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Identification of Alkylbenzene Sulfonate Surfactants Leaching from an Acrylonitrile Butadiene Rubber as Novel Inhibitors of Calcineurin Activity

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Calcineurin (CN) is a Ca^{2+}/calmodulin (CaM) dependent serine/threonine protein phosphatase and plays important role in several cellular functions in both higher and lower eukaryotes. Here we report inhibition of CN by linear alkylbenzene sulfonate. The clue to the finding was obtained while identifying the inhibitory material leaching from acrylonitrile butadiene rubber used for packing. Using standard dodecylbenzene sulfonate (C_{12}-LAS), we obtained strong inhibition of CN with a half maximal inhibitory concentration of 9.3 μM, whereas analogs such as *p*-octylbenzene sulfonate and SDS hardly or only slightly affected CN activity. Three alkaline phosphatases, derived from shrimp, bacteria, and calf-intestine, which exhibit similar enzymatic activities to CN, were not inhibited by C_{12}-LAS at concentrations of up to 100 μM. Furthermore, C_{12}-LAS did not inhibit Ca^{2+}/CaM-dependent myosin light chain kinase activity when tested at concentrations of up to 36 μM. The results indicate that C_{12}-LAS is a potent selective inhibitor of CN activity.

Key words: surfactants; alkylbenzene sulfonates; acrylonitrile butadiene rubber; calcineurin; inhibitors

Synthetic polymer products, such as PET bottles and various reusable and disposable plastic containers, are utilized for their convenience in several areas of the food, pharmaceuticals, and medical industries, but numerous chemical substances including synthetic materials, emulsifiers, plasticizers, and antioxidants, are used in the manufacture of these polymer products, and some compounds leaching from them may affect the health of living organisms, including humans. For example, bisphenol A is a raw material used to manufacture synthetic polymers, and it is known to be responsible for immune dysfunction, breast cancer, and prostate tumor multiplication.[1–3] Branched 4-nonylphenol, a raw material used to make anionic and non-ionic surfactants, and has been found to damage the DNA of sperm, lymphocyte, and MCF-7 breast-cancer cells.[4] Recent papers report that it is not only bisphenol A but also antimony that leaches from polycarbonate and polyethylene terephthalate containers into food and beverages.[5] Furthermore, additional harmful compounds, including nonylphenolethoxylate, di (2-hydroxyethyl) methyldodecyl ammonium, and 9-octadecenamide, have been reported to be leached from plastics.[6] These compounds are used routinely in the plastic manufacturing industry as dye-solubilizing agents, processing agents, and slip agents respectively. Nonylphenolethoxylate has been reported to inhibit the activity of mitochondria respiratory chain complex I at a half maximal inhibitory concentration (IC_{50}) of about 4 μM,[5] and di (2-hydroxyethyl) methyldodecyl ammonium was reported to inhibit both monoamine oxidase A and monoamine oxidase B at μM levels. Octadecenamide was also found to inhibit monoamine oxidase B at such levels.[6]

Calcineurin (CN, also known as protein phosphatase 2B) is a member of the serine/threonine protein phosphatase family. It is activated by Ca^{2+} and calmodulin (CaM), and constitutes a hetero-dimer comprised of a catalytic A-subunit (CN-A) of about 60 kDa, which contains the CaM-binding site, and a regulatory B subunit (CN-B) of about 19 kDa, which contains the Ca^{2+}-binding site.[7,8] Three isoforms of CN-A (α, β, and γ) have been identified. Their amino acid sequences are highly conserved among various organisms. The α isoform is distributed dominantly in neurons. The β isoform is broadly distributed, and the γ isoform is specifically expressed in the testis. In addition, two

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Abbreviations: CN, calcineurin; CaM, calmodulin; NBR, acrylonitrile butadiene rubber; HPLC, high-performance liquid chromatography; LAS, linear alkylbenzene sulfonate; IC_{50}, half maximal inhibitory concentration; C_{12}-LAS, linear dodecylbenzene sulfonate; TLC, thin-layer chromatography; pNPP, *p*-nitrophenylphosphate; pNP, *p*-nitrophenol; MLCK, myosin light-chain kinase; PAGE, poly-acrylamide gel electrophoresis; AP, alkaline phosphatase; PMSF, phenylmethylsulfonyl fluoride; COSY, chemical shift-correlated spectroscopy; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple-bond correlation

isoforms of CN-B have been identified: ubiquitously produced CN-B1, and CN-B2, which has been detected only in the testis.[9] CN has been implicated in a wide variety of biological responses, including lymphocyte activation, neuronal and muscle development, neurite outgrowth, morphogenesis of vertebrate heart valves, and the control of transcription even in yeast.[10] It has also been found that the immunosuppressant drugs, FK506 and cyclosporine A inhibit CN *via* immunophilins.[11–13]

Previously, during the course of screening pharmacological targets of CN, we found that CN inhibitory material was leached from rubber laboratory rings.[14] This suggested that the CN inhibitory substance derived from synthetic polymer products might influence bioecological systems. Unfortunately, identification of the CN inhibitor was unsuccessful due to limited availability of the material.

In the present study, we assessed the CN inhibitory effects of various polymer products after extraction with ethanol. The results showed that acrylonitrile butadiene rubber (NBR) contained largest amounts of CN inhibitory materials. We purified two species of CN inhibitors from the extracts, and structural analysis by MS and NMR revealed that they were analogs of linear alkylbenzene sulfonate (LAS). Using standard linear dodecylbenzene sulfonate (C_{12}-LAS), we confirmed that this compound strongly inhibited CN activity. To our knowledge this is the first report that C_{12}-LAS leached from NBR has CN inhibitory effect.

Materials and Methods

Materials. Silica gel 60 for column chromatography (0.06–0.20 mm) and for thin-layer plates (TLC, 0.25 mm, 20 × 20 cm) was purchased from Merck (Darmstadt, Germany). A Quick Start Bradford Protein assay kit, AG-X8, and DEAE BIO-GEL A were from Bio-Rad Laboratories (Hercules, CA). Sodium linear dodecylbenzene sulfonate (C_{12}-LAS), sodium *p*-octylbenzene sulfonate, and SDS were from Wako Pure Chemicals (Osaka, Japan). DEAE Sepharose Fast Flow was from GE Healthcare Bio-Science (Uppsala, Sweden). Sodium benzene sulfonate, *p*-nitrophenylphosphate (pNPP), and *p*-nitrophenol (pNP) were from Sigma (St. Louis, MO). A CN phosphatase assay kit, BML-AK804, was from Enzo Life Science (Farmingdale, NY).

Enzymes and proteins. Enzymes and proteins were purchased from the following companies; bovine brain CN (2,500–3,210 U/mg of protein, Sigma) and bovine testis CaM (40,000 U/mg of protein, Sigma); shrimp alkaline phosphatase (Roche Diagnostics, Basel, Switzerland); *Escherichia coli* alkaline phosphatase (Takara BIO, Shiga, Japan), and calf intestine alkaline phosphatase (Toyobo, Osaka, Japan). Our definitions of units of commercial available enzymes were in accordance with the manufacturers' descriptions. Rat brain CN, used in the quantitative determination of CN inhibitory activity, was partially purified using a method similar to one previously described up to the step of DEAE cellulose column chromatography.[15,16] The enzyme obtained was applied to a second DEAE BIO-GEL A column chromatography, and then CN was eluted by a linear concentration gradient of NaCl (0 to 0.5 M). One unit (U) of rat brain CN was defined as the amount of enzyme catalyzing the formation of 1 μmol of pNP per min. The enzyme solution obtained was divided into several portions and kept frozen at −80 °C until use. Unless otherwise indicated, purified rat brain CN with a specific activity of about 64 mU/mg of protein was used only in the purification of inhibitors. Smooth-muscle myosin was purified from chicken gizzard,[17] and myosin light chain kinase (MLCK) was purified from bovine stomach,[18] as described previously. Protein concentrations were determined by means of a Quick Start Bradford Protein assay kit, with bovine serum albumin as standard.

CN activity assay. Two methods were used to assay CN activity. In method I, pNPP was used as substrate. The standard incubation mixture contained 100 mM HEPES-NaOH (pH 7.5), 1 mM $CaCl_2$, 5 mM $MgCl_2$, 1.5 mM pNPP, 0.2 mM $NiCl_2$, and 5 U each of bovine brain CN and bovine testis CaM in a final volume of 400 μL. Incubation was for 30 min at 37 °C. The reaction was terminated by adding 600 μL of 50 mM EDTA (pH 8.5). The pNP formed was quantified by measuring A_{410}. In method II, a CN assay kit, BML-AK804[19] was used. In this assay, synthetic phosphopeptide (Asp-Leu-Asp-Val-Pro-lle-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-pSer-Val-Ala-Ala-Glu) and human recombinant CN were used as substrate and enzyme respectively.

CN inhibitory activity assay. Semiquantitative assays (I and II) and quantitative assay were used.

Semiquantitative assay I: The standard incubation mixture contained 100 mM HEPES-NaOH, (pH 7.5), 1 mM $CaCl_2$, 5 mM $MgCl_2$, 3 mM pNPP, 0.2 mM $NiCl_2$, 5 U of bovine brain CN, and 10 U of bovine testis CaM in the presence of ethanol extracts (1 μL) in a final volume of 200 μL. After incubation at 37 °C for 60 min, the reaction was terminated by adding 800 μL of 1 M Na_2CO_3, and then the A_{410} of the mixture was measured.

Semiquantative assay II: The ingredients of the incubation mixture, the incubation conditions, and the determination of Pi were as described above for CN assay method II, except that 0.5 μL of ethanol extract was added to the incubation mixture.

Quantitative assay: The ingredients of the incubation mixture were the same as those used for CN assay method I, except that 1.5 mM pNPP and 1.8 mU of purified rat brain CN were used. After incubation at 37 °C for 30 min, the reaction was terminated by adding 800 μL of 0.2 M EDTA (pH 8.5). CN activity was determined by measuring the A_{410} of the reaction mixture. By all three methods, control experiments were performed using ethanol instead of ethanol extracts. By serial 2-fold dilution of the ethanol extracts, the amounts of the extracts that achieved 50% inhibition were determined. One unit of inhibitory activity was defined as the amount of inhibitor causing 50% inhibition of the control activity, which was determined in the absence of an inhibitor.

Alkaline phosphatase (AP) activity assay. For shrimp AP, the standard incubation mixture contained 94 mM Tris–HCl (pH 9.0), 5 mM $MgCl_2$, 1 mM pNPP, and 4 U of shrimp AP in a final volume of 800 μL. After incubation at 37 °C for 60 min, the reaction was terminated by adding 200 μL of 0.2 M EDTA (pH 8.5). The pNP formed was quantified by measurement of A_{410}. For the *E. coli* AP and calf intestine AP assays, 0.8 and 2 U of AP were used instead of shrimp AP, respectively.

MLCK activity assay. Bovine MLCK (final concentration 0.074 μM) and CaM (final concentration 0.12 μM) were mixed with varying concentrations of C_{12}-LAS (0 to 36 μM), and then 1 μM (final concentration) of chicken gizzard myosin was added in a reaction buffer that contained 20 mM Tris–HCl (pH 7.5), 60 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP, 0.1 mM $CaCl_2$, and 1 mM dithiothreitol. The reaction was carried out in a final volume of 20 μL at 25 °C for 20 min. After terminating the reaction by adding an equal volume of a sample buffer containing 6 M urea, 14 mM 2-mercaptoethanol, and 50 mM Tris–HCl (pH 7.5), aliquots were analyzed by urea-glycerol poly-acrylamide gel electrophoresis (PAGE) as described previously.[20,21]

Statistical analysis. The data were recorded as mean ± standard deviation and were analyzed by one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test by SPSS (version 17.0; SPSS, Chicago, IL).

Screening for CN inhibitory materials in polymer products. The presence of CN inhibitory substances was screened for using polymer products for daily, industry and laboratory use (Table 1). Shredded polymers (0.2 g each) were soaked in 2 mL of ethanol and kept for 48 h at room temperature. After soaking, the CN inhibitory effects of the extracts obtained were determined by the two semiquantitative assays (see above).

Purification of CN inhibitory compounds from NBR. Twenty g of the sample (NBR) were shredded into pieces and soaked in 100 mL of

Table 1. CN Inhibitory Effects of Substances Leached from Polymer Products

Sample no.	Product name (company)	Material	CN inhibitory activity (%)	
			Assay I	Assay II
1	Electric cable cover (M corporation)	Unknown	73.1	78.5
2	Electric cable cover (Y corporation)	PVC	84.1	95.5
3	Electric cable cover (T corporation)	PVC	100.8	98.0
4	Electric cable cover (X corporation)	Unknown	39.8	ND
5	Electric cable cover (J corporation)	CLPE	ND	93.0
6	Packing of water purifier (N corporation)	Unknown	4.3	13.2
7	Packing of water pipe (K corporation)	EPDM	78.1	ND
8	Packing of bottle (W corporation)	PE	88.4	ND
9	Centrifuge cap (F corporation)	HDPE	98.6	ND
10	Centrifuge tube (F corporation)	PP	92.6	ND
11	Test tube (S corporation)	HDPE	96.6	ND
12	Centrifuge tube (S corporation)	PP	98.5	ND
13	Rubber band (D corporation)	NR	26.7	ND
14	Sterile syringe rubber (T corporation)	TPE	ND	114.2
15	Packing for water cleaner (A corporation)	Unknown	ND	114.8
16	Rubber tube (X corporation)	PU	ND	107.1
17	Packing of water pipe (K corporation)	NBR	11.0	1.6
18	Packing of bottle tube (A corporation)	PP	ND	111.9
19	Vial septum for HPLC (S corporation)	PTFE	ND	90.3
20	Cap for water bottle (J corporation)	PP	ND	100.4
21	Cable cover for HPLC (S corporation)	PVC	ND	101.2

Two semiquantitative assays, I and II, were used in the screening of CN inhibitory activities in the polymers. CN inhibitory activities were expressed as % of control activity, which was determined in the absence of inhibitor. *Abbreviations*: ND, not determined; PVC, polyvinyl chloride; CLPE, cross-linked polyethylene; EPDM, ethylene propylene rubber; PE, polyethylene; HDPE, high density polyethylene; PP, polypropylene; NR, natural rubber; TPE, thermoplastic elastomer; PU, polyurethane; NBR, nitrile butadiene rubber; PTFE, polytetrafluoroethylene

ethanol for 3 d at room temperature. The ethanol extracts were collected by filtration and dried under reduced pressure at 40 °C to obtain about 900 mg of red-brown residue. Part of this residue (600 mg) was dissolved in 8 mL of acetone/*n*-hexane (20:80, v/v) and applied to a silica gel column (2 × 8 cm) that had been equilibrated with acetone/*n*-hexane (20:80, v/v). The column was washed with 24 mL of acetone/*n*-hexane (20:80, v/v), 16 mL of acetone/*n*-hexane (50:50, v/v), 21 mL of acetone, and 20 mL of methanol/acetone (10:90, v/v). Finally, it was eluted by 20 mL of ethanol, and the solvent were removed under reduced pressure. The residue (11.7 mg) was dissolved in 220 µL of ethanol and subjected to TLC. A sheet of TLC plate gel (20 × 20 cm) was prewashed with a mixture of methanol and chloroform (10:90, v/v) and dried at room temperature. The condensed ethanol eluate containing the CN inhibitor was applied to the TLC plate and developed with chloroform/methanol/28% ammonium hydroxide (30:10:1, v/v). Sixteen bands were detected on the plate by brief exposure to UV light at 254 nm and at 366 nm (*Rf* values: 0.11, 0.31, 0.39, 0.43, 0.47, 0.52, 0.55, 0.56, 0.59, 0.63, 0.70, 0.73, 0.77, 0.82, 0.87, and 0.95). Among these a band (*Rf*: 0.31) was scraped off and mixed vigorously with 8 mL of ethanol, and the solutions were centrifuged at 2,400 *g* for 30 min. The supernatant solvent was evaporated, and in total 1,722 U (6.2 mg) of the CN inhibitor, as determined by the quantitative assay method, was obtained. Part of this (570 U, 2.1 mg) was dissolved in 200 µL of distilled water and divided into four parts. Each sample was subjected to reversed phase HPLC, as follows: The HPLC system used (Waters, Milford, MA) was comprised of one 2487 dual λ absorbance detector, one 1525 binary HPLC pump, and one 717 auto-sampler. In this system, an ODS column (A-307, ODS-AL, 6.0 × 150 mm, particle size 5 µm, YMC, Kyoto, Japan) was included. The following solutions (A and B) were prepared for chromatography: A, 1% trifluoroacetic acid in acetonitrile/water, 50:50 (v/v) and B, 1% trifluoroacetic acid in acetonitrile/water, 80:20 (v/v). After the column had been equilibrated with solution A for over 20 min at a flow rate of 1.5 mL, 50 µL of the sample solution was injected into HPLC over 50 min at a flow rate of 1.5 µL and started with solution A for 15 min, followed by a linear solvent gradient of 0 to 100% solution B for 33 min, and finally an isocratic flow with 100% solution B for 2 min, returned to solution A over 15 min.

The sample was separated into several peaks. Thirteen pivotal peaks were assigned and collected (labeled Hfr1–Hfr13) by monitoring A_{224}. The HPLC purification step was repeated for the remaining three parts of the TLC fraction, and the corresponding fractions, those eluted at similar retention times, were combined. Each of the combined fractions was evaporated to dryness under reduced pressure and redissolved in 260 µL of distilled water, and CN inhibitory activity was determined by quantitative assay. The solutions obtained from the residues of fractions Hfr3 and Hfr6 exhibited strong CN inhibitory activity and were evaporated to dryness again. We obtained 430 µg of dried material from fraction Hfr3 and 240 µg from fraction Hfr6. Aliquots were used for MS analysis, and the remaining residues were solubilized with methanol-d_4 (CD_3OD, 99.95 atom%D, Cambridge Isotope Laboratories, Woburn, MA) for NMR analysis.

MS and NMR. The negative ion mode electrospray ionization [ESI (−)] mass spectrum was recorded on a LCQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA). The following NMR spectra were recorded on an AVANCE 600 MHz spectrometer (Bruker Biospin, Faellanden, Switzerland): ^{1}H NMR chemical shift-correlated spectroscopy (COSY), ^{1}H-^{13}C NMR heteronuclear single quantum coherence (HSQC), and ^{1}H-^{13}C NMR heteronuclear multiple-bond correlation (HMBC). All spectra were recorded at 30 °C. Chemical shifts were referenced to internal peaks at δ_H 3.30 for CHD_2OD and at δ_C 49.0 for CD_3OD. The following data were obtained:

Hfr3: ESIMS m/z 311 $[M-H]^-$; ^{1}H NMR (CD_3OD, 600 MHz) δ_H 0.82 (3H, t, J = 7 Hz, –CH_3), 0.85 (3H, t, J = 7 Hz, –CH_3), 1.07 (2H, m, –CH_2), 1.15 (2H, m, –CH_2), 1.20–1.30 (8H, m, –CH_2), 1.55 (2H, m, –CH_2), 1.63 (2H, m, –CH_2), 2.54 (1H, m, –CH), 7.20 (2H, d, J = 8 Hz, –CH), 7.75 (2H, d, J = 8 Hz, –CH)

Hfr6: ESIMS m/z 325 $[M-H]^-$; ^{1}H NMR (CD_3OD, 600 MHz) δ_H 0.82 (3H, t, J = 7 Hz, –CH_3), 0.86 (3H, t, J = 7 Hz, –CH_3), 1.07 (2H, m, –CH_2), 1.15 (2H, m, –CH_2), 1.20–1.30 (10H, m, –CH_2), 1.55 (2H, m, –CH_2), 1.63 (2H, m, –CH_2), 2.54 (1H, m, –CH), 7.21 (2H, d, J = 8 Hz, –CH), 7.75 (2H, d, J = 8 Hz, –CH); ^{13}C NMR (CD3OD, 150 MHz) δ_C 14.3 (2C, –CH_3), 23.6–33.0 (5C, –CH_3), 28.7 (2C, –CH_2), 38.1 (2C, –CH_2), 47.1 (1C, –CH), 127.0 (2C, –CH), 128.6 (2C, –CH), 144.0 (1C, –C), 150.1 (1C, –C)

Results

Screening for CN inhibitory activity in polymer products

Previously, we noted that CN inhibitory compound leached from laboratory rubber rings used as test-tube

stoppers when screening for pharmacological targets of CN.[14] However, our attempt to purify and identify the inhibitor from the rubber was unsuccessful due to limited availability of the rubber ring. Hence we screened other polymers for CN inhibitory capacity using two semiquantitative assay procedures as described above in "Materials and Methods." As Table 1 indicates, the two products, no. 6 and no. 17, contained strong CN inhibitory activity as examined by multiple assay methods among the polymers tested. We chose to use product no. 17, which was made of acrylonitrile butadiene rubber (NBR), as the starting material for purification and identification of the inhibitor due to its easy availability.

It was not clear whether the inhibitor was just a surface contaminant of rubber, or was derived from the constituents of rubber. To test the first possibility, the following washing was performed: Sample no. 17 (1.25 g) was soaked in 12.5 mL of water (the water/rubber ratio was same as that used for ethanol extraction, see below), and sonicated for 3 min at 3 watts by ultrasonic processor (Sonics Vibra-Cell, Sonics & Materials, Newtown, CT). CN inhibitory activity in the washing water was then determined by quantitative assay, but no CN inhibitory activity was detected in the water extract. Thus it appeared that the inhibitor was not derived from the surface, but rather from the inside of the NBR. We tested extraction of the inhibitor from shredded NBR with both water and ethanol. No inhibitory activity was detected in the water extracts (data not shown). In contrast, a significant inhibitory effect was detected in the ethanol extracts. Hence we decided to use ethanol extracts as the starting material for inhibitor purification.

Purification of CN inhibitor from NBR

About 900 mg of residue was obtained from 20 g of shredded NBR by ethanol extraction. Using one-third of the sample, preliminary experiments were carried out to determine the chromatographic properties of the CN inhibitor. First we tested by silica gel column chromatography, in which the CN inhibitor was separated into two fractions. The first fraction (30% of inhibitory activity) was loosely bound to a column equilibrated with acetone/*n*-hexane (20:80, v/v), and eluted with 20 to 50% acetone in *n*-hexane. The second fraction (70% of inhibitory activity) was strongly bound to the column, and was not eluted with 50% acetone in *n*-hexane, 100% acetone, nor 10% methanol in acetone, but with 100% ethanol. We decided to purify the inhibitor from the latter fraction, because it represented the major inhibitory activity of the extracts. The ethanol eluate was evaporated to dryness. The residue was easily soluble in water, and the solution obtained was foamy, suggesting that it was surfactant nature. The re-dissolved solution was adsorbed to an anion exchanger AG-X8 (bicarbonate form) resin, and it was eluted by increasing concentrations of ammonium bicarbonate, indicating that the inhibitor contained anionic residues in the molecule. Next we tested for a good TLC developing solvent system using the above silica gel chromatography fraction. We determined that the basic solvent system (chloroform/methanol/28% ammonium hydroxide, 30:10:1, v/v) was better than another frequently used one (pyridine/chloroform/formic acid/water, 70:30:16:8, v/v). Using these results, we developed a purification procedure with reversed-phase HPLC as the final step, as described in "Materials and Methods."

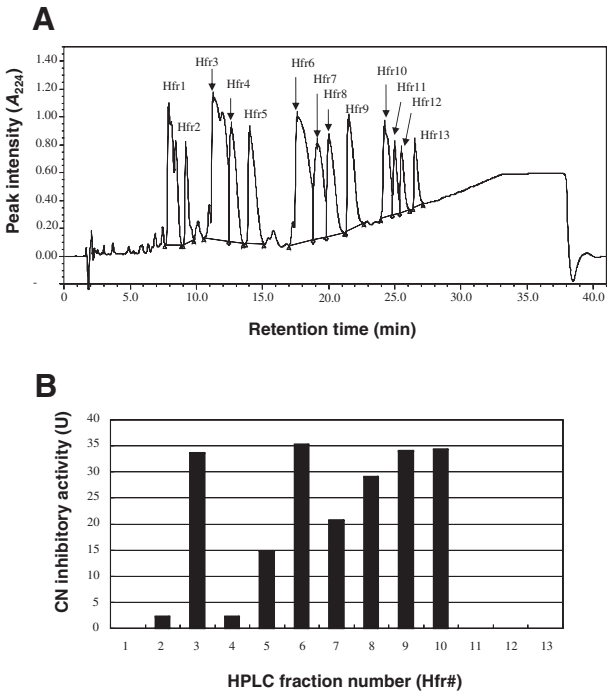

Fig. 1. Representative HPLC Chromatogram of TLC-Purified Compounds (A), and CN Inhibitory Activity of HPLC Fractions (B).

A, Fifty μL of the TLC fraction dissolved in aqueous solution was subjected to reversed-phase HPLC as described in "Materials and Methods." Thirteen pivotal peaks were assigned (Hfr1–Hfr13) by measuring A_{224}. B, CN inhibitory activities for the 13 HPLC fractions (Hfr1–Hfr13) were determined by quantitative as described in "Materials and Methods."

As shown in Fig. 1A, HPLC chromatography separated the sample from TLC into several A_{224} peaks and 13 pivotal peaks (labeled Hfr1–Hfr13) were collected. As Fig. 1B indicates, several fractions showed high CN inhibitory activity of these Hfr3 and Hfr6 were used for structural analysis by MS and NMR spectrometry.

Structural analysis of the CN inhibitor

The ESI(−)-MS spectra of Hfr3 and Hfr6 showed the $[M-H]^-$ ion at m/z 311 and 325 respectively. From this, the molecular masses of acid forms of Hfr3 and Hfr6 were estimated to be 312 and 326 respectively. The ^{1}H NMR spectrum of Hfr3 was essentially the same as that of Hfr6. In the ^{1}H NMR spectrum of Hfr6, two aromatic protons (δ_H 7.75, 7.21), an aliphatic methine (δ_H 2.54), nine methylenes (δ_H 1.63, 1.55, 1.30–1.07), and two aliphatic methyls (δ_H 0.86, 0.82) were observed. From the COSY data, two partial structures were assembled: a 1,4-disubstituted benzene ring and an aliphatic chain. In the HMBC spectrum, a correlation between an aromatic proton (δ_H 7.21) and an aliphatic methine carbon (δ_C 47.1) was observed. Based on these MS and NMR data, Hfr3 and Hfr6 were identified as undecylbenzene sulfonate and dodecylbenzene sulfonate respectively (Fig. 2). The possible reasons why alkylbenzene sulfonate was present in NBR are discussed below.

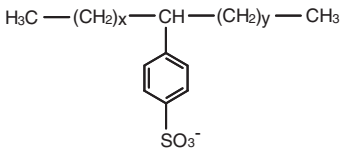

Fig. 2. Chemical Structures of the Compounds in Fractions Hfr3 and Hfr6.

The letters x and y indicate numbers of the methylene group. In Hfr3 (undecylbenzene sulfonate), $x + y = 8$, and in Hfr6 (dodecylbenzene sulfonate), $x + y = 9$.

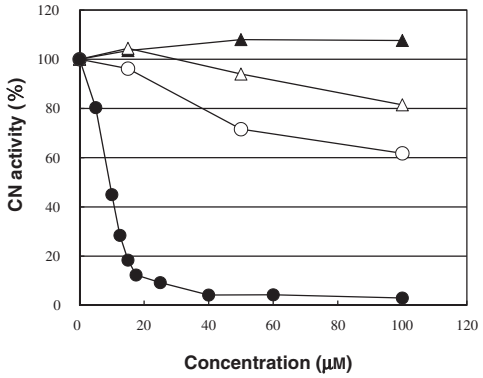

Fig. 3. Inhibition of CN Activity by C_{12}-LAS and Its Analogs.

CN activity was assayed by method I using the standard incubation mixture, as described in "Materials and Methods." Various inhibitors were added at the indicated concentrations. The symbols for the inhibitors used are as follows: solid circle, C_{12}-LAS; hollow circle, SDS; solid triangle, benzene sulfonate; hollow triangle, *p*-octylbenzene sulfonate. The results were expressed as % of control activity, which was determined in the absence of inhibitors.

C_{12}-LAS inhibited bovine brain CN

We noticed that the structures of two CN inhibitors, undecylbenzene sulfonate and dodecylbenzene sulfonate, looked similar to the principal ingredients of anion surfactant LAS. They are widely used not only in soap, but also in emulsifiers. In general, LASs are used as mixtures of alkyl side-chains of varying lengths (C_{10}–C_{14}). In order to confirm the CN inhibitory effect of alkyl benzene sulfonate, we obtained standard C_{12}-LAS together with sulfonate and sulfate analogs from a commercial source, and investigated their effects on bovine brain CN (Fig. 3). Under standard conditions, 20% and over 50% inhibition were obtained with 5 and 10 μM C_{12}-LAS respectively. Almost complete inhibition was obtained with 40 μM and higher concentrations of C_{12}-LAS. The IC_{50} value of C_{12}-LAS was estimated to be 9.3 μM. Benzene sulfonate at concentrations, up to 100 μM did not inhibit CN activity. *p*-Octylbenzenesulfonate, which has a shorter alkyl side-chain than C_{12}-LAS, exhibited weaker inhibition (20% at 100 μM). SDS, known as a strong protein denaturing reagent, inhibited CN only slightly. At 50 and 100 μM, inhibition was 28% and 38% respectively.

Since CN catalyzes the hydrolysis of pNPP to pNP and Pi in a manner similar to other phosphatases, we proceeded to examine the effects of C_{12}-LAS on bovine brain CN and a range of APs using pNPP as substrate. As Fig. 4 indicates, C_{12}-LAS inhibited CN selectively. Inhibition was obvious at 10 μM, and strong at 15 μM, but virtually no inhibition was observed with APs from

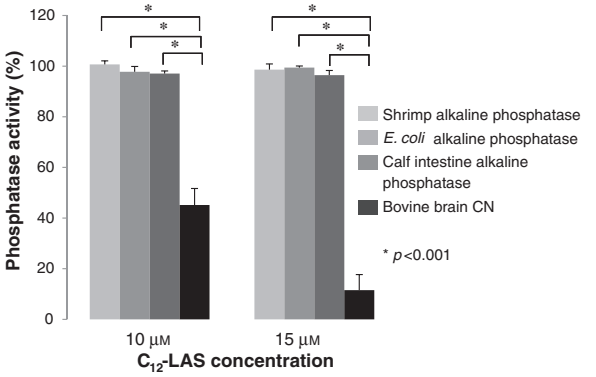

Fig. 4. Effect of C_{12}-LAS on the Activities of Various Phosphatases.

The activity of each phosphatase was expressed as % of control activity. The activities of three alkaline phosphatases were determined using standard incubation mixtures. The CN was assayed by method I as described in "Materials and Methods." The indicated amounts of inhibitors were added to the reaction mixture. Results are expressed as a mean $(n = 3) \pm SD$. Error bar represents SD. Statistical analysis was conducted as described in "Materials and Methods."

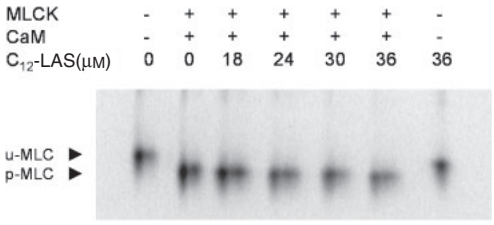

Fig. 5. Effect of C_{12}-LAS on MLCK Activity.

MLCK was assayed using a standard incubation mixture, as described in "Materials and Methods." The mixture contained C_{12}-LAS (0, 18, 24, 30, and 36 μM) as an inhibitor. After the reaction, the aliquots were analyzed by urea-glycerol PAGE. Arrows indicate the positions of unphosphorylated (u-MLC) and phosphorylated (p-MLC) myosin light-chain bands. The symbols (+ and −) indicate the presence and the absence of MLCK and CaM.

shrimp, *E. coli*, or calf intestine at these concentrations of C_{12}-LAS. We tested higher concentrations of C_{12}-LAS (20, 30, 50, and 100 μM) with those APs, but no inhibition was observed (data not shown).

Figure 5 shows the effect of C_{12}-LAS on MLCK. Since MLCK is a Ca^{2+}/CaM-dependent enzyme, like CN, we were interested to determine whether C_{12}-LAS inhibits this enzyme as well. The results clearly indicated that C_{12}-LAS did not inhibit MLCK activity when tested up to 36 μM. C_{12}-LAS was thus found to be a strong and specific inhibitor of CN.

Discussion

In the present investigation we identified alkylbenzene sulfonate leaching from NBR products as an inhibitor of CN activity. After screening of the polymer materials for CN inhibition, we found that ethanol extracts from NBR showed the strongest inhibition. The CN inhibitor was eluted in two fractions by silica gel column chromatography of the NBR ethanol extracts. The major peak of the inhibitor was more firmly bound to the column, and was thus eluted with a more polar solvent than the minor peak. We purified the inhibitor from the major peak and identified it. The minor peak

was not further investigated. We also noted that several peaks of the CN inhibitor emerged on reversed-phase HPLC, and they were not identified either. The purified inhibitors were identified as undecylbenzene sulfonate and dodecylbezene sulfonate by MS/NMR. We speculate that other unidentified peaks detected by HPLC might be other alkylbenzene sulfonates having different lengths of alkyl side-chain. This is supported by the fact that LAS is the most widely used surfactant among emulsion polymerizers.[22] LAS is commonly used as a surfactant in commercial and industrial applications, including emulsifiers, laundry detergents, surfactant-cleansing agents, and dyeing auxiliaries, with annual global production of four million tons around the world.[23] After use, most LAS is discarded into rivers with wastewater, and it is considered to be a high-priority pollutant. It is evident that this detergent causes toxicity in aquatic environments by adversely affecting a range of organisms. The toxicity of LAS against various freshwater species including bacteria, algae, insect, fishes, and amphibians, was studied by Canton and Slooff.[24] The acute toxicity of the compound differed greatly with the organism tested, up to a factor of 1,000. Yamamoto *et al.* reported on LAS aquatic environmental risk assessment in Japanese rivers.[25] LAS has been reported to cause biochemical impairment of the activity of several hydrolytic enzymes, including plant acid phosphatases,[26] fish phosphatases and succinic dehydrogenase,[27] microbial lipase,[28] fish phosphatases (APases and acid phosphatases),[29] and algae acid phosphatase.[30] It is not known whether LAS is incorporated into mammalian cells. Hence, further environmental biology research is necessary. If LAS is incorporated into cells, it probably inhibits various enzymes, including CN, and might cause severe damage to mammals.

Purification of the CN inhibitor leached form NBR enabled us to identify alkylbenzene sulfonates as the inhibitor. By standard C_{12}-LAS, we were able to confirm this identification. To the best of our knowledge, this is the first report of CN inhibition by LAS. Based on inhibitory mechanism, CN inhibitors can be classified into two groups. The first group are immunosuppressants, such as cyclosporine A and FK506, which indirectly inhibit CN *via* immunophilin.[12] The second group are direct inhibitors such as retinoids,[31] gossypol,[32] and unsaturated fatty acid.[33] C_{12}-LAS did not inhibit the CaM-dependent protein kinase MLCK concentrations at which CN activity was nearly completely inhibited. Thus C_{12}-LAS appears to inhibit CN by acting directly on one or both of its catalytic subunits, CN-A and CN-B, but not on CaM. This suggests that LAS belongs to the second group of CN inhibitors. Further study is necessary to confirm this.

C_{12}-LAS is a selective inhibitor of CN, since C_{12}-LAS did not inhibit APs, as determined by hydrolysis of pNPP to Pi and pNP at concentrations of up to 100 μM of LAS. A previous study by Tanaka *et al.* found that acid phosphatase from wheat germ was the most sensitive to LAS among various enzymes examined.[26] The IC_{50} of C_{15}-LAS to the enzyme was 11 ppm (this corresponds to about 28 μM by the average M_r of C_{15}-LAS). Our preliminary experiments with partially purified rat CN indicated that the longer the LAS alkyl-chain was, the stronger the inhibition displayed. The IC_{50} values for C_{12}-LAS, C_{13}-LAS, and C_{14}-LAS were 13.5, 5.7 and 2.9 μM respectively, suggesting that CN is the most sensitive to LAS among the various phosphatases we tested. It would be interesting to investigate the effects of LAS on other protein phosphatases.

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