Genotoxicity induced by saponified coconut oil surfactant in prokaryote systems

Tirzah Braz Petta, Silvia Regina Batistuzzo de Medeiros, Eryvaldo Soares Tabosa do Egito and Lucymara Fassarella Agnez-Lima

Departamento de Biologia Celular e Genetica, Centro de Biociencias and 1Departamento de Farmacia, Centro de Ciencias da Saude, Universidade Federal do Rio Grande do Norte, Campus Universitario, Lagoa Nova, 59078-970 Natal, RN, Brazil

Surfactants are amphiphilic substances with special properties and chemical structures that allow a reduction in interfacial tension, which permits an increase in molecule solubilization. The critical micelle concentration (CMC) is an important characteristic of surfactants that determines their aggregate state, which is generally related to its functional mechanism. In this work the genotoxic potential of saponified coconut oil (SCO), a surfactant obtained from Cocos nucifera, was analyzed using prokaryote systems. DNA strand breaks were not observed after treatment of a plasmid with SCO. Negative results were also obtained in strains TA100 and TA104 with treatment doses below the CMC. The cytotoxic, antioxidant and mutagenic effects of SCO were analyzed employing a series of cell-free and bacterial assays such as in vitro plasmid treatment, the Ames test, the SOS Chromotest and a forward mutagenesis test using CC104 strains of Escherichia coli, which also allow an analysis of antioxidant potential. The results suggest both mutagenic and antioxidant properties of SCO, which could be influenced by its aggregation state.

Introduction

Surfactants are substances that are adsorbed in liquid–liquid, liquid–gas and solid–liquid interfaces because of their chemical structure and properties, which reduce interfacial tension. They reduce superficial tension by increasing the solubility of apolar substances widely used in several branches of industrial production. Such compounds are presented as amphiphilic molecules, which have two parts of different polarities in their structures: a polar hydrophilic head group (ionic or non-ionic) and an apolar hydrophobic hydrocarbon chain. This property provides an ideal characteristic for the solubilization of many molecules. Surfactants can be classified as follows, according to the charge that originates when they dissociate in water: anionic, cationic, non-ionic and zwitterionic (for reviews see Maniasso, 2001; Torchilin, 2001; Zana, 2002).

Toxicological and genotoxicity assays with chemical compounds are useful in food, pharmaceutical and cosmetic products and have been recommended by regulatory agencies world wide prior to commercialization. Concerning surfactants, there is little information about their genotoxic risk. In this work, saponified coconut oil (SCO) was used as a chemical model to explain the behavior of amphiphilic molecules in biological systems related to their mutagenesis potential. SCO is an anionic surfactant obtained by saponification of triglycerides extracted from Cocos nucifera and is a compound widely used in the formulation of many cosmetic and hygiene products. It is made up of a mixture of triglycerides, in which caprylic, capric, lauric, miristic, palmitic, esteratic and oleic acids are prevalent (Hlongwane et al., 2001; de Castro Dantas et al., 2003).

Besides the composition of SCO, the critical micelle concentration (CMC) is an important characteristic for understanding its properties. Micelles are molecular clusters possessing both hydrophilic and hydrophobic phases that are generated by surfactants that associate spontaneously in aqueous solutions starting at a certain concentration, termed the CMC. Due to their conformation, micelles have the potential to solubilize a wide range of insoluble compounds in aqueous solutions. Each surfactant has its own CMC, the CMC of SCO being ~7.28 mM (Lucena Neto, 1999; de Castro Dantas et al., 2002, 2003).

In this work, the cytotoxicity and genotoxic potential of SCO were analyzed employing a series of cell-free and bacterial assays such as in vitro plasmid treatment, the Ames test, the SOS Chromotest and a forward mutagenesis test using CC104 strains of Escherichia coli, which also allow an analysis of antioxidant potential. The results suggest both mutagenic and antioxidant properties of SCO, which could be influenced by its aggregation state.

Materials and methods

SCO samples

The surfactant was obtained from coconut oil (Rio Grande do Norte regional product) with the following chemical composition (%): octanoic acid (7.6), decanoic acid (7.3), lauric acid (48.2), myristic acid (8.0), stearic acid (3.8), oleic acid (5.0) and linoleic acid (2.5). The saponification reaction was carried out using sodium hydroxide (NaOH) in a stoichiometric reaction (1:1 ratio). The extraction and saponification of the coconut oil were done by the laboratory of Dr T.N.de Castro Dantas (Surfactant Laboratory, Chemistry Department, UFRN, Brazil), which kindly supplied SCO samples for this work.

Plasmid and bacterial strains

The plasmid pBluescript II (pRLKS; Stratagene) is derived from pUC19 and has a ColE1 replication origin and an ampicillin resistance gene. Escherichia coli SOS Chromotest tester strains PQ35 and PQ37 have the following genotypes: sfrA::Mud(Ap lac) cts, lacΔU169, mal, galE, galY, PhoC, rfa, F−, thr, leu, his, pyrD, thi, trp::MUC−, str13000::Tn10, rpoB, uvrA− for PQ35; uvrA− for PQ37 (Quiildardet and Hofnung, 1985). The Salmonella typhimurium strains used in the Ames test were: TA98 (his D3052, rfa, ΔuvrB, bio−, pKM101 Ap+), TA100 (his, G46, rfa, ΔuvrB, bio−, pKM101 Ap+) and TA104 (his D428, rfa, ΔuvrB, bio−, pKM101 Ap−). While strain TA98 detects frameshift mutations, TA100 and TA104 detect base pair substitutions (Maron and Ames, 1983).
The genotype of Escherichia coli strain CC104 is ara, Δgtp-lac5, rpsL, [F lacI378, lacZ461, proA− B−], str−, while CC104mutMmutY is as CC104 with mutY::kan, mutM::tet (Cupples and Miller, 1989; Michaels et al., 1992).

Analysis of the occurrence of breaks in plasmid DNA

Plasmid samples were treated with SCO and incubated for 1 h at 37°C. Samples were applied to 0.7% agarose gels containing ethidium bromide (20 µg/ml) and electrophoresis was performed in TBE buffer (89 mM Tris–borate and 2 mM EDTA) at 80 V. Gels were photographed in a Kodak EDAS290 system. Breaks in plasmid DNA were evaluated through the presence of Form III (linear) and Form II (relaxed circular), since breaks in the phosphodiester chains promote relaxation of the tension of Form I (superhelical).

Forward mutagenesis assay

Cultures of CC104 and CC104mutMmutY were grown in Luria broth (LB) agar medium (1% NaCl, 1% tryptone, 0.5% yeast extract) supplemented with streptomycin (50 µg/ml) for CC104 or tetracycline (50 µg/ml) and kanamycin (50 µg/ml) for CC104mutMmutY at 37°C in the presence of different SCO concentrations until stationary phase. For cytotoxicity analysis, cultures were diluted to 105 cells/ml and 100 µl was plated on solid LB medium. The plates were incubated for 16–20 h at 37°C and colonies were counted. The survival rate was established as a percentage, as the ratio between the number of colonies after treatment and the number of colonies formed by the untreated control.

Mutagenesis analysis was performed by screening rifampicin-resistant colonies. CC104 strains are sensitive to this antibiotic, which binds to the β subunit of RNA polymerase (the product of the rpoB gene), thus blocking transcription. Missense mutations in the rpoB gene can lead to antibiotic resistance. Therefore, direct mutagenesis can be determined (Michaels et al., 1992; Cavalcante et al., 2002). Aliquots of 100 µl of treated cultures (without dilution) were plated on LB agar medium supplemented with rifampicin (100 µg/ml) and incubated for 16–20 h at 37°C, the colonies were counted and the mutation rate established as the ratio between the number of colonies in medium with rifampicin and total plated.

SOS Chromotest

This assay was performed according to the procedure described by Quillardet and Hofnung (1985). Exponential phase cultures of strains PQ35 and PQ37, grown in LB medium at 37°C, were diluted 1:9 in fresh medium. Aliquots of 100 µl of treated cultures (without dilution) were plated on LB agar medium supplemented with rifampicin (100 µg/ml) and incubated for 16–20 h at 37°C, the colonies were counted and the mutation rate established as the ratio between the number of colonies in medium with rifampicin and total plated.

Ames assay

These assays were performed according to the plate incorporation procedure described by Maron and Ames (1983), without metabolic activation. The cytotoxicity screening was performed with strain TA98 using a range of SCO concentrations. Due to its toxicity, strains TA98, TA100 and TA104 were treated with SCO at concentrations of 62.5, 125, 250 and 500 ng/plate. Negative and positive controls were used simultaneously in each experiment. The positive controls used were 4-nitroquinoline-N-oxide (0.5 µg/plate) for TA98, sodium azide (5 µg/plate) for TA100 and methyl methanesulfonate (250 µg/plate) for TA104. His− revertants were counted after 2 days incubation at 37°C. Mutagenicity was evaluated as the increase in number of histidine-independent revertant colonies. A test substance was considered mutagenic if it induced a 2-fold increase in the number of mutants at least one concentration when compared with the corresponding negative controls. A concentration–effect relationship was also taken as an indication of mutagenic potential.

Results

The results obtained after in vitro treatment of plasmid DNA with different concentrations of SCO are shown in Figure 1. The gel analysis shows that the test compound was unable to induce breaks in the plasmid phosphodiester chain, since changes in Form I concentration were not observed after treatment.

Treatment of wild-type and repair-deficient CC104 strains with SCO showed moderate cytotoxicity (Figure 2), mainly to the wild-type strain (CC104), whose survival was reduced by ~50% at the highest SCO dose. In the forward mutagenesis assay with CC104 strains no increase in mutation frequency was observed (Table I). Conversely, a decrease in the mutagenesis ratio was observed in comparison with the negative control at the lowest concentration in the repair-deficient strain (CC104mutMmutY). This strain presents not only a deficiency in enzymes repairing oxidative damage to DNA (FPG and MutY glycosylase), but also a mutator phenotype, with increased mutagenesis after treatment with oxidative agents (Cavalcante et al., 2002). The reduction in mutation frequency after treatment suggests that the presence of SCO could block or inhibit oxidative lesions generated by endogenous bacterial metabolism.

This antioxidant effect of SCO in a repair-deficient strain was found for the lowest concentration, which was below its...
and variance analysis, when compared with untreated control. SCO (strains with SCO. be related to the cytotoxic effect. which is above the SCO CMC, micelles are present, which can and dimers can be observed, while at the highest concentration, In fact, at low SCO concentrations the presence of monomers Fig. 3. aValues statistically significant (P < 0.05) by the Tuckey statistical test and variance analysis, when compared with untreated control.

Table I. Mutagenesis frequencies induced by SCO in bacterial rpoB gene

<table>
<thead>
<tr>
<th>SCO (µg/ml)</th>
<th>CC104 (×10⁻⁶)</th>
<th>CC104mutMnuTY (×10⁻⁵)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.70 ± 0.56</td>
<td>1.71 ± 1.3</td>
</tr>
<tr>
<td>0.0001</td>
<td>0.88 ± 0.45</td>
<td>0.48 ± 0.42</td>
</tr>
<tr>
<td>1</td>
<td>0.88 ± 0.63</td>
<td>0.36 ± 0.29</td>
</tr>
<tr>
<td>1000</td>
<td>0.71 ± 0.37</td>
<td>1.27 ± 1.38</td>
</tr>
</tbody>
</table>

The mutagenesis frequencies represent a cumulative set of at least seven independent experiments. *Values statistically significant (P < 0.05) by the Tukey statistical test and variance analysis, when compared with untreated control.

Fig. 3. Analysis of SOS induction after treatment of PQ35 and PQ37 strains with SCO.

CMC value. Therefore, it is possible that the molecular aggregation state can interfere with the antioxidant potential of SCO. In fact, at low SCO concentrations the presence of monomers and dimers can be observed, while at the highest concentration, which is above the SCO CMC, micelles are present, which can be related to the cytotoxic effect.

After treatment of bacterial strains PQ37 (uvrA⁻) and PQ35 (uvrA⁺) it was observed that SCO was unable to induce SOS function, since an alteration of 0.5 was not observed in the IF with respect to the negative control sample (Figure 3).

Results obtained after treatment of strains TA98, TA100 and TA104, without metabolic activation, are shown in Table II. SCO was not mutagenic for strain TA98, which allows frame-shift mutations to be detected, however, it demonstrates mutagenic potential for strains TA100 and TA104, which permit base substitutions to be detected. The SCO concentrations used in the Ames test were below its CMC, where the monomeric form is predominant, since concentrations above its CMC were very toxic to Ames strains (data not shown). The effects of SCO can also be attributed to the aggregative state of the molecule in this case. The monomeric state may be involved in mutagenesis induction while the micellar state may be related to cytotoxicity.

Discussion

The results obtained in this work are important in elucidating the mutagenic characteristics of SCO, as a model of surfactant interaction with bacteria. This compound was unable to induce strand breaks in plasmid DNA in vitro or an SOS response in bacteria. Besides that, SCO presented possible antioxidant characteristics, as seen in repair-deficient strain CC104mutMnuTY. However, SCO demonstrated a positive response in the Ames test not only for strains that detect base substitution mutations, like TA100 and TA104, but also cytotoxic potential for bacteria.

In S.typhimurium strains with a rfa deficiency (Maron and Ames, 1983) the test compounds can easily penetrate bacterial cells and reach the bacterial genome. On the other hand, in the rfa⁺ background of strains CC104 and CC104mutMnuTY (Michaels et al., 1992) SCO may accumulate adjacent to the bacterial membrane. This could explain why SCO was mutagenic for both strain TA100 and TA104, while it was not so for strains CC104 and CC104mutMnuTY. At the concentrations used in the Ames test SCO micelles are not found in solution. Thus, the mutagenic potential found in the Ames test can be attributed to the monomeric form of SCO. This species may also be responsible for the antioxidant effect of SCO, as seen in a decrease in mutagenicity in CC104mutMnuTY strains, probably due to membrane interactions, which would result in an effect on metabolic oxidative processes such as lipidic peroxidation.

The strain C104mutMnuTY shows a mutator phenotype attributed to a deficiency in repair of 7,8-dihydro-8-oxoguanine, a major DNA lesion, which is induced by endogenous oxidative stress (Michaels et al., 1992). The decrease in mutagenesis in strain CC104mutMnuTY after treatment with the lowest concentration of SCO may be related to the interaction of SCO monomers with cell membranes, perhaps reducing the formation of oxidative molecules and inducing a cellular antioxidant effect.

Research on surfactants and membrane solubilization showed that anionic surfactants interact more with lipid bilayers. The nature of this interaction can be determined by the surfactant properties, the CMC, and also the properties of the membrane components. Solubilization involves hydrophobic and ionic interactions between the surfactant chains and membrane proteins/lipids. This phenomenon shows a direct correlation between the CMC and molecule diffusion. In fact, the lower the CMC of the surfactant, the lower the speed of molecular diffusion into the cell interior (for a review see Jones, 1999). While SCO has a CMC of 7.29 mM, SDS has a CMC of 0.58 mM (Jones, 1999). Therefore, SCO has a higher diffusion speed in the cell. This property could explain the cytotoxic potential obtained for the CC104 (Figure 2) and Ames strains (data not shown) treated with SCO at a concentration above the CMC, at which micelles are predominant. It can be supposed that the interaction between the membrane and the surfactant occurs with formation of mixed micelles. This would

Table II. Number of S.typhimurium revertants induced by SCO, without metabolic activation

<table>
<thead>
<tr>
<th>SCO (ng/plate)</th>
<th>TA100</th>
<th>TA104</th>
<th>TA98</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>21 ± 7.33</td>
<td>405.3 ± 33</td>
<td>68.33 ± 6.88</td>
</tr>
<tr>
<td>62.5</td>
<td>165 ± 9.33a</td>
<td>1356.66 ± 118.3a</td>
<td>107.66 ± 60.88</td>
</tr>
<tr>
<td>125</td>
<td>306.5 ± 49.66a</td>
<td>1263.33 ± 328.33a</td>
<td>55 ± 4</td>
</tr>
<tr>
<td>250</td>
<td>564.5 ± 79a</td>
<td>1148.66 ± 129.83a</td>
<td>65.33 ± 6.88</td>
</tr>
<tr>
<td>500</td>
<td>962.5 ± 50a</td>
<td>1081.66 ± 64.16a</td>
<td>79 ± 15.33</td>
</tr>
<tr>
<td>Positive controlb</td>
<td>87.66 ± 68.16a</td>
<td>871.66 ± 109.66a</td>
<td>150.33 ± 27b</td>
</tr>
</tbody>
</table>

The data are the mean of three independent experiments. *Significant increase in mutagenesis.

bPositive controls: 4-nitroquinoline-N-oxide (0.5 µg/plate) for TA98; sodium azide (5 µg/plate) for TA100; methyl methanesulfonate (250 µg/plate) for TA104.
promote the formation of pores in the membrane bilayer leading to cell death.

Extrinsic membrane proteins exist in the cell membrane bilayer, which are bound by ionic and/or hydrophilic interactions. In some cases this occurs through anchors covalently linked to hydrophobic groups. This kind of interaction may also occur with surfactants (Seflon and Buss, 1987; Jones, 1999). Therefore, it can be supposed that similar interactions occur between SCO and the cell membrane. First, monomers could penetrate the bilayer membrane until saturation occurred; then mixed micelles between lipids and surfactant could be formed. In the end, formation of pores could allow the penetration of extracellular molecules into the intracellular compartment. The cytotoxicity of SCO could be attributed to this mechanism.

The bacterial SOS Chromotest with *E. coli* is widely used as a genotoxicity screen. The results obtained in this assay can be compared with those obtained in the Ames test, but the ability of the latter to identify carcinogens is much higher than that of the SOS Chromotest. Therefore, the results of the SOS Chromotest and of the Ames test complement each other (Quillardet et al., 1982; Rosenkranz et al., 1999). The SOS Chromotest is based on SOS response induction, which is activated in the presence of DNA damage and leads to replication blockage (for reviews see Cox, 2001; Janion, 2001). In our analysis SCO was demonstrated to be mutagenic in the Ames test, but not in the SOS Chromotest, suggesting that the DNA lesions involved in mutations induced in strains TA100 and TA104 do not characterize replication blockage. Similar results for the SOS Chromotest were obtained with various non-ionic surfactants, such as Tween 20 and 80, Triton X-305 and Tyloxyapol (Raabe et al., 1990).

Hrábk et al. (1982) published a work in which damage to the DNA synthesis machinery and to the membrane of human lymphocytes were analyzed with respect to the electric charge of some surfactants. Their results revealed that the effects were more intense after treatment with cationic surfactants (CPB), as opposed to anionic (NaDOC and SDS) and non-ionic (NP-40) surfactants, which showed decreased harmful potential after treatment of human lymphocytes. The anionic surfactant had only a moderate cytotoxicity for lymphocytes, so it may be useful in several areas, due to its high dissolution capacity and to its moderate effect on cellular integrity (Hrábk et al., 1982). However, their work did not discuss the aggregative state of the surfactants, which could interfere with the mechanism of action.

**Conclusion**

The results obtained in this work are relevant to the investigation of surfactant interactions with prokaryotic systems, using an anionic surfactant as a model. The results were indicative of cytotoxic, antioxidant and mutagenic properties of SCO. While the antioxidant and mutagenic potentials of SCO can be related to its monomer state, the cytotoxicity may be induced by the micellar state. This indicates that the monomeric and self-aggregated states of this compound are related to its interaction with cellular systems and, consequently, mechanisms of action. Thus, because surfactants have great potential in different industrial processes, a detailed study is necessary before they are used.

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