ELSEVIER

Journal of Chromatography A, 662 (1994) 263–267

JOURNAL OF CHROMATOGRAPHY A

High-performance liquid chromatographic purification of sodium bis(2-ethyl-1-hexyl)sulphosuccinate from commercial preparations containing near-UV absorbing and fluorescent impurities

Ettore Bismuto*, Gaetano Irace

Dipartimento di Biochimica e Biofisica, Seconda Università di Napoli, Via Costantinopoli 16, 80138 Naples, Italy

(First received July 19th, 1993; revised manuscript received October 20th, 1993)

Abstract

Commercial sodium bis(2-ethyl-1-hexyl)sulphosuccinate (Aerosol OT, AOT), a widely used surfactant forming reversed micelles in non-polar solvents, was found to contain some impurities with fluorescence characteristics, *i.e.*, absorption and emission spectra, similar to those of the tryptophan residues in proteins. Fluorescence lifetime determinations in the frequency domain confirmed the similarity between the emission decay of contaminants and that of tryptophan in proteins. A preparative procedure for the purification of commercial AOT by HPLC is described.

1. Introduction

Reversed micelles are spheroidal aggregates that are formed when certain surfactants are dissolved in apolar solvents. The polar head groups are directed towards the interior of the aggregate, thus forming a polar core that can solubilize water (water pool) [1]. Reversed micelles are capable of solubilizing a variety of proteins through encapsulation in the microaqueous phase. The observation that the catalytic activity of enzymes is retained in these systems has stimulated great interest as they provide a very useful model for studying the conformation and activity of biopolymers in membrane-like environments and has opened up new possibilities for investigations on enzymes at low temperatures [2]. Moreover, reversed micelles have been shown to provide new potential for technological applications [3].

The most common and best defined surfactant is the Aerosol OT (AOT), *i.e.*, sodium bis(2-ethyl-1-hexyl)sulphosuccinate. AOT molecules at a 3% concentration in hydrocarbon solutions are completely associated into uniformly sized micellar assemblies, each containing 23 AOT molecules. The addition of water to the micelle interior produces spheroidal particles. The structural and chemical homogeneity of such reversed micellar systems of AOT make them particularly convenient for studying biomembrane mimetic phenomena [4].

It has been reported that AOT preparations contain impurities as a result of the manufactur-

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(93)E1201-A

ing processes [5]. AOT is often prepared by diesterification of maleic or fumaric acid with 2-ethylhexanol. The diester is then sulphonated with sodium hydrogensulphite. Possible impurities are sodium hydrogensulphite, various acidic monoesters as a result of incomplete esterification and 2-ethylhexanol. The existence of aromatic species has also been reported. The presence of acidic impurities may significantly affect reactions in microemulsions where pH is important. Aromatic impurities interfere with spectroscopic investigations, in particular in fluorescence studies. Although the quality of commercial AOT has been much improved, impurities at the 0.1% level are not easy to identify and their subsequent removal can pose considerable problems. In this paper, we report the existence of fluorescent impurities in commercial preparations of AOT with emission properties resembling those shown by tryptophan residues in proteins [6–8]. An HPLC procedure is suggested in order to remove these impurities from the AOT preparations.

2. Experimental

2.1. Chemicals and reagents

Sodium bis(2-ethyl-1-hexyl)sulphosuccinate (AOT) was obtained from Sigma (St. Louis, MO, USA). Solvents were of HPLC grade from Fluka (Buchs, Switzerland). Deionized water was generated in a Milli-Q plus system (Millipore).

2.2. Chromatographic apparatus

The chromatographic apparatus was a Beckmann System Gold 126 equipped with a diode-array detector module. A Beckmann ultrasphere ODS (d_p 5 μm) column (25 cm × 4.6 mm I.D.) was used for analytical separations. The preparative column (25 cm × 10 mm I.D.) was packed with Bio-Sil C_{18} HL silica (d_p 40–63 μm) from Bio-Rad (Hercules, CA, USA).

2.3. Spectral measurements

Absorption spectra were recorded with a Perkin-Elmer Lambda Array 3840 UV–Vis spectrophotometer. Steady-state fluorescence spectra were obtained using a Perkin-Elmer Model MPF-66 spectrofluorimeter.

2.4. Fluorescence lifetime determination

Lifetime measurements were made using a multi-frequency cross-correlation phase and modulation fluorimeter (ISS, Urbana, IL, USA) with a 300-W xenon lamp [9]. The emission was observed through a longwave pass filter (WG 330) with a cut-off wavelength at 330 nm to avoid Raman emission. The modulation frequency was variable from 1 to 200 MHz. A solution of *p*-terphenyl (Kodak) in cycloexane was placed in the reference cell to correct for colour error. A lifetime of 1.000 ns was assigned to the reference solution. At least twenty different modulation frequencies were used, and the data were collected until the standard deviations for each measurement of phase and modulation was below 0.25° and 0.004, respectively. The temperature of the solution was maintained constant at 18°C by using an external bath circulator. The observed phase shifts and demodulation values were analysed in order to obtain the lifetime of the emitting species according to the equations reported elsewhere [10].

3. Results and discussion

Inspection of the structure of aerosol OT (shown in the inset in Fig. 1) reveals the absence of chromophoric groups which may absorb and subsequently emit in the near UV region (270–350 nm). Nevertheless, commercial AOT shows the presence of near-UV-absorbing and -emitting components whose spectral properties are similar to those of the most common intrinsic protein chromophore, the indole moiety of the tryptophanyl residue. Fig. 1 shows the steady-state fluorescence spectrum of commercial AOT in methanol solution resulting from excitation at

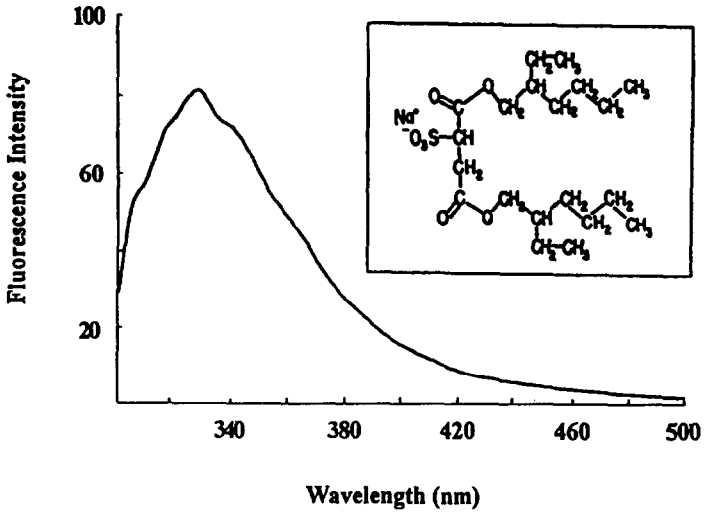

Fig. 1. Emission spectrum of commercial AOT dissolved in methanol. Excitation was at 290 nm. The sample absorbance at the exciting wavelength was less than 0.2. The inset shows the structure of AOT.

290 nm, a wavelength that is usually used to excite selectively the tryptophan residues in proteins. The shape of the emission spectrum is structured, suggesting the presence of aromatic impurities. The emission maximum, centred at about 330 nm, is coincident with that of tryptophan buried in the protein matrix.

The emission decay of commercial AOT preparations was investigated in the frequency domain. Fig. 2 shows the phase shifts and the demodulation factors *versus* the sinusoidal modulation frequency of the exciting light in the range 2–200 MHz [9]. The phase shift and the demodulation of the emission with respect to the exciting light are related to the fluorescence lifetime of the emitting species [10]. Table 1 shows the data fit obtained using a non-linear least-squares routine. The emission decay of commercial AOT in methanol is described by at least three discrete components with fluorescent lifetimes of 18.2, 4.4 and 0.7 ns, respectively. Table 1 also gives the results relative to the emission decays of reversed micelles formed by commercial AOT in isoctane and including an increasing water content. The average lifetime of the long-lived component is 11 ns whereas that of the second component is *ca.* 3 ns. This value is very close to that observed for the indole residue in several proteins [6–8] and may clearly affect the decay analysis of proteins included in AOT-formed reversed micelles. The very short lifetime

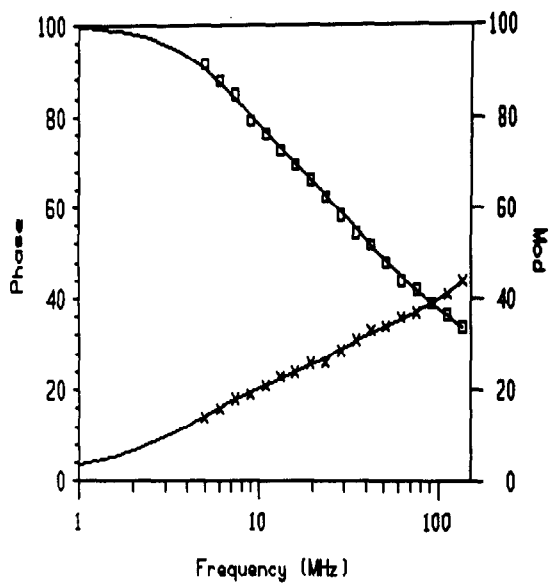

Fig. 2. Dependence of (×) phase shift and (□) demodulation factor on the modulation frequency of commercial AOT in methanol solution. Excitation was at 290 nm and emission was observed through a WG 330 filter. The absorbance of the sample at the exciting wavelength was less than 0.2. Solid lines were calculated from the tri-exponential fit parameters reported in Table 1.

Table 1
Emission decay analysis of commercial AOT as the sum of three exponentials

Sample	f_1	τ_1	f_2	τ_2	τ_3	χ^2
AOT, 0.5 *M* in methanol	0.37	18.2	0.33	4.38	0.70	2.13
Reversed AOT micelles, $R = 0$	0.36	11.2	0.37	2.52	0.60	1.98
Reversed AOT micelles, $R = 5$	0.33	10.9	0.41	1.75	0.05	1.85
AOT micelles, $R = 15$	0.27	11.7	0.55	2.61	0.02	2.20
Reversed AOT micelles, $R = 30$	0.40	8.2	0.46	2.14	0.03	2.01

f_i and τ_i are the fractions and the lifetimes (in nanoseconds), respectively. R is the H_2O:AOT molar ratio. The excitation wavelength was 295 nm and the temperature was maintained constant at 18°C.

component in Table 1 probably originates from weakly scattered light or other unpredictable instrumental contributions [11].

Recently there have been reports of studies of the dynamics of the water pool and enzymes encapsulated in reversed AOT micelles, most of which were based on the study of the emission decay [12–14]. Therefore, it appeared interesting to set up a rapid chromatographic procedure that eliminates the aromatic impurities that interfere with the emission decay of proteins.

The analytical separation of AOT from the near-UV-absorbing and -emitting impurities contained in the commercial preparations was carried out with a C_{18}-bonded silica column (25 cm × 4.6 mm I.D.). The column was eluted at a flow-rate of 0.7 ml/min with a binary linear gradient from 70 to 100% methanol in water in 40 min. All runs were performed at room temperature. Fig. 3 shows the chromatographic pattern of the separation detected by measuring the absorbance at 235 nm. The main peak centred at 5.34 min corresponds to the AOT molecule. Three other peaks are present in the chromatogram, at 3.9, 22.4 and 27.2 min. The absorption and emission spectra of the four fractions eluted from the column were recorded. The most important feature is that the main peak of the AOT preparation was virtually non-

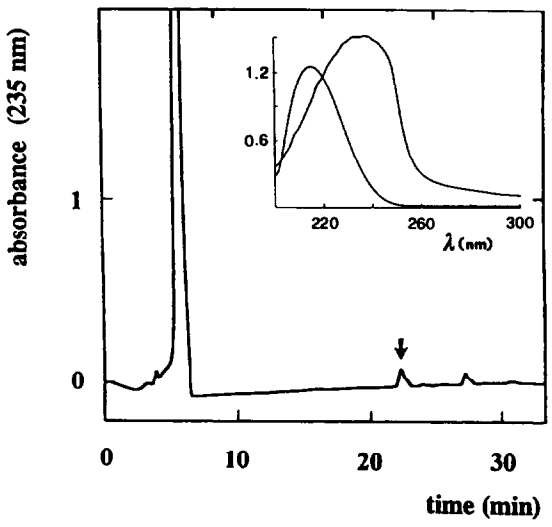

Fig. 3. Elution pattern of commercial AOT from the Ultrasphere ODS (5 μm) column (25 cm × 4.6 mm I.D.). The column was developed with a gradient from 60% to 100% methanol in water at a flow-rate of 0.7 ml/min. The inset compares the absorption spectra of commercial and HPLC-purified AOT. Both samples were dissolved in methanol–water (70:30).

fluorescent. Moreover, the absorption spectrum of purified AOT, shown in the inset in Fig. 3, revealed a strong decrease in near-UV-absorbing components compared with that of the commercial AOT in methanol solution. The absorption maximum of the purified AOT is shifted towards short wavelengths with a marked decrease in the near-UV region. The arrow in Fig. 3 indicates the fraction containing fluorescent material.

Large-scale AOT purifications from commercial preparations were performed on a chromatographic column (25 cm × 10 mm I.D.) packed with Bio-Sil C_{18} HL. The column was equilibrated with methanol–water (70:30). The flow-rate was maintained at 0.5 ml/min. Injections of 0.5–1 ml of commercial AOT [3.5 g per 10 ml of methanol–water (70:30)] were made. After the injection of the sample, the column was eluted with a linear gradient of from 70 to 100% methanol in water in 40 min and then with absolute methanol for 50 min. A typical elution pattern, obtained recording the absorbance at 235 nm, is shown in Fig. 4. As with the analytical separation, four components are resolved. The

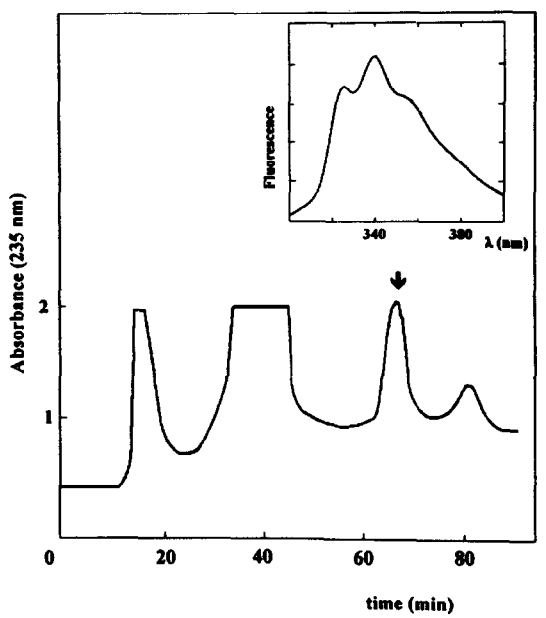

Fig. 4. Elution pattern of commercial AOT from the Bio-Sil C_{18} HL (40–63 μm) column (25 cm × 10 mm I.D.). The column was developed at a flow-rate of 0.5 ml/min with a gradient from 70 to 100% methanol in water in 40 min then with absolute methanol for 50 min. The emission spectrum of the AOT impurity corresponding to the peak marked with an arrow is shown in the inset.

inset in Fig. 4 shows the fluorescence spectrum of the third peak. It is worth noting that the first peak is much better separated from AOT using the preparative column. The collected main fraction corresponding to the purified AOT was vacuum dried. The recovery of AOT was 0.3 g for each run, an amount that is usually sufficient for performing a fluorescence lifetime determination of protein included in reversed micelles.

4. Acknowledgement

This research was supported by the Consiglio Nazionale delle Ricerche (Italy), grants 91.02478.CT14 and 92.02263.CT14.

5. References

[1] D. Langevin, in M.P. Pileni (Editor), *Structure and Reactivity in Reverse Micelles*, Elsevier, Amsterdam, 1989, pp. 13–42.
[2] P.L. Luisi, M. Giomini, M.P. Pileni and B.H. Robinson, *Biochim. Biophys. Acta*, 947 (1988) 209–246.
[3] R. Hilhorst, in M.P. Pileni (Editor), *Structure and Reactivity in Reverse Micelles*, Elsevier, Amsterdam, 1989, pp. 323–341.
[4] H.F. Eiche and Kvita, in P.L. Luisi and B.E. Straub (Editors), *Reverse Micelles*, Plenum Press, New York, 1984, pp. 21–35.
[5] P.D.I. Fletcher, A.M. Howe, B.H. Robinson and D.C. Steytler, in P.L. Luisi and B.E. Straub (Editors), *Reverse Micelles*, Plenum Press, New York, 1984, pp. 69–72.
[6] E. Bismuto, E. Gratton and G. Irace, *Biochemistry*, 27 (1988) 2132–2136.
[7] E. Bismuto and G. Irace, *Photochem. Photobiol.*, 47 (1989) 165–167.
[8] E. Bismuto, I. Sirangelo and G. Irace, *Arch. Biochim. Biophys.*, 287 (1991) 216–220.
[9] E. Gratton and M. Limkeman, *Rev. Sci. Instrum.*, 54 (1984) 294–299.
[10] E. Gratton, E. Limkeman, J. Lakowicz, B. Maliwal, H. Cherek and G. Laczko, *Biophys. J.*, 46 (1984) 479–486.
[11] B. Barbieri, F. De Piccoli and E. Gratton, *Rev. Sci. Instrum.*, 60 (1989) 3201–3206.
[12] E. Bismuto, I. Sirangelo and G. Irace, *Biophys. Chem.*, 44 (1992) 83–90.
[13] E. Bismuto, I. Sirangelo and G. Irace, *Biochim. Biophys. Acta*, 298-2 (1992) 83–90.
[14] V.N. Dorovska-Taran, C. Veeger and A.J.W.G. Visser, *Eur. J. Biochem.*, 211 (1993) 47–55.