Efficient Esterification of Sorbitan Oleate by Lipase in a Solvent-Free System

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ABSTRACT: The lipase-catalyzed esterification of sorbitan with oleic acid in a solvent-free system to form sorbitan oleate (commercial name Span80) was studied as a feasible approach aimed at meeting the demand for sugar alcohol-based surfactants. Screened results obtained from enzymatic synthesis of sorbitan oleate indicated that Novozym 435 had its highest catalytic activity in a solvent-free system. The introduction of a reduced-pressure system increased the production of sorbitan oleate to a maximum of 95% of theoretical, obtained from 0.2 mol sorbitan, 0.1 mol oleic acid, and 2.0 g lipase (6 wt% of sorbitan) in a solvent-free reaction mixture at optimal reaction conditions. Results obtained from lipase-catalyzed batch esterification reactions showed that more than 90% conversion of sorbitan oleate was maintained after 10 batches of esterification reactions, indicating excellent enzyme stability. Subsequent analysis by HPLC indicated that the product of enzyme-catalyzed esterification by the immobilized lipase contained a significantly greater amount of monoester (about 80%) compared to the composition obtained by chemical synthesis (about 50%).

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KEY WORDS: Batch reaction, esterification, Novozym 435 lipase, solvent-free system, sorbitan oleate.

Sorbitan esters, an important group of sugar-based, nonionic surfactants, are broadly used in various pharmaceutical, cosmetic, food, and beverage industries with several trade names, such as Span, Famodan, and Crill (1,2). Sorbitan esters are usually prepared by chemical esterification at 180–260°C in a solvent-free mixture of FA and molten anhydrous sorbitol with chemical catalysts, which yield numerous byproducts, including regioisomers of sorbitans and isosorbide. For example, as many as 65 components exist in food-grade Span20 (3). In addition, a lower content of sorbitan monooleate and elevated levels of complex chemical compounds do not satisfy consumers' demand for high-quality surfactants.

Currently, the enzymatic preparation of esters of sugars or sugar alcohols is considered a feasible alternative to conventional chemical synthesis (4–8). Although several studies on lipase-catalyzed esterification of sorbitol or sorbitan esters have been conducted by different methods using lipase (EC 3.1.1.8), including solvent-free processes or solid-phase systems (1,3,9–12), some factors, such as the solubility of sorbitan (or sorbitol) and oleic acid, the selection of reaction systems, and lower conversions, limit the use of the processes on a large scale (2,6,9,13,14).

The main objectives of this study were to (i) improve methods for the enzymatic synthesis of sorbitan monoesters using lipases as catalysts and (ii) to further investigate lipase-mediated esterification conditions in a feasible reaction system to obtain a high conversion on a multigram scale. The composition and characteristics of the final products synthesized by lipase were evaluated relative to the contents of sorbitan mono-, di-, and trioleates.

EXPERIMENTAL PROCEDURES

Materials. Sorbitol (Sigma Ultra) was obtained from Sigma Chemical Co. (St. Louis, MO) and a commercial sorbitan oleate (Span80) was provided by ICI (Middlesborough, United Kingdom). Oleic acid (>99%) was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Lipozyme IM (immobilized lipase from *Rhizomucor miehei*) and Novozym 435 (immobilized lipase from *Candida antarctica*) were donated by Novo Nordisk A/S (Bagsvaerd, Denmark); CCL (lipase from *C. rugosa*), RAL (lipase from *Rhizopus arrhizus*), and PPL (lipase from porcine pancreas) were purchased from Sigma Chemical Co.; CLL (lipase from *C. lipolytica*) was purchased from Genencor Biotechnology Co. (Palo Alto, CA); RCL (lipase from *R. chinensis*) was prepared in our laboratory (15). Other lipases were gifts from the Biotechnology Research Center of Toyama Prefectural University (Toyama, Japan). All other solvents and chemicals were of the highest purity available and purchased from Sigma Chemical Co.

Preparation of sorbitan. Sorbitol (45.5 g) was charged to a three-necked round-bottomed flask equipped with an agitator, condenser, and receiver, and vacuum was applied to a pressure of about 5 mm of mercury. Once sorbitol was made molten by heating to about 100°C using an oil bath, the reaction was initiated by addition of 455 mg of *p*-toluenesulfonic acid (H_3PO_4). The charge was heated to 120°C and maintained at this temperature for 60–80 min under a nitrogen atmosphere. When the product had a hydroxyl number of about 1300 (corresponding to a degree of anhydrization of about 1.1), the vacuum and heat were removed and the acid catalyst was neutralized with sodium hydroxide (120 mg) (16). The clear, syrupy sorbitan was then used directly for enzymatic esterification with oleic acid.

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Lipase-catalyzed esterification of sorbitan with oleic acid in a stoppered flask with added molecular sieves. Unless otherwise stated, a typical esterification reaction was carried out with the prepared sorbitan (16.4 g, 0.1 mol) and oleic acid (28.2 g, 0.1 mol) in a stoppered 500-mL round-bottomed flask. For reactions performed in solvent media, sorbitan and oleic acid were first dissolved in 200 mL of selected solvent and molecular sieves (4Å,12 g) were added. After addition of the biocatalyst (1 g lipase), esterification was carrried out at the corresponding optimal activity temperature of the biocatalyst in a shaker-incubator (150 rpm).

Lipase-catalyzed esterification of sorbitan with oleic acid under reduced pressure. The prepared sorbitan (16.4 g, 0.1 mol) and oleic acid (28.2 g, 0.1 mol) were added to a 500-mL two-necked round-bottomed flask equipped with an agitator, condenser, and receiver, and the condenser was connected to a vacuum pump. After addition of the biocatalyst (1 g lipase), lipase-catalyzed esterification was performed at 60°C under a pressure of about 50 mm of mercury.

Extraction of sorbitan esters. The reaction mixture was extracted three times with 100 mL *n*-hexane. The sorbitan esters produced and the unreacted oleic acid were dissolved in the *n*-hexane, and the enzyme and residual sorbitan were then separated from the hexane solution by filtration. The solvent was removed with a rotary evaporator to collect the residual material. The enzyme and residual sorbitan were transferred into 30 mL phosphate buffer (pH 7.0), and sorbitan was dissolved in the buffer. The immobilized enzyme was separated by filtration, washed three times with the same buffer, and finally washed with 30 mL *n*-hexane. The enzyme was then collected by vacuum filtration and stored in a desiccator for subsequent reaction.

The conversion of oleic acid was calculated based on the consumption of oleic acid in the reaction using the following equation:

$$\% \text{ conversion} = (1 - A/B) \times 100 \quad [1]$$

where A = moles of oleic acid in the reactant mixture, and B = initial moles of oleic acid.

HPLC analysis of the esterification products. A high-performance liquid chromatograph equipped with an on-column injector and UV spectrophotometer detector operating at 207 nm wavelength was used for the determination of sorbitan mono-, di-, and trioleates. A reversed-phase C18 column (HP ODS Hypersil, 5 µm particle size, 100 × 4.5 mm i.d.; Hewlett-Packard, Avondale, PA) was used with 2-butanol/water (88:12) as eluant at 25°C with a flow rate of 2 mL/min. Aliquots of enzyme reactions were solubilized in 2-butanol in a solution of 5% concentration and identified by HPLC as sorbitan mono-, di-, and trioleates under conditions described previously (10).

RESULTS AND DISCUSSION

Comparison of reaction media on the lipase-catalyzed esterification of sorbitan with oleic acid. To investigate the effects of the reaction media on lipase-catalyzed esterification of sorbitan with oleic acid, enzymatic reactions were performed in different reaction media (i.e., water, some selected organic solvents, and a solvent-free mixture) in stoppered flasks under the conditions described in the Experimental Procedures section. As shown in Figure 1, greater conversion (up to 43%) was obtained under the solvent-free condition catalyzed by Novozym 435 than was obtained by using water or organic solvents as reaction media, although organic solvents with different log*P* values (value of a logarithm to the partition coefficient of a given solvent between water and 1-octanol in a two-phase system) were used individually or as a mixture.

Oleic acid is a long-chain FA that is insoluble in water. Because the solubility of sorbitan in oleic acid and most organic solvents is very low, it is very difficult to obtain a homogeneous mixture between the substrates. In most instances, sorbitan tends to remain as a viscous semisolid at the bottom of the reaction vessel with oleic acid on top. With the synthesis of sorbitan oleate in a solvent-free system, it was observed that the initial insolubility between sorbitan and oleic acid was overcome gradually during the esterification reaction, eventually resulting in a viscous semisolid. This may be because sorbitan oleate is itself an amphipathic surfactant that has the ability to emulsify substrates.

The same reaction had been carried out with nine other common commercial lipases (immobilized or not; results not shown) from different sources for their ability to catalyze the esterification of sorbitan with oleic acid. Among them, Novozym 435 showed the highest synthetic activity for sorbitan oleate at 60°C after 72 h incubation and was therefore chosen for subsequent experiments.

Esterification for sorbitan ester production. It is well understood that water exerts a negative role in esterification reactions in a solvent-free system; thus, the most practically important factor affecting conversion during this process was

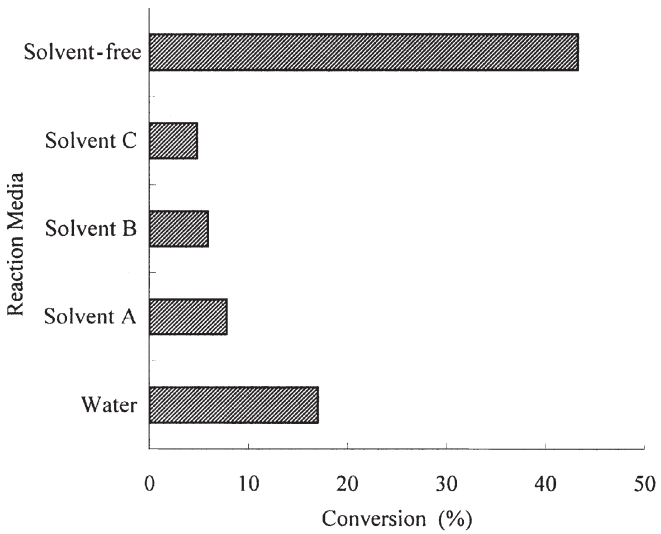

FIG. 1. Effects of reaction media (solvent-free, water, and solvents A, B, and C) on conversion in the esterification reaction with 0.2 mol sorbitan, 0.1 mol oleic acid, and 10% lipase (wt% of sorbitan) in a stoppered flask. Solvent A = *n*-hexane; Solvent B = *n*-hexane + *tert*-butanol (78:22, vol/vol); Solvent C = *N,N*-dimethylformamide.

the timely removal of water (11). Efficient removal of water can be achieved by many methods, and the addition of activated molecular sieves, which is a common method, was used here (15,17,18). However, in the esterification of sorbitan with oleic acid, no obvious improvement in conversion was observed by adding molecular sieves (data not shown), suggesting that it did not eliminate water effectively because the molecular sieves were covered by the viscous sorbitan during the process.

Evaporation under reduced pressure is an alterative method for water removal, although not all researchers obtain a high yield of esters with this method (2,13,19). The esterification reactions were conducted under reduced pressure at similar substrate levels compared with the systems in stoppered flasks. Figure 2 shows the time course of esterification of oleic acid with sorbitan in a stoppered flask and under reduced pressure, respectively. Esterification reached a maximum conversion of 90% after a 24-h reaction with 6.0% lipase (wt% of sorbitan) under reduced pressure. Moreover, the initial reaction rate under reduced pressure was also much faster than that in the stoppered flask. Accordingly, reduced pressure conditions were chosen for the synthesis of sorbitan oleate.

Meanwhile, some of the esterification reaction conditions were investigated in the solvent-free system under reduced pressure. Results shown in Figure 2 suggest that the amount of lipase added (6 wt% of sorbitan) efficiently converted up to 90% of the oleic acid in 48 h. As the mole ratio of sorbitan to oleic acid was increased from 0.5:1 to 3:1, the conversion increased (as shown in Table 1). In this system, as high as

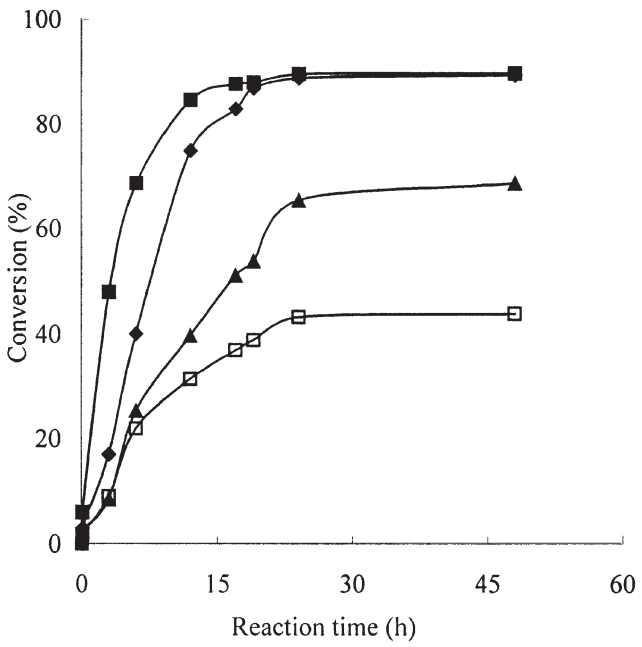

FIG. 2. Time course of the esterification reaction of oleic acid with sorbitan in a stoppered flask and with various amounts of added lipase. In a stoppered flask with 6% lipase (□); under reduced pressure with 6% lipase (◆), 9% (■), or 3% (▲) lipase. Reaction conditions: 0.2 mol sorbitan and 0.1 mol oleic acid at 60°C.

TABLE 1
Effects of Substrate Molar Ratio on Product Composition[a]

Effect	Substrate molar ratio (oleic acid/sorbitan) 2:1	1:1	1:2	1:3	Chemically catalyzed Span80[b]
Conversion (%)	62.7	77.8	90.2	90.7	
Monoesters (%)[c]	41.1	63.8	79.6	81.5	49.9
Diesters (%)[c]	47.7	25.7	10.3	9.6	36.2
Triesters (%)[c]	11.2	10.5	10.2	8.9	14.1

[a]Effects of the substrate molar ratio on product composition were investigated in a solvent-free system under reduced pressure for esterification of sorbitan with oleic acid with 6% lipase at 60°C for 48 h.
[b]Chemically synthesized. food-grade commercial Span80 was obtained from ICI (Middlesborough, United Kingdom).
[c]Percentages of mono-, di-, and triesters in the total ester population.

90.7% conversion and an 81% proportion of monoester were obtained with a 3:1 ratio of sorbitan to oleic acid, 6.0% (wt% of sorbitan) of lipase added, at a temperature of 60°C for about 48 h. There was no obvious difference in conversion between the mole ratios of 3:1 and 2:1, and the mole ratio of 2:1 was chosen for further investigation.

Batch esterification and lipase stability in the solvent-free system under reduced pressure. Considering the fact that maintaining and stabilizing the catalytic activity of a lipase in a synthetic process is of immense importance for its industrial application, repeated batch reaction experiments were performed under the same conditions by extracting the sorbitan ester products in each batch and repeating the reaction. After the 10th batch reaction cycle, all the mole conversions of sorbitan esters in each batch were greater than 92%, and the highest conversion was 96.8%, with one of the last batches reaching 96%. This obviously indicated that no decrease in conversion was observed. Furthermore, the residual synthetic activity of used lipase remained at more than 90% after the 10-h batch reaction cycles. These results indicate that the solvent-free system under reduced pressure is sufficiently efficient for the lipase-catalyzed synthesis of sorbitan oleate by batch reaction, and potentially suitable for application in the industrial production of sorbitan oleate on a large scale.

With repeated reactions, however, the proportions of mono-, di-, and triesters in the product varied from one batch to another. Figure 3 shows that the monoester proportion decreased and the di- and triesters increased during batch reactions, resulting in a 60% monoester content in the 10th batch product. It is reasonable to explain that the ratios of sorbitan mono-, di-, and triesters are dependent on the stereoselectivity of the biocatalyst, Novozym 435 lipase, in terms of its ability to act on the position of sorbitan. With repeated batch reactions, it is likely that the regioselectivity of lipase in the nonaqueous system declined gradually, although it remained active. This may be the reason for the decrease in the proportion of monoesters in the product with repeated reactions.

Evaluation of the composition of products. For comparison with the product made by traditional chemical synthesis, some specifications relevant to the emulsifying properties of

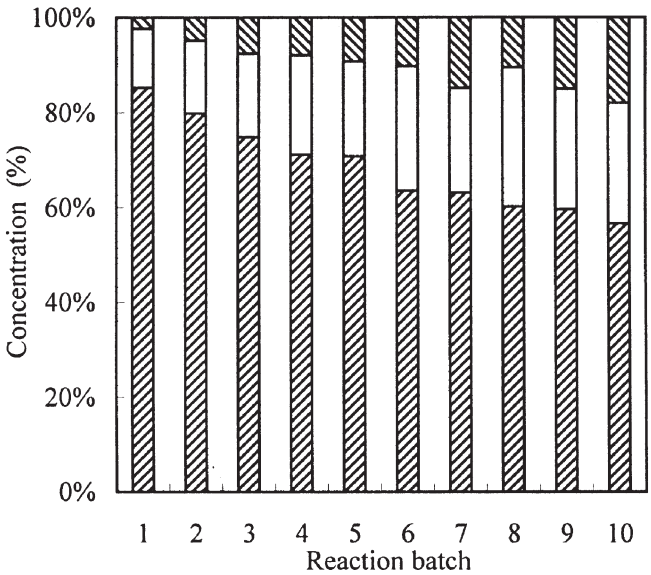

FIG. 3. Effect of the reaction batch on the ratio of mono- (▨), di- (□), and triesters (▧) of product in the solvent-free system under reduced pressure at 60°C with 0.2 mol sorbitan, 0.1 mol oleic acid, and 6% lipase.

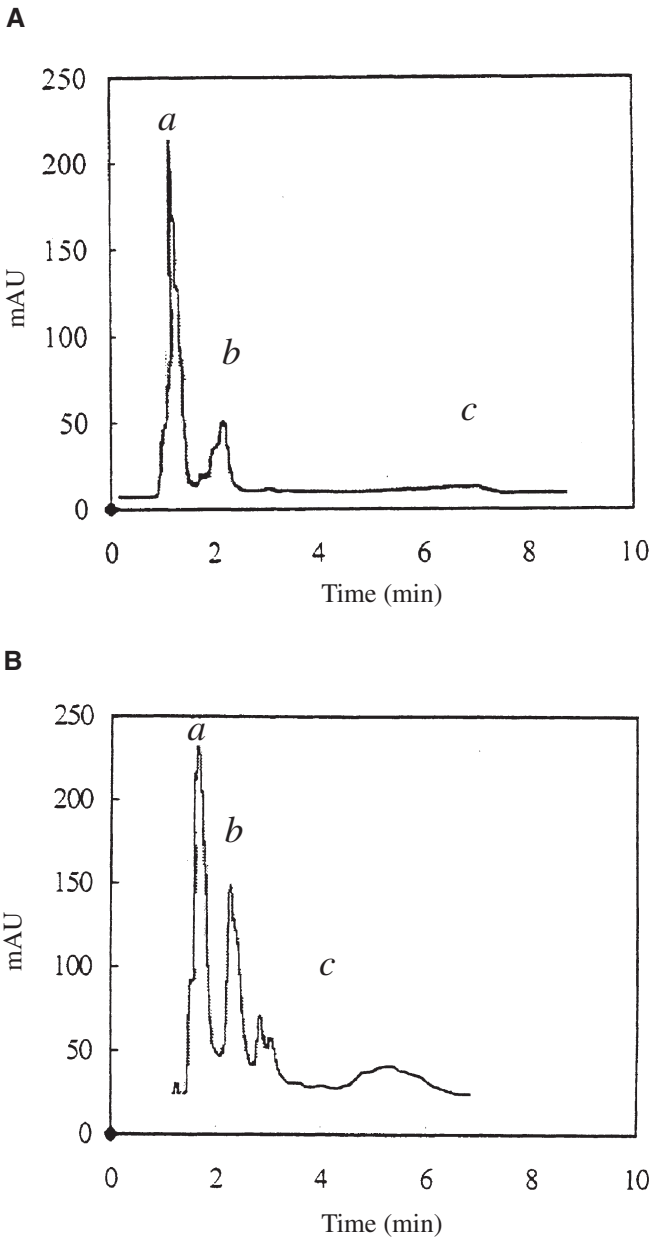

FIG. 4. High-performance liquid chromatograms of sorbitan oleate by (A) enzymatic synthesis or (B) chemical synthesis. *a*, Sorbitan monoester; *b*, sorbitan diesters; *c*, sorbitan triesters.

surfactants were determined by HPLC and titration methods in order to evaluate the quality of enzymatically synthesized sorbitan oleate. All analytical measurements, such as the hydrophile-lipophile balance, acid value, saponification, esterification, hydroxyl value, and so on, were identical to FAO/WHO standards (data not shown). Moreover, HPLC analysis of sorbitan esters indicated that sorbitan oleate obtained by lipase-mediated catalysis is characterized by a higher proportion of the monoester of oleic acid (Table 1) and by a lighter color (data not shown) than the chemically synthesized product. The monoester was the main ester component, and its content reached *ca.* 80% in the enzymatic product when the mole ratio of oleic acid and sorbitan was between 1:2 and 1:3. In contrast, the chemically synthesized product contained only 50% of the monoester. These results were attributed to an excess of hydroxyl donor (sorbitan) incorporated into the solvent-free system. The chemically synthesized sorbitan esters provided much more complicated profiles than those of the enzyme-synthesized ones when analyzed by HPLC (Figs. 4A, 4B).

Lipase-mediated synthesis of sorbitan oleate was demonstrated in a solvent-free system under reduced pressure, with the advantages of easy application and potential industrialization, efficient conversion from substrates, and a higher proportion of monoesters. Thus, the enzymatically prepared sorbitan oleates are possible substitutes for the chemically synthesized products that will satisfy the demand for high-quality surfactants in the near future.

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