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Sphingomyelinases in human, bovine and porcine seminal plasma

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The seminal plasma of man, boar and bull was found to have a sphingomyelinase (SMase) activity hydrolysing [*N-methyl*-^{14}C]sphingomyelin. The human and porcine enzymes had an acid pH optimum and were not influenced by divalent metal ions or chelating agents. They were closely similar with the lysosomal enzyme in many tissues. The bovine seminal plasma SMase was partially purified. The enzyme was a glycoprotein with pH optimum at 6.5, a broad p*I* 4.2–4.8 and molecular mass of 160 and 60 kDa, respectively, in native and SDS-PAGE. The enzyme was activated by Co > Mn > Cd > Ni and inhibited by chelating agents, Cu, Fe, Pb and Zn. The enzyme was clearly distinct from the acid lysosomal SMase and the previously described neutral Mg^{2+}-dependent and independent activities. It had a wide distribution in the bull reproductive tissues.

Sphingomyelinase; Enzyme purification; Enzyme characterization; Seminal plasma; (Bull, Boar, Human)

1. INTRODUCTION

Sphingomyelinase (SMase, EC 3.1.4.12) catalyzes the hydrolysis of sphingomyelin to ceramine and phosphorylcholine. Four SMase activities have been described in mammalian tissues: (i) a lysosomal enzyme with pH optimum at 5.0 is ubiquitously distributed [1–10], (ii) a neutral Mg^{2+}-dependent activity with pH optimum at 7.4 has been mainly found in the plasma membrane and microsomal fractions of brain tissue [10,11], (iii) a neutral Mg^{2+}-independent SMase is bound to myelin in rat brain [12] and (iv) a neutral Mn^{2+}(Mg^{2+})-activated membrane-bound enzyme has been identified in bovine adrenal medulla [13]. The acid SMase has been partially purified from rat and human brain [1,3,6] and human placenta [2,4,7,8], while the neutral Mg^{2+}-dependent SMase has been isolated from human brain [3] and rat liver [11]. Both the acid and neutral Mg^{2+}-dependent enzymes have been found in rat testis [14] and recently an Mg^{2+}-dependent neutral SMase in ram spermatozoa [15], while other male reproductive tissues have not been analysed. The present study was performed in the endeavor to determine the hydrolysis of labeled sphingomyelin by the seminal plasma samples of man, boar and bull. The bovine enzyme was analysed in greater detail because of its distinct enzymatic properties.

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2. MATERIALS AND METHODS

Human, bovine and porcine material was obtained as in [16,17]. Tissue samples were homogenized in 0.025 M imidazole-HCl buffer (pH 7.4), with 0.1% Triton X-100. Seminal plasma and spermatozoa were separated by centrifugation at 600 × *g* for 10 min. Aliquots of the seminal plasma were fractionated by gel filtration on a Sephacryl S300 superfine column (K 16/100) and prepacked Superose 6 column (Pharmacia), anion- and cation-exchange chromatography with Q Sepharose fast flow and S Sepharose fast flow columns (HR 10/10) attached to an Altex HPLC system and eluted with programmed gradients as well as an octyl-Sepharose CL-4B column (K 9/30) eluted with a decreasing ammonium sulfate gradient (1–0 M) and increasing Triton X-100 gradient (0–1.5%). Protein in the samples and chromatographic fractions was

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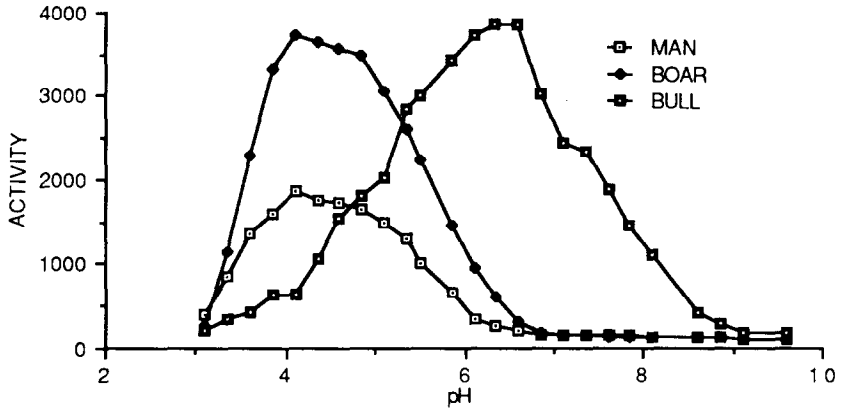

Fig.1. pH dependence of [*N-methyl*-^{14}C]sphingomyelin hydrolysis (cpm/sample) by seminal plasma samples of man, boar and bull.

measured according to Bradford [18]. The enzyme purity, molecular mass and p*I* were analyzed using the Pharmacia PhastSystem electrophoresis equipment with marker proteins and Coomassie brilliant blue for staining.

SMase activity was measured using bovine [*N-methyl*-^{14}C]sphingomyelin (58 mCi/mmol, Amersham) as substrate. The reaction mixtures in a total volume of 0.2 ml contained 1 nmol sphingomyelin (10000 cpm), an appropriate buffer (0.1 M acetate-HCl, pH 3.0–7.0; 0.1 M imidazole-HCl, pH 6.0–8.0; 0.1 M Tris-HCl, pH 7.0–9.5), metal ions and Triton X-100 (20 nmol). Following incubation at 37°C for 30–60 min, the reactions were stopped with 1 ml of $CHCl_3/CH_3OH/HCl$ (1000:1000:6) followed by the addition of 0.3 ml of 1 M HCl-1 mM EGTA solution. After vortex-mixing and centrifugation, a portion (0.4 ml) of the aqueous (upper) phase was removed for liquid scintillation counting to determine the release of radioactive phosphorylcholine. The hydrolysis of bis-*p*-nitrophenylphosphate (bis-p-NPP), *p*-nitrophenylphosphorylcholine (p-NPPC) and hexadecanoyl-*p*-nitrophenylphosphorylcholine (HD-p-NPPC) was measured spectrophotometrically [16].

3. RESULTS

The hydrolysis of labeled sphingomyelin by human and boar seminal plasma showed the highest activity at pH 4–5 (fig.1). This pattern was not influenced by the addition of divalent metal ions or EDTA. The bovine seminal plasma displayed sphingomyelin hydrolysis within the range pH 5–8 and optimum at pH 6.5 (fig.1). The presence of Mn^{2+} (1 mM) was required for maximum activity of bull SMase.

The effect of various modifier agents at 1 mM

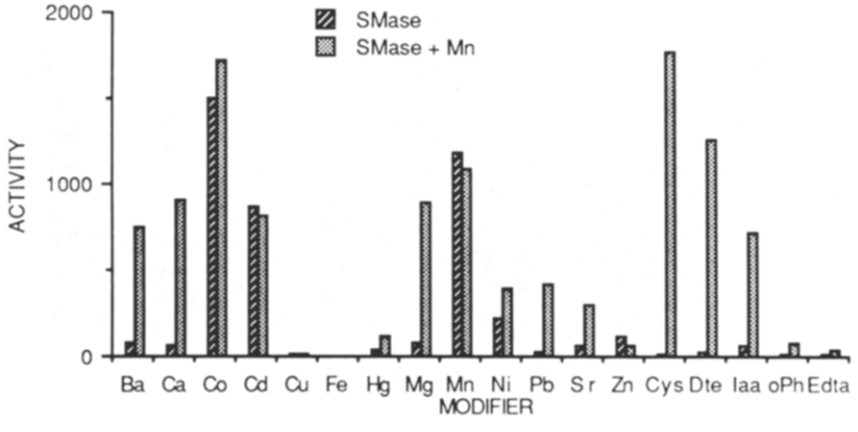

Fig.2. Effect of various modifiers (1 mM) on bull seminal plasma SMase (cpm/sample) in the absence and presence of Mn^{2+}. Cys, cysteine; Dte, dithioerythritol; Iaa, iodoacetamide; oPh, *o*-phenanthroline; Edta, EDTA.

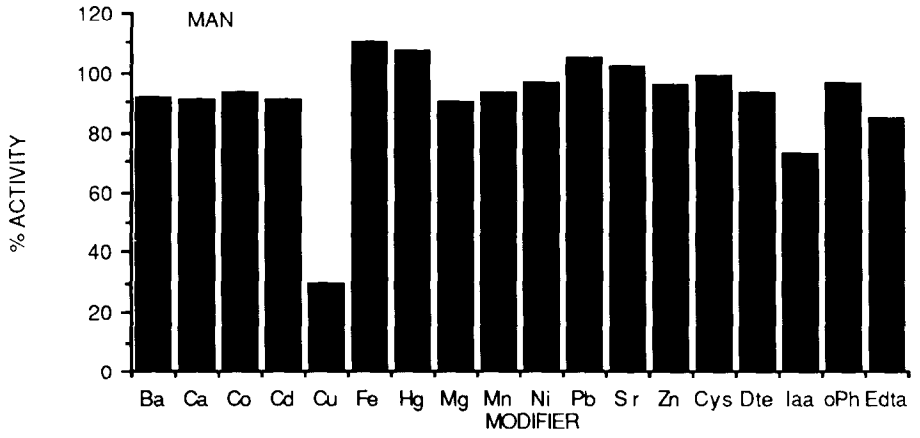

Fig.3. Percent effect of various modifiers (1 mM) at pH 4.0 on human seminal plasma SMase. For details see fig.2.

was tested on bull SMase at pH 6.5 (fig.2). It was found that Co^{2+}, Cd^{2+}, Mn^{2+} and Ni^{2+} were able to activate the reaction. In the presence of Mn^{2+} (1 mM) the chelating agents as well as some metal ions (Cu, Fe, Hg, Zn) were highly suppressive. A concentration-dependent response was found in the activation by Co^{2+} and Mn^{2+} with maxima being reached at 0.2 and 0.6 mM, respectively. The highest activation with Cd^{2+} and Ni^{2+} was obtained at 0.06–0.6 mM with subsequent suppression at higher concentrations. The human and boar enzymes were suppressed only by Cu^{2+}, while the other modifiers had no major effect (fig.3). The distribution of SMase activity (pH 6.5 with 1 mM Co^{2+}) in bovine reproductive tissues showed the highest levels in seminal plasma and epididymal cauda, but other reproductive organs were also reactive (fig.4).

The bull seminal plasma sample (20 ml) was utilized in the purification of SMase. Table 1 shows the sequential steps and a typical result of the purification gave a 12% yield and a purification coefficient of about 325. The modifier characteristics and pH optimum of the purified SMase were equal to those obtained with the seminal plasma. The purified preparation did not

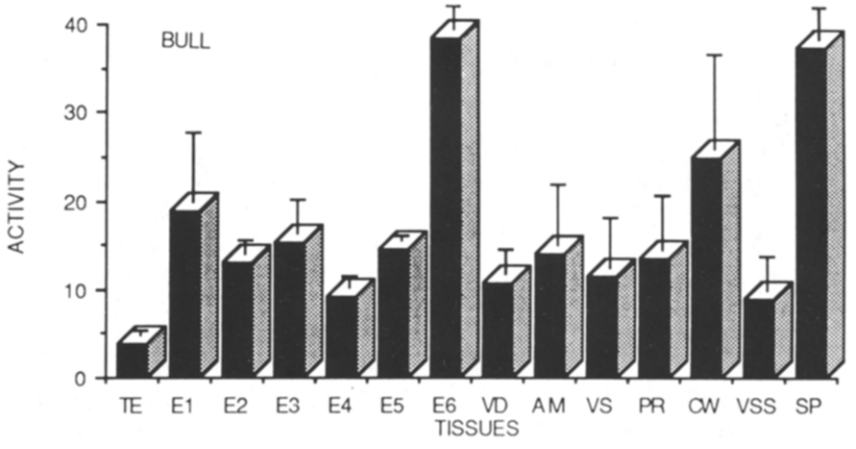

Fig.4. Hydrolysis rate (cpm/μg protein; mean ± SD) of [*N-methyl*-^{14}C]sphingomyelin (pH 6.5, 1 mM Mn^{2+}) by samples (n = 5) of bull testis (TE), six epididymal segments (E1–E6), vas deferens (VD), ampulla (AM), vesicula seminalis (VS), prostate (PR), Cowper's gland (CW), seminal vesicle secretion (VSS) and seminal plasma (SP).

Table 1

Purification of SMase from the bovine seminal plasma

Fraction	Total protein (mg)	Total activity (cpm)	Spec. act. (cpm/μg protein)	Purification (-fold)	Recovery (%)
Seminal plasma	2908.8	115038	39.5	1.0	100.0
Ammonium sulfate (40–60%)	1030.8	89686	87.0	2.2	80.0
Sephacryl S300	568.8	85480	150.3	3.8	74.3
Q Sepharose	30.1	48232	1601.7	40.5	41.9
S Sepharose	9.0	37138	4120.9	104.2	32.3
Con A-Sepharose	2.3	27588	11999.1	303.3	24.0
Superose 6	1.1	14130	12845.2	324.8	12.3

hydrolyse p-NPPC but was able to split HD-p-NPPC and bis-p-NPP.

The purified bovine SMase resulted in a major band at 160 kDa in a native PAGE gradient (8–25%) gel (fig.5A), while SDS-PAGE displayed a band at 60 kDa in addition to two minor bands at 16 and 95 kDa (fig.5B). A broad band was obtained at p*I* 4.2–4.8 in PhastGel IEF 3–9 (fig.5C). The enzyme remained active during 15 min thermal treatment at 55°C but lost about half of its activity at 65°C. The human and boar enzymes were clearly more sensitive to thermal treatment with 50% suppression already at 50°C.

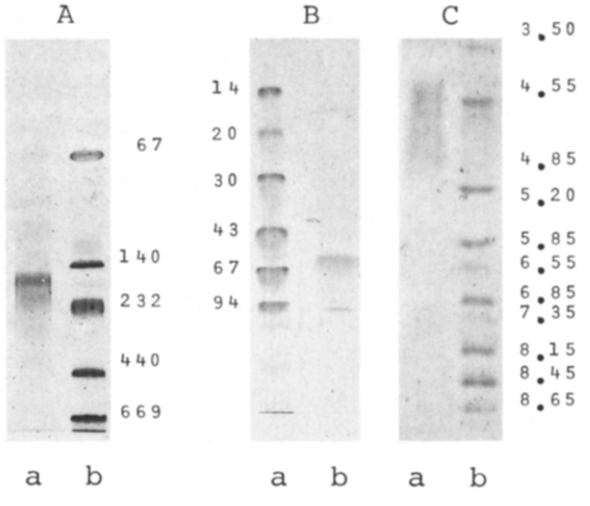

Fig.5. Gel electrophoresis pattern of the purified SMase from bull seminal plasma. (A) Native PAGE (gradient 8–25%): (a) sample; (b) high-molecular-mass proteins; (B) SDS-PAGE (gradient 8–25%): (a) low-molecular-mass proteins; (b) sample; (C) IEF (pH gradient 3–9): (a) sample; (b) p*I* standard proteins.

4. DISCUSSION

The present study indicates that human, porcine and bovine seminal plasma contains an active SMase. The human and porcine enzymes had an acid pH optimum and were not influenced by divalent metal ions or chelating agents. These are properties typical for the previously described lysosomal enzyme, with which the human and porcine SMases seem to be closely related. These enzymes, however, are secretory and may differ in some respects from the lysosomal activity. Detailed studies are required to disclose the differences. The bovine enzyme can be classified as a neutral enzyme with an obvious requirement for divalent metal ions in activation. However, it appears to be distinct from the previously described neutral SMases in its activation by Co^{2+}, Cd^{2+}, Mn^{2+} and Ni^{2+}. On the other hand, Mg^{2+} was ineffective with the bull seminal plasma enzyme, although it has been the major activator of the previously reported neutral SMases [10,11,13–15]. The latter enzymes have also been linked to particulate structures, while in the bovine seminal plasma SMase is clearly soluble.

The bovine SMase had a native molecular mass of 160 kDa, while in SDS-PAGE a value of 60 kDa was obtained for the major band. This indicates that the enzyme may consist of subunits. The enzyme had a broad band at pH 4.2–4.8 in IEF and was attached to the Con A-Sepharose column. This indicates that the enzyme is a glycoprotein and may vary in the amount of sugar residues. It was also strongly attached to the octyl-Sepharose column, suggesting the prevalence of hydrophobic groups also typical for the lysosomal

acid SMase [4,5,7–10]. Previously acid SMases have been found to hydrolyse synthetic substrates including p-NPPC, HD-p-NPPC and bis-p-NPP [2,4,6,8,10,19,20]. The last two substrates were also split by the bovine enzyme. In this respect it differs from the neutral brain SMase [20]. The bovine SMase was also clearly distinct from the previously identified Co^{2+}-activated phospholipase C-like enzyme hydrolysing p-NPPC [16]. The tissue distribution of SMase was also totally different from the latter activity and the two enzymes could be easily separated during fractionations.

Sphingomyelin concentrations in spermatozoa and seminal plasma have been extensively studied in different species (see [22] for references). Its relative amount seems to vary markedly from species to species and changes have been recorded in spermatozoa during epididymal transit, capacitation and acrosome reaction. The secretory enzyme is potentially able to attack sphingomyelin in the outer leaflet of sperm plasma membrane. The physiological importance of the enzyme still remains to be explored, but since the surface phenomena are very essential for the functional maturation of spermatozoa in the epididymis and after ejaculation, the contribution of SMase should also be taken into account.

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