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Purification of sphingomyelinase to apparent homogeneity by using hydrophobic chromatography

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Placental sphingomyelinase has been purified to apparent homogeneity by a procedure that makes extensive use of hydrophobic interaction chromatography on sphingosylphosphocholine–CH-, octyl-, hexyl- and Blue-Sepharoses. Enzyme purification is about 10000–14000-fold over starting extract with excellent yield (usually greater than 28%). Purification of bis-4-methylumbelliferyl phosphate phosphodiesterase activity generally paralleled that of sphingomyelinase during the final stages of the procedure. The enzyme also hydrolysed bis-*p*-nitrophenyl phosphate, but at a lower rate compared with bis-4-methylumbelliferyl phosphate. A single major protein was observed under non-denaturing conditions. Sphingomyelinase, denatured by reduction and alkylation, is composed of a major polypeptide chain with an apparent molecular weight of 89100 on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Two minor lower-molecular-weight components were consistently obtained at 47500 and 30700. These results were also obtained after maleoylation of the reduced and alkylated sample. The enzyme contains a blocked-*N*-terminal amino acid. An extensive search for contaminating enzymes revealed the presence of minor amounts of acid phosphatase, which were removed from the final enzyme sample. The highly purified enzyme is stable for several weeks when stored with Triton X-100 at 4°C. The pure enzyme aggregates under denaturing and electrophoretic conditions and special care must be taken to ensure that hydrophobic bonding of the protein is decreased as much as possible. The reproducibility and large scale of this procedure should facilitate further study on the structure and kinetic properties of the enzyme.

Sphingomyelinase (sphingomyelin phosphodiesterase, EC 3.1.4.12) occurs in most mammalian tissues as a lysosomal hydrolase (Kanfer *et al.*, 1966). Additional separate species occur in plasma membranes (Hostetler & Yazaki, 1979) and the central-nervous-system myelin (Yamaguchi & Suzuki, 1978). These other sphingomyelinases are distinct from the lysosomal form and possess unique properties.

Until recently purification of the lysosomal enzyme has been attempted by a number of groups with only marginal success (Pentchev *et al.*, 1977; Yamaguchi & Suzuki, 1977; Callahan *et al.*, 1978). Pentchev *et al.* (1977) achieved a high degree of purification of a very small portion of the total tissue enzyme pool. They also reported the resolution of two polypeptides in this fraction, which were assigned apparent molecular weights of 36800 and 28300 respectively. However, it is unclear whether these species are representative of the composition of the total tissue enzyme pool or reflect the composition of a single isoenzymic form of the enzyme. A human brain sphingomyelinase was partially purified by Yamaguchi & Suzuki (1977). The preparation contained two separable components, both of which possessed lysosomal sphingomyelinase activity. The two components, purified 800-fold and 2500-fold, differed in apparent molecular weight, but appeared to be structurally related. The preparations were not homogeneous, and possessed properties of both the lysosomal and plasma membrane sphingomyelinases, which Rao & Spence (1976) have shown to be distinct separable enzymes. We (Callahan *et al.*, 1978) described a procedure for obtaining 1500–1800-fold purification of the lysosomal enzyme from placenta, but, as in the other methods, obtained very low yield and homogeneity was not achieved.

In the present work we describe a reproducible facile procedure for purification of the enzyme to

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apparent homogeneity. The major feature of the method is the extensive use of hydrophobic chromatography, which takes advantage of the lipophilic properties of sphingomyelinase.

Materials and methods

Reagents

Sphingomyelin was tritiated by catalytic reduction (New England Nuclear) and purified by chromatography on silicic acid (Unisil; Clarkson Chem. Co.). Sphingosylphosphocholine was prepared from sphingomyelin and purified as previously described (Callahan *et al.*, 1980). The compounds bis-4-methylumbelliferyl phosphate, bis-*p*-nitrophenyl phosphate, 4-methylumbelliferyl pyrophosphate, *p*-nitrophenyl phosphate, thymidine 3′-phosphate *p*-nitrophenyl ester, thymidine 5′-phosphate *p*-nitrophenyl ester and α-methyl mannoside (type II) were obtained from either Sigma Chemical Co. or Koch–Light. Hexyl-agarose and a hydrophobic chromatography kit (Kit I) were obtained from Miles Laboratories. Octyl-Sepharose, Blue Sepharose, CH-Sepharose, Sepharose CL-6B, Sephadex G-25 and Con A-Sepharose (concanavalin A–Sepharose) were products of Pharmacia. Bio-Beads SM-2 and econo-columns of various sizes were obtained from Bio-Rad. Other chemicals were reagent-grade products from a variety of suppliers.

General procedures

All procedures were performed at 4°C unless otherwise stated. Protein determinations were carried out by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard. Enzyme samples were concentrated either by ultrafiltration by using an Amicon cell fitted with YM-10 diaflo membranes, or by the Amicon CH-4 Hollow Fiber apparatus with a nominal cut-off at mol.wt. 10000. Radioactive samples were analysed on a Beckman model LS-255 scintillation counter. Fluorescence measurements were made on a Turner model 430 fluorimeter and absorbance measurements were made on a Unicam model SP. 1800 spectrophotometer.

Triton X-100 assay

Triton X-100 was quantified by the method of Garewal (1973). In our hands the assay was linear in the concentration range 0.1–2.0mg of Triton X-100/ml at 622nm (maximum at longer wavelength) and was linear in the concentration range 0.5–8.0mg of Triton X-100/ml when the absorbance of the complex was read at 660nm. The absorbance of the Triton X-100 complex is totally unaffected by protein at either 622nm or 660nm.

Preparation of sphingosylphosphocholine–CH-Sepharose

Sphingosylphosphocholine (500mg) was dissolved in 50ml of water. To this was added 50ml of CH-Sepharose that had been first washed with 0.5M-NaCl followed by copious amounts of water. The pH of the slurry was adjusted to 5.0 with 1M-$NaHCO_3$. To this was added 1.92g of 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide hydrochloride (0.1M final concentration) and the mixture was stirred slowly at room temperature. The pH was maintained between 4.5 and 6.0 for 1h by the dropwise addition of 1M-HCl, after which time the reaction was stirred overnight without further pH adjustment. The gel was then washed with water, 0.5M-NaCl and finally copious amounts of 25mM-citrate/50mM-phosphate/0.5M-NaCl, pH4.3.

Enzymic assays

All assays were carried out at 37°C. One unit of activity represents hydrolysis of 1nmol of substrate/h and the specific activity represents units/mg of protein. Sphingomyelinase was assayed as previously described (Callahan *et al.*, 1975, 1980) with [^{3}H]sphingomyelin. With bis-4-methylumbelliferyl phosphate the assay was carried out by the method of Besley (1978), except that substrate concentration was 2.5mM instead of 5.0mM. Hydrolysis of bis-*p*-nitrophenyl phosphate, 4-methylumbelliferyl pyrophosphate and thymidine 5′-phosphate *p*-nitrophenyl ester was measured by the method of Callahan *et al.* (1974*a*,*b*). Acid and alkaline phosphatase were determined with *p*-nitrophenyl phosphate (dicyclohexylammonium salt) by the methods of Vanha-Perttula (1970) and Sakiyama *et al.* (1979) respectively. Other details are provided in the text.

Purification

Human placentae were frozen at −20°C immediately after delivery and stored for about 1 month before use. The placentae were then thawed overnight at 4°C and the cords were removed. Tissue was ground in a meat grinder and then homogenized in a Waring blender for 3min in 3vol. of 25mM-citrate/50mM-phosphate, pH4.5, containing 0.25% Triton X-100, 1mM-$CaCl_2$, 1mM-$MgCl_2$ and 1mM-$MnCl_2$. The supernatant fluid recovered from the crude homogenate by centrifugation for 1h at 11000*g* was loaded on a Con A-Sepharose column (150ml, 3.0cm × 20.6cm), pre-equilibrated in the above buffer, at a flow rate of 125ml/h. The column was then washed with 2 litres of 25mM-maleic acid, pH6.5, containing 0.5M-NaCl and 0.25% Triton X-100, to elute non-specifically bound proteins. The column was then eluted with two pools (500ml each) of 25mM-maleic acid, pH6.5, containing 0.5M-NaCl, 0.25% Triton X-100 and 0.5M-

α-methyl mannoside. One pool was recycled during the day (8h) and the second overnight (16h). Solid $(NH_4)_2SO_4$ (175.7g/litre, 0–30%) was added to the pooled eluates, allowed to stand for 1 h and then centrifuged for 1h at 25000*g*. To the supernatant from this step solid $(NH_4)_2SO_4$ (195.0g/litre, 30–60%) was added. It was allowed to stand for 1h and the bulk of the enzyme was recovered by centrifugation for 1h at 25000*g*. The precipitate, resuspended in a minimum volume of 10mM-maleic acid, pH6.5 (40ml) was dialysed against 5 litres of resuspension buffer (two changes). The sac contents were rapidly filtered through a Bio-Bead SM-2 column (20g) five times to remove almost all traces of Triton X-100.

The enzyme sample was then applied directly to a column (40ml, 2.7cm × 7.0cm) of sphingosylphosphocholine–CH-Sepharose. The column, pre-equilibrated in 10mM-maleic acid, pH6.5, was loaded at a flow rate of 30ml/h, washed with 150ml of the above buffer pH containing 0.1M-NaCl, and finally eluted with a gradient (500ml) from 0 to 0.5% Triton X-100 in the above buffer. Fractions (10ml) were collected, and the tubes were assayed for sphingomyelinase, bis-4-methylumbelliferyl phosphodiesterase, acid phosphatase and Triton X-100 content. Fractions containing the bulk of the sphingomyelinase activity were pooled (140ml), dialysed against 10mM-maleic acid, pH6.5 (5 litres, changed three times), and then applied directly to an octyl-Sepharose column (1.0cm × 20cm; 20ml), pre-equilibrated with 10mM-maleic acid, pH6.5, 10% saturated with $(NH_4)_2SO_4$. The column was washed with equilibration buffer (100ml), followed by 10mM-maleic acid, pH6.5, containing 0.5% Triton X-100. Fractions (5ml) were collected and assayed as above.

The octyl-Sepharose pool was then applied directly to a hexyl-Agarose column (20ml, 1.0cm × 20.0cm) that had been pre-equilibrated with 10mM-maleic acid, pH6.5, containing 0.5% Triton X-100. The column was washed with same buffer containing 0.5mM-NaCl. Since only contaminating hydrophobic proteins stick to this column, no special elution step was required. Bound protein was eluted by 10mM-maleic acid, pH6.5, containing 0.5M-NaCl and 0.5% Triton X-100. Fractions (5ml) were collected, and assayed as described above. The fractions with the bulk of the enzyme activity were pooled (100ml) and approximately one-half (45ml) of the enzyme pool was applied directly to a Blue Sepharose column (1.0cm × 30cm; 20ml) pre-equilibrated with 25mM-maleic acid containing 25mM-NaCl, pH6.5. Under these conditions no overloading occurred. The column was washed with 100ml of the above buffer. The enzyme was then eluted with the above buffer containing 0.5% Triton X-100. Enzyme activity was recovered quantitatively and was very stable, retaining full activity for at least 3 weeks.

Regeneration of columns

Sphingosylphosphocholine–CH- and octyl-Sepharose and hexyl-Agarose were regenerated by washing with the following sequence of buffers: water (1 bed vol.), ethanol (1 bed vol.), butan-1-ol (2 bed vol.), ethanol (1 bed vol.), water (1 bed vol.) and running buffer (2 bed vol.). Blue Sepharose was washed with 25mM-maleic acid/0.5M-NaCl and 0.5% Triton X-100 at pH6.5, followed by equilibration buffer as described above.

Sphingomyelinase binding to various alkyl-Sepharoses

Sphingomyelinase (800 units/2.0ml) in 0.1M Tris/acetate buffer, pH5.0, containing 0.2% Triton X-100, was applied to each of six mini-columns (hydrophobic chromatography Kit I) containing respectively 2ml of 0-, 2-, 4-, 6-, 8- and 10-carbon-chain alkyl-agarose that had been pre-equilibrated with 0.1M-Tris/acetate, pH5.0. The columns were each eluted with 2ml of equilibration buffer, and each collected in a pool that was assayed for enzymic activity and protein. The columns were stripped by washing with 2M-NaCl.

Polyacrylamide-gel electrophoresis

(*a*) *Non-sodium dodecyl sulphate method.* Native sphingomyelinase was subjected to electrophoresis in 7.5% running gels with Tris/glycine, pH8.3, by the Buffer 3 method of Smith (1968). The gels were stained with Coomassie Blue and destained with 7.5% (v/v) acetic acid/5% methanol (v/v) in water.

(*b*) *Sodium dodecyl sulphate/polyacrylamide-gel-electrophoresis methods.* The general procedure was the standard control (Method 2) of Weber *et al.* (1972), except that where guanidine hydrochloride was omitted, it was replaced by 2% sodium dodecyl sulphate. In addition, the reduced and alkylated enzyme was precipitated and washed twice with aq. 80% acetone at −20°C instead of dialysis. Enzyme solutions were reduced in 50mM-dithioerythritol and alkylated with 150mM-iodoacetic acid. It is imperative that the two-step alkylation procedure be followed with a final pH of 10.5–11.0. After re-acidification to pH7.2, electrophoresis was carried out in 10% gels. Molecular-weight assignments were obtained from mobilities of standard proteins run simultaneously. The standards were from the low-molecular-weight kit (Pharmacia), which includes phosphorylase *b* (mol.wt. 94000), bovine serum albumin (mol.wt. 67000), ovalbumin (mol.wt. 43000), carbonic anhydrase (mol.wt. 30000), soyabean trypsin inhibitor (mol.wt. 20100) and α-lactalbumin (mol.wt. 12400).

Maleoylation of amino groups

Maleoylation of the reduced and alkylated enzyme was by the method of Butler & Hartley (1972). Purified protein (0.345 mg) was dissolved in 0.20 ml of 8 M-guanidine hydrochloride in 0.1 M-Na_2CO_3, pH 8.6. To this was added enough dithioerythritol (1.60 mg) to make the solution 50 mM. The sample was boiled for 4 min, followed by incubation at 37°C for 2 h. To this was added 6.8 mg of iodoacetic acid (sodium salt), a 3-fold excess over the concentration of dithioerythritol. The pH was 9. After 10 min, one drop of mercaptoethanol was added to stop the reaction and the mixture was cooled in an ice/water bath. To the above solution one drop of phenolphthalein and one drop of 1 M-NaOH, to raise the pH slightly, were added. Four 0.025 ml portions (0.1 ml total) of maleic anhydride solution (4 mg total) were added, along with 1 M-NaOH, as needed, to keep the pH above 8 (pink-coloured solution) through the addition. The mixture was stirred vigorously. After 10 min, the reaction fluid was dialysed against 100 ml of 8 M-urea, containing a small amount of mixed bed resin to keep cyanide ion formed during the breakdown of urea at a low concentration. This step removed guanidine. The sample was then dialysed against fresh 8 M-urea (200 ml), pH 7.5, for 30 min. The sample solution was colourless. The sample was then finally dialysed for 36 h against 0.25 M-phosphate containing 0.1% sodium dodecyl sulphate, pH 7.2 (200 ml), with one change and electrophoresed as described above.

N-terminal analysis

Purified enzyme was dialysed against water (five changes of 5 litres) and then freeze-dried. The dried residue was dissolved in concentrated formic acid, transferred to a tared tube and redried and weighed. The white powder (0.58 mg) was dissolved in formic acid/5% H_2O_2 at −5°C for 2 h. The reaction solution was quickly diluted with 40 vol. of ice-cold water and freeze-dried. The dried residue was then dansylated, hydrolysed (16 h) and chromatographed by the method of Gray (1972).

K_m determination

The K_m was determined by using five concentrations of substrate, ranging from 62 μM to 9.2 μM, such that evenly spaced reciprocals were obtained above and below the K_m. The data were analysed by both the Eisenthal and the Lineweaver–Burk methods (see Segel, 1975).

Results

Binding of sphingomyelinase to alkyl-Agaroses

The binding of sphingomyelinase to each of a series of alkyl-Agaroses is presented in Fig. 1. The results show that at a carbon chain length of six (hexyl-Agarose), sphingomyelinase passes straight through the column, whereas 75% of the protein remains bound. However, all the sphingomyelinase activity and 85% of the protein binds to octyl-Agarose. Only about 40% of the protein and sphingomyelinase (results not shown) are recovered from the octyl- or decyl-Agarose columns by eluting with 2 M-NaCl as recommended. Total recovery of enzyme is achieved when detergent is applied.

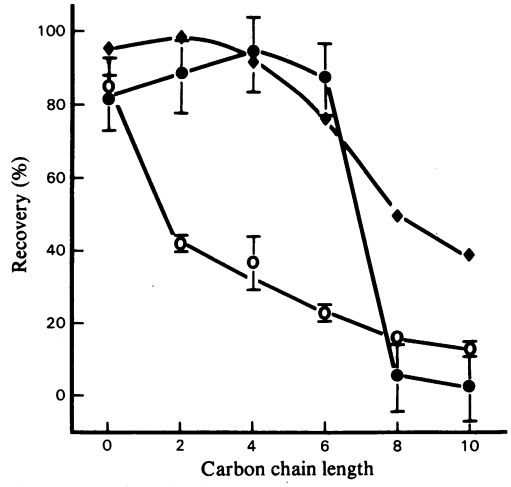

Fig. 1. *Binding of sphingomyelinase to alkyl-Agaroses*
Partially purified enzyme was applied to each column, which were washed and eluted as described. Unbound protein (○) progressively declined, whereas sphingomyelinase (●) remained essentially unadsorbed below the 8-carbon chain length. Total protein (♦) and enzyme recovered from unbound and eluted fractions also declined at this point.

Purification of sphingomyelinase

The initial steps have been well established and include Con A-Sepharose chromatography followed by precipitation between 30 and 60% $(NH_4)_2SO_4$ (Callahan *et al.*, 1978). Before sphingosylphosphocholine–CH-Sepharose chromatography, it is critical to decrease the Triton X-100 concentration. Removal of Triton X-100 at this stage did not cause significant loss of enzymic activity. With more highly purified protein, however, a concomitant loss of enzymic activity, protein and Triton X-100 was noted with each successive pass through the column. Sphingomyelinase is eluted from the sphingosylphosphocholine–CH-Sepharose column with a linear Triton X-100 gradient (Fig. 2). The unbound fraction contained a large amount of acid phosphatase activity, protein, and some activity towards the substrate bis-4-methylumbelliferyl phosphate. The majority of the sphingomyelinase and bis-

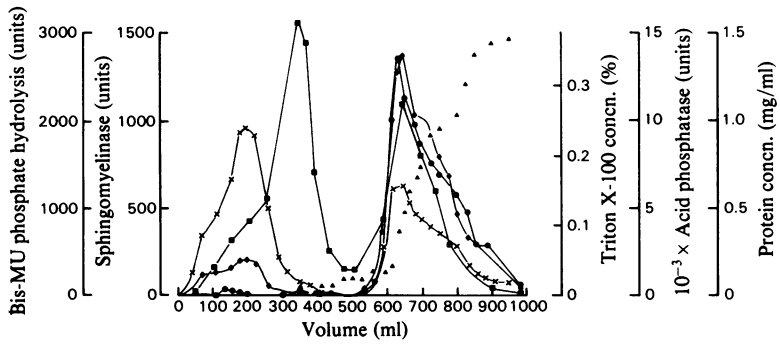

Fig. 2. *Chromatography of sphingomyelinase on sphingosylphosphocholine–CH-Sepharose*
Details are provided in the text. The bulk of sphingomyelinase (●) and bis-4-methylumbelliferyl (Bis-MU) phosphate activities (♦) bound to the support and were eluted with a Triton X-100 gradient (▲). A large amount of protein (■), acid phosphatase (×) and a small portion of the bis-4-methylumbelliferyl phosphate activity was not bound by the column. Recovery was 78%. One unit of bis-4-methylumbelliferyl phosphate hydrolysis represents 1 nmol hydrolysed/h per ml; 1 unit of acid phosphatase is the amount required to hydrolyse 1 nmol of substrate/h per ml. Units of sphingomyelinase are defined in the text.

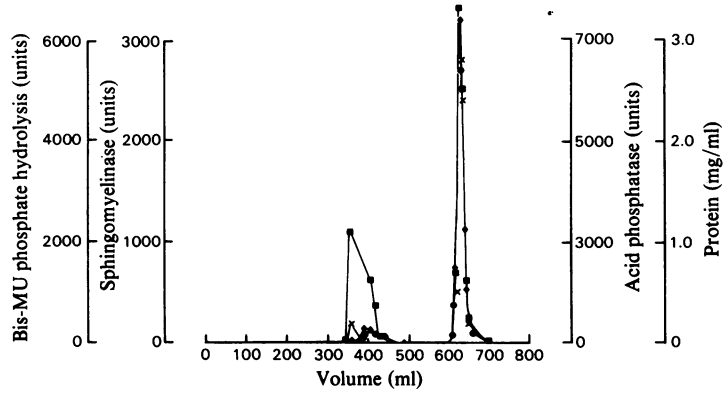

Fig. 3. *Chromatography of sphingomyelinase on octyl-Sepharose*
The effluent from the sphingosylphosphocholine–CH-Sepharose was applied to the column. The bulk of the protein (■), sphingomyelinase (●) and bis-4-methylumbelliferyl (Bis-MU) phosphate hydrolysis (♦) activities was bound. Elution buffer was applied at 580 ml. Protein, sphingomyelinase and some acid phosphatase (×) contamination was co-eluted. Recovery of enzyme was 98%. The various units of activity are defined in the legend to Fig. 2.

4-methylumbelliferyl phosphate activity was eluted at approx. 0.1–0.2% Triton X-100. The pooled enzyme, applied directly to an octyl-Sepharose column, was bound and eluted in a single sharp peak (Fig. 3), separated from the bulk of the protein, which remained bound to the column even after elution with 0.5% Triton X-100. The fractions containing sphingomyelinase activity were applied directly to the hexyl-Agarose column. The enzyme was eluted in the void volume, whereas other more hydrophobic proteins were eluted with buffer containing high-concentration salt (Fig. 4). Highly purified sphingomyelinase recovered at this stage retains all of its original activity when stored at 4°C, in the presence of 0.5% Triton X-100, for a period of at least 4 months.

A representative purification procedure is presented in Table 1. The final purification was about 12200-fold with good yield (39%). The procedure was quite reproducible and 2.8–3.6 mg of protein was obtained per preparation. Specific activities ranged from 49000–56000 nmol/h per mg of protein and purification varied from 10000 to 14000. Yield of enzyme units was 28–40%. A single major band of protein was found on disc-gel electrophoresis (Fig. 5). Enzyme activity with sphingomyelin, bis-4-methylumbelliferyl phosphate and bis-*p*-nitrophenyl phosphate was coincident with

Table 1. *Purification procedure for placental sphingomyelinase*

Data are for eight placentas. Enzyme activities quoted represent average values from linear assays carried out at three or more protein concentrations and in optimum assay conditions. Abbreviation used: SPC, sphingosylphosphocholine.

	10^{-3} × Activity with sphingomyelin as substrate (units)	Protein (mg)	Specific activity (units/mg of protein)	Recovery of sphingomyelin (%)	Purification (fold)		Bis-4-methylumbelliferyl phosphate/sphingomyelin ratio
					Sphingomyelin as substrate	Bis-4-methylumbelliferyl phosphate as substrate	
Homogenate	929.4	218980	4.3	—	1.0	1.0	2.34
Supernatant fluid	486.1	117263	4.4	100	1.0	0.9	2.18
Con A-Sepharose adsorbed	520.5	1124	463	107	108	124	2.68
$(NH_4)_2SO_4$ fractionation							
0–30%	19.8	185	107	4	25		
30–60%	444.0	451	984	91	229	210	2.13
SPC–CH-Sepharose	362.8	148	2451	75	570	294	1.20
Octyl-Sepharose	237.0	58.5	4050	49	942	543	1.34
Hexyl-Agarose	189.5	3.6	52639	39	12242	8195	1.55

the major protein band (results not shown). Sphingomyelinase activity was also detectable in the diffusely stained region ahead of the enzyme on these gels, presumably due to the presence of Triton X-100. In control experiments, we observed marked smearing of the gel pattern when high amounts (0.1–1.0%) of Triton X-100 were included in gel buffer.

Recovery of bis-4-methylumbelliferyl phosphate generally paralleled sphingomyelinase. During the early steps a bis-4-methylumbelliferyl phosphate/sphingomyelin enzyme activity ratio of approx. 2–3 was found. After removal of a significant amount of phosphodiesterase activity on sphingosylphosphocholine–CH-Sepharose chromatography, a new constant ratio (approx. 1–1.5) was established.

It should be noted here that the absolute value of the bis-4-methylumbelliferyl phosphate/sphingomyelinase activity ratio varied from preparation to preparation, but was always greater than 1.0. This can be seen in Table 2. The reasons for this are unclear, but may be related to the kinetic properties with bis-4-methylumbelliferyl phosphate as substrate. This is mentioned later.

Enzyme purity

The final enzyme preparations were tested for the presence of contaminating enzymes (Table 2). Enzyme purified through the hexyl-Agarose stage was contaminated with small amounts of acid phosphatase, acid pyrophosphatase and phosphodiesterase activities. No activity was detected against 12 other lysosomal hydrolases. The sphingomyelinase was also inactive against bis(monoacyl-*sn*-glycero)phosphate (S. Huterer, personal communication), and other phospholipids such as phosphatidylcholine and phosphatidylinositol (results not shown). To decrease the contaminants even further, chromatography on Blue Sepharose (Fig. 6) was employed. Sphingomyelinase was completely recovered from this column with removal of virtually all contaminating enzyme.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Electrophoresis of the enzyme in sodium dodecyl sulphate/polyacrylamide gels provided reproducible results only after the enzyme was reduced with dithioerythritol and alkylated at pH 11.0 as described in the Materials and methods section (Fig. 7). Enzyme was resolved into a single major component at an apparent molecular weight of 89 100 with two minor components appearing at 47 500 and 30 700 respectively. A distinct region of protein staining was also visible from mol.wt. 71 500 to 79 000. This area was diffuse and contained some additional bands, but was much less prominent than the mol.wt.-89 100 component. This distribution was confirmed

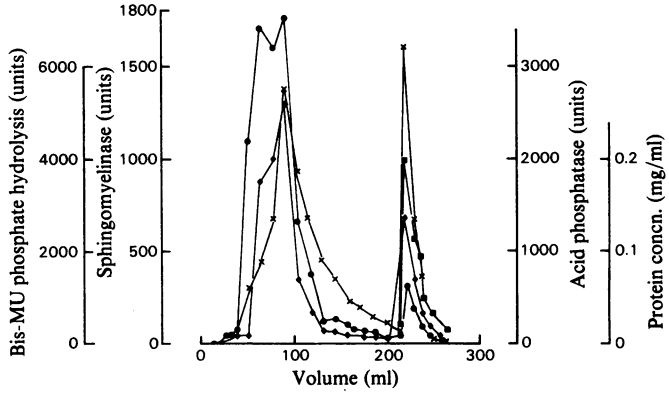

Fig. 4. *Chromatography on hexyl-Agarose*

The peak of enzyme from the octyl-Sepharose column is placed on the hexyl-Agarose. Virtually all the protein (■) is bound, but not sphingomyelinase (●) and bis-4-methylumbelliferyl (Bis-MU) phosphate hydrolysis (♦) activities. A portion of the acid phosphatase (×) was co-eluted with sphingomyelinase. A small amount of sphingomyelinase binds to the column and can be eluted. Protein content of the unadsorbed fraction was so low that it could not be quantified accurately. The various units of activity are defined in the legend to Fig. 2.

Table 2. *Enzymic activities in sphingomyelinase preparations*

Contaminating enzymes in two representative preparations carried through the Hexyl-agarose stage are shown. The specific activities of sphingomyelinase represent 12200- and 13000-fold purification respectively.

Enzyme*	Substrate	Concn. (mM)	pH	Specific activity (units/mg of protein) Prep. 17	Prep. 19
Sphingomyelinase	Sphingomyelin	0.17	5.0	52639	49068
	Bis-4-methylumbelliferyl phosphate	2.5	5.0	81583	130533
	Bis-*p*-nitrophenyl phosphate	5.0	5.0	26861	29387
Acid phosphatase	*p*-Nitrophenyl phosphate	10.0	5.0	123639†	44693
Acid pyrophosphatase	4-Methylumbelliferyl pyrophosphate	5.0	5.0	31333	46080
Alkaline phosphodiesterase	Thymidine 5′-phosphate *p*-nitrophenyl ester	30.0	9.0	8472	0

* No activity of the following enzymes was detected: α- and β-galactosidase, α- and β-glucosaminidase, α- and β-glucosidase, β-galactocerebrosidase, α-fucosidase, α-galactosaminidase, α-mannosidase, arylsulphatase A, alkaline phosphatase.

† For example, this represents 0.087% of the initial activity present and a 65-fold enrichment over the initial extract.

Fig. 5. *Polyacrylamide-gel electrophoresis of native enzyme*

A representative sample (30μg) of purified enzyme (prep. 19) was electrophoresed on 7.5% gels as described in the text. The major band of protein coincided with bis-4-methylumbelliferyl phosphate hydrolysis and bis-*p*-nitrophenyl phosphate hydrolysis activities (results not shown).

after maleoylation (Fig. 7). The appearance of additional bands in the maleoylated enzyme is likely due to peptide breakdown resulting from the rigorous alkylation conditions. Less rigorous reduction and alkylation conditions lead to inadequate penetration into gels due to aggregate formation and to the appearance of as many as 30 protein bands in the molecular-weight region from 70000 to 90000.

N-terminal analysis

The two-dimensional t.l.c. employed in the *N*-terminal analysis is shown in Fig. 8. The dansyl (5-dimethylaminonaphthalene-1-sulphonyl) deri-

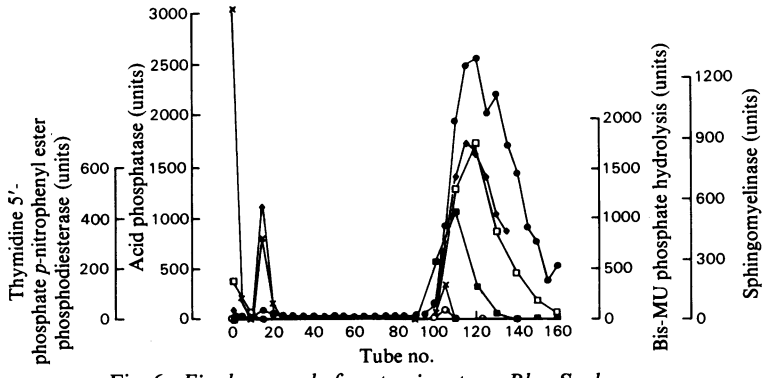

Fig. 6. *Final removal of contaminants on Blue Sepharose*

The highly purified enzyme from hexyl-agarose contains small amounts of contaminants such as acid phosphatase (x) and thymidine 5'-phosphate *p*-nitrophenyl ester phosphodiesterase (one unit is the amount of enzyme converting 1 nmol of substrate/h per ml) measured at pH 5.0 (■) and at pH 9.0 (○). The vast majority of acid phosphatase does not bind and appears in the breakthrough portion of the elution. Sphingomyelinase (●). bis-4-methylumbelliferyl (Bis-MU)phosphate hydrolysis (♦) and bis-*p*-nitrophenyl phosphate hydrolysis (□) are coincident and were eluted in virtually quantitative yield. Units not defined in this legend are defined in the legend to Fig. 2.

vatives of the protein revealed only one very intense dansyl-lysine spot and neighbouring spots, which most likely derive from dansyl hydroxide and dansyl-ammonia, all side products of the dansylation procedure. The plate was otherwise devoid of any spots that could be interpreted as dansylated amino acids. This essentially negative result indicates that there may be a blocked *N*-terminus. There was more than twice (0.575 mg) the recommended amount of protein, which is reflected in the high intensity of the dansyl-lysine spot formed with the ε-amino groups of the protein. This result also serves to illustrate the degree of purity achieved, since there is no contaminant present that contains a reactive *N*-terminal amino acid.

pH optimum and K_m values

Pure sphingomyelinase displayed a pH optimum of 4.6 and an apparent K_m of 25 μM. With bis-4-methylumbelliferyl phosphate a pH optimum of 4.8–5.0 was seen, but a K_m value could not be determined because of a deviation from Michaelis–Menten kinetics at substrate values above 2.5 mM. Bis-*p*-nitrophenyl phosphate hydrolysis showed a pH optimum of 4.8–5.0 and an apparent K_m value of 14.5 mM.

Discussion

Placental sphingomyelinase has been purified 12 200-fold in good yield by a procedure that utilizes the hydrophobic properties of the enzyme. The preference for specific alkyl-chain lengths is exhibited by the results with the series of alkyl-Agaroses and large-scale preparation procedures. The use of sphingosylphosphocholine–CH-Sepharose was also important for purification. When this column adsorbent was omitted, purification of only 4000–5000-fold was achieved. In addition, since the enzyme is eluted from this column at low Triton X-100 concentrations, it must be used before chromatography on either octyl- or hexyl-Agaroses, which use high (0.5%) Triton X-100 concentrations. This is a very important point since the detergent could be removed from the $(NH_4)_2SO_4$ fractions by Bio-Bead treatment with no loss of enzyme activity, whereas the purified enzyme, obtained after hexyl-Agarose, bound to the beads along with the detergent and could not be recovered without substantial loss of activity.

We (Callahan *et al.*, 1980) have recently shown that Triton X-100 binds reversibly to sphingomyelinase and the acidic isoelectric point of the enzyme, which is shifted to higher values by binding of the detergent (Besley, 1976), can also be restored on removal of the detergent. The hydrophobic domains in the enzyme where these interactions occur are not known and further work is needed to clarify this point. The hydrolysis of a hydrophobic substrate and binding and elution from a substrate-analogue column suggest that at least a portion of the hydrophobic domain is at or near the active site. The hydrophobic properties of the enzyme also lead to aggregation of the enzyme which make gel-electrophoretic runs very complex and difficult to interpret. It was only after repeated trials that satisfactory electrophoretograms were obtained. The electrophoresis procedures described

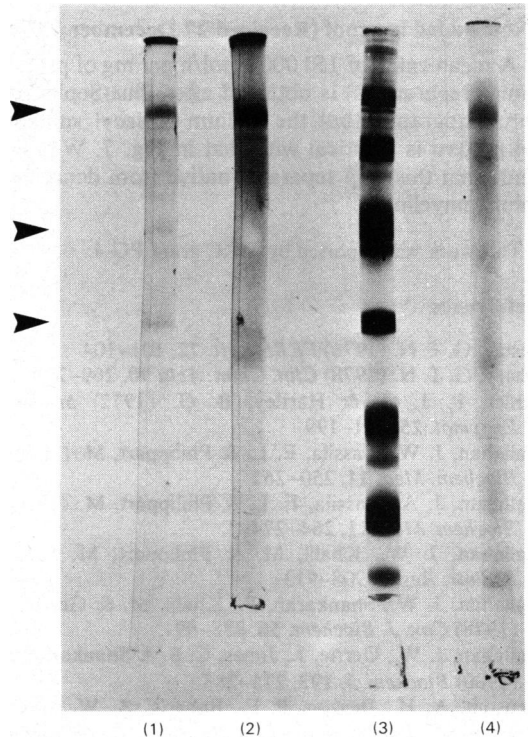

Fig. 7. *Polypeptide composition of pure sphingomyelinase*

Pure enzyme was reduced, alkylated and maleoylated as described and run on 10% sodium dodecyl sulphate/polyacrylamide gels as described in the text. Lane 1 (40 μg) shows the reduced and alkylated acid phosphatase free enzyme obtained from Blue Sepharose (prep. 16); lane 2 contains the maleoylated polypeptides (120 μg). Lane 4 contains a hexyl-Agarose fraction (13000-fold purification; prep. 19, 80 μg) that has been reduced and alkylated only. Arrows indicate the major (mol.wt. 89100) and two minor (mol.wt. 47500 and 30700) polypeptides. A faint diffusely stained region (mol.wt. approx. 71500–79000) can be seen in lanes 1 and 2. Further details are provided in the text. Lane 3 shows the molecular-weight standards.

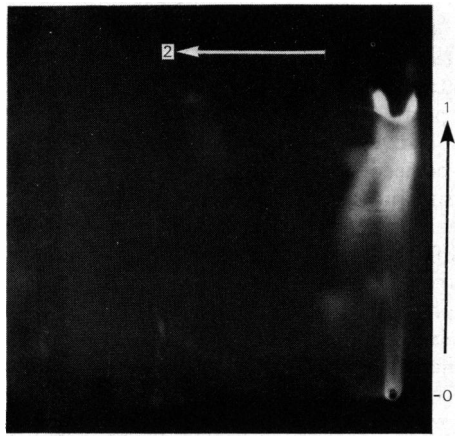

Fig. 8. *Thin-layer chromatogram of dansylated terminal amino acids*

A portion of the sample (575 μg) shown in lanes 1 and 2 of Fig. 7 was analysed as described in the text. The prominent crescent-shaped area represents the ε-amino groups of lysine. The other area contains by-products of the dansylation procedure. No dansylated *N*-terminal amino acid could be recognized.

by Takayama *et al.* (1964) for mitochondrial membrane proteins and the one used by Pentchev *et al.* (1977) for sphingomyelinase gave poorer results and were abandoned.

Sphingomyelinase purification to virtual homogeneity is achieved at purification values of 12000–14000. The final specific activity is approx. 51600 nmol/h per mg. This degree of purification is comparable with or better than that of Pentchev *et al.* (1977), but the final specific activity is 3-fold lower than in their report. This discrepancy may be due to differences in substrate, since they used a [^{14}C]-choline-labelled sphingomyelin, or in the assay conditions, since Pentchev *et al.* (1977) reported using suboptimal amounts of substrate, or to difficulties encountered in accurately quantifying very low amounts of proteins. Furthermore, since these workers purified only a small selected fraction of the total pool it may not have catalytic properties representative of the bulk of the enzyme.

The major polypeptide of sphingomyelinase had an apparent molecular weight on sodium dodecyl sulphate/polyacrylamide gels of 89100. Two distinct yet minor components were resolved at apparent-molecular-weight values of 47500 and 30800. It should be noted that the enzyme isolated by Pentchev *et al.* (1977) was reported to contain two separate polypeptides with apparent molecular weights of 36800 and 28300 respectively. The minor components observed in our preparations (47500 and 30800 respectively) could represent the low-molecular-weight components observed by these workers and indicate the presence of a separate minor species of sphingomyelinase. The origin of the faintly staining diffuse region at a molecular weight of 72500 remains unclear, but could be due to incomplete dissociation of the enzyme–Triton X-100 complex during reduction and alkylation (Fig. 7). As noted previously, up to 30 protein-staining bands (mol.wt. 70000–90000) could be seen on electrophoresis of the purified enzyme under denaturing conditions. We also experienced problems with reproducibility. Only after alkylation included the ε-amino group of lysine at high pH were reproducible gel patterns obtained. The molecular weight for pure sphingomyelinase has been reported to be

290000 (Pentchev *et al.*, 1977), but Gatt & Gottesdiner (1976) found this value varied according to concentrations of Triton X-100. Thus the polypeptide molecular-weight values reported here must be considered approximate and further analysis will be needed to define the native molecular weight, the subunit and polypeptide structure of the protein. The presence of a blocked *N*-terminal further complicates and limits estimation of the number of polypeptide chains.

Sphingomyelinase clearly displays heterogeneity as expressed by the isoelectric-focusing pattern (Callahan *et al.*, 1980), which is influenced markedly by Triton X-100. The present data suggest that true isoenzymic forms may exist, since at least one major and at least two minor polypeptide chains were resolved. It is possible that the major polypeptide-staining region on sodium dodecyl sulphate/polyacrylamide gels could consist of more than a single species of polypeptide. An analogous situation has been demonstrated recently for polypeptides derived from placental hexosaminidase B (D. Mahuran & J. A. Lowden, 1981).

Finally, the hydrolysis of bis-4-methylumbelliferyl phosphate and bis-*p*-nitrophenyl phosphate by sphingomyelinase is worthy of comment. At least 50% of the activity towards bis-4-methylumbelliferyl phosphate can be clearly resolved from sphingomyelinase on sphingosylphosphocholine–CH-Sepharose, leading to a degree of purification that is about 50% of sphingomyelinase. This has been a consistent finding. During the latter stages of purification activity of sphingomyelinase was always coincident with bis-4-methylumbelliferyl phosphate and bis-*p*-nitrophenyl phosphate (Figs. 2–5). We conclude that both synthetic substrates can be hydrolysed by sphingomyelinase. However, crude extracts of tissues contain other (non-sphingomyelinase) enzymes that can also hydrolyse these substrates and data on crude extracts must be interpreted with caution. The hydrolysis of bis-4-methylumbelliferyl phosphate by purified sphingomyelinase does not obey Michaelis–Menten kinetics and additional studies are needed to define this further (C. S. Jones & J. W. Callahan, unpublished work). Hydrolysis of bis-*p*-nitrophenyl phosphate is generally about 35% of that displayed with bis-4-methylumbelliferyl phosphate, suggesting that it is a poorer substrate for the enzyme.

Bis-4-methylumbelliferyl phosphate activity has been used in the diagnosis of certain types of Niemann–Pick disease (Fensom *et al.*, 1977; Besley, 1978). Our own results confirm decreased bis-4-methylumbelliferyl phosphate hydrolysis in fibroblast extracts from types A and B, but we believe that at present these data are only suggestive and do not replace assays based on hydrolysis of the natural substrate.

Note added in proof (Received 27 December 1980)

A mean value of 151000 nmol/h per mg of protein (four preparations) is obtained after Blue-Sepharose chromatography, but the sodium dodecyl sulphate gel pattern is identical with that in Fig. 7. We conclude that this step separates native from denatured sphingomyelinase.

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