Enzymatic Synthesis of Polyglycerol–Fatty Acid Esters in a Solvent-Free System

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ABSTRACT: The enzymatic transesterification of fatty acid methyl esters from vegetable oil and polyglycerol has been successfully performed in the presence of Lipozyme without any solvent. The optimal conditions to obtain a mixture with lipophilic tensioactive properties were found to be a molar ratio of fatty acid methyl esters and polyglycerol of 1.33:1 at 60°C. Evaporation of the methanol produced in this reaction increased the yield of the reaction. Moreover, for this reaction, adsorption of the polyol on silica gel prior to the transesterification allowed the completion of the reaction and improved the kinetic properties. Hydrophile–lipophile balance and surface activity of the polyglycerol esters were measured to determine the emulsifying properties of these molecules.
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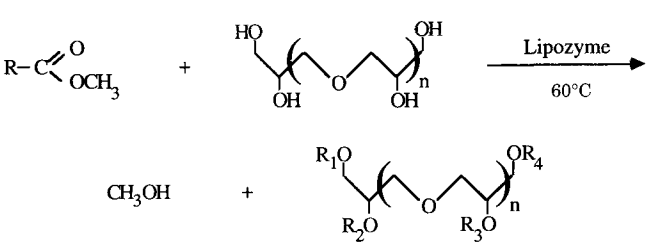
KEY WORDS: Lipase, polyglycerol–fatty acid esters, solvent-free system, transesterification.

Oleochemical products have numerous advantages compared to their mineral counterparts. They are renewable and cause less damage to the environment than petroleum chemicals, the availability of which is finite. These aspects support the interest in surfactants produced from natural fats and oils. Polyol–fatty acid esters are part of these products. These nonionic surfactants are widely used in various applications such as cosmetics, medicines, textile processing and food emulsifiers. The ester bond of these molecules can be easily hydrolyzed, thus improving the biodegradation of the product.

Polyol–fatty acid esters are synthesized from a polyol and a lipidic substrate. However, this reaction presents a major problem. The polarity of both substrates is so different that it results in poor micscibility and inefficient kinetics. Moreover, industrial production of surfactants by chemical synthesis usually requires high temperature and/or high pressure which sometimes leads us to the degradation of the polyol moiety. On the other hand, enzymatic synthesis presents several advantages such as enzyme specificity and mild conditions, thereby avoiding undesirable side reactions and by-products. At low water concentrations, lipases (EC 3.1.1.3) are able to catalyze that type of reaction (1,2). Several enzymatic attempts to synthesize polyol–fatty acid esters for nonionic surfactant production have focused the attention of chemists in recent years. Polar organic solvents are often used to solubilize the hydrophilic and lipophilic substrates (3–7), thus increasing the toxicity of the reaction medium and generally reducing enzymatic activity. Another way to produce these molecules is the modification of the hydrophilic moiety, through alkylation (3,8,9) or acetal formation (10), to increase its miscibility with the lipid phase. The protection and deprotection of the polar head involves some additional reactions. Other methods of production are biphasic systems (11) or micellar media (12), but yields are often low and/or the separation of products is not easily achieved. Finally, it is possible to use a solubilizing agent to form a complex with the sugar (13–16). In this case, organoboronic acids are used. This process requires an additional step and increases the toxicity of the media.

In this paper we describe the synthesis of polyol–fatty acid esters in a solvent-free system to obtain a nontoxic process. The enzyme is immobilized to facilitate its recovery and reuse. The substrates have been chosen according to economical constraints. The polar substrate of the reaction is polyglycerol. Actually, the glycerine supply may rise rapidly in the near future due to the development of biodiesel fuel and also to the expansion of oleochemical products manufactured from natural fats and oils. Thus polyglycerol, resulting from the polymerization of glycerol, may replace molecules such as propylene glycol, sorbitol or other polyols as the hydrophilic moiety of surfactants. On the other hand, the lipophilic part of our system consists of methyl esters of vegetable oil (rapeseed and sunflower). The reaction is a transesterification (Scheme 1) that produces polyglycerol–fatty acid esters and methanol, which is evaporated at the temperature



SCHEME 1

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of the reaction. To scale up this process, methanol could be evaporated and then condensed again. The reaction between oleic acid and polyglycerol was not studied because this reaction is an equilibrium between synthesis and hydrolysis. The kinetics are not complete, and a purification step is then necessary.

EXPERIMENTAL PROCEDURES

Materials. All reactions were catalyzed with Lypozyme IM60, a 1,3-specific lipase from *Mucor miehei* immobilized on a macroporous anion exchange resin, from Novo Nordisk Bioindustrie S.A. (Fontenay sous Bois, France). Silica gel 100 (70–230 mesh ASTM) from Fluka (Buchs, Switzerland) was used. Methyl esters of oleic sunflower (77.8% oleic acid methyl ester) were supplied by Robbe (Compiègne, France). Diglycerol was obtained from Solvay (Solingen, Germany). Penta- and octaglycerol were supplied by I.F.P. (Paris, France). All other chemicals were purchased from Aldrich-Chimie (Strasbourg, France).

Synthesis of polyglycerol–oleic acid esters. Equal amounts (w/w) of polyglycerol and silica gel were stirred mechanically. Different amounts of adsorbed polglycerol were suspended in oleic acid methyl ester 99% (1 mL, 3 mmol). The reaction was started by the addition of 50 mg Lypozyme. The mixture was shaken at a temperature of 60°C. Five µL of the reaction medium was withdrawn and analyzed by gas chromatography (GC) at various time intervals.

Synthesis of polyglycerol–oleic sunflower fatty acid esters. Equal amounts (w/w) of polyglycerol and silica gel were mechanically stirred. Different amounts of adsorbed polyglycerol were suspended in methyl esters of oleic sunflower (10 mL) with 500 mg Lypozyme. The reaction medium was mixed by a magnetic stirrer at 60°C. At various time intervals, 5 µL of the reaction medium was withdrawn and analyzed by GC.

Recovery of products. At the end of the reaction, the mixture was vacuum-filtered. The liquid part, containing the esters of polyglycerol, was separated from the solid mixture of Lipozyme and silica gel which can be re-used.

Preparation of trimethylsilyl derivatives for GC analysis. The reaction medium (5 µL) was dissolved in 0.5 mL of N,N-dimethylformamide that contained a determined amount of internal standard (lauric acid butyl ester). 1,1,1,3,3,3-Hexamethyldisilazane (0.5 mL) was added. The mixture was stirred and left for 30 min with occasional shaking. An aliquot of 0.5 µL/min was directly injected into the GC.

GC analysis. Trimethylsilyl derivatives of the product were injected on a Varian (Fernando, CA) 3400 gas chromatograph equipped with a flame-ionization detector, a compact low-thermal mass on-column capillary injector and a Varian 4290 data integrator. The GC was fitted with a 12 m x 0.32 mm x 0.25 µm SGE BPX 5 column (Ringwood, Victoria, Australia). Nitrogen was used as the carrier gas with a flow rate of 30 mL/min. The detector temperature was 380°C. The column temperature was set to 150°C for a 1-min threshold and was then programmed at 10°C/min to 350°C which was maintained constant until the last compound was eluted.

Hydrophile–lipophile balance (HLB) determination. The HLB was determined by the water number method described previously (17).

Measurement of surface tensions. Surface tension was measured in distilled water at 25°C with a Dognon Abribat tensiometer. Different amounts of surfactant were mixed with 50 mL distilled water. The solution was left for 24 h in the tensiometer vessel before measurement. A platinum plate was immersed in the solution. The vessel was lowered while simultaneously increasing the tension of the dynamometer. The force required to pull the plate up from the water surface was taken as the apparent surface tension. Real surface tension was obtained by applying correction factors.

RESULTS AND DISCUSSION

At first, the parameters of the reaction between pure oleic acid methyl ester and diglycerol were optimized. Lipozyme is supplied with an optimal temperature of 60°C. This temperature is chosen for this reason and also because at 60°C, the methanol issued from the reaction is evaporated from the medium, avoiding the eventual denaturation of the enzyme by a "water stripping" phenomenon (18). This phenomenon is due to desorption of the water bound to the enzyme and required to maintain its catalytically active conformation in the polar organic phase. Evaporation of the methanol also improved the shift of the reaction to the right-hand side (Scheme 1) because the reverse reaction was not possible. Other lipophilic substrates longer than methyl ester were not efficient for that kind of reaction. The leaving group produced by the reaction of an ester with a larger alkyl group could not be evaporated under our conditions. A purification step of the final reaction medium was then necessary to remove the nucleophile produced. One of the advantages of our system was the simple recovery of the products from the complete transesterification by filtration, which removed the enzyme and the solid support of the hydrophilic substrate.

Berger *et al.* (19) showed that for a successful enzymatic esterification of glycerol, the hydrophilic substrate (glycerol) has to be adsorbed onto a solid support prior to its use. For polyglycerol–fatty acid ester production, comparison of reaction rates with the hydrophilic substrate adsorbed and nonadsorbed on silica gel was performed (Fig. 1). The time at which the methyl ester concentration equaled half the initial concentration was 10 h with the diglycerol adsorbed and 36 h with the diglycerol nonadsorbed prior to use. Moreover, for adsorbed diglycerol the transesterification reaction was complete, whereas the yield was not more than 88% after 150 h with nonadsorbed diglycerol. Thus, the adsorption process allowed for completion of the reaction with faster kinetics. Berger *et al.* (19) suggests that this process creates an interphase that is involved in the lipase mechanism. With the enzyme adsorbed onto a solid support, the interphase necessary for the lipase mechanism already exists. Another hypothesis

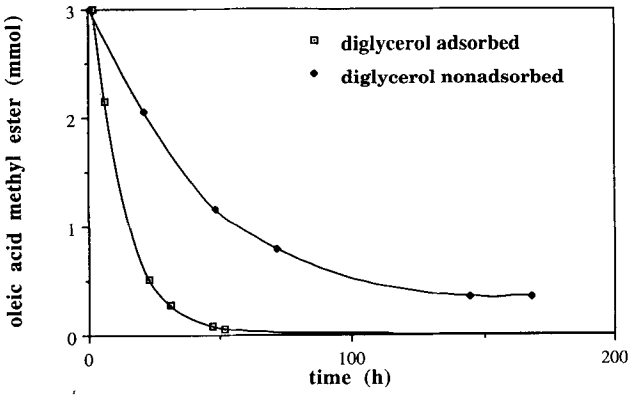
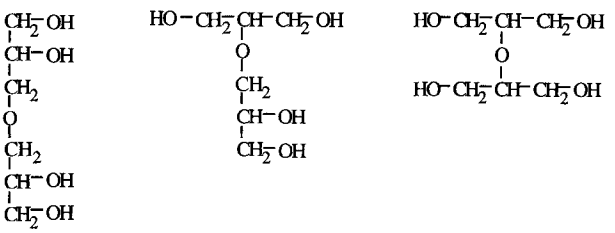


FIG. 1. Time course of the synthesis of diglycerol–oleic acid ester from oleic acid methyl ester (3 mmol) and diglycerol (1.8 mmol) with 50 mg Lipozyme.

could be proposed–the hydroxyl groups at the surface of the silica, called silanol groups (Si-OH), could form hydrogen bonds with the OH groups of the polyol during the adsorption process. The polarity of the polyol is then lowered, and the miscibility with the lipid phase is increased. Without a difference in polarity, transesterification between adsorbed polyglycerol and fatty acid methyl ester is then feasible. Moreover, the solid support could influence the hydrophilic substrate conformation, which makes access to the active-site of the enzyme possible. Diglycerol, the hydrophilic substrate of the reaction, was prepared by formation of an ether bond from two hydroxyls of two glycerols. The ether bond could be formed between two primary hydroxyl groups but also between a primary and a secondary and between two secondary OH groups. Diglycerol is then a mixture of three isomers (Scheme 2). Moreover, it has been previously shown (20) that a 1,3-specific lipase is able to catalyze the synthesis of triglycerides by isomerization through acyl migration from a primary alcohol to a secondary alcohol. Indeed, this isomerization phenomenon was observed in our experiments because chromatographic analysis of the final reaction medium gave a mixture of mono-, di-, tri- and tetraesters. The addition of a lipid chain is not only done to the primary hydroxyl groups; because of the diglycerol isomers and the "acyl migration" phenomenon, the final reaction composition is a mixture of isomers. The surface-active properties of the synthesized surfactant depend on the composition of this final mixture. The factor that determines this composition is the molar ratio between methyl ester and diglycerol at the beginning of the reaction.



SCHEME 2

We define the ratio as the initial number of fatty acid methyl ester molecules as compared to the initial number of diglycerol molecules. Several reactions with different substrate ratios were carried out to synthesize mixtures with different compositions and different emulsifying properties. Because of the high molecular weight and high boiling point of tri- and tetraesters of diglycerol, only the mono- and diesters were identified by GC. Owing to the impossibility of having pure isomer samples of each diglycerol–fatty acid ester, an arbitrary unit was adopted to compare the monoesters with each other and the diesters with each other for the different reaction conditions. The time course of the synthesis of polyglycerol–fatty acid esters shows a decrease in the amount of mono- and diesters of digycerol after reaching a maximum value (Fig. 2). This decrease is due to the phenomenon of acyl migration. Mono- and diesters of diglycerol produced are then consumed to a higher degree of substitution. The reaction was complete with an initial ratio of 1.33:1, and the final mixture was mainly composed of mono- and diesters of diglycerol, whereas a ratio of 4:1 produced more-substituted diglycerol esters after complete reaction. (Fig. 2). Indeed, a ratio of 4:1 corresponded to one molecule of methyl ester for one free hydroxyl of diglycerol. Thus, at the end of the reaction, each hydroxyl group had reacted with a methyl ester

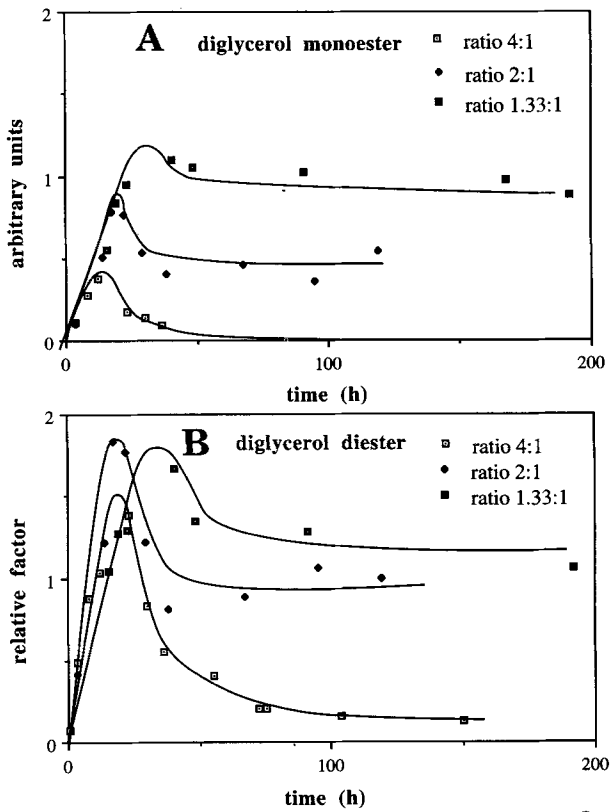


FIG. 2. Synthesis of diglycerol–oleic acid esters (A, monoesters; B, diesters) with different initial molar ratios of oleic acid methyl ester and diglycerol. (Due to the lack of standards for diglycerol esters, the arbitrary unit is defined as the peak area of one monoester and one diester of diglycerol divided by the peak area of lauric acid butyl ester used as internal standard.)

molecule. With an initial ratio of an amount of methyl esters favoring diglycerol, the reaction was not complete, and the final medium contained substrates to be removed. With more diglycerol at the beginning of the reaction favoring methyl esters, the viscosity of the solution was increased and problems of substrate diffusion occurred.

A scale-up of the reaction was made to determine the surface-active properties of the different polygylcerol–fatty acid ester mixtures synthesized. Methyl esters from sunflower oil were used as the lipid phase. Diglycerol was chosen as the hydrophilic part. The optimized reaction conditions, determined at the lower scale, were used. Two different ratios of substrates were tried to have different final reaction mixtures (ratio 4:1 and 1.33:1). The ability to reduce the surface tension of water and HLB measurements were used to determine the surfactant properties of the different mixtures. At an emulsifier concentration of 0.01% (vol/vol), both mixtures of ratio 4:1 and 1.33:1 significantly reduced the surface tension of water (Table 1). Nevertheless, the substrate ratio of 1.33:1, producing more mono- and diesters of diglycerol, had the properties of a lipophilic surfactant. An HLB less than 4 suggests a water-in-oil emulsifier. Determination of the critical micelle concentration (CMC) (the concentration of surfactant above which micelles of surfactant appear in the media, making emulsification possible) was performed (Fig. 3). A low CMC was found allowing emulsification to take place at low surfactant concentration (0.008%). On the other hand, the ratio of 4:1 produced esters of diglycerol with a lipophilic moiety too high compared to the hydrophilic one; thus, it had no more surfactive properties.

To synthesize an emulsifier with more hydrophilic properties, diglycerol was substituted with pentaglycerol and octaglycerol as the hydrophilic substrates. With an increased number of hydroxyl groups, polyglycerol conferred a larger polar head on the surfactant molecule, thus theoretically increasing the HLB number. Table 1 shows that the amphiphiles synthesized from penta- and octaglycerol had almost the same properties as those produced from diglycerol. The reason for this result is that polyglycerols used as substrates for the reaction were actually mixtures of cyclic and linear molecules with penta- or octaglycerol, respectively. Indeed, the final

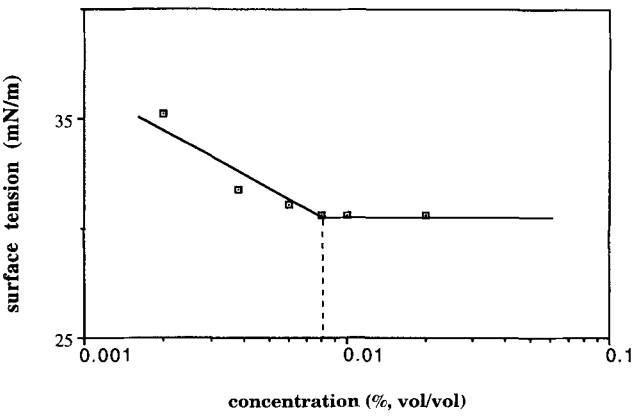


FIG. 3. Effect of concentration of diglycerol–fatty acid esters on reduction of water surface tension. Determination of the critical micelle concentration (0.008%; vol/vol).

composition of polyglcerols, chemically synthesized by polymerization of glycerol, was determined theoretically from the amount of water produced by the reaction. This method was not efficient in producing pure linear polyglycerol of several glycerol units. To produce an emulsifier with more hydrophilic properties and thus to increase its solubility in water, it seems more sensible to reduce the carbon chain-length of the lipid substrate, due to the difficulty of synthesizing linear polyglycerol with more than two glycerols in the skeleton.

In conclusion, a mixture of "natural" polyglycerol–fatty acid esters was synthesized by means of a nontoxic process. The yield of the reaction was complete when the hydrophilic substrate was adsorbed onto silica gel prior to the reaction. The surfactant mixture was then obtained by filtration of the reaction medium to remove the enzyme and polyol adsorbent. Best results, in terms of the lipophilic surfactant, were obtained with a molar ratio of fatty acid methyl ester to diglycerol of 1.33.1.

TABLE 1
Surface-Active Properties and Hydrophile–Lipophile Balance (HLB) of Different Mixtures of Polyglycerol–Fatty Acid Esters

	Surface tension (mN/m) 0.01% (vol/vol)	HLB
Water	72	—
Sunflower methyl esters	34.9	—
Diglycerol (ratio 4:1[a])	36.9	—
Diglycerol (ratio 1.33:1)	29.6	<4
Pentaglycerol	29.7	<4
Octaglycerol	30.7	<4

[a]Definition of ratio in Results and Discussion section.

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