Purification, characterization, and expression of rat intestinal alkaline sphingomyelinase

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Abstract Intestinal alkaline sphingomyelinase (SMase) has physiological roles in the digestion of sphingomyelin (SM) and clinical implications in colonic carcinogenesis. In the present work, the enzyme from rat has been purified 1,589-fold with 11% recovery by elution of the intestine with bile salt, precipitation of the proteins by acetone, and several types of chromatographies. Its molecular mass was 58 kDa and optimal pH was 9 to 9.5. Under the optimal conditions, the V_{max} was 930 μmol/h/mg and K_m was about 1.25 mM. The enzyme could hydrolyze phosphatidylcholine at pH 7.4 in the presence of Ca^{2+}; the rate was about 8% of that for SM. The activity against SM was dependent on bile salt. Taurine conjugated bile salts were much more effective than glycine conjugated ones, and the most effective bile salts were taurocholate and taurochenodeoxycholate. 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and Triton X100 (TX100) had no stimulatory effects. Unlike neutral SMase, intestinal alkaline SMase was not Mg^{2+} dependent, not inhibited by EDTA, and not inhibited by glutathione. The enzyme was stable during incubation with temperatures up to 50°C and in pHs from 7 to 10. Trypsin and chymotrypsin had no effects on its activity, and 10 mM dithiothreitol reduced its activity by 25%. A specific antibody against the enzyme was developed, and Western blot showed that the enzyme was expressed in the intestine but not in other organs. In conclusion, we purified a potentially important SMase in the intestine with several properties different from neutral SMase.—Cheng, Y., Å. Nilsson, E. Tömquist, and R-D. Duan. **Purification, characterization, and expression of rat intestinal alkaline sphingomyelinase. *J. Lipid Res.* 2002.** 43: **316–324.**

Supplementary key words sphingomyelin • bile salt • digestion • glutathione • Triton X100

Hydrolysis of sphingomyelin (SM) generates multiple molecules that regulate cell proliferation, differentiation, and apoptosis (1, 2). The hydrolysis is initiated by sphingomyelinase (SMase), which cleaves the phosphocholine head group from SM. Based on the optimal pH, three types of SMases have been identified. Acid SMase is a lysosomal enzyme with an optimal pH around 5 and neutral SMase is a membrane-bound enzyme with an optimal pH of 7.5 (3). Both acid and neutral SMases have been purified and cloned recently (4–8). In the intestinal tract there is another type of SMase that was discovered by Nilsson in 1969 (9). The enzyme prefers alkaline pH and was termed alkaline SMase (10). Unlike the widely distributed acid and neutral SMases, alkaline SMase is specifically distributed in the intestinal mucosa, with high activity localized at the middle small intestine and low activity in the colon (9, 10).

Intestinal alkaline SMase may have physiological roles in the digestion of dietary SM (11, 12), as dietary SM is not hydrolyzed by pancreatic juice, and SM digestion occurs mainly in the middle and lower small intestine, where alkaline SMase is abundant. The enzyme may also have protective roles in tumorigenesis in the colon (13, 14). Feeding mice with SM or ceramide reduced the number of colonic aberrant crypt foci and inhibited the promotion of colon carcinoma induced by 1,2-dimethylhydrazine (15–18). We previous reported that in human colonic adenoma, colonic carcinoma, and familial adenomatous polyposis, the activities of alkaline SMase were decreased by 50%, 75%, and 90%, respectively (19, 20). In the present work, the rat intestinal alkaline SMase has been purified and several properties characterized. An antibody has been developed, and expression of the enzyme in various organs was studied.

MATERIALS AND METHODS

Materials

Sprague Dawley rats (~250 g) were obtained from Mollegård (Ry, Denmark). Bovine milk SM was provided by Dr. Lena Nyberg at Skåne Dairy Association and labeled with [N-^{14}C-CH_3]-choline by the methods of Stoffel (21). The specific activity of labeled SM, [^{14}C]SM, was 56 μCi/mg. [N-^{14}C-CH_3]choline-labeled phosphatidylcholine, [^{14}C]PC, was purchased from Du Pont

Abbreviations: CDC, chenodeoxycholate; CMC, critical micelle concentration; GC, glycocholate; GDC, glycodeoxycholate; GCDC, glycochenodeoxycholate; HIC, hydrophobic interaction chromatography; SM, sphingomyelin; SMase, sphingomyelinase; TC, taurocholate; TCDC, taurochenodeoxycholate; TDC, taurodeoxycholate; TUDC, tauroursodeoxycholate; TX100, Triton X 100; UDC, ursodeoxycholate.

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NEN (Boston, MA), and the specific activity was 50 Ci/mmol. Phenylmethylsulfonyl fluoride (PMSF), benzamidine, anti-chicken IgY conjugated with alkaline phosphatase, cholate, taurocholate (TC), taurodeoxycholate (TDC), taurochenodeoxycholate (TCDC), glycocholate (GC), glycodeoxycholate (GDC), glycochenodeoxycholate (GCDC), Triton X 100 (TX100), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), dithothreitol (DTT), ceramide, and oxidized and reduced glutathione were from Sigma Co. (St. Louis, MO). Neutral SMase from *Streptomyces sp.* was from ICN Biomedicals (Aurora, OH). Ursodeoxycholate (UDC) and tauroursodeoxycholate (TUDC) were provided by Dr. Falk Pharma GmbH (Freiburg, Germany). DEAE Sepharose, Sephadex G 25 (PD10) column, and Phenyl Sepharose 6FF were from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Biologic HR protein purification system, Econo High Q anion exchange chromatography cartridge (HQ), and Pre-packed SE gel chromatography column were purchased from Bio-Rad (Sundberg, Sweden). YM 30 filtration membrane and Centricon 30 with 30 kDa molecular mass cut-off were purchased from Amicon (Beverly, MA).

Purification of intestinal alkaline SMase

Thirty rats fasted overnight were anesthetized with diethyl ether. A segment of small intestine from 10 cm below pylorus to 10 cm above the cecum was cannulated. This part of small intestine contains high alkaline SMase activity (22). The segment was eluted twice with 25 ml 0.9% NaCl containing 1 mM benzamidine, 1 mM PMSF, and 3 mM TDC, which serves to dissociate the alkaline SMase from the intestinal brush border (22). The eluted solution was centrifuged at 5,000 *g* at 0°C, and the supernatant was concentrated by ultrafiltration through a YM 30 membrane. The proteins were precipitated by adding acetone to 50% (v/v) at temperatures below −10°C. After centrifugation at 8000 *g* for 15 min at 0°C, the protein pellets were dissolved in 50 ml of 20 mM Tris-HCl buffer containing 1 mM benzamidine and 0.075 M NaCl (pH 8.2). The non-dissolved proteins were removed by centrifugation at 15,000 *g* for 15 min at 0°C. The samples were loaded on a DEAE Sepharose column (2.6 × 10 cm; bed volume, 53 ml), which had been equilibrated with the same buffer to dissolve the proteins. The column was washed with 150 ml of the buffer at a rate of 4 ml/min, followed by elution with the same buffer containing 0.5 M NaCl. The alkaline SMase activity was determined, and the fractions containing high SMase activity were pooled, concentrated, and desalted by PD10 column. The sample was then loaded on an HQ cartridge (bed volume, 5 ml) equilibrated with 20 mM Tris buffer containing 1 mM benzamidine, pH 8.2. After washing, the cartridge was eluted with a NaCl gradient from 0 to 0.25 M in 75 ml of the buffer and finally with the buffer containing 0.5 M NaCl at a rate of 2 ml/min. The SMase activities were determined, and the fractions with high activity were pooled and concentrated. The concentrated sample was supplemented with ammonium sulfate to 1 M and loaded on a Phenyl Sepharose column (1.3 × 8 cm; bed volume, 42 ml), which had been equilibrated with 20 mM Tris-Maleate buffer containing 1 M ammonium sulfate and 1 mM benzamidine (pH 7). The column was washed with 80 ml of the buffer, followed by an elution with a linear gradient of ammonium sulfate from 1 to 0 M in a volume of 120 ml at a rate of 1.5 ml/min. The fractions with high activity were concentrated by Centriprep 30 and applied on SE column (0.4 × 30 cm; bed volume, 15 ml), which was eluted at a rate of 0.2 ml/min with 30 mM Tris-HCl (pH 8.2) containing 0.15 M NaCl and 1 mM benzamidine. All the purification procedures except the first DEAE chromatography were programmed using the Bio-Logic HR system, and the protein concentrations were monitored by UV detector. The whole procedure was carried out at 4°C.

For checking the purity of the enzyme, the samples with high SMase activity from each step were subjected to 12% SDS-PAGE and the protein was stained by silver staining.

SMase assay

Activity of alkaline SMase was determined according to Duan and Nilsson (23). Briefly, 2–5 μl of samples were added in 50 mM Tris-HCl buffer containing 0.15 M NaCl, 2 mM EDTA, 10 mM TC (pH 9) (assay buffer), and 0.1 mM SM to a final volume of 80 μl. The reaction was started by addition of 20 μl of [^{14}C]SM (8,000 dpm, 80 pmol) followed by incubation at 37°C for 30 min. The reaction was terminated by adding 0.4 ml of chloroform-methanol (2:1, v/v) followed by centrifugation at 10,000 rpm for 10 s. An aliquot (100 μl) of the upper phase was taken, and the radioactivity was counted by liquid scintillation. Enzyme activity expressed as dpm appeared in the upper phase in the incubation time. When purification factors were determined, the percentage hydrolysis of substrate was calculated from the ratio of dpm in the upper phase to the total dpm added in the system. This percentage factor was applied to calculate the mass hydrolysis of total SM. Activity was expressed as μmol/h/mg enzyme protein. The procedure for neutral SMase assay was the same as for alkaline SMase except the assay buffer, which was 50 mM Tris-HCl containing 0.15 M NaCl, 4 mM Mg^{2+}, and 0.12% TX100 (pH 7.4).

Identification of ceramide

The ceramide was identified by HPLC according to Previati et al. (24). Briefly, alkaline SMase (5 ng) was incubated with 0.25 mg SM for 1 h. The lipid products in the organic phase were dried under nitrogen and dissolved in chloroform. Ceramide was coupled with methoxy-α-methyl-naphthaleneacetic acid and applied on a Nucleosil 5CN column. The ceramide peak was detected by a UV detector at 230 nm with the reference of standard ceramide.

Determination of hydrolytic capacity

To determine hydrolytic capacity of the enzyme, 0 to 400 nmol unlabeled SM dissolved in ethanol was mixed with 80 pmol [^{14}C]SM and dried under nitrogen. The assay buffer was added, and the solution was sonicated for 10 s. Purified intestinal alkaline SMase (3 ng in 5 μl) was added, and incubation was performed at 37°C for 30 min. The percentage of hydrolysis was calculated, and the mass hydrolysis of SM was determined. The Lineweaver-Burk plot was performed, and V_{max} and K_m were determined according to the intercepts on the axes.

Effect of alkaline SMase against PC

For examining the activity of the purified SMase against PC, two types of buffer were used. Type one is the assay buffer for alkaline SMase determination, and type two is 50 mM Tris-HCl buffer containing 4 mM Ca^{2+} and 10 mM TC, which is for the phospholipase C assay. In both buffers, alkaline SMase was incubated with 7,000 dpm of [^{14}C]SM or 7,000 dpm of [^{14}C]PC for 30 min. The control group contained only the buffer without the enzyme. The activity was expressed as fold change over control.

Characterization of purified intestinal SMase

Optimum pH of the enzyme was examined by assaying SMase activity in various buffers with different pHs, as described previously (10). Fifty millimolar Tris-Maleate buffer was used for pHs from 5 to 7, and 50 mM Tris-HCl buffer was used for pHs from 7.5 to 10. All the buffers above contained 0.15 M NaCl and 10 mM TC.

The effects of different bile salts were examined by assaying the enzyme activity in alkaline SMase assay buffer containing dif-

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ferent bile salts with different concentrations up to two times their critical micellar concentrations (CMC) (25). For comparison, the effects of CHAPS up to 20 mM and TX100 up to 0.4% on both alkaline and neutral SMases were determined.

Effect of Mg^{2+} on alkaline SMase or neutral SMase activity was studied by assaying the enzyme activity in the presence of 0–8 mM Mg^{2+}. The Mg^{2+}-free condition was assured by addition of EDTA to 2 mM.

Heat stability was examined by preincubation of the enzyme in the assay buffer at 0, 23, 42, 50, and 60°C for 1 h followed by assaying the remaining SMase activity. To study the stability of the enzyme against pH, the purified enzyme was added in 25 μl of buffers with different pH values and incubated for 1 h. After incubation, 10 μl of the mixture was taken and alkaline SMase activity was determined.

To study the resistance of the enzyme to trypsin and chymotrypsin digestion, the enzyme was added in 25 μl of 50 mM Tris-HCl buffer (pH 8) containing 4 mM Ca^{2+} and different concentrations of trypsin or chymotrypsin for 30 min at 37°C. After preincubation, 10 μl of the mixture was taken and SMase activity determined.

The effect of DTT was examined by determination of the enzyme activity with DTT ranging from 0 to 10 mM. For studying the effect of glutathione, alkaline or neutral SMase was preincubated in their assay buffers with either an oxidized or reduced form of glutathione for 15 min, and the remaining enzyme activities were determined.

Antibody preparation

The purified enzyme was subjected to 10% SDS-PAGE and stained by 0.1% Coomassie blue in water. The SMase band was excised, homogenized, and used to immunize a hen once a month for three times, by a modified method of Larsson and Sjöquist (26). Collection of eggs was started after the second injection. The antibody IgY against alkaline SMase was purified from the yolk based on the method of Polson et al. (27). The whole procedure was performed by AgriSera AB (Vännäs, Sweden), and a detailed description of the modification is not released from the company.

Western blot

Western blot using IgY was done according to Murata et al. (28). Various tissues were homogenized in a buffer containing 0.25 M sucrose, 5 mM $MgCl_2$, 0.15 M KCl, 50 mM KH_2PO_4, 1 mM PMSF, and 10 mM taurocholate, followed by sonication for 10 s. After centrifugation, 50 μg protein of each sample was subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane by electrophoresis. The membrane was washed with 20 mM TBS (pH 7.5) containing 7.5% dried milk and 0.05% Tween 20 and probed with IgY at 1:500 dilution in TBS buffer containing 2% dry milk and 0.25% TX100 for 2 h. The membrane was then rinsed with TBS buffer containing 0.05% Tween 20, followed by incubation with anti-IgY conjugated with alkaline phosphatase at 1:160,000 dilution for 2 h. The visualization of the band was performed using a kit from Bio-Rad Co.

RESULTS

Purification of intestinal alkaline SMase

The profile of the first step of purification, DEAE Sepharose chromatography, is shown in **Fig. 1**. Under these conditions, alkaline SMase was passed through the matrix, whereas a large proportion of proteins was retained. The retained proteins, after eluting by a buffer containing 0.5 M NaCl, had little SMase activity. The fractions with SMase activity were pooled, desalted, and subjected to three chromatographies, including HQ anion exchange chromatography, hydrophobic interaction chromatography (HIC), and SE chromatography (**Fig. 2**). The enzyme was retained on an HQ column and was eluted off by a NaCl gradient from 0 to 0.25 M (Fig. 2A). The activity peak largely corresponded to the second of six protein peaks eluted out by NaCl gradient. During HIC (Fig. 2B), SMase was retained in the column and eluted by a decreasing gradient of ammonium sulfate from 1 to 0 M. On SE gel chromatography (Fig. 2C), there are four protein peaks, and alkaline SMase was corresponded to the second protein peak.

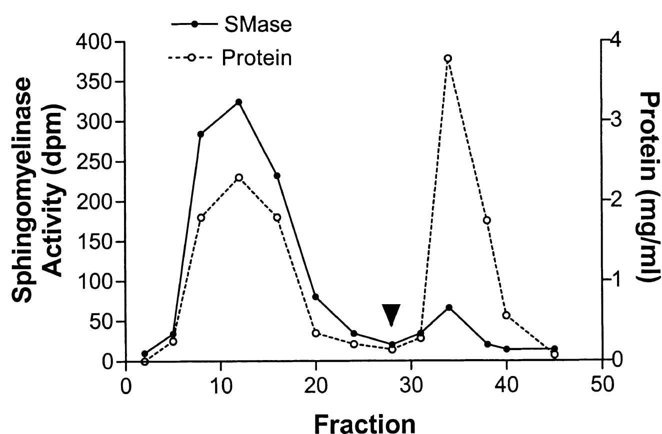

Fig. 1. Profile of DEAE Sepharose chromatography. The proteins precipitated from the intestine by acetone were dissolved in 20 mM Tris-HCl buffer containing 1 mM benzamidine and 0.075 M NaCl (pH 8.2) and loaded on a DEAE Sepharose column (2.6 × 10 cm). The column was eluted with 150 ml of the buffer and then with the same buffer containing 0.5 M NaCl, as indicated by the arrow. The flow rate was 4 ml/min, and the fraction was collected every 1.5 min.

The fractions with the highest alkaline SMase activity after each purification step were subjected to 12% SDS-PAGE, and the purity is shown in **Fig. 3.** A single protein band with a molecular mass of 58 kDa was demonstrated by silver staining in the final product with the highest activity.

The changes of specific activities after each step are shown in **Table 1**. The specific activity was increased by 1,589-fold and the yield was about 11%.

Hydrolytic capacity of intestinal alkaline SMase

Figure 4 shows the hydrolytic capacity of purified intestinal alkaline SMase in 50 mM Tris buffer containing 0.15 M NaCl, 10 mM TC, 2 mM EDTA, and different amount of SM. The hydrolytic rate increased rapidly with the increasing amount of the substrate. The V_{max} calculated is about 930 μmol/h/mg and K_m is 1.25 mM under the conditions.

The hydrolytic product was checked by HPLC. After incubation of the purified SMase with SM, a sharp peak corresponding to the standard ceramide was demonstrated (data not shown).

Comparison of the activity against SM and PC

Previous studies showed that acid and neutral SMase may have activity against PC and *p*-nitrophenyl phosphate

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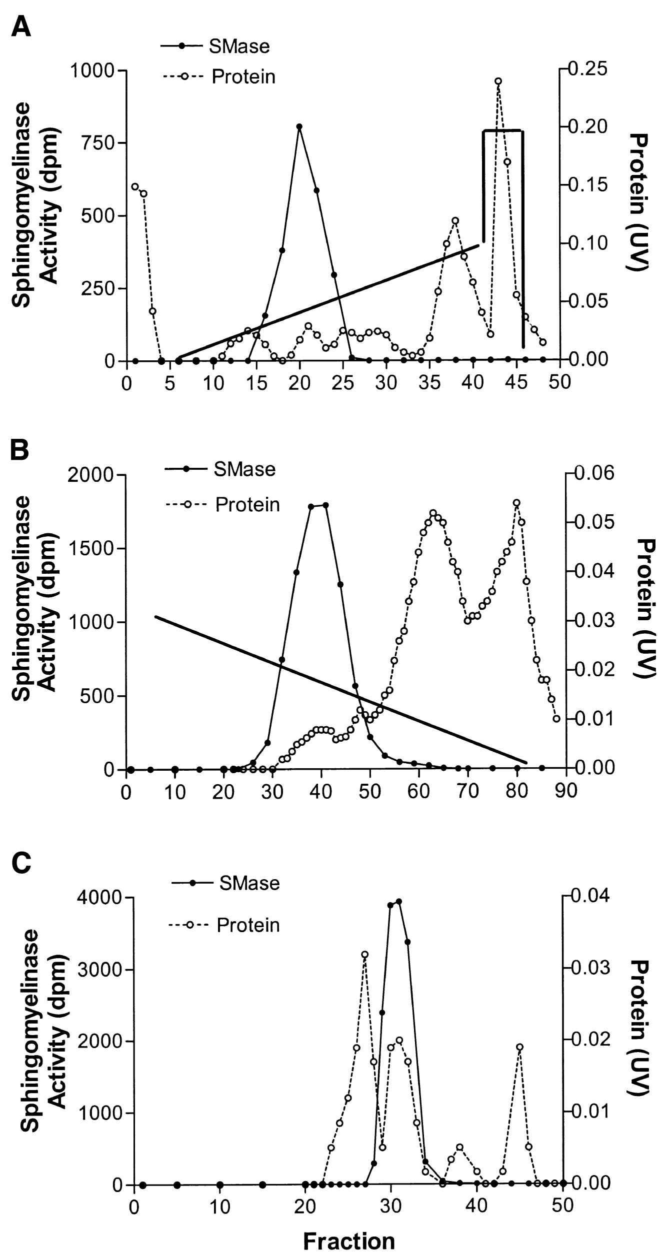

Fig. 2. Profiles of High Q (HQ) anion exchange chromatography, hydrophobic interaction chromatography (HIC), and SE gel chromatography on a BioLogic HR chromatography system. A: The samples after DEAE Sepharose chromatography were loaded on an HQ cartridge equilibrated with 20 mM Tris HCl and 1 mM benzamidine (pH 8.2). The cartridge was eluted with a gradient of NaCl from 0 to 0.25 M and finally with the buffer containing 0.5 M NaCl, as indicated by the bold line in the figure. The flow rate was 2 ml/min and the fraction size was 1.5 ml. B: The samples with high activity from HQ chromatography were supplemented with ammonium sulfate to 1 M and loaded on a Phenyl Sepharose 6 FF column equilibrated with 20 mM Tris-Maleate buffer containing 1 mM benzamidine and 1 M ammonium sulfate (pH 7). The column was eluted with a gradient of ammonium sulfate from 1 M to 0 M, as indicated by the bold line in the panel. The flow rate was 1.5 ml/min, and fractions were collected every min. C: The activity peak from the HIC column was loaded on a SE chromatography column equilibrated with 30 mM Tris-HCl buffer containing 1 mM benzamidine and 0.15 M NaCl (pH 8.2). The flow rate was 0.2 ml/min, and a fraction was collected each min. The figures are reproduced from the original profile on the chromatography system. For detailed information, see the Materials and Methods section.

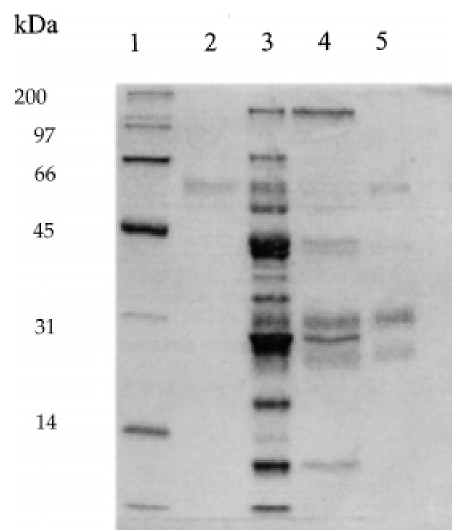

Fig. 3. Purity of the proteins after each purification step. Samples were subjected to 12% SDS-PAGE, and the protein bands were visualized by silver staining. Lane 1: standard proteins; lane 2: final sample after gel chromatography; lane 3: sample after DEAE chromatography; lane 4: sample after HQ chromatography; and lane 5: sample after hydrophobic interaction chromatography.

(29–32). We therefore compared the activity of the purified alkaline SMase against SM and PC. We found that the purified SMase had no activity against PC in assay buffer for alkaline SMase, but it could hydrolyze PC in a buffer which is optimal for PC-specific phospholipase C. The activity against PC was about 5–8% of that against SM.

Characterization of intestinal alkaline SMase

The pH optimum of the enzyme is shown in **Fig. 5**. The activity was negligible at pH below 6, but increased when pH was higher than 7. The optimal pH was 9–9.5. The activity at pH 7.5 was about 60% of the maximal activity at pH 9.5. We also assayed the enzyme activity at pH 7.5 in the presence of 4 mM Mg^{2+} and found that the presence of Mg^{2+} at pH 7.5 did not increase the activity significantly.

Previous studies have indicated that the activity of intestinal alkaline SMase is dependent on bile salt (9, 10). In

TABLE 1. Purification factors and specific activity at various purification stages

Step	Specific Activity	Total Protein	Total Activity	Purification	Yield
	μmol/h/mg	*mg*	*μmol/h*	*fold*	*%*
Origin	0.56	1274	713	1	100
Acetone	1.51	450	680	3	95
DEAE	3.69	140	517	7	73
HQ	17.5	18	315	31	44
HIC	284	0.6	170	507	24
SE	890	0.09	80	1,589	11

The samples for each purification stapes were saved at −20°C. The alkaline SMase activities were determined in the presence of 80 pmol [^{14}C]SM, and 20 nmol SM in the 50 mM Tris buffer containing 0.15 M NaCl, 2 mM EDTA, and 10 mM TC (pH 9.0).

HIC, hydrophobic interaction chromatography; HQ, High Q.

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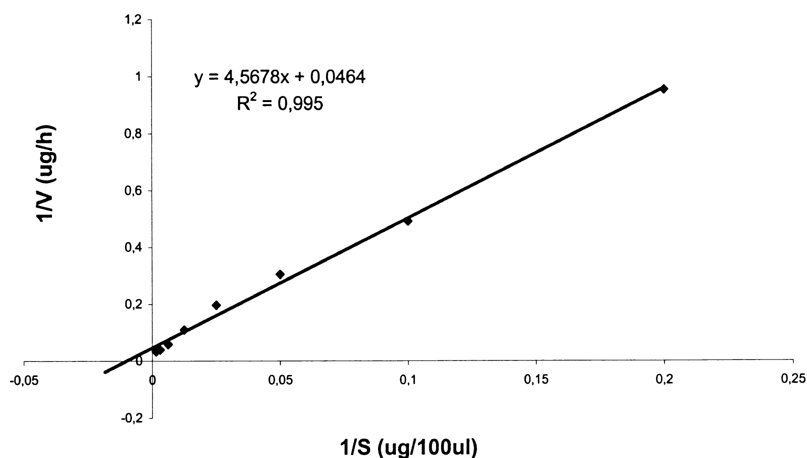

Fig. 4. Lineweaver-Burk plot to determine the hydrolytic capacity of alkaline sphingomyelinase (SMase). Purified SMase (3 ng) was incubated with different amount of unlabeled sphingomyelin (SM) together with [^{14}C]SM. The percentage of SM hydrolysis was calculated according to the radioactivity distributed in the upper and lower phases, and the hydrolytic rate of total SM was determined.

this article, the effects of 11 bile salts were examined. Generally speaking, all bile salts at monomeric concentrations increased the activity of alkaline SMase, and the maximal effects occurred around the CMC of the bile salts tested. At concentrations higher than CMC, the effects of bile salts decreased. The dose-dependent effects of TC, TCDC, and TDC are shown in **Fig. 6A**. Similar patterns were obtained for other bile salts. However, when the maximal effects of various bile salts were compared (Fig. 6B), significant differences were demonstrated. TC and TCDC were 6–30 times more effective than other bile salts in stimulating alkaline SMase activity. Glycine conjugated bile salts were significantly less effective than their corresponding taurine conjugated ones. We also found that CHAPS, the non-physiological detergent that has the identical steroid nuclei and similar CMC as TC but different side chains, did not have any stimulatory effect on alkaline SMase activity in concentrations ranging from 0 to 20 mM. However, CHAPS dose-dependently stimulated neutral SMase activity and 5 mM CHAPS increased neutral SMase activity by 60% (data not shown). TX100 alone had no stimulatory effects on alkaline SMase, and it inhibited TC-induced activation of alkaline SMase significantly (**Fig. 7A**). Replacing TC with 0.12% of TX100 in the assay buffer abolished the alkaline SMase activity (Fig.7B). However, at this concentration, TX100 is as effective as TC in stimulating neutral SMase activity.

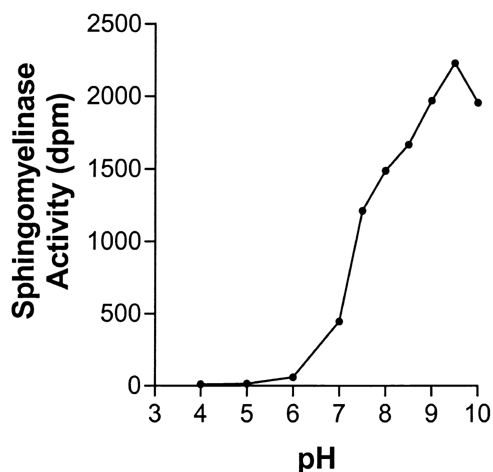

Fig. 5. Optimum pH of the purified SMase. The enzyme activity was determined in 50 mM Tris buffers of different pH values. Fifty micromolar Tris-Maleate buffer was used for pHs from 5 to 7, and 50 mM Tris-HCl was used for pHs from 7.5 to 10.

The influences of Mg^{2+} and glutathione, as well as the stability of the enzyme against pH and temperature, are shown in **Fig. 8**. As shown in Fig. 8A, alkaline SMase was effective in the Mg^{2+}-free condition and Mg^{2+} increased its activity slightly. However, the activity of neutral SMase was abolished in the Mg^{2+}-free condition, and the enzyme was fully activated by 2 mM Mg^{2+}. We also found that the activity of purified alkaline SMase was not significantly affected by Ca^{2+}, which is in agreement with our previous studies with crude mucosal homogenate (10).

When alkaline or neutral SMase was preincubated with glutathione at different concentrations for 15 min, we found that the reduced form of glutathione almost abolished neutral SMase activity at concentrations higher than 5 mM, but it had no significant effect on the activity of alkaline SMase (Fig.8B). The oxidized form of glutathione had no effect on either neutral or alkaline SMase activity. DTT only slightly affected alkaline SMase activity, and 10 mM DTT reduced the alkaline SMase by 25% (data not shown).

The short-term stability of the enzyme against temperature is shown in Fig. 8C. After preincubating the enzyme for 1 h at temperatures up to 50°C, the enzyme activity was generally preserved. However, the enzyme was denatured after incubation at 60°C, and activity decreased by 80% as compared with the activity from the samples kept on ice. After incubation of the enzyme in buffers with different pH for 1 h, the enzyme activity was not changed in buffers with pH values from 7 to 10. However, buffer of pH 6 greatly reduced the activity, and that of pH 5 almost abolished the activity (Fig. 8C).

After incubation of the enzyme with trypsin or chymotrypsin up to 1 mg/ml for 30 min, the enzyme activity was not decreased (data not shown). The results are in agreement with our previous findings with crude tissue homogenates (10).

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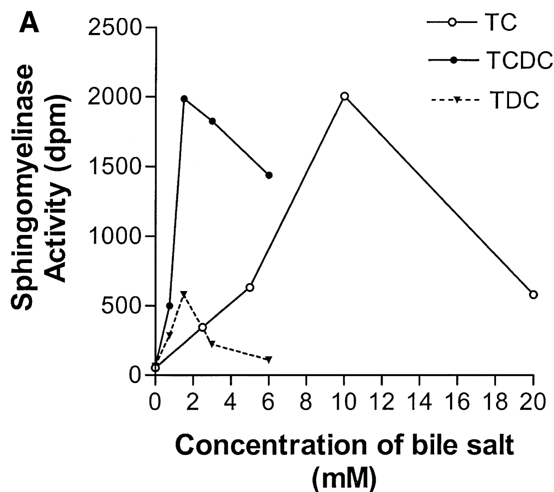


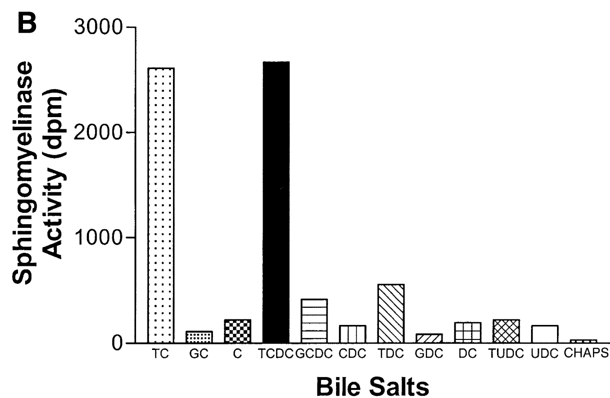
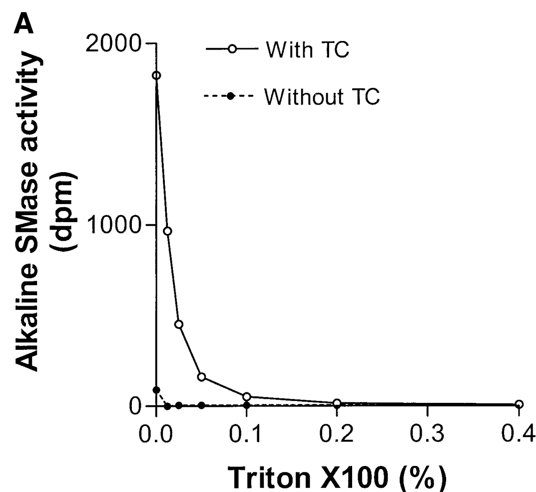


Fig. 6. Effects of bile salts on alkaline SMase activity. A: A representative figure showing the dose-dependent effects of three types of bile salts on alkaline SMase activity. The enzyme activity was determined in alkaline SMase assay buffer [50 mM Tris-HCl buffer (pH 9) containing 0.15 M NaCl and 2 mM EDTA] containing different concentrations of bile salts studied. B: Comparison of the stimulatory effects of different bile salts at critical micellar concentrations on alkaline SMase. C, cholate.



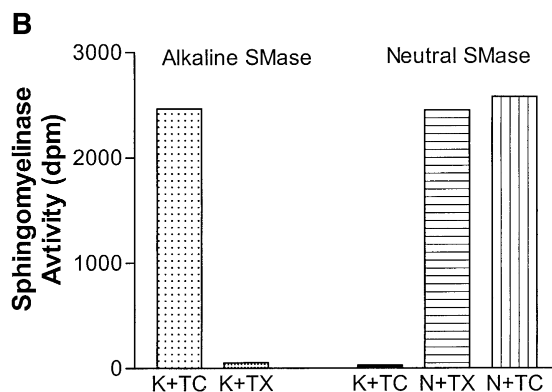


Fig. 7. Effects of Triton X 100 (TX) on alkaline and neutral SMase activity. A: Alkaline SMase was assayed in an alkaline SMase assay buffer with and without 10 mM taurocholate (TC) in the presence of different concentrations of TX100. B: The activities of alkaline and neutral SMase were determined in different buffers. On the left, alkaline SMase activity was assayed in the assay buffer containing 10 mM TC or 0.12% TX100. On the right, neutral SMase activity was assayed in alkaline SMase assay buffer (K) with 10 mM TC or in neutral SMase assay buffer [50 mM Tris-HCl, 0.15 M NaCl, and 4 mM Mg^{2+} (pH 7.4)] (N) with either 0.12% TX100 or 10 mM TC.

According to the data provided by the company that developed the antibody, 1:500 dilution of IgY was chosen in Western blot to study the expression of the enzyme in various tissues. As shown in **Fig. 9**, when the proteins from various tissues were probed with anti-SMase IgY, one major band at about 58 kDa and two smaller bands were demonstrated in the intestinal sample, whereas it was hardly visible in the samples of brain, heart, lung, liver, spleen, kidney, and pancreas. When the membrane was probed with preimmune IgY, no bands could be identified (data not shown).

DISCUSSION

Intestinal alkaline SMase was discovered at the end of the 1960s (9). Its potential biological importance had not been recognized until SM hydrolysis was found to be a novel signal transduction pathway related to cell growth and apoptosis (2, 33, 34). Recent studies have provided evidence that the enzyme may be implicated in colorectal tumorigenesis (14–16, 19, 20, 35). While acid and neutral SMase have been purified, characterized, and, recently, cloned, purification of alkaline SMase from intestinal mucosa and characterization of the enzyme in a purified form have not been reported. In this study, the enzyme from rat intestine has been purified and characterized. Special attention has been paid to distinguish it from neutral SMase. In addition, an antibody against the enzyme has been developed, and the expression of the enzyme in various tissues has been studied.

The first step of the purification was to dissociate the enzyme from the brush border by 6 mM TDC, which stimulated the dissociation of the enzyme from the mucosa (22). This procedure avoided homogenization of the mucosa membrane and enriches the enzyme in the starting materials. The proteins in the medium were precipitated by acetone and dissolved in 20 mM Tris buffer with a low

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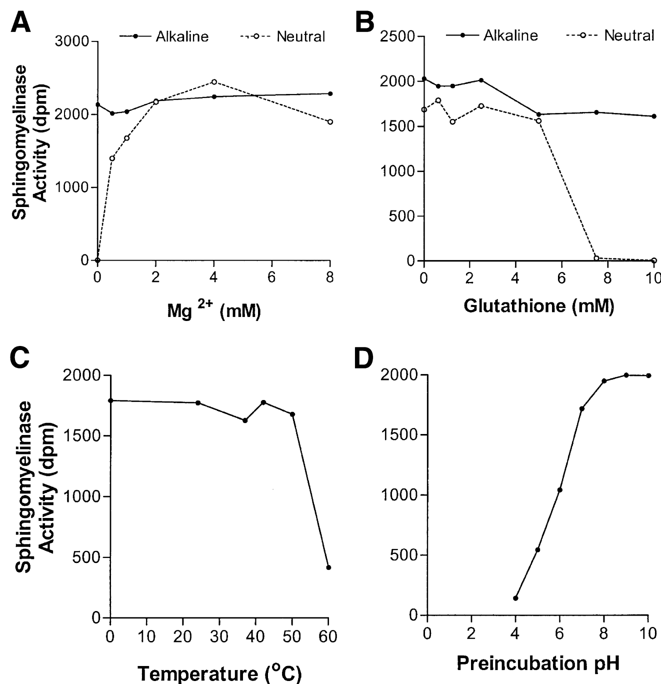

Fig. 8. Effects of magnesium and glutathione and stability of the enzyme against temperature and pH. A: Alkaline SMase and neutral SMase were assayed in the presence of different concentrations of Mg^{2+}. B: Alkaline and neutral SMases were preincubated with the reduced form of glutathione for 15 min, followed by determination of the remaining activity. C, D: Stability of intestinal alkaline SMase against temperature and pH. The purified enzyme was preincubated in the assay buffer with different temperatures for 1 h (C) or preincubated in 50 mM Tris buffers of different pH for 1 h (D). After incubation, the remaining activity was assayed in alkaline SMase assay buffer as described.

concentration of NaCl (0.075 M). Such a concentration of salt prevented the retention of the enzyme on DEAE Sepharose and, meanwhile, removed a large part of other proteins. Before going to the second step, due to a weak affinity of the enzyme on anion exchange matrix, the sample had to be desalted and high affinity anion exchange matrix had to be used. The binding of the enzyme on HQ matrix was still weak, and 0.06 M NaCl was able to elute the retained alkaline SMase from the column. For HIC, our preliminary study found that the enzyme was not retained on Butyl Sepharose and Octyl Sepharose, but was retained on Phenyl Sepharose matrix in neutral to alkaline pH. For running HIC, attention should be paid to the pH values of the buffer, because high pH values resulted in a poor retention of the enzyme and low pH would cause inactivation of the enzyme. After SE gel chromatography, a single band with a molecular mass of 58 kDa was identified by silver staining. The purification factor was 1,589, which was not high as compared with reports on the purification of acid and neutral SMase (30, 36, 37). This might be a consequence of the fact that eluting the brush border enzyme with bile salt enriched the enzyme to some degree in the starting materials.

The purified enzyme is specific against SM. Under optimal conditions it has no hydrolytic activity against PC. In

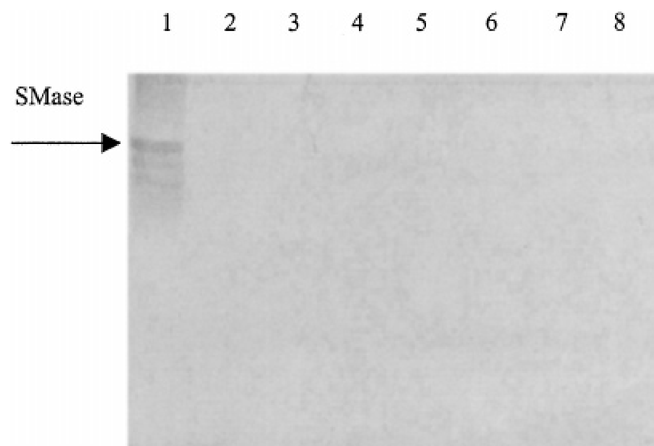

Fig. 9. Western blot showing expression of intestinal alkaline SMase in various organs of rats. Fifty micrograms of protein from homogenate of various organs was subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was probed with IgY against rat intestinal alkaline SMase and secondarily incubated with rabbit anti-IgY conjugated with alkaline phosphatase. Lane 1: rat small intestinal mucosal; lane 2: brain; lane 3: heart; lane 4: lung; lane 5: liver; lane 6: spleen; lane 7: kidney; and lane 8: pancreas.

the conditions favorable to PC-specific phospholipase C, alkaline SMase has weak activity against PC, which is less than 10% of its activity against SM at either pH 9 or pH 7.4. In addition, the enzyme has no activity against *p*-nitrophenylphosphate. These properties distinguish intestinal alkaline SMase from acid SMase purified from human placenta, which has phosphodiesterase activity against *p*-nitrophenylphosphate (30, 36).

The molecular mass of the purified enzyme is about 58 kDa, which is close to one type of neutral SMase reported recently (7). However the enzyme can not be classified as neutral SMase, because characterization demonstrated several properties clearly distinguishing it from neutral SMase. The optimal pH of the enzyme is 9 to 9.5, which is similar to previous studies with crude intestinal mucosal homogenate (9, 10). However, compared with the results from crude homogenate of intestinal mucosa, the activity of the purified enzyme at neutral pH is higher, being about 60% of that at pH 9. This indicates that some factors in the crude mucosal homogenate inhibit the activity of the enzyme at neutral pH. The independence of the enzyme to Mg^{2+} also distinguishes the enzyme from Mg^{2+}-dependent neutral SMase. Although Mg^{2+} slightly increased the activity of intestinal alkaline SMase, 2 mM EDTA failed to significantly reduce the activity of the enzyme, whereas it completely abolished the activity of neutral SMase. In addition, the reduced form of glutathione inhibited neutral SMase, but had no effect on the activity of alkaline SMase. The inhibition by glutathione of neutral SMase is in agreement with previous studies (7, 8, 38).

Although it is known that intestinal alkaline SMase is bile salt dependent, the specific actions of individual bile salts have not been closely compared. In this study, 11 bile salts were tested in concentrations up to two times their

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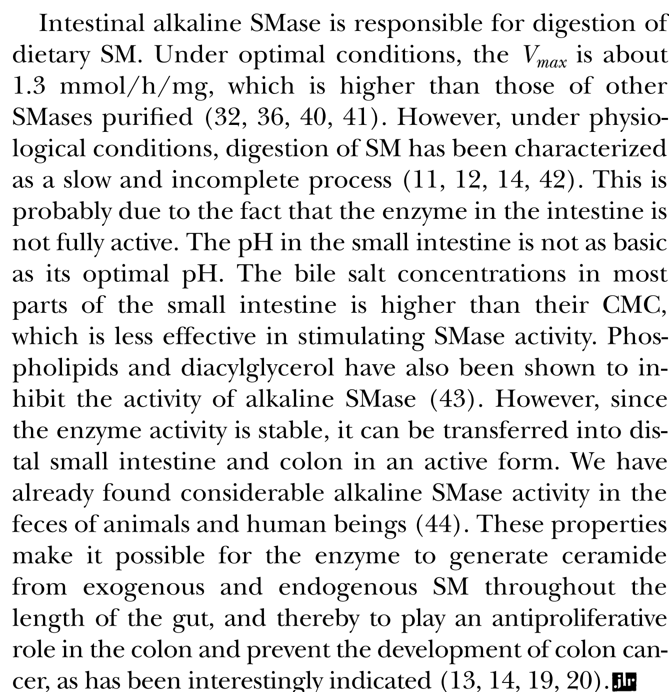
CMC for their effects. All bile salts increased alkaline SMase activity, but their efficacy varied greatly. It appears that the taurine conjugation is an important factor in stimulating SMase, as the three most effective bile salts are TC, TCDC, and TDC. If the effects of taurine conjugated bile salts are compared with their corresponding glycine conjugated ones, the effects of all taurine conjugated bile salts are more effective than glycine conjugated ones. If one compares the efficacy of TC, GC, cholate, and CHAPS, it is obvious that removing or replacing taurine from TC resulted in a marked reduction of its ability to activate alkaline SMase. These results imply a biological effect of the taurine conjugated bile salts on the enzyme, rather than a detergent effect. In addition, the α-hydroxyl group at position 7 might also be important. Absence of this group resulted in a significant reduction of their stimulatory effects on the enzyme activity. The dependence of intestinal alkaline SMase on bile salts is of specific importance, because bile salts are present in the intestine where the enzyme exists and therefore are the natural activators of the enzyme. Other types of detergents such as CHAPS and TX100 have no stimulatory effects on alkaline SMase at all. Unlike other types of SMase, intestinal alkaline SMase is the only form of SMase that requires physiological detergent for its activity. Based on this property, intestinal SMase can be called bile salt stimulated SMase.

In this study, we also showed different effects of TX100 on activities of alkaline and bacterial neutral SMases. TX100, which has been widely used in assays of neutral SMase, strongly inhibits alkaline SMase, whereas TC can equally activate both alkaline and neutral SMases. Because alkaline SMase has activity at neutral pH, it could be a problem to distinguish whether the activity is contributed by neutral or alkaline SMase, particularly when the activity is determined in cell or tissue homogenate. Using TX100 instead of TC in the neutral SMase assay and 2 mM EDTA in the alkaline SMase assay may be a simple method to diminish the overlap of the activities of the two enzymes under different pH values. This is important for the samples from gastrointestinal tract, where both neutral and alkaline SMase are present.

Generally speaking, intestinal alkaline SMase is relatively stable. Trypsin and chymotrypsin at concentrations as high as 1 mg/ml did not affect its activity, in agreement with previous studies with crude mucosal homogenate. The activity was largely unchanged after preincubation of the enzyme at temperatures up to 50°C. In addition, DTT, even at 10 mM, only decreased the activity by 25%, and glutathione had no effect on its activity. From these results, it appears that the critical structure of the active site of rat alkaline SMase is not related to peptide bonds formed by arginine or lysine or to disulfide bonds. However, the activity of the enzyme is labile at acid pH, and preincubation of the enzyme at pH lower than 7 resulted in a significant reduction of its activity.

The expression of the enzyme appears tissue-specific as Western blot identified a major band at 58 kDa and two smaller bands in the homogenate of small intestinal mucosa. The smaller bands may represent degraded forms of the enzyme. However, no positive band could be identified in the samples from other tissues. This may indicate that the enzyme is only expressed in the intestinal tissue or that its levels in other tissues are too low to be identified by the methods used in this article. We have measured the activities of three types of SMase in various organs and found acid and neutral SMase activities were generally identified in all tissues, but alkaline SMase activity was found only in the small intestine.



In human bile there is another type of alkaline SMase (39) that has been purified and characterized (32). Both alkaline SMases share several important properties, such as bile salt dependence, Mg^{2+} independence, and alkaline pH optimum. However, the molecular weight of human bile alkaline SMase is higher, and its activity at neutral pH as well as its specific activity are lower as compared with rat intestinal alkaline SMase. In addition, human bile alkaline SMase is retained on DEAE Sepharose matrix, but rat intestinal alkaline SMase is not. The mechanism behind these differences remains unclear.

Intestinal alkaline SMase is responsible for digestion of dietary SM. Under optimal conditions, the V_{max} is about 1.3 mmol/h/mg, which is higher than those of other SMases purified (32, 36, 40, 41). However, under physiological conditions, digestion of SM has been characterized as a slow and incomplete process (11, 12, 14, 42). This is probably due to the fact that the enzyme in the intestine is not fully active. The pH in the small intestine is not as basic as its optimal pH. The bile salt concentrations in most parts of the small intestine is higher than their CMC, which is less effective in stimulating SMase activity. Phospholipids and diacylglycerol have also been shown to inhibit the activity of alkaline SMase (43). However, since the enzyme activity is stable, it can be transferred into distal small intestine and colon in an active form. We have already found considerable alkaline SMase activity in the feces of animals and human beings (44). These properties make it possible for the enzyme to generate ceramide from exogenous and endogenous SM throughout the length of the gut, and thereby to play an antiproliferative role in the colon and prevent the development of colon cancer, as has been interestingly indicated (13, 14, 19, 20).

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