ASSAY OF SPHINGOMYELINASE ACTIVITY

Protocol for Protein Extraction

Stock Solution

1. Leupeptin/hydrochloride (FW 463.0, <0°C)
   1 mg/ml PBS -40°C
2. PMSF (phenylmethylsulfonyl fluoride, FW174.2)
   200 mM prepare with isopropyl alcohol/isopropanol, -20°C
3. Phosphate buffered saline (PBS), pH 7.4
4. Sucrose Buffer, pH 7.4
   20 mM Hepes (FW 238.3) 0.4766 g/100 ml DW
   1 mM EDTA 1 ml (100 mM) /100 ml
   255 mM Sucrose (FW 342.3) 8.729 g/100 ml
5. Tris/HCl, EDTA (TE) buffer, pH 7.5
   20 mM Tris/HCl (FW157.6) 0.315 g /100 ml DW
   1 mM EDTA 1 ml (100 mM) /100 ml

Solution

1. Homogenate buffer-1, pH 7.5
   TE buffer 20 ml/20 ml
   10 mM 2-Mercaptoethanol 1.4 ul (14.3 M, RT) /20 ml
   1 ug/ml Leupeptin 20 ul (1 mg/ml) /20 ml
   0.1 mM PMSF 10 ul (200 mM) /20 ml
2. Homogenate buffer-2, pH 7.4
   Sucrose buffer 40 ml/40 ml
   0.1 mM PMSF 16 ul (250 mM)/40 ml

Preparation of Homogenate from Small Bovine Coronary Arteries

1. isolate coronary arteries and place them in ice-cold PBS
2. remove the adherent fat, connective tissue, and endothelium
3. frozen with dry ice, grind into powder with grind bowl in liquid nitrogen
4. homogenize with a Teflon pestle glass homogenizer in homogenate buffer
   put homogenate into one glass tube
5. sonicate at #5, 20 seconds/time for 3 times
   aliquot into 1.5 ml eppendorf tubes
6. centrifuge for 5 min at 2940 g (6000 rpm) 4°C
   bring supernatant together and aliquot into eppendorf tubes
   take some supernatant for protein assay

* rotational speed(rpm)
  relative centrifugal force/field (RCF) = 1.12r(RPM/1000)^2 (xg)
  Ex: r=204 mm, rmp=3000, RCF=2060 g

7. frozen with liquid nitrogen, and store at -80°C

Preparation of microsomal and cytosolic fraction of rat myocardium

1. anesthetize the rat with Nembutal(pentobarbital sodium, 50 mg/ml) at 0.1 ml/100g
2. cut off the heart, wash with cold PBS, excise the left heart and chop into small pieces
3. transfer to glass mortar, add homogenization sucrose buffer (1 ml/left heart), usually 1 volumes buffer per volume of tissue
4. immerse the glass mortar in ice water to maintain a low temperature
5. homogenize with power-driven glass-Teflon homogenizer at speed 30-40 (~1800 rpm), pass through the sample 10 times, allowing 5-10 seconds per stroke
6. aliquot into eppendorf tubes (2 tubes per sample), sonicate for three times 20 seconds/time
   * sonicate can increase the yield of membrane fraction, 'cause it'll pull off the membrane bound protein
7. tissue homogenate subjected to low-speed centrifugation (1,000 g for 10 min at 4°C)
   pellet contains connective tissues, whole cells, nuclei, cytoskeletons, plasma membranes
   supernatant subjected to medium-speed centrifugation (10,000 g for 20 min at 4 °C)
   pellet contains mitochondria, lysosomes, peroxisomes
   supernatant subjected to ultra-centrifugation (100,000 g for 90 min at 4°C)
   the pellet contains microsomes
   the supernatant contains cytosolic soluble proteins
   * make sure the samples are well balanced before ultracentrifuge
1. aliquot the supernatant into four tubes
2. dissolve the pellet with about 600 ul sucrose buffer (PMSF 10 uM) total, aliquot into three tubes
3. frozen with liquid nitrogen, and store at -80°C

Protein Assay

Reagent

Bio-Rad Protein assay (4°C): take 100 ml protein assay concentrate, add 400 ml deionized water, and then filter the buffer.
Concentration range for the assay: 20-90 ug protein

Protocol

1. add 4 ml reagent into each cuvette
2. add standard 0, 25, 50, 100 ul BSA (22 mg/ml) into cuvettes #1-4 respectively
3. add 1-10 ul sample into #5-6 respectively
4. protein concentration is calculated as (sample OD/ standard OD) x 22/5 (mg/ml)

Ex: 25 ul BSA 50 ul BSA 100 ul BSA 5 ul sample 10 ul sample
0.309 0.543 0.910 0.300 0.534
standard OD = (0.309 + 0.543/2 + 0.910/4)/3 = 0.269
sample OD = (0.300 + 0.534/2)/2 = 0.284
protein con. = (0.284/0.269)x22/5 = 4.64 mg/ml

Sphingomyelinase(SMase) Assay

Stock

1. Glutathione/disodium salt (GSH, FW 656.6, desiccate, store at < 0°C)
   500 mM 0.3283 g/1.0 ml reaction buffer, -20°C
2. ^{14}C-Sphingomylin(25 uCi/ml, 55 mCi/mmol, 0.45 mM, 10 uCi/vial, -20°C)
   2.5 uCi/ml 100 ul original ^{14}C-SM + 900 ul methanol
   4 ul 2.5 uCi/ml ^{14}C-SM = 22200 dpm = 182,000 fmol
   cpm (counts per minute) = dpm/counting efficiency, 1 mCi=2.22x10^{9} dpm
3. Sphingomyelin (FW 820, <0°C)
   100 mM 100 mg/1.22 ml methanol, -40°C
   1:1000 dilution to 0.1 mM, add 4 ul as hot sphingomyelin
4. Sphingomyelinase (4°C)
   0.1 U/1 ul 50 U/0.44 ml
5. Reaction buffer
   Reaction buffer-1(Tris-1) *for N-SMase*
   20 mM Tris/HCl, pH 7.4 0.315 g/100 ml
   Reaction buffer-2(Tris-2) *for N-SMase*
   100 mM Tris/HCl, pH 7.4 1.576 g/100 ml
   0.05% Triton X-100 50 μl/100 ml
   5 mM Magnesium Chloride 0.102 g/100 ml
   Reaction buffer-3 *for A-SMase*

   Reaction buffer-4 *for A-SMase*
   100 mM Sodium Acetate(136.08), pH 5.0 1.361 g/100 ml

Solution

1. DL-Dithiothreitol (DTT, FW 154.2, 4°C) 500 mM
   0.0116 g/0.15 ml reaction buffer
   1:10 dilution with buffer to 50, 5, 0.5 mM
   final concentrations are 10, 1, 0.1, 0.01 mM
2. glutathione
   stock 500 mM, diluted with buffer to 250, 125, 50 mM
   final concentrations are 10, 5, 2.5, 1 mM
3. Sphingomyelinase
   1:10 dilution with buffer to different concentrations

N-SMasa Assay

1. Standard SMase

1. add solutions into 1.5 ml eppendorf tubes

	1	2	3	4	5	6	7	8	9	10	11
SMase (U)	0	0.3	0.1	0.03	0.01	0.003	0.001	0.0003	0.0001	0.00003	0.00001
SMase (μl)	0	3	1	1	1	1	1	1	1	1	1
Tris-1 (μl)	50	47	49	49	49	49	49	49	49	49	49
Tris-2 (μl)	46	46	46	46	46	46	46	46	46	46	46
14C-SM (μl)	4	4	4	4	4	4	4	4	4	4	4

↓ mix with vortex and centrifuge

2. incubate at 37 °C for 1 h, speed #5
   (take out and mix 30 min after incubation, and then put them back)

↓ vortex, centrifuge, transfer into glass tubes

3. add 1.5 ml chloroform-methanol (2:1) to stop reaction

↓ shake

   add 0.2 ml water

↓ <u>shake vigorously</u>, centrifuge at 6000 rpm x 5 min

4. take out as much as the upper phase into liquid scintillation tube

* the results may be better than just taking out a certain portion of the upper phase, but be careful not to stir up the lower phase

↓

5. do the same extraction by adding 0.2 ml water into glass tube
   * just to increase the yield

↓ <u>shake vigorously</u>, centrifuge at 6000 rpm x 5 min

6. take out as much as upper phase into liquid scintillation vial
   add 4 ml Ecolite, shake , then run program 2

2. Concentration-effect relationship

Homogenate from cow coronary arteries (6.8 μg/μl, 03/ 25/97)

	1	2	3	4	5	6	7	8	9	10
Homo (μg)	0	50	50	100	100	500	500	1000	1000	
Homo (μl)	0	7	7	15	15	74	74	147	147	0
Tris-1 (μl)	200	193	193	185	185	126	126	53	53	0

Tris-2 (μl)	46	46	46	46	46	46	46	46	46	46
14C-SM (μl)	4	4	4	4	4	4	4	4	4	4

3. Time course

Homogenate from cow coronary arteries (6.8 μg/μl, 03/ 25/97)

	1	2	3	4	5	6	7	8	9	10
Homo (mg)	0	500	500	500	500	500	500	500	500	
Homo (μl)	0	74	74	74	74	74	74	74	74	0
Tris-1 (μl)	200	126	126	126	126	126	126	126	126	0
Tris-2 (μl)	46	46	46	46	46	46	46	46	46	46
14C-SM (μl)	4	4	4	4	4	4	4	4	4	4
incub time (min)	60	30	30	60	60	90	90	120	120	120

4. Inhibition of SMase by GSH

Homogenate from cow coronary arteries (6.8 μg/μg, 03/ 25/97)

	1	2	3	4	5	6	7	8	9	10
Homo (μg)	0	500	500	500	500	500	500	500	500	
Homo (μl)	0	74	74	74	74	74	74	74	74	0
GSH (μl)	0	0	0	5	5	5	5	5	5	0
Tris-1 (μl)	200	126	126	121	121	121	121	121	121	0
Tris-2 (μl)	46	46	46	46	46	46	46	46	46	46
14C-SM (μl)	4	4	4	4	4	4	4	4	4	4

	1	2	3	4	5	6	7	8	9	10	11	12
Homo (μg)	0	500	500	500	500	500	500	500	500	500	500	
Homo (μl)	0	74	74	74	74	74	74	74	74	74	74	0
GSH (μl)	0	0	0	5	5	5	5	5	5	5	5	0
Tris-1 (μl)	200	126	126	121	121	121	121	121	121	121	121	0
Tris-2 (μl)	46	46	46	46	46	46	46	46	46	46	46	46
14C-SM (μl)	4	4	4	4	4	4	4	4	4	4	4	4

* NaAc, Homo and GSH were incubated at 37°C for 15 min, then add Tris-2 and 14C-SM buffer.

* GSH final concentrations are 10, 5, 2.5, 1 mM.

5. Inhibition of SMase by DTT

Homogenate from cow coronary arteries (6.8 μg/μg, 03/ 25/97)

	1	2	3	4	5	6	7	8	9	10	11	12
Homo (μg)	0	500	500	500	500	500	500	500	500	500	500	
Homo (μl)	0	74	74	74	74	74	74	74	74	74	74	0
DTT (μl)	0	0	0	5	5	5	5	5	5	5	5	0
Tris-1 (μl)	200	126	126	121	121	121	121	121	121	121	121	0
Tris-2 (μl)	46	46	46	46	46	46	46	46	46	46	46	46
14C-SM (μl)	4	4	4	4	4	4	4	4	4	4	4	4

* Tris-1, DTT and Homo were incubated at 37°C for 15 min, then add Tris-2 and 14C-SM buffer.

DTT final concentrations are 10, 1, 0.1, 0.01 mM.

Identification of Ceramide and Sphingosine by TLC

Stock Solution

1. C2 Ceramide (D-*erythro*-Sphingosine, N-Acetyl, MW341.5, -20°C)
   10 mM 5 mg/1.46 ml ethanol, aliquot to 50 ul/vial, -40°C
2. C16 Ceramide (D-*erythro*-Sphingosine, N-Palmitoyl-, MW537.9, -20°C)
   10 mM 5 mg/930 ul warm ethanol, aliquot to 50 ul/vial, -40°C
3. Sphingomyelin (FW 820, <0°C)
   100 mM 100 mg/1.22 ml methanol, -40°C
4. Sphingomyelinase (4°C)
   0.1 U/1 ul 50 U/0.44 ml
5. Sphingosine (D-erythro-Sphingosine-1-phosphate, MW397.5,)
   10 mM 1 mg/264 ul methanol, aliquot to 50 ul/vial, -40°C
6. Ammonium hydroxide (MW 35.05)
   28-30% (NH3)

Solution

1. C16 Ceramide
   1:50 dilution with chloroform to 0.2 mM 20 ul stock + 980 ul
   100 ul solution (0.2 mM) = 0.02 umol
2. C16 Ceramide
   1:50 dilution with chloroform to 0.2 mM 20 ul stock + 980 ul
3. Sphingomyelin
   1:10 dilution with methanol to 10 mM 40 ul stock + 360 ul
   1: 500 dilution with chloroform to 0.2 mM 2 ul stock + 998 ul
4. Sphingosine
   1:50 dilution with chloroform to 0.2 mM 20 ul stock + 980 ul
5. Solvent system
   I: chloroform:methanol:water:25% ammonium hydroxide (50:50:2:1)
   121 : 121 : 4.9 : 2.4 ml, total 250 ml
   II: chloroform:methanol:water:25% ammonium hydroxide (90:10:0.5:0.5)
   223 : 25 : 1.2 : 1.2 ml, total 250 ml

Protocol

Sample Preparation

Tube 1-2: Rat heart homogenate (2)(12/25/97, protein concentration is 14 ug/ul)
Tube 2-4: Rat kidney homogenate (protein concentration is 25 ug/ul)
Tube 5-6: Cow coronary artery homogenate (03/25.97, protein concentration is 6.8 ug/ul)
Tube 7-8: Standard sphingomyelinase (0.1 U/ 1 ul)

Cold-SM (10 mM)/Tris buffer bulk for 5 samples: 40 ul + 960 ul

	1	3	5	7
Homo (μg)	500*2	100*2	500*2	0.1*2
Homo (μl)	72	8	148	2
Tris-1 (μl)	128	192	52	198
Tris-3 (μl)	192	192	192	192
SM (μl)	8	8	8	8
Incub (min)	60			

1. After extraction, put the lower phase of the same sample together
2. dry under N2 gas
3. dissolve the sample with 100 ul chloroform

TLC Preparation

1. pour the mobile phase into TLC tank 10-15 min before development, make sure paper is saturated with mobile phase

Spotting

1. rinse syringes (250 ul) with chloroform or mobile phase 3x
2. load syringes with samples and standards, be sure to leave 50 ul of air space behind samples
3. fasten syringes with bar and pin
4. lower needles onto TLC plate gently
5. turn on N2 gas and spotter, make spots as smaller as possible
6. when finished, rinse syringes with chloroform or mobile phase 3x

from left to right:
0.2 mM Sphingomyelin 100 ul, 0.2 mM Sphingosine 100 ul,
Sample1 100 ul, Sample2 100 ul, …,
0.2 mM C16 Ceramide 100 ul, 0.2 mM C2 Ceramide 100 ul

Development
develop to 70% with solvent system I
dry under nitrogen
then develop to the top of the plate with solvent system II

Visualization
stain with iodine