Synthesis of Alkyl Glycoside Fatty Acid Esters in Non-Aqueous Media by *Candida* sp. Lipase

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Alkyl glycoside fatty acid esters were successfully synthesized by lipase-catalyzed transesterification of methyl glucoside, methyl glucoside, methyl galactoside and octyl glucoside with methyl oleate. The experiments were carried out in organic media with lipase enzymes from *Candida* sp. as biocatalysts. Time course and the effects of temperature, solvent type, substrate concentration, added water and of immobilized *vs.* nonimmobilized enzyme were studied. The optimal conditions for the enzymatic synthesis of alkyl glycoside fatty acid esters were: a molar ratio of alkyl glycoside and methyl oleate of 1:4, an immobilized lipase, SP382 from *Candida* sp.; benzene/pyridine (2:1, vol/vol) with no added water; temperature, 55°C; reaction time, 48 h; and shaking at 200 rpm. Acceptable levels of oleic acid incorporation (58.6–100 mol%) onto the alkyl glycosides were achieved.

KEY WORDS: Alkyl glycosides, alkyl glycoside fatty acid esters, biosurfactants, enzymatic synthesis, lipases, organic solvents, transesterification.

Carbohydrate fatty acid esters are used as surfactants in the food, detergent and cosmetic industry (1). Sugar esters of fatty acids are nonionic, odorless, tasteless and biodegradable and compare well in overall performance with other surface-active compounds in emulsification, detergency and related properties (2). Alkyl glycoside fatty acid esters (mono- and diesters) are excellent oil-in-water (o/w) emulsifiers and are also easily digestible (3,4). One advantage of biosurfactants (surfactants synthesized with biocatalysts such as enzymes) is that in addition to their efficacy, such products may still be considered "natural" (5).

Chemical synthesis of alkyl glycoside fatty acid esters has been carried out with free sugars, but prolonged heating at temperatures as low as 90°C causes caramelization, making product purification difficult (3,4,6). Alkylation converts reducing sugars with reactive C-1 anomeric centers to nonreducing sugars with less reactive anomeric centers (6). Monounsaturated fatty acids such as oleic acid, 18:1n-9, have been under extensive study due to their hypolipidemic and hypocholesterolemic properties (7–9). Previous reports on chemical synthesis of alkyl glycoside fatty acid esters involved the esterification of methyl glucoside with free fatty acids, with or without xylene, under CO_2 atmosphere with lead oxide, stannous soap and sodium hydroxide as catalysts to produce methyl glucoside fatty acid esters (mono- and dilaurates) (3). Albano-Garcia *et al.* (4) synthesized methyl glucoside esters of coconut fatty acids with anhydrous potassium soap as a catalyst at a temperature of 125–128°C. Akoh and Swanson (10) reported the one-stage synthesis of raffinose fatty acid polyesters by means of an alkali metal (2% sodium), and the solvent-free interesterification of trehalose octaacetate and sorbitol hexaacetate to make their fatty acid polyesters with sodium metal (1–2.5%) as the catalyst (11). In another study, Akoh and Swanson (6) reported the synthesis of alkyl glycoside and stachyose fatty acid polyesters.

Janssen *et al.* (12) and Seino *et al.* (13) reported enzymatic synthesis of carbohydrate esters in aqueous media. Except for the study of Therisod and Klibanov (14), there has been no report on the lipase-catalyzed synthesis of alkyl glycoside fatty acid esters in other organic media. Enzymatically synthesized alkyl glycoside fatty acid mono- and diesters may well be the answer to the consumer demand for a "natural" biodegradable emulsifier with potential health benefits, depending on the fatty acid incorporated. The potential for successful synthesis, application and consumption of "natural" alkyl glycoside fatty acid ester emulsifier appears to be great.

The aim of this study was to synthesize alkyl glycoside fatty acid esters by transesterification with lipases from *Candida* sp. as biocatalysts in organic media. The alkyl glycoside starting materials used were methyl α-D-glucopyranoside, methyl β-D-galactopyranoside and 1-O-octyl-β-D-glucopyranoside.

MATERIALS AND METHODS

Materials. 1-0-Methyl α-D-glucopyranoside (99% pure), 1-0-methyl β-D-galactopyranoside (98% pure), 1-O-octyl-β-D-glucopyranoside (98% pure), methyl oleate (70% pure) and ANS salt (8-anilino-1-napthalenesulfonic acid ammonium salt, 97% pure) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Immobilized nonspecific lipase SP382 (40 BIU/g) from *Candida* sp. was provided by Novo Nordisk Bioindustrials Inc. (Danbury, CT). BIU stands for Batch Interesterification Unit (*i.e.*, one μmole of incorporated palmitic acid into triolein per min under standard conditions as defined by the manufacturer). Nonimmobilized lipase from *Candida* sp. (85.7 U/mg) was supplied by Biocatalysts Ltd. (Wales, U.K.). Silica gel 60 thin-layer chromatography (TLC) plates were purchased from E. Merck (Darmstadt, Germany). Ryoto sugar ester (sucrose fatty acid ester, 50% mono-, 50% di-, tripolyester) was supplied by Mitsubishi-Kasei Food Corp. (Tokyo, Japan). All the solvents were of high-performance liquid chromatography (HPLC) grade and obtained from Fisher Scientific (Norcross, GA).

Transesterification method. In a typical synthesis of alkyl glycoside fatty acid ester, 25 mg of alkyl glycoside was combined with 114.5 mg of methyl oleate (mole ratio, 1:4) and 13.95 mg of SP382 lipase (*i.e.*, 10%, w/w, of reactants) in a screw-cap test tube. Three mL of benzene/pyridine (2:1) was added, and the mixture was incubated in an orbital shaking water bath at 55°C for 24 h at 200 rpm. All reactions were carried out in duplicate. A blank control with no enzyme was done with the same amount of substrate and reacted as described above.

Effect of different solvents. To five different samples containing 25 mg of methyl glucoside, 114.5 mg of methyl oleate and 10.95 mg of SP382 lipase was added 3 mL of

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pyridine, benzene, benzene/pyridine (2:1, vol/vol), hexane or *n*-heptane. The reaction mixtures were incubated at 55°C for 24 h at 200 rpm. The products were then extracted for analysis.

Immobilized vs. *nonimmobilized enzyme.* To methyl glucose and methyl oleate at a molar ratio of 1:4 was added 10% (w/w) of immobilized lipase SP382, or nonimmobilized powdered lipase from *Candida rugosa* and 3 mL of benzene/pyridine (2:1, vol/vol). The samples were incubated at 55°C for 24 h at 200 rpm and then extracted for analysis.

Extraction and analysis. The reaction mixtures were cooled, the enzyme filtered out and the filtrate dried under N_2. The residue was then redissolved in 1 mL of chloroform and dried by passing through a column of anhydrous sodium sulfate. TLC was used to purify and isolate the sugar esters. A 50-μL aliquot of the reaction mixture was co-plated with methyl glucoside, methyl oleate and a sugar ester standard (sucrose fatty acid ester consisting of 50% monoester, 50% di, tripolyester) on pre-coated silica-gel 60 plates which were activated by heating at 110°C for 1 h. The plates were developed with $CHCl_3$/MeOH/HoAc/H_2O (90:8:1.0:0.8, by vol), and the bands were visualized by spraying with 0.1% ANS salt and detection with ultraviolet light. To identify the glycoside ester band, a second plate was spotted, developed with the same solvent system, sprayed with sulfuric-acid dichromate (50% H_2SO_4 solution), and the bands were visualized as purple-violet spots on TLC plates after heating at 105°C for 5–10 min. The unreacted methyl oleate (R_f = 0.74) or free fatty acids (R_f = 0.60) gave yellow spots after heating at 105°C for 5–10 min. The bands corresponding to alkyl glycoside esters (R_f = 0.17, essentially monoesters) from the ANS-sprayed plates were scraped into a test tube to which a 10-μg solution of heptadecanoic acid (17:0) internal standard was added. The glucoside diesters had an R_f of 0.34, and the yield was less than 10%; therefore, they were not analyzed further. Three milliliters of 6% HCl in methanol (vol/vol) was added, and the mixture was reacted at 70–80°C overnight. The fatty acid methyl esters were extracted (2 times) with 2 mL hexane, dried over anhydrous sodium sulfate and evaporated, and the methyl esters were redissolved in 75 μL of methylene chloride. One microliter of this extract was analyzed by gas-liquid chromatography (GLC). An HP 5890 Series II gas chromatograph (Hewlett-Packard, Avondale, PA) was used. A DB 225 fused-silica capillary column of 30 m × 0.25 mm i.d. (J&W Scientific, Folsom, CA) was used and operated isothermally at 200°C. Injector and detector temperatures were set at 250°C, and helium was the carrier gas. The relative content of fatty acid methyl esters (FAME) as mol% was quantitated by an on-line computer with 17:0 as internal standard.

RESULTS AND DISCUSSION

Variable amounts of oleic acid (29.0–100.0 mol%) were incorporated into the alkyl glycosides from the methyl oleate starting material. The levels of incorporation reported here were greater than the level (27.7 mol% conversion) reported by Seino *et al.* (13).

Figure 1 shows the time course for the lipase-catalyzed synthesis of methyl glucoside oleic ester by immobilized *Candida* sp. lipase, SP382, in pyridine and benzene/pyridine (2:1, vol/vol) as organic solvents. The time course experiment with pyridine indicates that there is maximal incorporation of oleic acid (33.5 mol%) after 24 h under the experimental conditions described. It is possible that water present in the pyridine and/or in the enzyme may have caused hydrolytic reaction when pyridine was the alcoholysis medium. It should be noted that SP382 lipase contained 2% w/w water and that no additional water was added in this experiment. Another possible explanation is that the transesterification reaction may have attained equilibrium in 24 h and, therefore, prolonged reaction time beyond 24 h did not improve the oleic acid incorporation. Under aqueous conditions, enzymes will catalyze predominantly hydrolytic reactions, but when the medium is a dry organic solvent the reaction will be predominantly synthetic (15,16). Although no specific measures were taken in our experiments to remove water from the solvents, it is most likely that water introduced into the solvents, especially in pyridine, may have enhanced the hydrolytic reaction. Ergan *et al.* (17) reported the removal of water formed from acidolysis reactions by spontaneous evaporation, molecular sieves, vacuum and dry-air bubbling. They also demonstrated that the removal of water with molecular sieves from a solvent-free environment greatly enhanced triolein production from glycerol and oleic acid (17). With benzene/pyridine (2:1, vol/vol) the maximum incorporation of oleic acid (48.1 mol%) occurred after 48 h (Fig. 1), after which incorporation decreased rapidly, probably due to competition between hydrolysis and alcoholysis reactions. Therefore, to achieve good yields the reaction must be stopped after 48 h when benzene/pyridine is the alcoholysis medium.

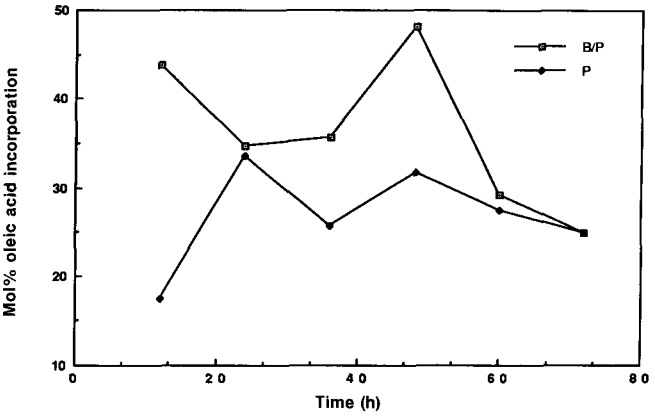

FIG. 1. Time course for the lipase-catalyzed synthesis of methyl glucoside fatty acid ester from methyl glucoside and methyl oleate with SP382 in pyridine (P) or benzene/pyridine (B/P) 2:1 (vol/vol). The reaction mixture was incubated in a shaking water bath at 55°C for 72 h at 200 rpm. A 200-μL aliquot of the reaction mixture was taken out every 12 h for analysis.

Table 1 shows the effect of several solvents on the incorporation of oleic acid into methyl glycoside. A comparison of the effects of different organic solvents in the synthesis of alkyl glycoside oleate shows that benzene/pyridine (2:1) gives the highest mol% incorporation of oleic acid (77.1 mol%). Hexane (68.5 mol%) was the next best solvent, followed by pyridine (64.3 mol%), *n*-heptane (29.0 mol%) and benzene (19.2 mol%). The effect of combining benzene and pyridine appeared to be synergistic

TABLE 1

The Effect of Different Solvents on Methyl Glucoside Oleate Synthesis with Lipase SP382 and Methyl Oleate

Solvent type	Mol% oleic acid incorporation
Benzene/pyridine	77.1
Hexane	68.5
Pyridine	64.3
n-Heptane	29.0
Benzene	19.2

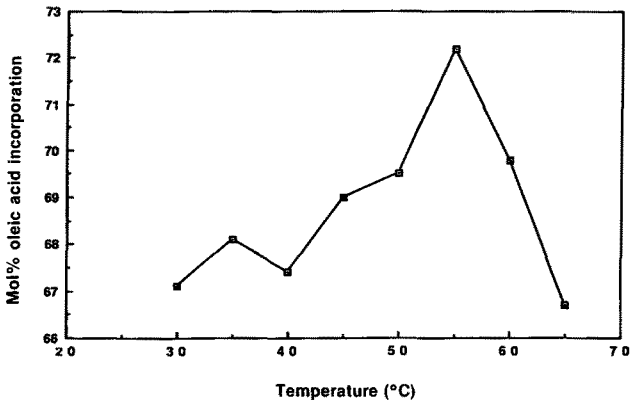

FIG. 2. Effect of temperature on the transesterification of methyl glucoside with methyl oleate, catalyzed by immobilized SP382 lipase. The solvent was benzene/pyridine 2:1 (vol/vol). The samples were incubated at different temperatures for 24 h at 200 rpm.

because when used alone they gave lower yields than when they were combined. Benzene/pyridine, consisting of both a polar and an apolar solvent, may be the best solvent for dissolving substrates such as alkyl glycosides, which are polar, and methyl oleate, which is nonpolar. Therisod and Klibanov (14) have reported the synthesis of monoacylated sugars in pyridine. However, for food applications we recommend that hexane be used as the reaction medium because it may be less toxic than pyridine or benzene. In addition, we found that hexane gave acceptable yields and incorporation (68.5 mol%) of oleate.

The effect of temperature on the transesterification of methyl glucoside with methyl oleate catalyzed by immobilized lipase SP382 is shown in Figure 2. Using benzene/pyridine as a solvent, the temperature profile indicates that 55°C is the optimum temperature for lipase SP382-catalyzed transesterification under the conditions described. About 72.2 mol% incorporation of oleic acid (18:1n-9) was observed at 55°C. There was an increase in the mol% incorporation of oleic acid as the temperature was increased from 30–55°C (Fig. 2). Increases in temperature apparently increased the amount of oleic acid incorporated, demonstrating the thermostability of the enzyme in organic media. Klibanov (18) reported increased thermostability of pancreatic lipase in organic solvent. However, our results showed that temperatures greater than 60°C resulted in lower degrees of incorporation of oleic acid. This may be due to enzyme inactivation. According to the manufacturer, SP382 is optimally used in low-water concentrations at 60°C or above in the absence of solvent.

Figure 3 illustrates the effect of varying methyl oleate

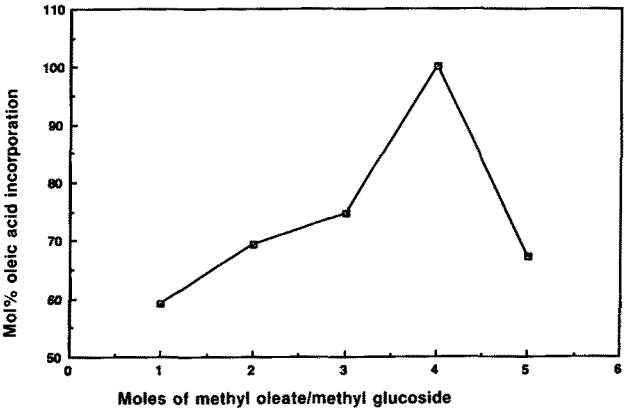

FIG. 3. The effect of varying the molar ratio of methyl oleate to methyl glucoside from 1 to 5 on methyl glucoside fatty acid ester synthesis. The reaction mixtures were incubated at 55°C for 24 h at 200 rpm.

TABLE 2

The Effect of Using Immobilized *vs.* Nonimmobilized Lipase on the Incorporation of Oleic Acid into Three Alkyl Glycosides

	Mol% incorporation	
Alkyl glycoside[a]	Immobilized	Nonimmobilized
Met. Glu.	76.5	34.2
Met. Gal.	71.5	30.2
Oct. Glu.	53.7	0.0

[a]Met. Glu., methyl glucoside; Met. Gal., methyl galactoside; and Oct. Glu., octyl glucoside.

concentration on the synthesis of methyl glucoside fatty acid ester. Molar ratios of methyl glucoside/methyl oleate was varied from 1:1 to 1:5, and the enzyme concentration was kept constant. The highest mol% incorporation (100 mol%) of oleic acid occurred when 4 moles of methyl oleate was reacted with 1 mole of methyl glucoside (Fig. 3). At molar ratios greater than 1:4, a sharp decrease in mol% incorporation was observed, possibly due to substrate inhibition or saturation of the enzyme active site.

When SP382 (immobilized lipase from *Candida* sp.) was compared to nonimmobilized lipase from the same organism, it was found that the immobilized lipase gave higher mol% incorporation of oleic acid than the nonimmobilized enzyme with all three alkyl glycosides (Table 2). Immobilization apparently helps spread the enzyme over a larger surface area so that the enzyme is exposed to a more homogeneous substrate concentration compared to nonimmobilized enzyme. Gray *et al.* (19) reported that the activities of *Candida rugosa* lipase immobilized on polyethylene EP-400 had activity twice that of the soluble lipase. They postulated that immobilization actually helps to expose the enzyme more efficiently to the substrate (19). Methyl glucoside oleate synthesized with SP382 showed a higher mol% incorporation (76.5 mol% oleic acid) compared to the nonimmobilized lipase (34.2 mol%). The percent incorporation of oleic acid was lowest in octyl glucoside as compared to methyl galactoside and methyl glucoside when immobilized lipase SP382 was the biocatalyst. The powdered enzyme showed little or no in-

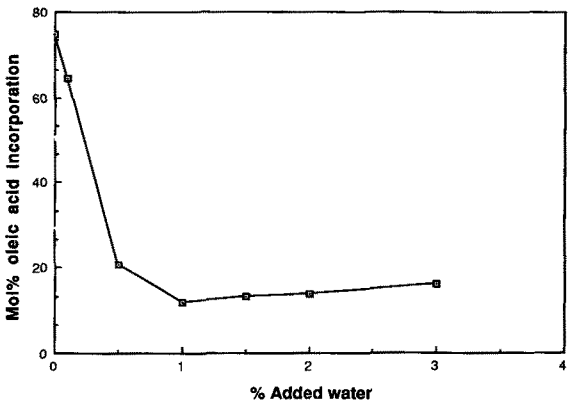

FIG. 4. The effect of added water on the incorporation of oleic acid into methyl glucoside with methyl oleate, SP382 lipase and benzene/pyridine (2:1, vol/vol). The reaction mixtures were incubated at 55°C for 24 h at 200 rpm, and the products were extracted for analysis.

corporation of oleic acid into the octyl glucoside. The reason for the low incorporation into octyl glucoside is not clear at this time and warrants further investigation. Water is necessary for all enzymes to maintain their proper three-dimensional conformation. SP382 lipase is provided with a water content of 2.0%. A study of the effect of added water on the incorporation of oleic acid into methyl glucoside revealed a sharp decrease in the incorporation of oleic acid beyond the level of 0.1% (w/w) added water (Fig. 4). At 1.5% (w/w) added water, the amount of incorporated oleic acid decreased from 74.6 to 13.3 mol%. This trend was expected because the presence of higher concentrations of water than needed by the enzyme drives the reaction toward hydrolysis rather than synthesis (20).

The optimal conditions for the synthesis of alkyl glycoside fatty acid esters under the conditions investigated are: a mole ratio of methyl glucoside/methyl oleate of 1:4; benzene/pyridine (2:1, vol/vol) as the solvent with no added water; a temperature of 55°C; a reaction time of 48 h; and immobilized *Candida* sp. lipase. The effect of other parameters such as enzyme source, enzyme concentration, enzyme reuse and different fatty acid sources on the synthesis of alkyl glycoside fatty acid esters are in progress in our laboratory.

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