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*Published in:*

Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids

*DOI:*

[10.1016/j.bbalip.2004.11.006](https://doi.org/10.1016/j.bbalip.2004.11.006)

2005

[Link to publication](#)

*Citation for published version (APA):*

Wu, J., Cheng, Y., Palmberg, C., Bergman, T., Nilsson, Å., & Duan, R.-D. (2005). Cloning of alkaline sphingomyelinase from rat intestinal mucosa and adjusting of the hypothetical protein XP\_221184 in GenBank. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, 1687(1-3), 94-102. <https://doi.org/10.1016/j.bbalip.2004.11.006>

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**Cloning of alkaline sphingomyelinase from rat intestinal mucosa and adjusting of the hypothetical protein XP\_221184 in GenBank**

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## **Abstract**

Intestinal alkaline sphingomyelinase (alk-SMase) digests sphingomyelin and the process may influence colonic tumorigenesis and cholesterol absorption. We recently identified the gene of human alk-SMase and cloned the cDNA (J Biol Chem 278:38528-38536, 2003). Cross-species screening of homology in GenBank found a hypothetical rat protein XP\_221184 with 491 amino acid residues, which shares 73% identity with human alk-SMase. Based on the cDNA sequence of the protein, we cloned a cDNA from rat intestinal mucosa by RT-PCR. The cloned cDNA encodes 439 amino acid residues 85% identical with those of human alk-SMase. It differed from the XP\_221184 cDNA in splice sites linking exons 2 and 3, and exons 3 and 4, respectively. In the translated protein sequence the predicted activity motif, sphingomyelin binding sites, and potential glycosylation sites in human alk-SMase are all conserved. Native alk-SMase was purified from rat intestine and subjected to proteolytic digestion followed by MALDI mass spectrometry and ESI tandem mass spectrometry. Seven tryptic peptides were found to match the cloned protein sequence. Transient expression of the cloned cDNA linked with a myc tag in COS-7 cells demonstrated high SMase activity, with an optimal pH at 9.0 and a specific dependence on taurocholate and taurochenodeoxycholate. The expressed protein reacted with both anti-myc and anti-human alk-SMase antibodies. Northern blotting of rat tissues revealed high levels of mRNA in jejunum but not in other tissues. In conclusion, we cloned rat alk-SMase cDNA from rat intestine, adjusted the putative rat alk-SMase in GenBank, and confirmed the specific expression of the gene in the small intestine.

## Introduction

Alkaline sphingomyelinase (alk-SMase) was first found in the intestinal tract of human, rat and pig by Nilsson [1]. The enzyme hydrolyses sphingomyelin (SM) in the gut to ceramide, which is in turn hydrolyzed by ceramidase to sphingosine. Both ceramide and sphingosine are important signalling molecules that inhibit cell proliferation and induce differentiation and apoptosis [2]. SM digestion in the gut may have physiological and pathophysiological implications. Previous studies found that supplement of SM and ceramide analogues in the diet inhibited the development of chemically induced colon cancer in mice [3,4]. We reported that the activity of alk-SMase, the most abundant SMase in the intestine, is significantly reduced in colon adenoma and carcinoma tissues [5,6]. Recently, we also identified a mutant alk-SMase mRNA which encodes an inactive alk-SMase in HT-29 cells, a poorly differentiated colon cancer cell line [7].

We have purified alk-SMase from human and rat intestine [8,9], identified the gene of human alk-SMase and cloned the human alk-SMase cDNA [10]. The human enzyme is a type of ecto-enzyme sharing 32% identity with the nucleotide phosphodiesterase (NPP) family. The two metal binding sites in NPP formed by 6 amino acid residues were conserved and the predicted active core of NPP was modified in human alk-SMase. Human alk-SMase has transmembrane domains at both N- and C-terminals. The N-terminal domain is a predicted signal peptide and that at the C-terminal is a signal anchor [10,11]. The enzyme is hooked on the brush border membrane with the C-terminal anchor. Pancreatic trypsin cuts the anchor and releases the enzyme into the intestinal lumen [11]. Human alk-SMase is a glycoprotein with five potential N-glycosylation sites. [10].

Based on the human alk-SMase sequence, BLAST searches in GenBank found a predicted rat protein XP\_221184, which contains 491 amino acid residues 73% identical with

those in human alk-SMase. This means that the mass of the hypothetical protein is greater than the mass of human alk-SMase, which is not in agreement with our previous findings that the mass of rat alk-SMase purified from small intestine was smaller than that of human enzyme (458 residues) [10]. Whether this predicted protein is correct and its cDNA does encode rat alk-SMase thus requires clarification. In the present work, on the basis of the cDNA sequence XP\_221184, we cloned rat intestinal alk-SMase from rat intestinal mucosa, compared the enzyme with human alk-SMase at molecular level, adjusted the sequence of rat alk-SMase, and studied its expression in gut and other tissues.

### **Materials and Methods**

**Materials.** Female Sprague-Dawley rats weighing about 200 g were obtained from Mollegaard, Denmark. COS-7 cells were purchased from American Tissue Culture Collection. SM was purified from bovine milk and labeled with [ $^{14}\text{C}$ -CH $_3$ ]choline ([ $^{14}\text{C}$ -SM]) [12]. Plasmid pCDNA4/TO/Myc-His B, lipofectamine<sup>TM</sup> 2000, Ready-To-Go RT-PCR Beads, mouse anti-myc antibody and all primers used were purchased from Invitrogen (Paisley, UK). Quickprep<sup>TM</sup> total RNA extraction kit, and GFX<sup>TM</sup> DNA and gel band purification kit, sheep anti-mouse IgG antibody conjugated with horseradish peroxidase, donkey anti-rabbit IgG antibody conjugated with horseradish peroxidase, *Gene Images* Random Prime Labeling Module and *Gene Images* CDP-*Star* detection module were obtained from Amersham Biosciences (Uppsala, Sweden). RNeasy<sup>TM</sup> RNA stabilization Reagent was purchased from QIAGEN GmbH (Hilden, German). Zeta-probe GT membrane was purchased from Bio-Rad Laboratories AB (Sundbyberg, Sweden). All cell culture media and other chemical agents used were purchased from Sigma Co (Stockholm, Sweden).

**RT-PCR.** Rats were fasted overnight and anesthetized by i.m. injection of a mixture of ketamin and xylazin (1:2). A 5 cm long segment of jejunum was cannulated and washed with 20 ml PBS. The segment was then tied in both ends and about 1 ml of RNeasy RNA

stabilization reagent was injected into the lumen. The segment was removed and intestinal mucosa was scraped. Total RNA was extracted and purified by Total RNA Extraction kit. Rat alk-SMase cDNA was amplified by Ready-To-Go RT-PCR Beads using the total RNA as template, oligo (dT)<sub>12-18</sub> as first-strand primer, and sense primer 5'atggtaccgaaagcatggggcactcagctgtcct3' and antisense primer 5'atgcggccgcctacgaccttggccagaccg3'. The RT-PCR program was 42°C 30 min for reverse transcription, 95 °C 5 min to inactivate reverse transcriptase, followed by 95°C 25 sec, 55°C 30 sec, and 72°C 180 sec for 35 cycles. The RT-PCR products were isolated electrophoretically on 1% agarose gel and the 1.3 kb DNA fragment was purified by GFX DNA purification kit. The DNA fragment was digested with KpnI/Not I and constructed into similarly digested pcDNA4/TO/myc-His B plasmid. The cDNA inserts in the recombinant plasmid were sequenced by Cybergene (Huddinge, Sweden) using sense 5'cgcaaatggcggttaggcgtg3' and antisense 5'tagaaggcacagtcgagg3'primers of the vector.

**Transient expression.** COS-7 cells were cultured in DMEM medium with 2 mM glutamine and 4500 mg/l glucose, containing 100 IU/ml penicillin, 10 µg/ml streptomycin and 10% heat inactivated fetal calf serum. At about 90% confluence, the cells were transfected with 4 µg of the constructed plasmid with rat alk-SMase cDNA in the presence of lipofectamin 2000. Control cells were transfected with the mock plasmid in a similar way. After 48 h, an aliquot of the cell culture medium was collected. The cells were scraped and centrifuged. The cell pellets were lysed in 100 µl buffer containing 50 mM Tris-HCl, 1 mM PMSF, 2 mM EDTA, 0.5 mM dithiothreitol, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 10 mM taurocholate as described previously [10].

**Alk-SMase assay.** The alk-SMase activity was determined according to Duan and Nilsson [13]. Briefly, 5 µl sample was incubated in 95 µl 50 mM Tris-HCl buffer, containing 0.15 M NaCl, 2 mM EDTA, 10 mM taurocholate (assay buffer), 0.8 µM [<sup>14</sup>C-SM] and 100 µM SM

for 30 min. The reaction was stopped by adding 0.4 ml of chloroform/methanol (2:1, v/v) and the cleaved phosphocholine in the upper phase was determined by liquid scintillation counting. The protein concentration was analyzed by a kit obtained from Bio-Rad using bovine albumin as standard.

**Western blotting.** Western blotting of expressed alk-SMase was performed as described elsewhere [10]. In brief, the cell lysate containing 50 µg cellular proteins was subjected to 10% SDS-PAGE and then transferred to nitrocellulose membrane electrophoretically. After blocking, the membrane was probed with anti-myc (1:10000) or anti-human SMase antibody (1:5000) for 1.5 h and then reacted with anti-mouse IgG antibody (1:50000) or anti-rabbit IgG antibody (1:50000) conjugated with horseradish peroxidase for 1.5 h. The alk-SMase bands were identified by ECL advanced reagents and the emitted light was recorded on Kodak X-ray film.

**Identification of tryptic peptides from alk-SMase purified from rat intestine.** Rat intestinal alk-SMase was purified in our laboratory as described [9]. The enzyme preparation was subjected to 10% SDS-PAGE and stained with Coomassie blue. The stained protein band was cut from the gel and placed in an Eppendorf tube for in gel digestion [14]. Washing was carried out in 0.2 M ammonium bicarbonate containing 50% acetonitrile. The protein was reduced using dithiothreitol and alkylated with iodoacetamide, followed by in-gel digestion with 0.5 µg of trypsin (Promega, modified) in 0.2 M ammonium bicarbonate overnight at 37°C [14]. The tryptic peptides were extracted using acetonitrile in 0.1% trifluoroacetic acid, first at 60% and then at 40%. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry and liquid chromatography electrospray ionization (ESI) tandem mass spectrometry of tryptic fragments were carried out using a Voyager DE Pro instrument (Applied Biosystems) and a QTOF ultima API instrument (Waters), respectively.



Identification was based on a comparison of theoretical mass values and sequences for tryptic fragments derived from the cDNA sequence and those found in the in-gel digest [15].

**Enzyme characterization.** To identify the optimal pH of the expressed enzyme, the SMase activity was determined in buffers of various pH, as described previously [9]. Since previous studies have shown that bile salts at critical micellar concentration has maximal stimulatory effect on alk-SMase [9], the effect of bile salt on alk-SMase activity was determined at the following critical micellar concentrations: 10 mM for taurocholate (TC) and glycocholate (GC), 1.5 mM for taurochenodeoxycholate (TCDC), 6 mM for taurodeoxycholate (TDC) and glycodeoxycholate (GDC). As a comparison, the effect of 0.12% Triton X-100 was also examined.

**Northern blotting.** Rats were anesthetized by i.m. injection of a mixture of ketamin and xylazin (1:2). Tissue samples of stomach, duodenum, jejunum, ileum, colon, liver, pancreas, brain, lung, heart, spleen, kidney, and skeleton muscle were removed and put into 1ml of *RNAlater* RNA stabilization Reagent. Total RNA was extracted and purified and 20 µg of total RNA from each tissue was separated by electrophoresis and transferred to Zeta-probe GT nylon membrane by capillarity and fixed for 0.5 h at 80 °C. The membrane was stained with methylene blue to show 28S and 18S rRNAs as an index for equal amount of total RNA loaded. The membrane was photographed and destained with 0.2 x SSC containing 1% SDS. The cloned rat alk-SMase cDNA was cleaved from constructed plasmid with KpnI/NotI, and labeled with fluorescein according to the instruction of the manufacturer. The RNA transferred to the nylon membrane was then hybridized with the labeled probe at 65°C overnight. After washing and blocking, the membrane was probed with anti-fluorescein antibody conjugated with alkaline phosphatase (1:5000). The labeled mRNA was identified by CDP-*Star* detection module and the emitted light was recorded on Kodak X-ray film.

## Results

**Cloning of rat alk-SMase cDNA.** Using primers designed on the basis of the GenBank sequence XP\_221184, RT-PCR identified a 1.3 kb cDNA fragment from rat intestinal mucosa. The cDNA was cloned into a mammalian expression vector with a myc tag and sequenced. The results are shown in Fig. 1. The reading frame contains 1320 bp, which encodes a protein with 439 amino acid residues. To examine whether this gene product is alk-SMase, rat alk-SMase purified from rat small intestine [9] was subjected to trypsin digestion followed by MALDI mass spectrometry and ESI tandem mass spectrometry. The masses of seven tryptic peptides and sequence information for five of these were determined and found to match theoretical data for tryptic fragments derived from the amino acid sequence of the cloned cDNA.

**Transient expression of the cDNA and characterization of the expressed protein.**

To further confirm that the cloned cDNA encodes alk-SMase, the cDNA with a myc tag was expressed in COS-7 cells. As shown in Fig. 2, transient expression resulted in a sharp increase of alk-SMase activity in COS-7 cells from zero to 60 nmole/h/mg (upper panel). The activity in the cell culture medium (middle panel) was also increased from zero to 6.3 nmole/h/ml, compared with the cells transfected with the mock vector. Western blotting identified a 58 kD protein cross-reacting with anti-myc and anti-human alk-SMase antibodies in the COS-7 cells transfected with cloned rat alk-SMase cDNA, but not in the cells transfected with the mock vector (bottom panel).

Being similar to the characteristics of the purified rat alk-SMase from rat intestinal mucosa [9], the expressed enzyme showed highest SMase activity at pH 9.0 (Fig. 3, upper panel). Another specific property of intestinal alk-SMase is the selective dependency of the enzyme activity on TC and TCDC [9]. This was also the case for the expressed enzyme as shown in the lower panel of Fig. 3. TC and TCDC are the most effective bile salts to activate alk-SMase. Also in good agreement with previous findings was that the taurine conjugated

bile salts were more effective than the glycine conjugates (Fig.3, lower panel). Like the rat and human enzymes earlier purified from intestine, the activity of the expressed rat-SMase was completely inhibited by Triton X 100.

**Sequence comparison of the expressed rat alk-SMase with human alk-SMase and the hypothetical protein XP\_221184.** A comparison of the cloned rat alk-SMase with human alk-SMase and the protein XP\_221184 is shown in Fig. 4. The cloned rat alk-SMase shared 85% identity with human alk-SMase, which is higher than the corresponding value i.e. 73% for XP\_221184. The putative active motif TMTSPCH (residues 71-78), the amino acids forming the metal ion binding sites (D38, D198, H202, D245, H246, and H352), and the potential N-glycosylation sites (99,120,145,167,266) in human alk-SMase are all conserved in the cloned enzyme as well as in XP\_221184. However, one segment containing 18 amino acid residues, from H414 to G431, was found in human alk-SMase but not in rat enzyme and XP\_221184.

When the cloned rat alk-SMase was compared with the predicted XP\_221184 protein, two sequences in XP\_221184 were not found in the cloned rat enzyme. The first is the sequence from V133 to Q187 and the second is that from S398 to P403. These two sequences were not present in human alk-SMase either. There is a tryptic site (Arg) at residue 342 for rat alk-SMase and 343 for human alk-SMase. Mass spectrometry on purified rat alk-SMase verified the existence of the Arg in the native enzyme. But the Arg is not presented in the predicted protein XP\_221184.

We then examined rat genomic DNA sequence (NW\_047902) and found that the cloned rat alk-SMase cDNA differed from XP\_221184 at four splice sites, which link exons 2 and 3, and exons 3 and 4. As shown in Fig 5, in the cDNA of XP\_221184, the exons 2 and 3 were predicted to be linked with sites 737 and 1875, and exons 3 and 4 were linked with sites 2645 and 4508. Our cloned alk-SMase from intestinal mucosa showed that the exons 2 and 3 are

actually linked with sites 695 and 1998 and exons 3 and 4 are linked with sites 2624 to 4478. It is the difference between the native splice sites and those predicted sites that caused the addition of two segments in XP\_221184, deletion of Arg 342, and increasing of the translated protein mass.

In the analysis by TMPred, we found that rat alk-SMase, similar to human alk-SMase, has one transmembrane domain in both N (G2-V19) and C (Q421-V439) terminals (Fig 6). However, the sequences forming the hydrophobic domains in rat alk-SMase differ significantly from those in human alk-SMase (Table 1). It is notable that in both human and rat alk-SMase, there is an Arg upstream the C-terminal transmembrane domain (position 421 for rat and 440 for human). The significance of this tryptic site will be discussed later.

**Alk-SMase expression in rat organs.** To identify the expression of the alk-SMase gene in different organs, Northern blotting of total RNA from the organs of the gastrointestinal tract as well as from other organs was performed. As shown in Fig 7, high levels of alk-SMase mRNA was identified in jejunum. By this method, alk-SMase mRNA was hardly detected in stomach, duodenum, ileum, colon, liver, and pancreas, and neither in other tissues including brain, heart, lung, spleen, kidney, and skeletal muscle (data not show).

## Discussion

Intestinal alk-SMase is an enzyme that has recently gained increasing attention due to its potential roles in digestion of dietary SM, cholesterol absorption, and colonic carcinogenesis [16-19]. The enzyme is located on the surface of the brush border membrane as an ecto-enzyme and it hydrolyses both endogenous and exogenous SM in the intestinal tract [8,10]. Although the activity of the enzyme has been found in the intestinal tract of many species, purification of the enzyme from intestine was not successful until recently [8,9]. Up to date, only the cDNA of human alk-SMase has been cloned [8] and no cloning of the enzyme directly from normal intestinal tissue has been reported. The present study, for the first time,

cloned the enzyme from rat intestinal tract, clarified a discrepancy between a predicted alk-SMase protein in GenBank and the native purified enzyme, and compared the rat enzyme with the human form at molecular levels.

The following pieces of evidence indicate that the cDNA that we cloned does code for rat intestinal alk-SMase. First, the cDNA was cloned directly from rat intestinal mucosa and the amino acid sequence shares 85% identity with human alk-SMase, which is higher than for the predicted protein XP\_221184. All the critical domains of alk-SMase including the predicted active core, the metal binding sites which we predicted to be the site for SM binding [10], and the glycosylation sites are conserved. The rat enzyme lacks a sequence containing 18 amino acid residues from H414 to G431 present in human alk-SMase, which is in good agreement with our previous finding that rat intestinal alk-SMase is about 2 kDa smaller than human alk-SMase [8]. Secondly, MALDI mass spectrometry and ESI tandem mass spectrometry of proteolytic fragments from the native enzyme purified from intestinal mucosa identified seven tryptic peptides that match the translated protein sequence from the cloned cDNA. Thirdly, expressing the cloned cDNA in COS 7 cells demonstrates high SMase activity with alkaline pH optimum. The enzyme activity, similar to purified alk-SMase from human and rat, was specifically dependent on TC and TCDC, and inhibited by other detergents such as Triton X 100 or CHAPS. Such a dependency on physiological detergents and an inhibition by other non-physiological detergents have never been reported for any other type of SMase. Finally, the expressed enzyme with a myc-tag cross-reacted with both anti-myc and anti-human alk-SMase antibodies in Western blotting. Both the cDNA and amino acid sequences have been submitted to GenBank as rat intestinal alk-SMase (AY 568760).

As mentioned above, XP\_221184, although sharing 73% homology with the human alk-SMase sequence, is probably not a cDNA sequence representing the protein present in native

rat intestinal tract coding for rat alk-SMase. RT-PCR in this study did not identify a product, which is greater than the one coding for our cloned alk-SMase. Other evidence against the presence of the XP\_221184 cDNA in the intestine comes from tryptic digestion and mass spectrometry of fragments of purified native rat alk-SMase from small intestine. Seven peptides matched the cloned enzyme sequence and the last one has M/Z 1937.96, corresponding to the sequence from I326 to R342. Because the cleavage site R342 is not present in the predicted XP\_221184 protein sequence, if rat alk-SMase is derived from XP\_221184 cDNA, this tryptic segment would not be identified (Fig. 1 and 4). By analysis of the genomic sequence, we showed that the presented sequence of XP\_221184 is a consequence of a misprediction of splice sites. The misprediction results in an insert of a sequence containing 55 amino acid residues and causes other alterations including the deletion of R342 in XP\_221184. Whether XP\_221184 is present in other organs or in other pathological conditions is unknown. Since RT-PCR did not identify a larger product and Northern blotting did not show positive bands in other organs, the possibility of the presence of XP-221184 cDNA is small.

The cloning of rat alk-SMase cDNA identifies, for the first time, the full sequence of the enzyme. TMpred analysis found that the enzyme is also an ecto-enzyme with a signal peptide at the N-terminal and a signal anchor at the C-terminal. Although the amino acid residues forming the transmembrane domains in the rat enzyme differ from those in the human enzyme, an Arg residue is similarly located just upstream the C-terminal anchor in both enzymes. This residue has been suggested to be important for release of the enzyme from mucosal membrane to the intestinal lumen, as we recently showed that pancreatic trypsin rapidly cleaves the enzyme, presumably by attacking the Arg residue upstream of the C-terminal anchor [11]. In addition, we also found a segment of 18 amino acid residues (H414 to G431) located upstream of the C-terminal anchor in human alk-SMase but not in rat alk-

SMase. The function of this domain is likely to be nonessential for SMase activity. Due to the location upstream of the predicated C-terminal transmembrane anchor, it may serve as an arm for the ecto-enzyme. The question whether the length of the arm affects exposure of the enzyme to the substrate both in the lumen and in the membrane, or alternatively affects the stability of the enzyme on the brush border needs further investigation.

Expression of alk-SMase is tissue specific. High activity was previously found in the middle of the jejunum in many species and additionally in human bile [1,20,21]. Western blotting using an antibody against rat alk-SMase has shown the presence of the protein only in the intestine not in other organs [9]. In the present study, Northern blotting only found high expression of the enzyme in jejunum and not in other organs including the liver. The results confirm the previous findings in Western blotting and imply that the low protein levels of alk-SMase in many organs other than intestine is a consequence of inhibited transcription, not a reduced translation rate. High levels of alk-SMase mRNA was previously found in human liver [10] but was not found in rat liver in this study. The result is also in agreement with previous findings that alk-SMase activity is only found in human bile not in the bile of rat or many other examined species [22].

### **Acknowledgement**

The study was supported by grants from the Swedish Cancer Foundation, the Swedish Research Council, the Albert Pålsson Foundation, the Crafoord Foundation, the Gunnar Nilsson Cancer Foundation, and the Foundation of Lund University Hospital.

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Table 1. The amino acid residues forming the transmembrane domains at N- and C-terminals of human and rat intestinal alk-SMase.

	N-terminal Inside to outside	C-terminal Outside to inside
Human	GLAVLLTVALATLLAPGAGA-21	<i>R440</i> -PLLVMGLLGTVILLSEV457
Rat	RGLAVLLTVALATLLATL-19	<i>R420</i> -QHHLVVVLMGILTGLAKVV439

## Figure legends

**Fig. 1.** Nucleotide and amino acid sequences of rat alk-SMase. The figure shows the reading frame of the cloned rat alk-SMase cDNA from intestine and the translated amino acid sequence. The numbers of nucleotides are shown on the right side and those of the amino acids on the left side. MALDI mass spectrometry and ESI tandem mass spectrometry after trypsin digestion was performed on rat alk-SMase purified from intestinal mucosa. Seven tryptic segments were found to match the translated enzyme sequence, as underlined in the figure. The amino acid residues identified in 5 of the 7 tryptic digests by ESI tandem mass spectrometry were bolded.

**Fig. 2.** Alk-SMase in COS-7 cells after transient expression. COS-7 cells were transfected with cloned rat alk-SMase cDNA constructed in pcDNA4TO/Myc-His B. The control cells were transfected with the plasmid alone. 48 h after transfection, the alk-SMase activities in both cell-free extract (upper panel) and cell culture medium (middle panel) were determined. The results are mean  $\pm$  SE from three experiments. In the bottom panel, the cell-free extract was subjected to Western blot with anti-myc and anti-human alk-SMase antibodies.

**Fig 3** Characterization of rat alk-SMase expressed in COS-7 cells. SMase activity in cell-free extract from rat alk-SMase transfected COS-7 cells was determined in buffers with different pH values (upper panel). In the lower panel, the activities were determined in the assay buffer optimal for alk-SMase in the presence of various bile salts at critical micellar concentrations. Results are mean  $\pm$  SE from three separate experiments.

**Fig 4** Multiple alignment of cloned rat alk-SMase, human alk-SMase and the rat hypothetical protein XP\_221184. The cloned rat alk-SMase sequence was compared with those of human alk-SMase (AY230663 in GenBank) and the protein XP\_221184. Identities are indicated as dashes. Dots indicate the residues not existing. The putative activity motif is marked with \*,

the potential glycosylation sites are marked with  $\alpha$ , and the residues forming the metal binding sites are marked with #.

**Fig. 5.** The difference in splice sites between the cloned rat alk-SMase and the hypothetical protein XP\_221184. The cDNA sequences of cloned rat alk-SMase and the protein XP\_221184 were examined in relation to the rat genomic sequence (NW\_047902). The solid horizontal bars indicate exons and the thin lines indicate introns. The numbers of different splice sites between the cloned protein and the predicted protein are indicated.

**Fig. 6.** Comparison of the predicted transmembrane domains of rat and human alk-SMases analyzed by TMpred. The N-terminal domain is from inside to outside and the C-terminal domain from outside to inside. The X axis is the amino acid sequences of the enzyme and the Y axis is the hydrophobicity of the residues.

**Fig. 7.** Expression of alk-SMase in organs of rat gastrointestinal tract. Total RNAs were extracted from different organs of rat gastrointestinal tract and 20  $\mu$ g RNA was subjected to Northern blotting. The cloned rat alk-SMase cDNA was labeled with fluorescein and the hybridization bands were reacted with anti-fluorescein antibody conjugated with alkaline phosphatase. The positive bands were visualized using a *Gene Images CDP-Star* detection module. The low panel shows the levels of 28S and 18S RNAs stained with methylene blue in the samples loaded.

Fig1

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1 atggggcactcagctgtcctcctctctgtggctctagtcacccctcccagcttgtgtgact 60
 1 M G H S A V L L S V A L V I L P A C V T
61 gggggtcctgtccaaaggcagcagcagcataagcttctccttgtatccttcgatggcttc 120
 21 G G P V Q R Q Q Q H K L L L V S F D G F
121 cgctggaactatgaccaagacgtggaaacccctaacctggactccatggctcaggaagga 180
 41 R W N Y D Q D V E T P N L D S M A Q E G
181 gtgaaagctcgctacatgactcctgcctttgtcaccatgaccagcccctgccactttaca 240
 61 V K A R Y M T P A F V T M T S P C H F T
241 ctgggtcacccggcaaatacatcgagaacccatgggggtgggtccacaacatgttctataacacc 300
 81 L V T G K Y I E N H G V V H N M F Y N T
301 accaacaaggtgagggctgccctaccatgccacgcttgggatccagaggtgggtgggataac 360
101 T N K V R L P Y H A T L G I Q R W W D N
361 ggcagcataccatctggatcacagcccagaggcagggcttaaaaaccggctccttcttc 420
121 G S I P I W I T A Q R Q G L K T G S F F
421 taccctggtgggaacgtcacctaccaaggagaggccgtgacgatgagccggaaggaaggc 480
141 Y P G G N V T Y Q G E A V T M S R K E G
481 gtcttacacaattacaaaaacgagacagagtggaggggcaaagtggacacagtgtatgaag 540
161 V L H N Y K N E T E W R A N V D T V M K
541 tgggtcacggaggaggatgtgtccctgggtcactctctactttggggagccagactccact 600
181 W F T E E D V S L V T L Y F G E P D S T
601 ggccacaagtatggccctgagtcgccagagaggaaaggacatgggtgaaacaggtagacagg 660
201 G H K Y G P E S Q E R K D M V K Q V D R
661 acggtgggctacctccgggacagcatcaagcgccaccacctcacagacagcctcaacctg 720
221 T V G Y L R D S I K R H H L T D S L N L
721 ataatcacctcggaccacggcatgacgaccgtcaacaagaaggccagcgacttgggtcgag 780
241 I I T S D H G M T T V N K K A S D L V E
781 ttccacaagttccccaacttcaccttcagggacattgagttcgaactcttggactacggg 840
261 F H K F P N F T F R D I E F E L L D Y G
841 cccaacgggatgctgatccccaaggaagggatgctggagaaggtatacagtgtcctcaag 900
281 P N G M L I P K E G M L E K V Y S V L K
901 gatgcccaccccaggctgcatgtgttacaagaaggaggacttcccgaagacctttcactac 960
301 D A H P R L H V Y K K E D F P K T F H Y
961 gccaaacaaccccaggatcacatccctgctcatgtacagtgcctcggctatgtcattcat 1020
321 A N N P R I T S L L M Y S D L G Y V I H
1021 gggagagtgaacgtccagttcaacagtggtgagcatggtttcgacaaccaagacatggac 1080
341 G R V N V Q F N S G E H G F D N Q D M D
1081 atgaagaccatcttccgggctgtgggtcccgacttcaaggcaggcttggagtgagccc 1140
361 M K T I F R A V G P S F K A G L E V E P
1141 ttcgagagcgtccatgtgtacgagctcatgtgtcagctgctgggcattgtgcccagagccc 1200
381 F E S V H V Y E L M C Q L L G I V P E P
1201 aacgatgggcatccaggcgtcctgcaacccatgctccgggtcaggatctccgctcagcagg 1260
401 N D G H P G V L Q P M L R S G S P L S R
1261 cagcaccacttgggtgggtggtgttgatggggatcctaacgggtctggccaaggtcgataaa 1320
421 Q H H L V V V L M G I L T G L A K V V - 439

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Fig2

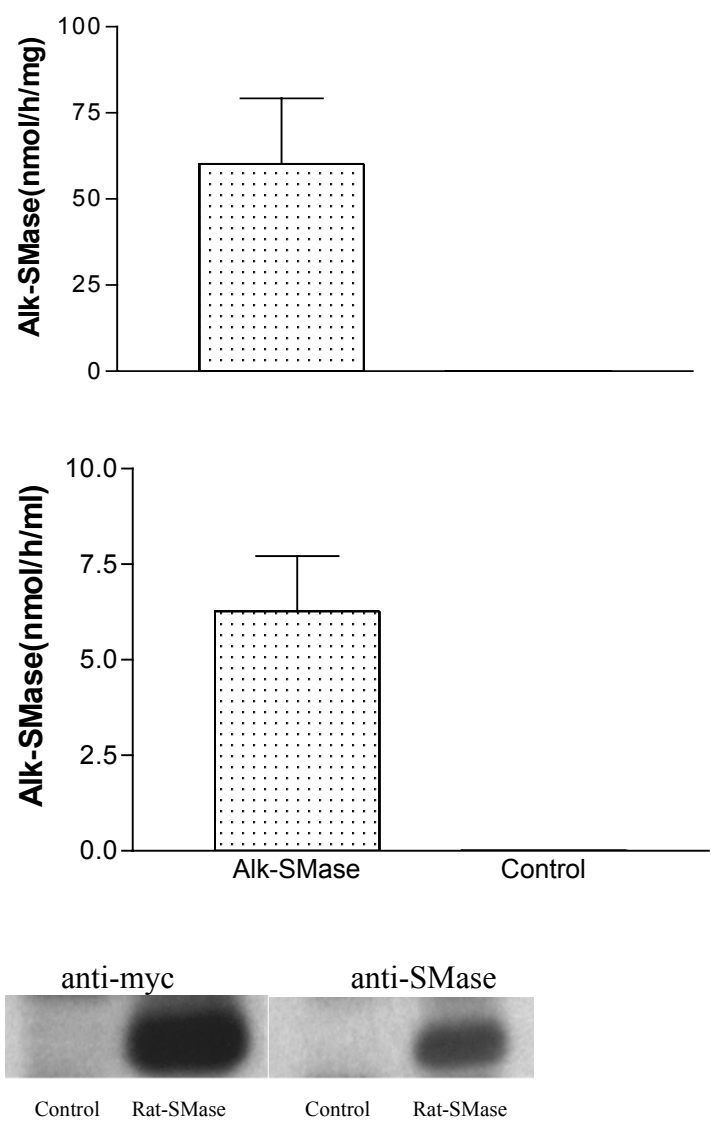


Fig 3

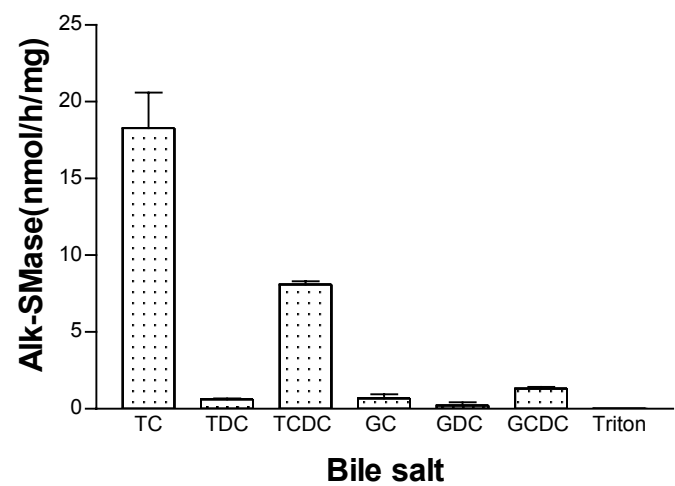
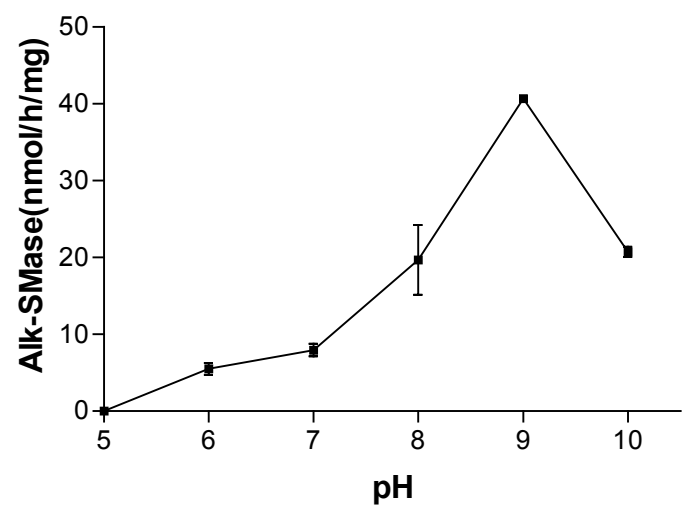


Fig 4

			#		
Rat	1	MGHSAVLLSVALVILPACVTGGPVQRQQ.QHKLLLVSFDFGRWNYDQDVE	49		
Human	1	MRGP-----T---AT-L-PGA-A---S-GS-N-----D	50		
XP_221184.2	1	-----R-----	49		
		*****			
Rat	50	TPNLDSMAQEGVKARYMTPAFVTMTSPCHFVLVTGKYIENHGVVHNMFYN	99		
Human	51	-----A-RD-----Y--	100		
XP_221184.2	50	-----	99		
Rat	100	TTNKVRLPYHATLGIQRWWDNGSIPIWITAQRQ.....	132		
Human	101	--S-K-----V-----	133		
XP_221184.2	100	-----VRRPARRETEAVTNTKG	149		
Rat	133	.....GLKTGSFFYPGG	144		
Human	134	.....--RA-----	145		
XP_221184.2	150	FKAVGKENEVAQTEKQASSGEGARQDKESWTLHPLTLQ-----	199		
Rat	145	NVTYQGEAVTMSRKEGVLHNYKNETEWRANVDVMKWFTEEDVSLVTLYF	194		
Human	146	-----V---R---IA-----I---A-----LD-----	195		
XP_221184.2	200	-----	249		
		# #			
Rat	195	GEPDSTGHKYGPESQERKDMVKQVDRTVGYL RDSIKRHHLTD SLNLIITS	244		
Human	196	-----R---P---RE---R-----E---A-N---R-----	245		
XP_221184.2	250	-----	299		
		##			
Rat	245	DHGMTTVNKKASDLVEFHKFPNFTFRDIEFELLDYGPNGLIPKEGMLEK	294		
Human	246	-----D-RAG-----L---R---	295		
XP_221184.2	300	-----	349		
Rat	295	VYSVLKDAHPRLHVYKKEDFPKTFHYANNPRITSLLMYS DLGYVIHGRVN	344		
Human	296	--DA-----K-----A---EA-----V-P-----I-	345		
XP_221184.2	350	-----...	396		
		#			
Rat	345	VQFNSGEHGFDNQDMDMKTIFRAVGPSFKAGLEVEPFESVHVYELMCQLL	394		
Human	346	---N-----K-----R-----R--	395		
XP_221184.2	397	-SGLLQP-----	446		
Rat	395	GIVPEPNDGHPGVLQPML.....RSGSPLSRQHHLVV	427		
Human	396	-----A---LAT-L---HTESALPPDGRPTLLPKG--AL-P-SRPL--M	445		
XP_221184.2	447	-----	479		
Rat	427	VLMGILTGLAKVV	439		
Human	446	G-L-TVIL-SE-A	458		
XP_221184.2	480	-----	491		



Fig. 5.

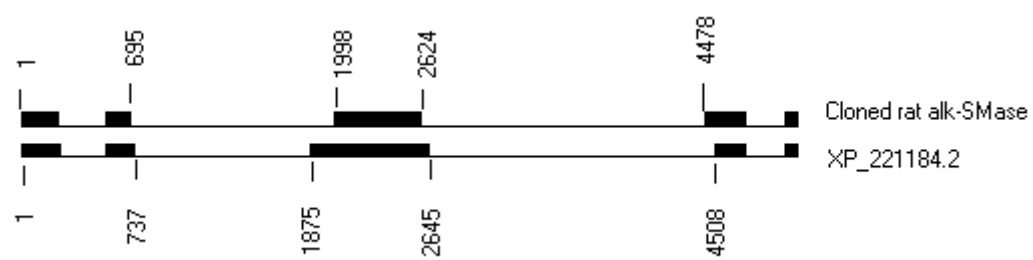


Fig. 6

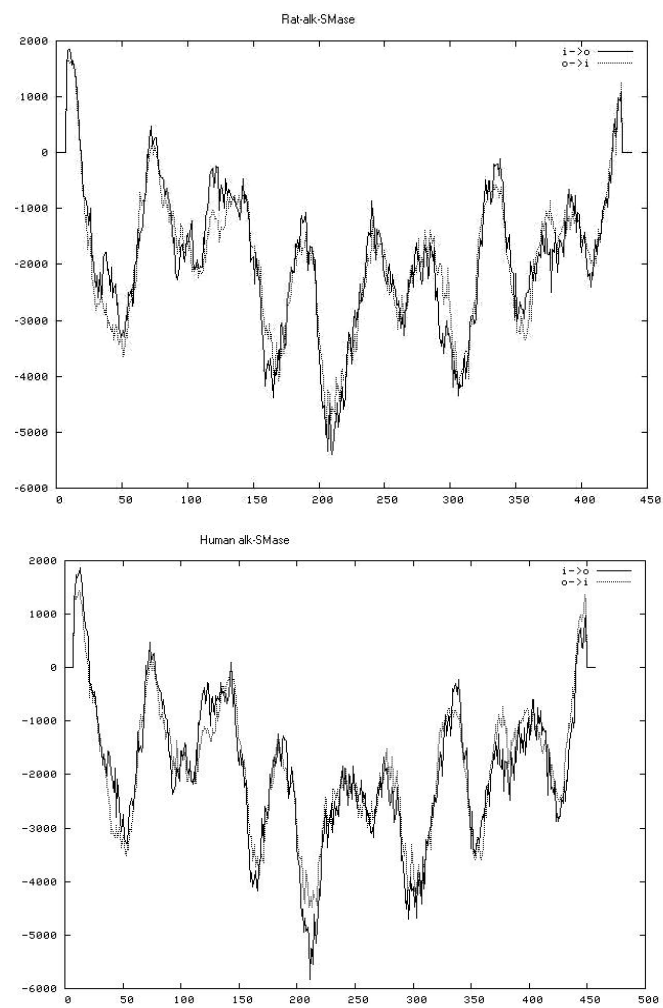


Fig 7

