Characterization of Enzymatically Prepared Biosurfactants

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ABSTRACT: Various fatty monoesters of sugars and sugar alcohols were prepared enzymatically in organic solvent. Water produced during esterification was removed by refluxing through a dessicant under reduced pressure. Surface properties of these esters such as surface and interfacial tensions and their ability to stabilize emulsions at 30°C were evaluated: oleate esters of glucose, fructose, and sorbitol show similar behavior in reduction of surface and interfacial tensions, and values for the critical micelle concentration are about $8 \cdot 10^{-5}$ M. It was also observed with sorbitol esters that the shorter the alkyl chain, the higher the critical micelle concentration. Generally, emulsions prepared with the emulsifier dissolved in the water or in the oil phase lead to oil-in-water or water-in-oil emulsions, respectively. Sorbitol monolaurate significantly increased the stability of oil-in-water emulsions, with only 5% separation of the phases after 48 h at 30°C, compared to 10% for chemically prepared sorbitan monolaurate under the same conditions. Sorbitol monoerucate was very efficient in stabilizing water-in-oil emulsions, with only 1% separation of the phases.

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KEY WORDS: Critical micelle concentration, emulsifier, emulsion stability, enzymatic esterification, lipase, nonionic surfactant, sugar alcohol esters, sugar esters, surface activity.

For several years, there has been great interest in the enzymatic preparation of fatty esters of carbohydrates. This type of amphiphilic molecule constitutes an interesting group of nonionic surfactants. It is made from renewable, inexpensive, and readily available feedstocks. Completely biodegradable, it is harmless to the environment, is nontoxic, is a nonskin-irritant, is odorless and tasteless, and forms normal food products after human and animal digestion. For all these reasons, there are plenty of potential applications in many diverse areas, such as in environmental, pharmaceutical, food, cosmetic, and detergent products (1,2). Besides chemical synthesis that involves high temperatures, which lead to colored mixtures as final products and contain numerous isomers and side products, which are highly dehydrated and cyclized in the case of sugar alcohols (3,4), there are two different approaches to realizing the synthesis of sugar esters by enzymatic processes. One process uses organic solvents (5–11) to solubilize both substrates. The other process is based on the esterification of sugar derivatives under solvent-free conditions at reduced pressure (12–15). In a previous paper (16), we described a method, a combination of both approaches: enzymatic esterification was performed between unmodified sugars (or sugar alcohols) and fatty acids in organic solvent, so that the contact between the starting materials, which have opposite polarities, is improved. Water generated by the reaction is continuously removed by refluxing through a dessicant under reduced pressure. Under these conditions, esterification was favored, and water activity required by the enzyme was maintained. Various fatty esters of sugars or sugar alcohols could be easily obtained by this method from readily available starting materials, such as glucose, fructose, or sorbitol, and lauric acid (one of the main constituents of coconut and palm kernel oils), oleic acid (the major constituent of peanut and olive oils), or erucic acid (a major fatty acid present in rapeseed oil). We now report that these molecules thus formed are efficient surfactants that can easily reduce surface and interfacial tensions and stabilize emulsions.

EXPERIMENTAL PROCEDURES

Materials. Novozyme 435 (type-B lipase from *Candida antarctica*, immobilized on an acrylic resin) was a gift from Novo Industries (Bagsvaerd, Denmark). The purity of sorbitol (BDH), glucose (Sigma, St. Louis, MO), fructose (Sigma), and xylitol (Fisher Scientific, Nepean, Ontario, Canada) was over 98%. Oleic acid (*cis*-9-octadecenoic acid), erucic acid (*cis*-13-docosenoic acid), lauric acid (dodecanoic acid), and caprylic acid (octanoic acid), all supplied by Sigma, were more than 99% pure. 2-Methyl-2-butanol, used for the different syntheses, was supplied by Aldrich Chemical Co. (Milwaukee, WI) and was more than 99% pure. Sorbitan monolaurate and sorbitan monooleate were the commercially available SPAN 20 and SPAN 80 (ICI Americas Inc., Wilmington, DE), respectively, and both were purchased from Sigma. The water, used in the measurement of surface and interfacial tensions, was purchased from Anachemia and was high-purity glass-distilled. Xylene was a mixture of the *ortho*, *meta*, and *para* isomers, supplied by Fisher Scientific. All the solvents used in chromatography were high-performance liquid chromatography (HPLC) grade.

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Enzymatic esterification of sugars or sugar alcohols. Synthesis of glucose monoleate, fructose monooleate, xylitol monooleate, sorbitol monooleate, sorbitol monoerucate, sorbitol monolaurate, and sorbitol monocaprylate were carried out with Novozym 435 directly from sugars or sugar alcohols and fatty acids in molar ratio 1:1, according to the method previously described (16). Contact between both substrates was increased by dissolving them in a common solvent (2-methyl-2-butanol), and water generated by the reaction was continuously removed by vaporizing the solvent rich in water under reduced pressure and recycling the dry solvent in the reaction medium after passing it through a water trap. Continuous elimination of water displaces the equilibrium of the reaction toward synthesis, while keeping the minimum quantity of water required by the biocatalyst to be active. Totally dried enzymes are usually inactive (17). Under these conditions, appreciable monoester yields, in the order of 70%, can be obtained after 24 h. Operating conditions and yields obtained are briefly summarized in Table 1.

HPLC analysis. All HPLC analyses were carried out as described previously (16). Briefly, HPLC analyses were performed on a reversed-phase column (CSC-S Inertsil ODS2, 5 μm, 25 cm × 4.6 mm; CSC, Montreal, Quebec, Canada) maintained at 20°C on a Waters system (Waters Scientific, Mississauga, Ontario, Canada), and all chromatograms were monitored at 205 nm. Elution of sorbitol, glucose, fructose, and xylitol monooleates was performed with a mixture of acetone/acetonitrile/10^{-3} M acetic acid (45:45:10, vol/vol/vol) at 2 mL/min.

Purification. Purification of monoesters was achieved by chromatography on silica gel, according to the method previously described (16). Monoesters were eluted with a mixture of chloroform/methanol/water (64:10:1, vol/vol/vol). The final purity of each monoester was higher than 99% by HPLC determination.

Determination of surface and interfacial tensions. Surface tension in water and interfacial tension between water and xylene were measured with a Fisher Surface Tensiomat (model 21; Fisher Scientific) according to the du Nouy method by using a series of aqueous solutions at various concentrations of biosurfactant at room temperature (20°C). The solutions were aged at room temperature before each measurement until equilibrium was reached between adsorption and desorption of the amphiphilic molecules of biosurfactants at the interface. Measurements were repeated three times, and the mean value was taken.

Determination of emulsion stability. The ability of the synthesized biosurfactants to stabilize emulsions, oil in water (o/w) or water in oil (w/o), was assessed as follows: 3 volumes of the surfactant solution (diluted in water or in xylene as the oil phase) were mixed with 2 volumes of the other phase (xylene or water) with a Polytron mixer (Kinematics, Luzern, Switzerland) for 5 min. Each emulsion contained 0.25 or 0.5% (wt/vol) surfactant as specified. The emulsion formed was transferred to a graduated cylinder and kept at 30°C, and the separation of the phases was measured as a function of time. The emulsion is an o/w emulsion if the continuous (outer) phase can be diluted with water, and it is a w/o emulsion if it can be diluted with xylene.

RESULTS AND DISCUSSION

Surface and interfacial tensions. A surface-active agent has the characteristic property to adsorb at low concentration onto the surface or interface of a system that is constituted by two immiscible phases and, as a result, to lower the surface or interfacial tension. Curves of the reduction of surface tension vs. molar concentration in water are shown in Figure 1 for monooleate esters of various sugars or sugar alcohols and in Figure 2 for sorbitol monoesters of fatty acids with different chainlengths. The presence of small quantities of any synthesized sucroester considerably reduces the surface tension of water and compares well with chemically synthetized sorbitan esters. Each surfactant can be characterized by its critical micelle concentration (CMC), efficiency, and effectiveness. CMC can be determined from the inflexion of the surface tension vs. the concentration curve. In general, the CMC of a biosurfactant in an aqueous medium decreases as the number of carbon atoms in the hydrophobic group increases to about 16, while above 18 carbons, CMC remains unchanged with an increase of the chainlength (18,19). Efficiency corresponds to the concentration of surfactant required to produce a sig-

TABLE 1
Synthesis of Sucroesters from Substrates in Equimolar Ratio; Conditions as Described in the Experimental Procedures Section

Biosurfactant	Acyl moiety	Hydroxyl moiety	Reaction time (h)	Monoester yield (% w/w)
Glucose monooleate	Oleic acid	α-Glucose	24	78.2[a]
Fructose monooleate	Oleic acid	β-Fructose	24	58.1[a]
Xylitol monooleate	Oleic acid	Xylitol	24	54.2[a]
Sorbitol monooleate	Oleic acid	Sorbitol	24	58.4[a]
Sorbitol monocaprylate	Caprylic acid	Sorbitol	7	42[b]
Sorbitol monolaurate	Lauric acid	Sorbitol	7	50[b]
Sorbitol monoerucate	Erucic acid	Sorbitol	7	40[b]

[a]Before purification on silica column.
[b]Isolated monoester after purification on silica column.

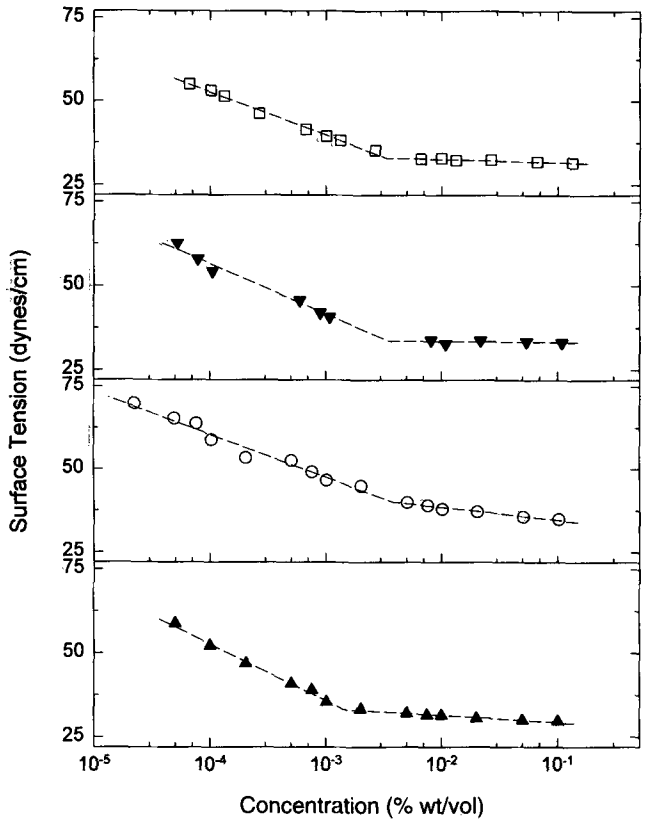

FIG. 1. Reduction of surface tension by monooleate esters of fructose □, glucose ▼, sorbitol ○, and xylitol ▲ as a function of concentration.

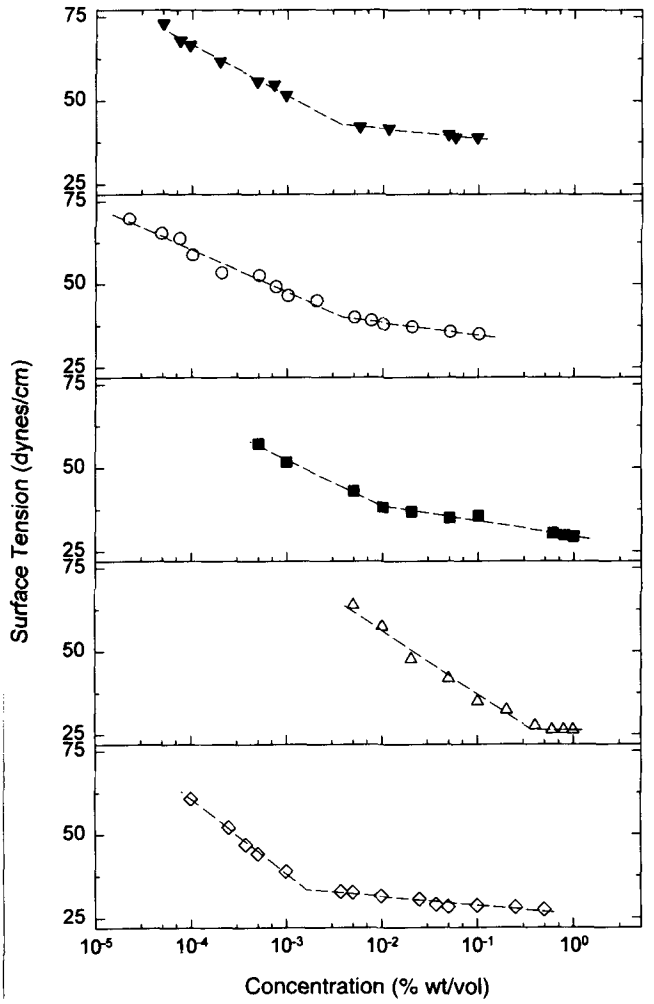

FIG. 2. Reduction of surface tension by sorbitol monoesters of erucic acid ▼, oleic acid ○, lauric acid ■, caprylic acid △, and sorbitan monolaurate ◇ as a function of concentration.

nificant reduction in the surface tension equivalent to 20 dynes/cm (18,20). Effectiveness of a surfactant corresponds to the minimum value to which it can depress the surface tension of water. Values of CMC, efficiency, and effectiveness, found for each synthesized biosurfactant, as well as those previously obtained by Matsumura *et al.* (21) for alkyl glucosides with alkyl chains ranging from 8 to 12 carbons, are reported in Table 2. CMC values obtained for the oleate esters of glucose, fructose, and sorbitol, which have similar hydrophilic character, remain around $8 \cdot 10^{-5}$ M, while xylitol monooleate, which has a smaller hydrophilic group, leads to a lower CMC. As expected, the effect of the hydrophobicity of the alkyl chain is clear: comparing esters of sorbitol with different chainlengths, the shorter the alkyl chain, the higher the CMC. Moreover, values obtained for sorbitol monooleate and sorbitol monoerucate are close, and show no influence of the chainlength above 18 carbons. Values obtained for lauric and caprylic sorbitol esters are close to those obtained for alkyl glucosides. The minimum value that can be achieved with the alkyl esters of sorbitol increases with chainlength: sorbitol monoerucate decreases the surface tension to 39 dynes/cm, while sorbitol monocaprylate decreases the surface tension to 26 dynes/cm.

Analyses of the interfacial properties of each synthesized biosurfactant are reported in Figures 3 and 4. As observed for surface tension, the presence of surfactant at low concentration decreases interfacial tension between water and xylene. In all cases, interfacial tension drops to almost zero, as with chemically synthesized surfactants, but the exact concentration required depends on the nature of the enzymatically prepared surfactant (biosurfactant). Again, biosurfactants with long chainlengths are more potent at low concentrations than those with shorter chainlengths. Reduction of interfacial tension obtained for sorbitol monooleate compares nicely with those obtained by Chopineau *et al.* (6) with surfactants prepared from sorbitol and triglycerides from apricot seed oil, an oil rich in oleic acid.

Emulsion stability. Another classic test for a surfactant is its ability to help form emulsions. Different emulsions were prepared, as described in the Materials and Methods section, from water and xylene with various concentrations of surfactant in one of the two phases. These emulsions were allowed to stand at 30°C, and the percentage of phase separation was measured as a function of time. Results are reported in Figure 5 for the emulsions prepared with 10 mL water and 15 mL

TABLE 2
Effect of Biosurfactant on Surface Tension

Monoester	CMC[a]	Efficiency[a]	Effectiveness[a]
Sorbitol monocaprylate	$1.2 \cdot 10^{-2}$ M	$4.3 \cdot 10^{-4}$ M	26.3 dynes/cm
Sorbitol monolaurate	$2.5 \cdot 10^{-4}$ M	$2.1 \cdot 10^{-5}$ M	29.4 dynes/cm
Sorbitol monooleate	$8.6 \cdot 10^{-5}$ M	$7.2 \cdot 10^{-6}$ M	35.0 dynes/cm
Sorbitol monoerucate	$7.4 \cdot 10^{-5}$ M	$1.4 \cdot 10^{-5}$ M	39.0 dynes/cm
β-Fructose monooleate	$7.6 \cdot 10^{-5}$ M	$1.8 \cdot 10^{-6}$ M	31.6 dynes/cm
α-Glucose monooleate	$8.9 \cdot 10^{-5}$ M	$3.4 \cdot 10^{-6}$ M	33.2 dynes/cm
Xylitol monooleate	$3.5 \cdot 10^{-5}$ M	$2.1 \cdot 10^{-6}$ M	29.7 dynes/cm
β-Glucose monocaprylate (21)	$2.0 \cdot 10^{-2}$ M	—	30.5 dynes/cm
β-Glucose monolaurate (21)	$1.5 \cdot 10^{-4}$ M	—	27.3 dynes/cm
Sorbitan monooleate (SPAN 20)[b]	$4.5 \cdot 10^{-5}$ M	5.7 • 10–6 M	27.5 dynes/cm

[a]As defined in text.
[b]Sigma Chemical Co. (St. Louis, MO).

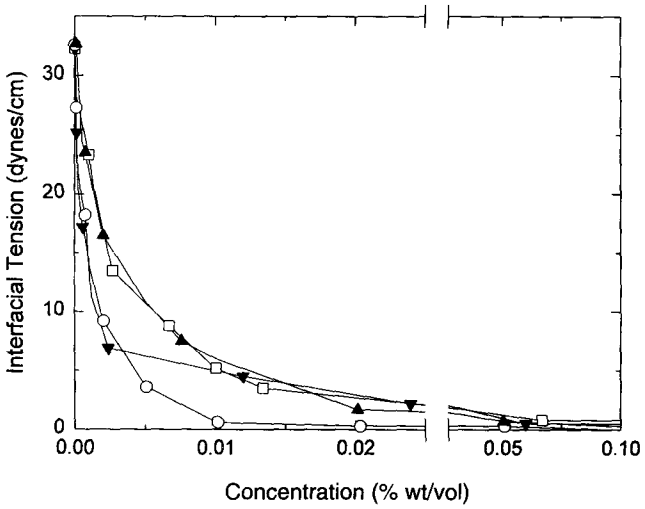

FIG. 3. Reduction of interfacial tension between xylene and water by monooleate esters of fructose □, glucose ▼, sorbitol ○, and xylitol ▲ as a function of concentration.

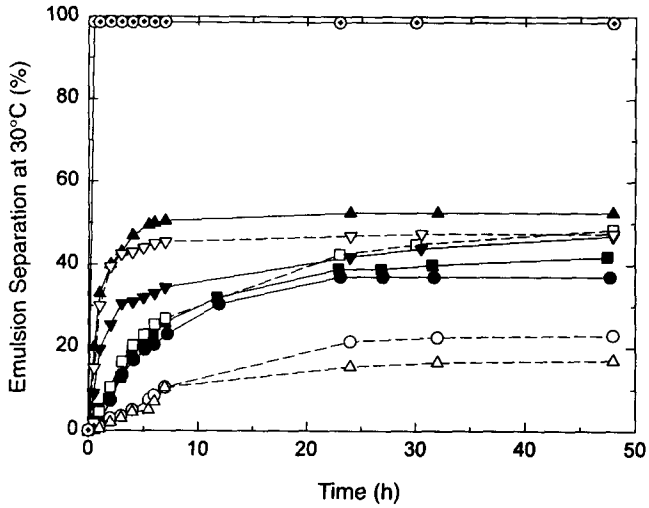

FIG. 5. Stabilization of emulsions at 30°C by sorbitol monoerucate ○, sorbitol monooleate □, sorbitan monooleate △, sorbitan monolaurate ▽, and without surfactant ⊕. [Emulsions are prepared with 10 mL water and 15 mL of a solution of surfactant dissolved in xylene at 0.5% wt/vol (closed symbols) and 0.25% wt/vol (open symbols)].

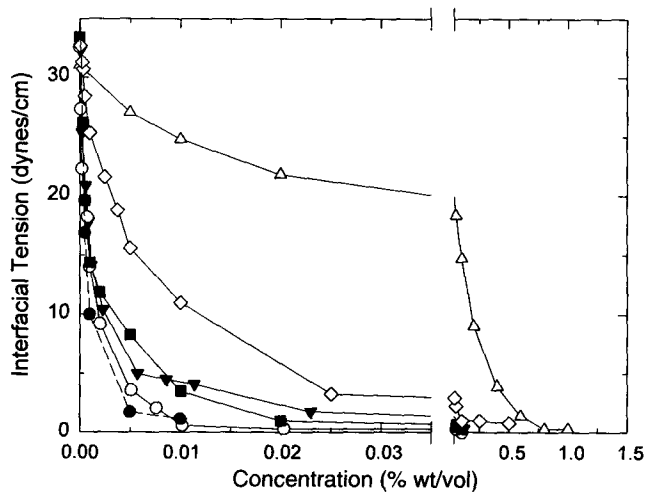

FIG. 4. Reduction of interfacial tension between xylene and water by sorbitol monoesters as a function of concentration: sorbitol monoesters of erucic acid ▼, oleic acid ○, lauric acid ■, caprylic acid △, and sorbitan monolaurate ◇. Surfactant enzymatically synthesized from sorbitol and apricot seed oil ● [results taken from Ref. (6)].

xylene containing the emulsifier, and in Figure 6 for the emulsions prepared with 10 mL xylene and 15 mL of an aqueous solution of surfactant. Compared with emulsions that were prepared without any surfactant, in which the separation of the phases was complete after only a few minutes, those obtained with the biosynthesized surfactants showed good stability, with less than 50% separation of the phases after 48 h at 30°C. It is known that the type of emulsion formed by water and oil depends primarily on the nature of the surfactant and, to a minor extent, on the process used for preparing the emulsion and the relative proportion for oil and water present. In general, o/w emulsions are produced by emulsifying agents that are more soluble in the water than in the oil phase, whereas w/o emulsions are produced by emulsifying agents that are more soluble in the oil than in the water phase (19). Emulsions that were produced with emulsifier dissolved in water led to o/w emulsions, except with sorbitol monoerucate and sorbitol monooleate, which led to w/o emulsions, probably as a result of the strong hydrophobic character of these

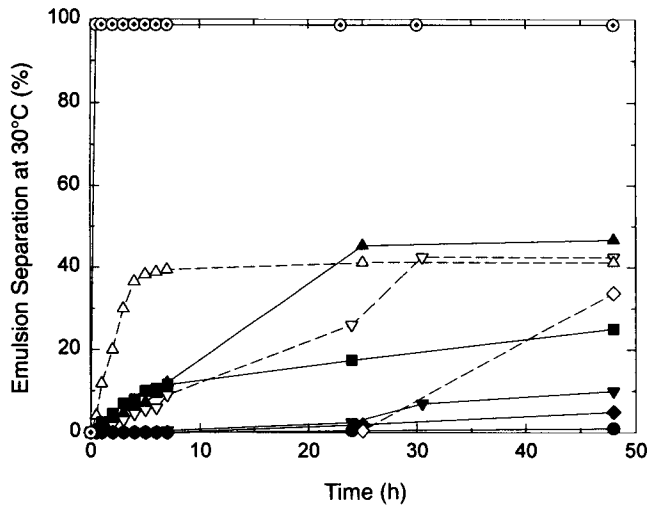

FIG. 6. Stabilization of emulsions at 30°C by sorbitol monocaprylate ▲, sorbitol monolaurate ◆, sorbitol monooleate ■, sorbitol monoerucate ●, sorbitan monolaurate ▼, and without any surfactant ⊕. [Emulsions are prepared with 10 mL xylene and 15 mL of an aqueous solution of surfactant at 0.5% wt/vol (closed symbols) and 0.25% wt/vol (open symbols)].

two molecules. On the other hand, emulsions produced with emulsifier dissolved in xylene led to w/o emulsions, except with the sorbitan monooleate at 0.25%, which led to an o/w emulsion. The type of emulsion obtained with sorbitol monooleate at 0.25% was not determined. Sorbitol monolaurate stabilizes o/w emulsion well, with only 5% separation of the phases after 48 h, while sorbitol monoerucate stabilizes w/o emulsions with only 1% separation. These results show that the biosynthesized surfactants have emulsifying and stabilizing properties that compare well with chemically synthesized surfactants of the related family of sorbitan esters. The choice of biosurfactant is dictated by the type of emulsion desired.

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