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Gum arabic microcapsules as protectors of the photoinduced degradation of riboflavin in whole milk

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ABSTRACT

Microcapsules (MC) made with gum arabic (GA) as shell material without and with β-carotene (βc) as core material were prepared by the spray-drying technique. The effect of these MC on the photodegradation of riboflavin (Rf) in whole milk by fluorescent daylight lamp irradiation was evaluated at a storage temperature of 4°C. The additions of 1.37 mg/mL of MC without βc (MC-GA) and with 0.54 μg/mL of βc (MC-βc-GA) decreased the apparent first-order rate constant of Rf photodegradation by approximately 26 and 30%, respectively. A systematic kinetic and mechanistic analysis of the results indicates that the global protective effect of the MC is mainly due to the combination of quenching of the electronically excited triplet state of Rf and scavenging of the photogenerated reactive oxygen species, such as singlet molecular oxygen, superoxide radical anion and hydroxyl radical. A minor contribution to the photoprotective effect can be also associated with the inner-filter effect exerted by the MC, which partially blocks the direct excitation of Rf. These results allow us to conclude that photodegradation of Rf in milk can be considerably reduced by the addition of small amounts of MC, avoiding large losses in the nutritional value of milk.
Key words: riboflavin photodegradation, β-carotene, gum arabic, reactive oxygen species

INTRODUCTION

The oxidative stability of milk and its products is of great importance for the dairy industry. It is well known that some of the photoinduced degradation process in milk occurs mainly because of the presence of vitamin B_2 [riboflavin (**Rf**)], which acts as a photosensitizer, absorbing environmental light to generate electronically excited states of the flavin, in particular the triplet excited state (^{3}**Rf***; Montenegro et al., 2007). In the presence of triplet molecular oxygen ($^3O_2$) or electron donors, or both, the ^{3}Rf* is able to generate several reactive oxygen species (**ROS**), such as superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$), and singlet molecular oxygen ($^1O_2$) either by electrotransfer (type I mechanism) or energy-transfer (type II mechanism) reactions (Massad et al., 2004; Skibsted, 2010). These photogenerated ROS have a deleterious effect in milk, as they have a large degree of reactivity and may cause oxidation of proteins, vitamins, and lipids, with the collateral production of low-molecular-weight volatile compounds, responsible for off-flavor and nutritional quality loss in milk (Bradley, 1980; Mortensen et al., 2003; Mestdagh et al., 2011). In turn, the oxidative stability of milk depends on a delicate balance between the anti- and prooxidants processes (Halliwell and Gutteridge, 1999), which are influenced by several factors, such as the unsaturation degree of FA, content of transition metals, and antioxidant molecules (**AOx**; Kristensen et al., 2004). Adding AOx to scavenge harmful ROS species or molecules with the capability of quenching ^{3}Rf*, avoiding the formation of ROS, seem to be suitable strategies to avoid the undesired photoinduced off-flavor of milk.

Currently, natural AOx are preferred to synthetic ones to be added to milk to avoid or lessen toxicological side effects. Among these types of molecules, the lipid-soluble carotenoids (**CAR**) show exceptional nutritional and health-promoting properties, such as provitamin A activity and scavenging activity against ROS, in particular very efficient quenching of $^1O_2$, promoting the prevention and (or) reduction of human diseases associated with oxidative stress (Burton and Ingold, 1984; Liebler, 1993).

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However, the highly nonpolar properties of CAR preclude their direct utilization in aqueous media, where they aggregate and precipitate with the complete loss of their antioxidant properties. Among the several approaches for CAR vehiculization and controlled delivery in aqueous food matrices, spray-dried microencapsulation with edible biopolymers as coating material is a suitable and economical feasible method (Rodriguez-Huezo et al., 2004; Barbosa et al., 2005; Gharsallaoui et al., 2007). In this process, the biopolymer wall acts as a physical permeable barrier to diffusion of oxygen and other molecules (Edge et al., 1997; Bustos et al., 2003) and allows the stabilization, transport, and controlled delivery of CAR into the aqueous media (Rodríguez-Huezo et al., 2004; Barbosa et al., 2005).

In a previous study, we evaluated the effect of lycopene microencapsulation by spray drying with a gum arabic (**GA**)-sucrose (8:2) mixture in the Rf-mediated photosensitized degradation of vitamins A and D_3 in skim milk, using white fluorescence lamps as a light source, as the visible absorption band of Rf overlaps with the blue-shifted emission of the fluorescent light (Montenegro et al., 2007). The results indicated that the addition of 6.5 mg/mL of this microencapsulated skim milk produced a reduction of approximately 45% of the photosensitized degradation rate of both vitamins.

In a more recent study, we evaluated the $^1O_2$-quenching capacity of microcapsules (**MC**) of GA (**MC-GA**) or maltodextrin containing natural AOx molecules such as CAR or tocopherol derivatives (Faria et al., 2010). The results indicated that the $^1O_2$-quenching efficiency by the AOx in MC was strongly dependent on the lipophilicity degree of the AOx, being more efficient in the polar ones due to compartmentalization effects of the AOx in the core of the MC that modified the accessibility of $^1O_2$. Additionally, it was demonstrated that empty MC of GA were efficient quenchers of $^1O_2$, due to the interaction with amino acid residues (Trp, His, and Met, among others) of the protein moiety present in this glycoprotein (Mahendran et al., 2008). Later, for the same series of microencapsulated AOx studied before, Rodrigues et al. (2012) analyzed the antioxidant activity against both reactive oxygen and nitrogen species, such as peroxyl radical ($ROO^{\bullet}$), hydrogen peroxide (H_2O_2), $HO^{\bullet}$, and peroxynitrite anion ($ONOO^-$). They found that the scavenging capacities were influenced by the wall material and by the type of antioxidant molecule, being in all cases higher for the microcapsules with GA than with maltodextrin.

Due to the relevance of Rf photochemistry in milk, the objective of this work was the evaluation of the efficiency of MC-GA itself and of MC-GA containing the provitamin A precursor β-carotene (**βc**) as scavengers of ROS generated by fluorescent light photosensitization of Rf in whole milk.

MATERIALS AND METHODS

Materials and Chemicals

β-Carotene (98% purity), Rf (≥98%), sodium azide (**NaN_3**), superoxide dismutase (**SOD**), nitro blue tetrazolium (**NBT**), 2-deoxy-D-ribose, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid [Trolox (**TX**); 99.5% purity], and SDS were supplied by Sigma-Aldrich (St. Louis, MO). Food-grade GA (molecular weight = 3.5×10^5 g/mol) was purchased from Colloides Naturels Brasil (São Paulo, Brazil). Acetate buffer was from Merck Química Argentina S.a.i.c. (Buenos Aires, Argentina); $FeCl_3$, H_2O_2, KH_2PO_4, K_2HPO_4, ascorbic acid, thiobarbituric acid (**TBA**), TCA, hydroxylamine hydrochloride (**HAHC**), all analytical grade, were obtained from Biopack Productos Químicos (Buenos Aires, Argentina); and HPLC-grade solvents: methanol, hexane, 1-butanol, ultrapure water, and glacial acetic acid were from Merck KGaA (Darmstadt, Germany; LiChrosolv). The milk samples were prepared from whole-milk powder of a recognized Argentinean trademark.

Preparation of MC

The MC were prepared with GA as shell material (MC-GA) and with β-carotene (MC-βc-GA) by using a laboratory-scale spray-dryer system (Labplant SD-04; Labplant UK Ltd., Huddersfield, 123 UK), under the following working conditions: aspersion nozzle diameter of 0.7 mm, air pressure of 5 kgf/cm^2, and air flow rate of 30 mL/min, entrance and exit air temperatures of 170 and 110°C, respectively. Gum arabic solutions [30% (wt/vol) of soluble solid] were prepared in water at 45°C and kept under continuous stirring until the temperature reached 30°C. β-Carotene was dissolved in dichloromethane and a small aliquot was added to the GA aqueous solution. The mixture was stirred at 7,000 rpm for 30 min to obtain an emulsion. Afterward, the emulsion was diluted with water to obtain a 20% (wt/vol) GA solution. The emulsion was placed in the spray-dryer chamber, maintaining slow agitation during the spray-drying process. The microcapsules obtained were immediately stored in a glass bottle under N_2 atmosphere at −18°C to avoid βc degradation.

Quenching of ^{3}Rf* by MC and Laser Flash Photolysis Experiments

Generation and detection of ^{3}Rf* were performed by laser flash photolysis (LFP) experiments using the third harmonic at 355 nm of a neodymium-doped yttrium alu-

minum garnet (Nd:YAG) Minilite II laser with 7 ns of full width at half maximum (FWHM) from Continuum Inc. (Santa Clara, CA). Triplet-to-singlet difference transient absorption spectra of Rf (35 μ*M*) in Ar-saturated phosphate buffer (pH 7.4) solutions were recorded with the m-LFP 112 laser-flash photolysis apparatus from Luzchem Research Inc. (Ottawa, ON, Canada) linked to a 300-MHz Tektronix TDS 3032B digital oscilloscope (Tektronix Inc., Beaverton, OR) for signal acquisition, as described in Montenegro et al. (2007).

Scavenging of Free Radical Species by MC in Buffer Solution

Hydroxyl radicals ($HO^{\bullet}$) were generated via the Fenton reaction at pH 7.4 (Aruoma, 1994). In the presence of deoxyribose, the $HO^{\bullet}$ reacts, producing malondialdehyde (MDA) among other products, which formed a pinkish adduct in the presence of TBA, allowing its quantification by UV-visible spectroscopy (Gutteridge, 1981). The $HO^{\bullet}$ scavenging effect of the MC was investigated as follows: the reaction was performed in 50 m*M* phosphate buffer (pH 7.4) containing 10 m*M* deoxyribose, 100 m*M* H_2O_2, 1 m*M* $FeCl_3$, and 5 m*M* EDTA in the presence and absence of the MC samples. The reaction started with the addition of ascorbic acid in a final concentration of 5 m*M*. The reaction mixture was incubated for 1 h at 37°C in a water bath. Then, 1% (wt/vol) TBA and 2.8% (wt/vol) cold TCA were added and heated to boiling temperature (95–100°C) for 20 min to cause the colored adduct to form, of which the absorbance was measured at 532 nm. Trolox was used as antioxidant reference.

The scavenging of anion superoxide $O_2^{\bullet-}$ was determined according to the method described by Sabu and Kuttan (2002) using NBT as colorimetric reagent. The assay is based on the $O_2^{\bullet-}$ generation by HAHC autoxidation, which reduces NBT to nitrite ($NBT^{+\bullet}$). In the presence of water, the organic radical-cation $NBT^{+\bullet}$, produces the stable cation monoformazan (MF^{+}), which absorbs at 560 nm. The competitive scavenging of $O_2^{\bullet-}$ by the MC was studied as follows: in a test tube, the desired MC concentration was weighed and dissolved in 150 m*M* sodium carbonate buffer (pH 10) containing 1.8 m*M* NBT, 1 m*M* EDTA, and 6 m*M* HAHC. The samples were incubated for 1 h at 37°C and afterward, the absorbance of MF^{+} was measured at 560 nm. The enzyme SOD was used as antioxidant reference.

The percentage radical scavenging (**%S**) by the MC was calculated using Equation 1:

$$\%S = \left(1 - \frac{A_X}{A_0}\right) \times 100, \qquad [1]$$

where A_0 is the absorbance of the control and A_X is the absorbance in the presence of MC-βc-GA, MC-GA, or the reference compound. The results were expressed as Trolox equivalent antioxidant capacity (**TEAC**) for $HO^{\bullet}$ and SOD equivalent antioxidant activity ($\mathbf{EAA_{SOD}}$) for $O_2^{\bullet-}$ according to the ratio between initial slopes of the %S graphs. The assays were performed in triplicate and the results are reported with the respective standard deviation. Absorbance measurements were performed using a 1-cm optical path quartz cuvette on an Analytik Jena Specord S600 diode array spectrophotometer (Analytik Jena AG, Jena, Germany) at room temperature.

Milk Photooxidation

A recognized Argentinean brand of whole-milk powder supplemented with iron (as slightly soluble ferric phosphate, of low bioavailability and possibility to catalyze oxidation reactions, leading to significant changes in the nutritional value of milk) and vitamin C was reconstituted with ultrapure water (resistivity <18.2 MΩ·cm at 25°C) according to the manufacturer's indications. The milk photodegradation was performed by placing 500-mL aliquots in glass bottles (cutoff of 310 nm), which were stored at shelf temperature (4°C) under 1,000-lx fluorescent lamps for 120 h. Milk aliquots were randomly sampled at different times for Rf determination. A control was also performed in darkness under the same conditions. The photodegradation assay was divided into 2 protocols called Kinetic I and Kinetic II, respectively, where Kinetic I was performed with the addition of 1.37 mg/mL of MC-GA to milk and Kinetic II with the addition of 1.37 mg/mL of MC-βc-GA to milk. Details of each protocol procedure are given in the following section.

Protocols for HPLC Quantification of Rf in Milk

Kinetic I. The sample preparation consisted in a hot acid extraction based on the method of the Association of Official Analytical Chemists (AOAC, 1984; method 970.65). Briefly, 5 mL of milk sample was treated with 5 mL of 0.1 *M* HCl and autoclaved at 121°C for 30 min. Later, the pH was adjusted to 4.5 with 1.25 *M* sodium acetate and the final volume of 25 mL was reached by addition of sodium 0.2 *M* acetate buffer. The samples were filtered through 0.45-μm membrane filters before being injected into the HP1050 series HPLC apparatus (Agilent Technologies Inc., Palo Alto, CA) equipped with an analytical Phenomenex Synergi RP80A Fusion 4U C_{18} column (150 × 3.0 mm; Phenomenex Inc., Torrance, CA) with a precolumn. The signals were recorded with a fluorescence detector operated at an excitation

wavelength of 453 nm and emission wavelength of 580 nm. The mobile phase used was a mixture of water, methanol, and glacial acetic acid (ratio of 65:35:0.1) at a flow rate of 0.8 mL/min. This procedure was carried out in duplicate for each sample.

An external calibration curve with authentic Rf was performed with a concentration range of 0.1 to 3.0 μg/mL. Linear regression equations and correlation coefficients were calculated ($y = 36.85261x + 1.03471$; $R^2 = 0.99$; $n = 8$) and 2 independent replicates were performed per concentration.

Kinetic II. To overcome the HPLC partial overlapping of matrix interferences with Rf, a different extraction method of Rf was used, which was based on the solid-phase extraction method described by Ashoor et al. (1985). We used a C_{18} extraction cartridge (Sep-Pack; Agilent Technologies Inc.). A mixture of 0.02 *M* sodium acetate buffer at pH 4.0 and methanol (1:1, vol/vol) was used as the eluent solution. The sample was subsequently concentrated on a rotary evaporator and filtered with a 0.45-μm membrane filter before injecting into the HPLC equipment. The chromatographic analysis was performed under identical conditions used in the Kinetic I protocol, except for the use of 1 diode array detector at 270 nm. This procedure was carried out in duplicate for each sample. The Rf was quantified by using an external standard calibration curve, following the same concentration range and procedure as previously described for Kinetics I. Linear regression equations and correlation coefficients were calculated ($y = 30.723x + 0.9668$; $R^2 = 0.98$; $n = 8$).

The level of Rf found in milk, by both protocols, before light exposure is in agreement with data reported in the literature (e.g., 0.8–3.0 μg/mL; Muñoz et al., 1994).

Determination of the Rf Photooxidative Mechanism in Milk

Riboflavin-mediated photosensitized generation of $^1O_2$ and $O_2^{\bullet-}$ and the self-degradation of Rf in milk were evaluated by analysis of the degradation rate of Rf in the absence and presence of a specific quencher or scavenger for each ROS, such as NaN_3 (1 m*M*) for $^1O_2$ (Wilkinson et al., 1995) or SOD (5 μg/mL) for $O_2^{\bullet-}$ (Criado et al., 1996). The remaining Rf concentration was determined as a function of the irradiation time with the same protocol described for Kinetic I (see above).

Statistical Analysis

The experimental data were processed and analyzed with OriginPro 8 software (OriginLab Software Corp., Northampton, MA) for kinetic analysis. The experiments were done in duplicate (Rf photooxidation assays) and triplicate (ROS scavenging and ^{3}Rf*-quenching assays) and subjected to statistical ANOVA (one-way ANOVA) with a significance level of $P < 0.05$. These calculations were performed using the Statgraphics Centurion XV program (StatPoint Technologies Inc., Warrenton, VA). Data were presented as mean value ± standard deviation.

RESULTS AND DISCUSSION

Photoprotection of Rf in Milk by MC-GA

Figure 1 shows the degradation kinetics of Rf determined by HPLC (see the Protocols for HPLC Quantification of Rf in Milk section) in whole milk stored in dark and light conditions at 4°C, without and with the addition of 1.37 mg/mL of MC-GA and MC-βc-GA. Under dark conditions, the flavin was not degraded during the observed time range, whereas under illumination with fluorescent light, Rf was consumed following first-order kinetics, according to the equation $[Rf] = [Rf]_0 \times \exp(-k_{pd}t)$, where $\boldsymbol{k_{pd}}$ represents the global first-order rate constant of Rf photodegradation and t is the storage time (Table 1).

As compared with the milk samples without additive, the presence of MC significantly reduced the photodegradation of the flavin, and the photoprotection percentage of Rf ($\boldsymbol{\%PP_{Rf}}$) can be calculated using Equation 2:

$$\%PP_{Rf} = \left(1 - \frac{{k_{pd}}^{MC}}{{k_{pd}}^{0}}\right) \times 100, \quad [2]$$

where ${k_{pd}}^0$ and ${k_{pd}}^{MC}$ are the photodegradation rate constants of Rf in the absence and presence of MC in milk, respectively. In the present case, the addition of 1.37 mg/mL of MC produced a $\%PP_{Rf}$ of 26 (±4) and 30% (±5%) for MC-GA and MC-βc-GA, respectively. Under the studied conditions, the presence of 0.54 μg of βc/mL produced a slight increment in the photoprotection of Rf.

It is well established that photosensitization of Rf in milk samples generates ROS as singlet molecular oxygen ($^1O_2$) and anion superoxide ($O_2^{\bullet-}$). To explore the participation degree of different photogenerated ROS in the degradation of Rf in milk, degradation kinetics of the flavin were analyzed by HPLC in the presence of specific deactivators of ROS, such as NaN_3 for $^1O_2$ and SOD for $O_2^{\bullet-}$, respectively (Serrano et al., 2013).

Figure 2 shows the effect on the first-order kinetics of Rf photodegradation of the addition of 1 m*M* NaN_3 or 5 μg/mL SOD, which are concentrations large enough to

produce almost total deactivation or scavenging of both ROS. Therefore, by comparing the observed first-order rate constants of Rf (k_{pd}) without and with NaN_3 or SOD, the reduction of k_{pd} was observed in the presence of both additives, resulting in the calculation of $\%PP_{Rf}$ = 71 and 17% for NaN_3 and SOD, respectively, indicating that the main Rf degradation pathway is caused by reaction with $^1O_2$. The remaining 12% can be caused by additional degradation mechanisms of Rf, which could involve other ROS, such as $HO^\bullet$ or H_2O_2. These species can be generated in milk either by microbial metabolism (H_2O_2) or by Fenton reaction for $HO^\bullet$ (Ito et al., 2003). In particular, in our experiments, the generation of $HO^\bullet$ via the Fenton reaction was favored, as the milk sample was commercially fortified with ferric phosphate as iron source, and with vitamin C, which can act as a catalyst for the Fenton reaction.

Table 1. Global first-order rate constant of riboflavin (Rf) photodegradation (k_{pd}) in whole milk without and with addition of 1.37 mg/mL of microcapsules (MC) of gum arabic (MC-GA) and MC-GA with β-carotene (MC-βc-GA) at 4°C

Sample	$k_{pd}/10^{-3}(h^{-1})$ with MC-GA	$k_{pd}/10^{-3}(h^{-1})$ with MC-βc-GA
Milk + light	30 ± 5	25 ± 4
Milk + MC + light	22 ± 2	18 ± 6
Milk in the dark	0.3 ± 0.1	0.2 ± 0.1

The possibility of an additional photoprotection mechanism by inner filter effect by the MC in the region where Rf absorbs ambient light (380–500 nm) can be ruled out because in a previous study (Montenegro et al., 2007), we demonstrated by diffuse reflectance measurements that skim milk with 6.5 mg/mL of MC-GA containing lycopene only contributed <2% of the photoprotection. In the present case, a lesser concentration of MC was used and, therefore, the inner-filter effect was expected to be negligible.

In addition to evaluating the photodegradation of Rf in milk as the result of the combination of several

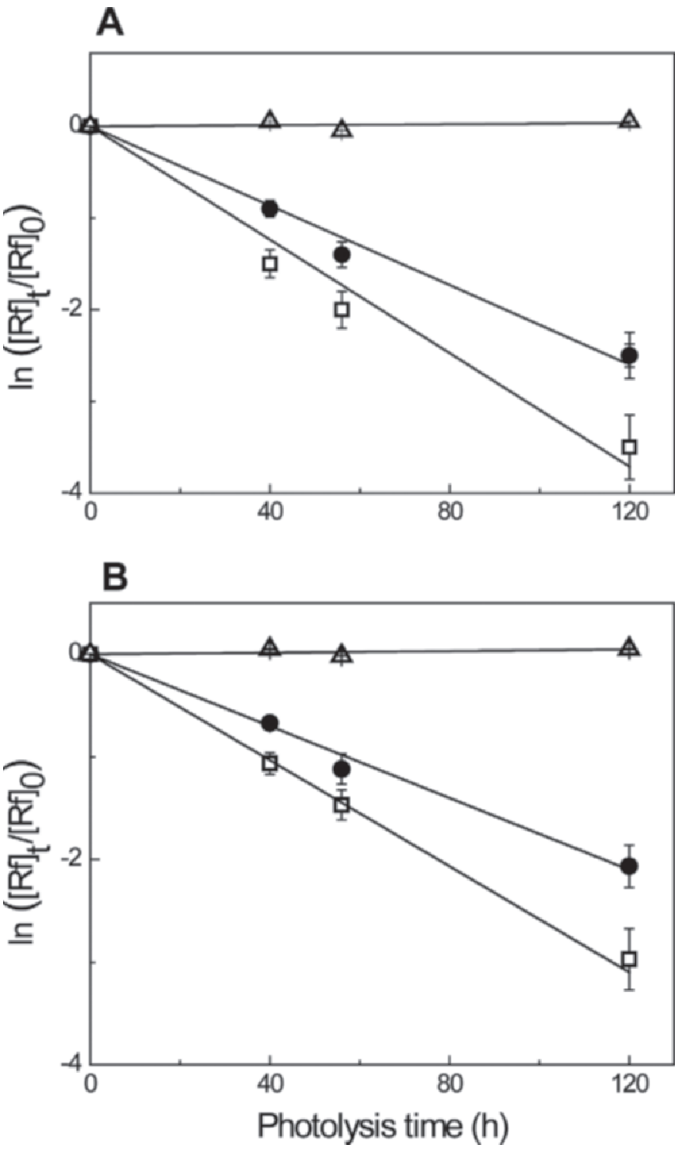

Figure 1. Riboflavin (Rf) degradation kinetics in reconstituted powdered whole milk stored at 4°C under light and dark conditions, obtained from HPLC analysis. (A) With addition of 1.37 mg/mL of microcapsules (MC) of gum arabic (MC-GA); (B) with addition of 1.37 mg/mL of MC-GA with β-carotene (MC-βc-GA). t = storage time; □ = milk + light; ● = milk + MC + light; Δ = milk in the dark. Error bars represent the SD calculated in duplicate experiments.

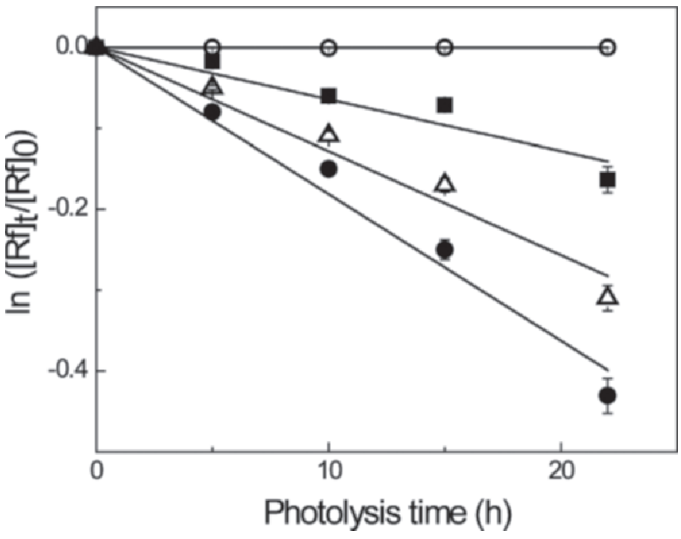

Figure 2. Riboflavin (Rf) degradation kinetics in milk samples with and without addition of 0.1 *M* sodium azide (NaN_3) and 0.5 mg/mL superoxide dismutase (SOD), stored under conditions of light and dark at 4°C, obtained by HPLC measurements. t = storage time; ○ = milk in the dark (negative control); ● = milk + light (positive control); Δ = milk + SOD; ■ = milk + NaN_3. Error bars represent the SD calculated in duplicate experiments.

ROS in different proportions, we evaluated the specific photoprotective effect of both spray-dried MC-GA and MC-βc-GA preparations toward the ^{3}Rf* and ROS in aqueous phosphate buffer solutions, as described in the following sections.

Quenching of ^{3}Rf* by GA and MC-GA in Buffer Solutions

In previous work, we demonstrated that ^{3}Rf* was almost not quenched by lycopene in 30 m*M* Triton X-100 aqueous micelle solutions (Montenegro et al., 2007). Similar results were obtained by Cardoso et al. (2007), who studied the deactivation of ^{3}Rf* by ascorbate, CAR, and tocopherols in aqueous food model systems, demonstrating that βc, lycopene, and crocin were not able to quench ^{3}Rf*. Therefore, in the present work, only the interaction of ^{3}Rf* with GA was analyzed by laser flash photolysis. Direct evidence of ^{3}Rf* quenching by GA in N_2-saturated phosphate buffer solutions was obtained by monitoring the typical transient absorption of ^{3}Rf* at 720 nm after laser excitation at 355 nm, using transient absorption spectroscopy (Figure 3).

We evaluated the increases in the decay rate k_T ($= 1/\tau_T$), where τ_T is the lifetime of the triplet state, of the ^{3}Rf* monitored at 710 nm as the GA concentration was increased (Equation 3), where $k_T^0 = (\tau_T{}^0)^{-1} = (27\ \mu s)^{-1}$ is the decay rate of the triplet state in the absence of GA, and $^3k_Q{}^{GA}$ is the bimolecular quenching rate constant of ^{3}Rf* by GA:

$$k_T = k_T^0 + {}^3k_Q^{GA}[GA]. \quad [3]$$

The inset of Figure 3 shows a linear dependency of Equation 3, allowing the calculation of $^3k_Q{}^{GA} = 1.6\ (\pm 0.3) \times 10^9\ M^{-1}s^{-1}$. Therefore, GA is able to quench ^{3}Rf* with almost a diffusion-controlled quenching rate, in a similar fashion to the quenching of ^{3}Rf* by free amino acids, such as His and Tyr [e.g., $^3k_Q{}^{His} = 3.8(\pm 0.5) \times 10^8 M^{-1}s^{-1}$ and $^3k_Q{}^{Tyr} = 1.8(\pm 0.8) \times 10^9\ M^{-1}s^{-1}$] as studied in this work (data not shown), in accordance with previous reported values (Cardoso et al., 2004; Huvaere and Skibsted, 2009). As GA is a heteropolysaccharide containing about 2% polypeptide (Mahendran et al., 2008), the quenching effect of ^{3}Rf* by GA can be associated with the presence of amino acid residues, such as His and Tyr, in the protein moiety of GA. Assuming the same quenching efficiency of ^{3}Rf* by GA forming MC, the shortening of the flavin triplet lifetime (~21 μs) by the addition of 1.37 mg/mL MC-GA allows the calculation of the quenching fraction of ^{3}Rf* (f_T) by GA (i.e., $f_T = 1 - \tau/\tau_0 = 0.2$), indicating that approximately 20% of the Rf photoprotection observed in the presence of MC-GA can be associated with the quenching of ^{3}RF*, which in turn, avoids in the similar extension the generation of ROS.

Scavenging of $HO^\bullet$ and $O_2^{\bullet-}$ by the MC in Buffer Solution

Figure 4 shows the %S of $HO^\bullet$ and $O_2^{\bullet-}$ by MC and TX or SOD as reference AOx, respectively. It can be observed that at MC concentrations (1.37 mg/mL) used in the experiments of milk photooxidation, MC-GA or MC-βc-GA produce more efficient scavenging of $O_2^{\bullet-}$ than of $HO^\bullet$. In this case, for the calculation of the TEAC and EAA_{SOD} values, the experimental data of the plots of %S versus [AOx] (*M*) was fitted with a second-order polynomial function, and the slopes of the initial linear region obtained for the MC were divided for those of TX or SOD, and the respective TEAC and EAA_{SOD} were obtained (Table 2). The results show that MC-βc-GA was an about 1.5 to 1.7 times more efficient scavenger than the "empty" MC-GA. This effect can be attributed to the scavenging ability of βc as a consequence of the presence of an extended conjugated double bond system in its structure (Mortensen et al., 2001), which reduces the energy barrier for reaction with free radicals, such as $ROO^\bullet$, $OH^\bullet$, and $O_2^{\bullet-}$. However, the data of Table 2 also indicates that the scavenger activity of MC-GA for both ROS is not negligible, in agreement with antioxidant effects attributed to GA (Al-Majed et al., 2003; Trommer and Neubert, 2005).

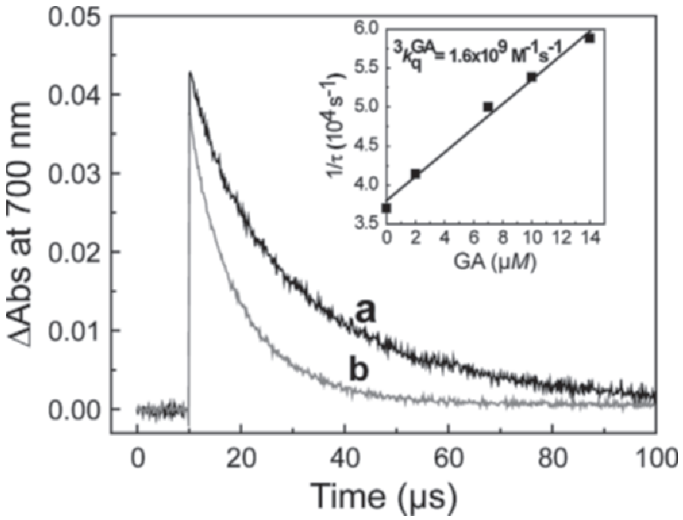

Figure 3. Transient absorption decay at 720 nm of the triplet excited state of riboflavin (^{3}Rf*) after laser pulsed excitation at 355 nm of Rf in N_2-saturated phosphate buffer solutions as a function of the gum arabic (GA) concentration: (a) 0 μ*M*; (b) 14 μ*M*. Inset: Stern-Volmer plot for the quenching of ^{3}Rf* by GA (Equation 3 in the text). τ = lifetime of the triplet state; $^3k_Q{}^{GA}$= biomolecular quenching rate constant of ^{3}Rf* by GA.

Mechanistic Analysis of Photooxidation and Photoprotection in Milk

Depending on the light exposition, presence of molecular oxygen ($^3O_2$), and concentration of dissolved substrates (Q), the Rf is capable of promoting the oxidation of various milk constituents through photosensitized ROS generation (Massad et al., 2004). In this process, the light absorption promotes the sensitizer (Rf, in ground state) to the electronically excited singlet and triplet states ^{1}Rf* and ^{3}Rf*, respectively (steps 1 and 2 in Figure 5). In the presence of electron donor molecules (as MC), the ^{3}Rf* can be quenched by an electron-transfer process to yield radical species (step 3 in Figure 5; Heelis, 1982). In this work, we demonstrate that MC-GA is an efficient quencher of ^{3}Rf*; considering that GA concentration is the same in both microencapsulated (MC-GA and MC-βc-GA) and that βc is not able to quench ^{3}Rf*, the fraction quenching of ^{3}Rf* would be the same both cases.

Furthermore, in aerobic conditions, the ^{3}Rf* is quenched by ground-state oxygen ($^3O_2$) through energy-transfer (type II mechanism) and electron-transfer (type I mechanism) reactions to produce singlet molecular oxygen $^1O_2$ (step 4 in Figure 5) or the superoxide radical anion species ($O_2^{\bullet-}$; step 5 in Figure 5), respectively (Skibsted, 2010; Massad et al., 2004). In aqueous media, the capacity of ^{3}Rf* to generate $^1O_2$ is 60 times greater than that of $O_2^{\bullet-}$, suggesting that the main ROS formed during milk light-induced oxidation is $^1O_2$. In turn, $^1O_2$ can interact with electron donor molecules (as MC) through physical or reactive processes, or both (steps 6 and 7 in Figure 5). In this context, taking into account the values of the total quenching rate constant of $^1O_2$ (k_t) by MC-GA and MC-βc-GA (2.7×10^7 and 5.1×10^7 $M^{-1}s^{-1}$, respectively) reported by Faria et al. (2010) and the CAR acting as a singlet oxygen quencher, by a physical mechanism (step 6), MC-βc-GA could have a greater quenching effect approximately twice that of MC-GA. Moreover at neutral pH, $O_2^{\bullet-}$ may generate H_2O_2 by interaction with radical species of Rf (Rf*; step 8 in Figure 5; Lu et al., 1999). In the presence of metals [e.g., Fe(II)], H_2O_2 can produce the hydroxyl radical ($HO^{\bullet}$) by the Fenton reaction (step 9 in Figure 5; Sutton and Winterbourn, 1989). Finally, the ROS $O_2^{\bullet-}$ and $HO^{\bullet}$ may react with electron donor molecules (as MC) through steps 9 and 10 in Figure 5. In the in vitro assays performed in buffer solution in the current work, the MC at the concentrations in which they were added to the milk showed moderate ability to deactivate both oxygen radicals ($O_2^{\bullet-}$ and $HO^{\bullet}$), being 3 times more effective against $O_2^{\bullet-}$ and MC-βc-GA being more efficient than MC-GA.

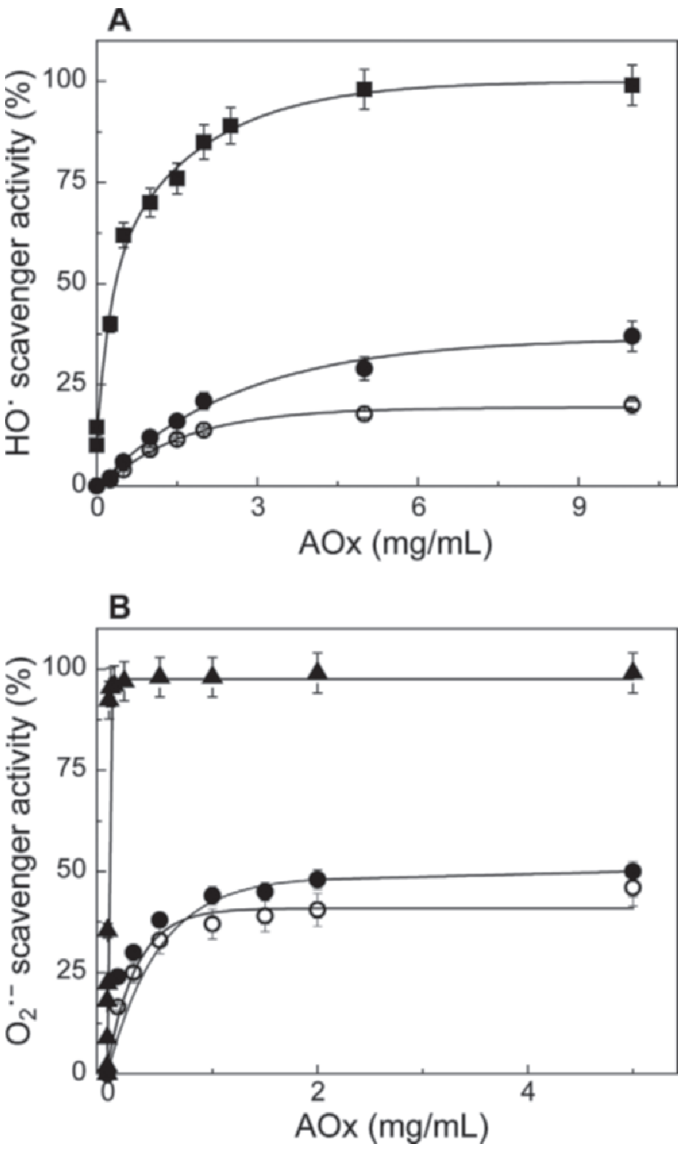

Figure 4. Percentage scavenging activity of radicals $HO^{\bullet}$ (A) and $O_2^{\bullet-}$ (B) by microcapsules (MC) of gum arabic (MC-GA) and MC-GA with β-carotene (MC-βc-GA). ○ = MC-GA; ● = MC-βc-GA; ■ = Trolox (TX); ▲ = superoxide dismutase (SOD); AOx = antioxidant molecules. Error bars represent the SD calculated in triplicate experiments.

Table 2. Trolox equivalent antioxidant capacity (TEAC) for $HO^{\bullet}$ and superoxide dismutase (SOD) equivalent antioxidant activity (EAA_{SOD}) for $O_2^{\bullet-}$ with addition of microcapsules (MC) of gum arabic (MC-GA) and MC-GA with β-carotene (MC-βc-GA)

MC	TEAC for $HO^{\bullet}$	EAA_{SOD} for $O_2^{\bullet-}$
MC-βc-GA	0.52 ± 0.06	0.11 ± 0.02
MC-GA	0.30 ± 0.05	0.07 ± 0.01

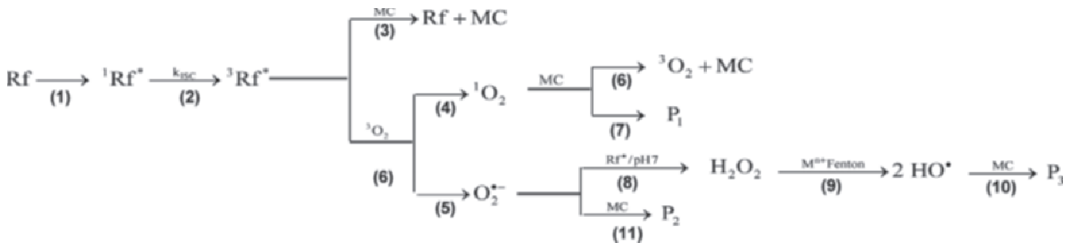

Figure 5. Possible riboflavin (Rf) photooxidation and photoprotection mechanisms in milk. $^1Rf^*$ = electronically excited singlet state of Rf; $^3Rf^*$ = electronically excited triplet state of Rf; k_{ISC} = rate constant of intersystem crossing; MC = microcapsule; P_1 = oxidation product 1; P_2 = oxidation product 2; P_3 = oxidation product 3; Rf* = riboflavin radical; M^{n+} = transition metal. Steps 1 through 10 are described in the text.

CONCLUSIONS

Both MC-βc-GA and MC-GA can act as efficient protectors of the self-photoinduced degradation of Rf. This protective effect can be explained mainly by the capability of GA to quench the $^3Rf^*$, avoiding the subsequent reactions of the triplet state of the flavin with molecular oxygen to produce different ROS. The deactivation mechanism of $^3Rf^*$ is associated with its interaction with solvent-exposed amino acid residues of the proteinaceous moiety of GA. Furthermore, the MC showed interesting scavenging properties, also associated with the complex nature of GA. The encapsulation of βc improved the ROS scavenging properties of the MC, probably by easier accessibility of the photogenerated ROS to the localization site of βc in the MC. Altogether, the results indicate that GA-based MC containing CAR are efficient antioxidants to preserve milk from the deleterious effect of Rf sensitization by ambient blue light, extending its nutritional value under illuminated conditions.

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