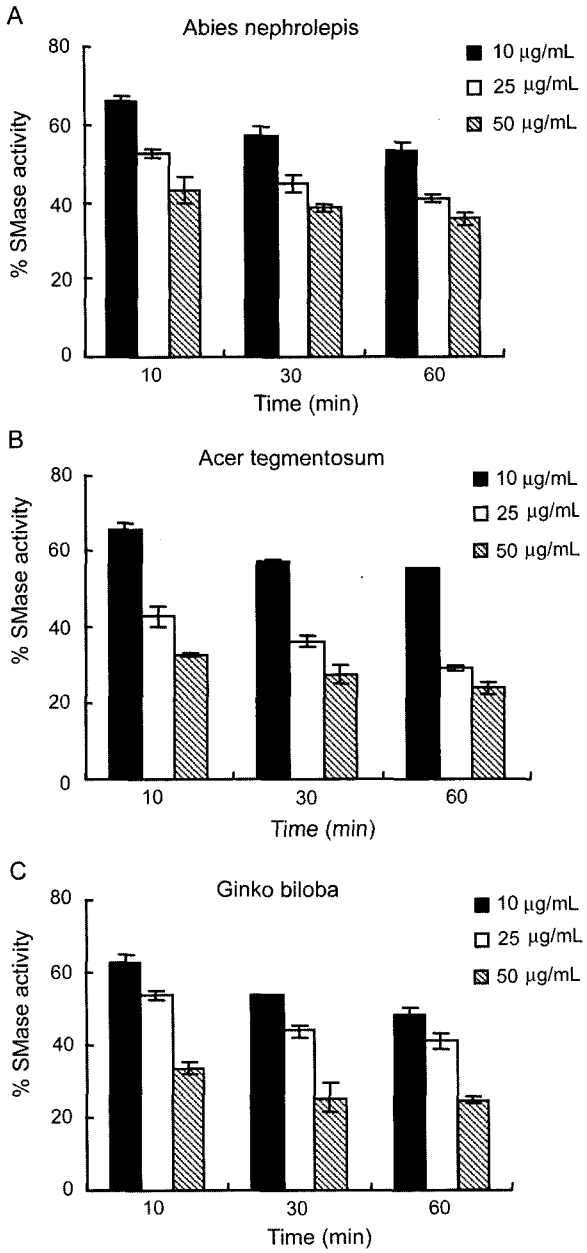

Fig. 3. Time-dependent inhibition of natural plant extracts on the purified *N*-SMase activity. The purified bovine brain *N*-SMase activity (5,000 cpm/10 μL aliquot) was incubated with the different concentrations of inhibitors at 37°C for the indicated times, and then the activity was measured as described in "Materials & Methods". Each data point represents the mean±S.E.M. of three independent experiments.

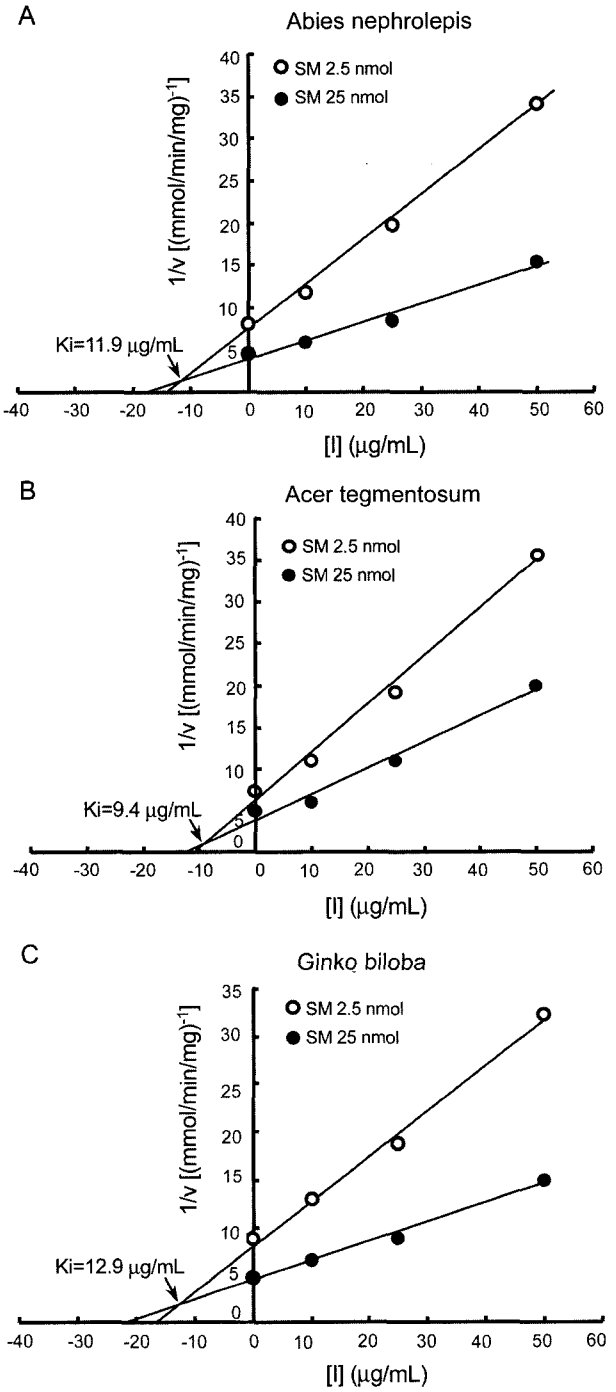

Fig. 4. Determination of the inhibitory pattern of natural plant extracts on the *N*-SMase activity. The *N*-SMase activity was assayed for 20 min at 37°C in the presence of the indicated concentration of inhibitor and 2.5 nmol (○) and 25 nmol (●) of SM as described in "Materials & Methods". Shown are values from one experiment representative of three independent experiments producing similar results.

Analysis of the inhibitory pattern of the plant extracts on the *N*-SMase activity

To determine the inhibitory pattern on *N*-SMase by extracts from *Abies nephrolepis*, *Acer tegmentosum*, and *Ginkgo biloba*, Dixon plots were constructed from the hydrolysis rates of the substrate ([*N*-methyl-^{14}C]SM of 25 and 250 μM) induced by *N*-SMase at various concentration of extracts. Fig. 4 illustrates that the apparent K*i* of *Abies nephrolepis* and *Acer tegmentosum* and *Ginkgo biloba* extracts was 11.9 μg/mL, 9.4 μg/mL, and 12.9 μg/mL respectively. Thus, all of the extracts are apparently competitive. This result supports a direct binding of these

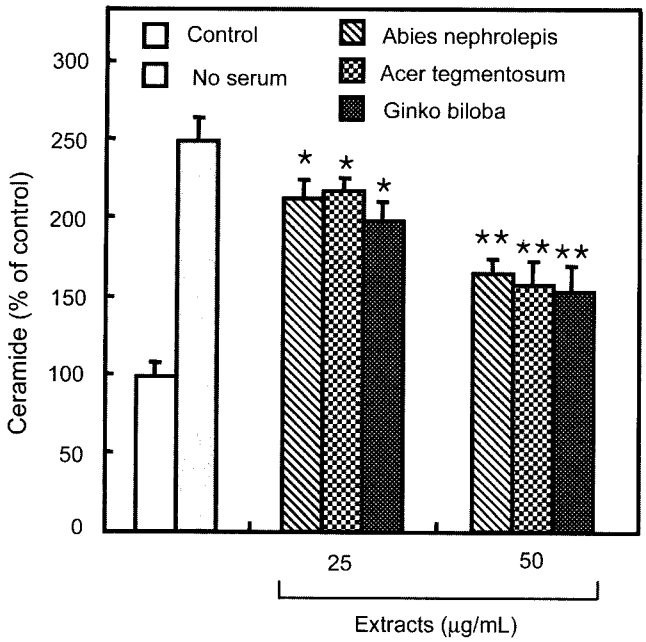

Fig. 5. Evaluation of the inhibitory effect of the natural plant extracts on the production of ceramide induced by serum deprivation in SH-SY5Y cells. SH-SY5Y cells were washed once with PBS and incubated in DMEM supplemented with 10% FBS and 1 μCi/mL [^{3}H]palmitic acid. As described in "Materials & Methods", the cells were washed with PBS and exposed to the serum deprivation with each of the indicated concentrations of the extracts. Data are mean±S.E.M. of three independent experiments (Student's *t*-test; *P<0.05, **P<0.001 vs. control).

extracts to the enzyme.

Evaluation of the inhibition of the plant extracts on the production of ceramide induced by serum deprivation in SH-SY5Y cells

Furthermore, to examine whether these extracts can penetrate into the SH-SY5Y cells and block an activation of the *N*-SMase induced by serum deprivation, we labeled the cells with [^{3}H]palmitic acid and exposed them to serum deprivation. The effect on the ceramide generation was measured. As shown in Fig. 5, serum deprivation leads to a 2.5-fold increase in the production of ceramide. The inhibition was dose-dependently by all the extracts in the neuronal cell line. These results suggest that each of these extracts could contain a chemical inhibitor which can penetrate the cells, competitively inhibit the activity of the *N*-SMase and hence lead to the decrease in the ceramide production.

DISCUSSION

In this study, we report the inhibitory effects of natural plant extracts on the activity of the *N*-SMase partially purified from bovine brain, and the production of ceramide induced by serum deprivation. To examine the inhibitory effect of domestic plant extracts on *N*-SMase, we highly purified *N*-SMase from bovine brain. Although many research groups have attempted to identify membrane-bound forms of *N*-SMase from different sources including rat brain (Maruyama *et al.*, 1989; Carre *et al.*, 1989), little is known about their precise primary structures and biological functions. Further studies should be performed to identify and characterize the enzymes. In this context, it will be important to screen and identify a specific inhibitor for the *N*-SMase.

A thoroughly screening of domestic plant extracts of Korea, with a preparation of brain *N*-SMase, identified three candidates from extracts of *Abies nephrolepis*, *Acer tegmentosum*, and *Ginkgo biloba* as effective inhibitors. Nara *et al.* (1999a, b) reported that scyphostatin, a constituent of *Trichopeziza mollissima*, inhibits *N*-SMase in rat brain microsomes. Nakane *et al.* (2000) demonstrated that lethal forebrain ischemia stimulates sphingomyelin (SM) hydrolysis and ceramide generation (SM pathway) in the gerbil hippocampus. *N*-SMase is mainly found in brain tissues (Liu *et al.* 1998; Bernardo *et al.*, 2000) and thus its active role in the occurrence of insults in the brain could act as a critical mediator in neuronal cell death during ischemia and reperfusion. We found that serum deprivation induces cell death through an apoptotic process in neuronal cells including SH-SY5Y cells with a concomitant production of ceramide and reduction of SM. This suggests a SM pathway, where the SM hydrolysis facilitated by *N*-SMase activation. (Jung *et al.* unpublished data). Thus, it is possible that the purified *N*-SMase from bovine brain may serve as a mediator for the ceramide production responsible for the neuronal apoptosis. To verify this possibility, the creation of a selective and potent inhibitor of *N*-SMase and its application in blocking the SM pathway will contribute to clarify the role of N-SMase in neuronal death. In further studies, we should isolate an active ingredient to inhibit the *N*-SMase activity from each of the natural plant extracts and examine whether such components specifically inhibit the *N*-SMase activity compared with other isoforms of *N*-SMase.

In summary, this study shows that the methanol extracts of *Abies nephrolepis*, *Acer tegmentosum*, and *Ginkgo biloba* revealed the inhibitory action on the 60 kDa and salt-extractable *N*-SMase, one isoform of various *N*-SMases, not only *in vitro* but also in a cellular model, probably *via* a competitive inhibition. In further study, if the active ingredients could be successfully purified from each of the extracts, they will be very potent competitive inhibitors and useful to identify a critical role of the *N*-SMase in the various types of cellular responses including neuronal apoptosis.

REFERENCES

Bernardo, K., Krut, O., Wiegmann, K., Kreder, D., Micheli, M.,

Schäfer, R., Sickman, A., Schmidt, W. E., Schroder, J. M., Meyer, H. E., Sandhoff, K., and Kronke, M., Purification and characterization of a magnesium-dependent neutral sphingomyelinase from bovine brain. *J. Biol. Chem.*, 275, 7641-7647 (2000).

Bligh, E. G. and Dyer, W. J. A., Rapid method of total lipid extraction and purification. *J. Can. J. Biochem. Biophysiol.*, 37, 911-917 (1959).

Chatterjee, S., Neutral sphingomyelinase. *Adv. Lipid Res.*, 26, 25-47 (1993).

Jayadev, S., Liu, B., Bielawska, A. E., Lee, J. Y., Nazaire, F., Pushkareva, M., Obeid, L. M., and Hannun, Y. A., Role for Ceramide in Cell Cycle Arrest. *J. Biol. Chem.*, 270, 2047-2052 (1995).

Strum, J. C., Small, G. W., Pauig, S. B., and Daniel, L. W., 1-beta-D-Arabinofuranosylcytosine stimulates ceramide and diglyceride formation in HL-60 cells. *J. Biol. Chem.*, 269, 15493-15497 (1994).

Jung, S. Y., Suh, J. H., Park, H. J., Jung, K. M., Kim, M. Y., Na, D. S., and Kim, D. K., Identification of multiple forms of membrane-associated neutral sphingomyelinase in bovine brain. *J. Neurochem.*, 75, 1004-1014 (2000).

Liu, B., Hassler, D. F., Smith, G. K., Weaver, K., and Hannun, Y. A., Purification and characterization of a membrane bound neutral pH optimum magnesium-dependent and phosphatidylserine-stimulated sphingomyelinase from rat brain. *J. Biol. Chem.*, 273, 34472-34479 (1998).

Spence, M. W., Burgess, J. K., and Sperker, E. R., Neutral and acid sphingomyelinases: Somatotopographical distribution in human brain and distribution in rat organs. A possible relationship with the dopamine system. *Brain Res.*, 168, 543-545 (1979).

Nara, F., Tanaka, M., Hosoya, T., Suzuki-Konagai, K., and Ogita, T., Scyphostatin, neutral sphingomyelinase inhibitor from a discomycete, *Trichopeziza mollissima*: taxonomy of the producing organism, fermentation, isolation, and physicochemical properties. *J. Antibiot.*, 52, 525-530 (1999a).

Nara, F., Tanaka, M., Masuda-Inoue, S., Yamasato, Y., Doi-Yoshioka, H., Suzuki-Konagai, K., Kumakura, S., and Ogita, T., Biological activities of scyphostatin, a neutral sphingomyelinase inhibitor from a discomycete, *Trichopeziza mollissima*. *J. Antibiot.*, 52, 531-535 (1999b).

Nyberg, L., Duan, R. D., Axelson, J., and Nilsson, A., Identification of an alkaline sphingomyelinase activity in human bile. *Biochim. Biophys. Acta*, 1300, 42-48 (1996).

Obid. L. M., Linardic, C. M., karolak, L. A., and Hannun, Y. A., Programmed cell death induced by ceramide. *Science*, 259, 1769-1771 (1993).

Okazaki, T., Bielawska, A., Bell, R. M., and Hannun, Y. A., Role of ceramide as a lipid mediator of 1,25-dihydroxyvitamin D_3-induced HL-60 cell differentiation. *J. Biol. Chem.*, 265, 15823-15831 (1990).

Okazaki, T., Bielawska, A., Domae, N., Bell, R. M., and Hannun, Y. A., Characteristics and partial purification of a novel cytosolic, magnesium-independent, neutral sphingomyelinase activated in the early signal transduction of 1α,25-dihydroxyvitamine D_3-induced HL-60 cell differentiation. *J. Biol. Chem.*, 269, 4070-4077 (1994).

Quintern, L. E., Weitz, G., Nehrkorn, H., Tager, J. M., Schram, A. W., and Sandhoff, K., Acid sphingomyelinase from human urine: purification and characterization. *Biochim. Biophys. Acta*, 922, 323-336 (1987).

Schiessel, S. L., Schuchman, E. H., Williams, K., J., and Tabas, I., Zn^{2+}-Stimulated Sphingomyelinase Is Secreted by Many Cell Types and Is a Product of the Acid Sphingomyelinase Gene. *J. Biol. Chem.*, 271, 18431-18436 (1996).

Schutze, S., Potthof, K., Machleidt, T., Berkovic, D., Wiegmann, K., and Kronke, M., TNF activates NF-kappa B by phosphatidylcholine-specific phospholipase C-induced "acidic" sphingomyelin breakdown. *Cell*, 71, 765-776 (1992).

Segui, B., Bezombes, C., Uro-Coste, E., Medin, J. A., Andrieu-Abadie, N., Auge, N., Brouchet, A., Laurent, G., Salvayre, R., Jaffrezou, J. P., and Levade, T., Stress-induced apoptosis is not mediated by endolysosomal ceramide. *FASEB J.*, 14, 36-47 (2000).

Spence, M. W., Sphingomyelinase. *Adv. Lipid Res.*, 26, 3-23 (1993).

Spence, M. W. and Burgess, J. K., Acid and neutral sphingomyelinase of rat brain. Activity in developing brain and regional distribution in adult brain. *J. Neurochem.*, 30, 917-919 (1978).

Spence, M. W., Byers, D. M., Palmer, F. B., St, C., and Cook, H. W., A new Zn^{2+}-Stimulated sphingomyelinase in fetal bovine serum. *J. Biol. Chem.*, 264, 5358-5363 (1989).

Yoshimura, S., Banno, Y., Nakanishi, Y., Takenaka, K., Sakai, H., Nishimura, Y., Sakai, N., Shimizu, S., Eguchi, Y., Tsujimoto Y., and Nozawa, Y., Ceramide formation leads to caspase-3 activation during hypoxic PC12 cell death. Inhibitory effects of Bcl-2 on ceramide formation and caspase-3 activation. *J. Biol. Chem.*, 273, 6921-6925 (1998).

Tepper, C. G., Jayadev, S., Liu, B., Bielawska, A., Wolff, R. A., Yonehara, S., Hannun, Y. A., and Seldin, M. F., Role for Ceramide as an Endogenous Mediator of Fas-Induced Cytotoxicity. *Proc. Natl. Acad. Sci.*, 92, 8443-8447 (1995).

Venable, M. E., Lee, J. Y., Smyth, M. J. Bielawska, A., and Obeid, L. M., Role of ceramide in cellular senescence. *J. Biol. Chem.*, 270, 30701-30708 (1995).