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Biocatalytic acylation of carbohydrates with fatty acids from palm fatty acid distillates

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Abstract Palm fatty acid distillates (PFAD) are by-products of the palm oil refining process. Their use as the source of fatty acids, mainly palmitate, for the biocatalytic synthesis of carbohydrate fatty acid esters was investigated. Esters could be prepared in high yields from unmodified acyl donors and non-activated free fatty acids obtained from PFAD with an immobilized *Candida antarctica* lipase preparation. Acetone was found as a compatible non-toxic solvent, which gave the highest conversion yields in a heterogeneous reaction system without the complete solubilization of the sugars. Glucose, fructose, and other acyl acceptors could be employed for an ester synthesis with PFAD. The synthesis of glucose palmitate was optimized with regard to the water activity of the reaction mixture, the reaction temperature, and the enzyme concentration. The ester was obtained with 76% yield from glucose and PFAD after reaction for 74 h with 150 U ml^{-1} immobilized lipase at 40°C in acetone.

Keywords Carbohydrate fatty acid esters · *Candida antarctica* lipase · Palm fatty acid distillates

Introduction

Carbohydrate fatty acid esters are composed of mono- or oligosaccharides esterified with fatty acids of various chain lengths. The esters are non-ionic surfactants and can be employed in foods [14], detergents, and cosmetics [12], as well as in the pharmaceutical and biomedical industries [13, 15]. These compounds can be synthesized from renewable resources and are non-toxic, non-allergenic, and biodegradable [10]. The chemical synthesis of carbohydrate fatty acid esters is hampered by the severe reaction conditions resulting in high energy consumption, formation of unwanted side products due to low selectivity, and the use of toxic solvents [11, 22].

Alternatively, carbohydrate fatty acid esters can be synthesized enzymatically (For a review, see [19]). A regioselective synthesis of mono- and oligosaccharide fatty acid esters has been reported using lipases or proteases in various organic solvents [7, 9, 16, 18, 20, 22]. The yield of reversed hydrolase reactions in organic solvents is strongly affected by the type of solvent used, the water content of the reaction mixture, the stability of the enzyme in the solvent, and the solubility of the substrates [5, 8].

Fatty acid distillates from palm oil (PFAD) are by-products of the refining process of crude palm oil. About 4% of the total palm oil is obtained as palm fatty acid distillates (PFAD), which is mainly used as a raw material for soap and candle manufacturing, cosmetics, toiletries, and pharmaceutical products [17]. The synthesis of several carbohydrate esters using palm fatty acids has been reported recently, however, with low yields using a lipase from *Mucur miehei* [17].

In this paper, we describe the biocatalytic acylation of carbohydrates in high yields by a lipase from *Candida antarctica* using PFAD as a suitable source of fatty acids for the synthesis of carbohydrate fatty acid esters.

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Materials and methods

Chemicals and enzymes

Lipase from *C. antarctica* immobilized on macroporous acrylic resin (Novozym SP435, 10 U mg^{-1}) was a kind gift of Novo Nordisk A/S (Bagsvaerd, Denmark). Acetone, 2-methyl-2-butanol, *tert*-butanol, methyl-*tert*-butylether (MTBE), and *n*-hexane were purchased from

Merck (Darmstadt, Germany). D-Glucose, L-ascorbic acid, D-fructose, D-galactose, D-sorbitol, and α-methyl-D-glucose were purchased from Fluka (Buchs, Switzerland). Bis(trimethylsilyl)-trifluoroacetamid (BSTFA) and trimethylsilylimidazol (TMSI) were from Macherey-Nagel (Düren, Germany).

Samples of PFAD were a kind gift of the Department of Chemical Engineering, Prince of Songkla University, Thailand. The crude PFAD mixture was partially purified by dissolving 200 g in 500 ml *n*-hexane at 45°C, followed by cooling to 4°C for 30 min. The precipitate formed was recovered by filtration, washed several times with chilled *n*-hexane, and dried in a dessicator. Analysis of the precipitate by gas chromatography showed a fatty acid composition of palmitate (94.1%), oleate (4.65), and stearate (1.25%).

Synthesis of carbohydrate fatty acid esters

The reaction mixtures contained purified PFAD (100 mM palmitate) and acyl acceptors (D-glucose, D-fructose, D-galactose, D-sorbitol, L-ascorbic acid, and α-methyl-D-glucose) (100 mM) in a 5.0 ml organic solvent (acetone, 2-methyl-2-butanol, *tert*-butanol, MTBE, or *n*-hexane). Molecular sieves (pore diameter 30 nm, 1.0 g) were added to remove the water formed in the reaction. The water activity (a_w) of the enzyme and substrate preparations was adjusted by incubation with saturated aqueous salt solutions, LiBr ($a_w = 0.07$), LiCl ($a_w = 0.11$), CH_3COOK ($a_w = 0.23$), K_2CO_3 ($a_w = 0.43$), and $Mg(NO_3)_2$ ($a_w = 0.53$) for 3 days at room temperature.

The reaction was started by adding the immobilized *C. antarctica* lipase preparation (150 U ml^{-1}) to the reaction mixture. The reaction was carried out at 45°C on a shaker with 400 rpm. The reactions were followed by removing aliquots (10 µl) during a reaction time of 96 h which were stored at −20°C for later analysis. All reactions were carried out at least in duplicate.

For the purification of 6-*O*-palmitoyl-α-D-glucopyranose, the immobilized enzyme and the molecular sieves were removed from the mixture by filtration after 72 h of reaction. The organic solvent was evaporated under reduced pressure and hexane was added to precipitate the product. The remaining glucose was removed by washing the precipitate several times with distilled water followed by drying in a dessicator for 24 h. The product (100 mg) was dissolved in 10 ml of a mixture containing chloroform/methanol/formic acid (50:10:1 v/v/v) and 2 g of silica gel was added. The solvent was removed under reduced pressure and the silica gel was transferred into a 10 ml disposable syringe. The remaining fatty acids were eluted with 15 ml of a mixture containing chloroform/methanol (95:5 v/v). The product was finally eluted with 15 ml of a mixture containing chloroform/methanol/formic acid (50:10:1 v/v/v) and the solvent was evaporated under reduced pressure [10].

Analysis of the reaction products

For the quantitative analysis of the reaction products by thin layer chromatography with flame ionization detection (TLC/FID), the samples (10 µl) were spotted on Chromarod-s-II rods (Iatron Laboratories, Tokyo, Japan) and developed with a mixture containing chloroform/methanol/formic acid (50:10:1 v/v/v). The rods were dried in an oven at 105°C for 5 min before analysis. A TLC/FID analyzer (Iatron Laboratories) was used with an H_2 flow rate of 150 ml min^{-1}, an airflow rate of 700 ml min^{-1}, and a scanning speed of 30 s rod^{-1}. The reaction products were also analyzed by gas chromatography as described previously [9]. Product yields were calculated with respect to percentage of carbohydrate consumed.

^{1}H NMR spectra of 6-*O*-palmitoyl-α-D-glucopyranose were recorded on a 250 MHz Bruker Avance spectrometer (Karlsruhe, Germany) in CD_3OD. Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry was carried out on an Axima QIT spectrometer (Shimadzu Deutschland GmbH, Duisburg, Germany). Calculated for $C_{22}H_{42}O_7Na(M + Na^+)$ 441.42, found 441.07.

Results and discussion

Results of the gas chromatographic analysis of the products obtained with the *C. antarctica* lipase after 72 h of reaction with glucose and PFAD are shown in Fig. 1. The main peak with a retention time of 4.9 min corresponded to 6-*O*-palmitoyl-α-D-glucopyranose, while the two minor peaks with 5.5 and 5.6 min retention time presumably represent the stearate and oleate esters of glucose, respectively. The identity of the product was confirmed by ^{1}H NMR and comparison with published data [9] and by MALDI-TOF spectrometry where the expected mass of 6-*O*-palmitoyl-glucopyranose was found. These results confirm the previously reported ability of *C. antarctica* lipase to catalyze the synthesis of carbohydrate fatty acid esters from underivatized carbohydrates and free fatty acids. The enzyme was found to catalyze selectively a monoacylation at the 6-position of glucose [1, 3, 16].

Due to the different polarity of the acyl donor and acceptor and the limited stability of enzymes in organic solvents, a suitable solvent has to be selected to dissolve the substrates without compromising enzymatic activity [4, 5, 8]. Pyridine, which has been previously employed as a solvent, may inactivate the enzyme and is not compatible with applications in the food industry [21]. Hexane, acetone, ethyl methylketone, and *tert*-butanol have also been used in spite of the low solubility of carbohydrates in these solvents [8, 9, 22]. When we compared acetone, MTBE, *tert*-butanol, and 2-methyl-2-butanol as organic media for the synthesis of PFAD glucose ester, the highest yields were obtained with acetone as solvent after a reaction time of 72 h (Fig. 2). Similar results have

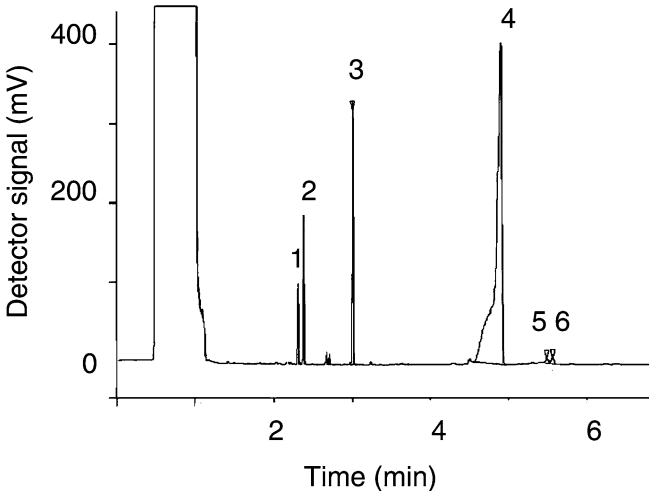

Fig. 1 Gas chromatographic analysis of the reaction products obtained by incubation of PFAD and glucose with *Candida antarctica* lipase. *Peak 1* α-D-glucose, *peak 2* β-D-glucose, *peak 3* octyl-β-D-glucoside (standard), *peak 4* glucose palmitate, *peak 5* glucose stearate, *peak 6* glucose oleate

been previously reported for the synthesis of palmitate esters of glucose, mannitol, and sorbitol in acetone [1, 2]. Since the carbohydrates are only partially soluble in acetone, the reaction is performed in a heterogeneous system where most of the substrate is suspended in the reaction mixture. The use of acetone as organic solvent for the synthesis of carbohydrate esters has the advantage that it can be easily removed from the reaction system for product recovery [1].

The yield of the enzyme-catalyzed synthesis reaction in organic media is greatly dependent on the amount of water present in the reaction system [6]. When the synthesis of PFAD glucose ester in reaction mixtures with different water activities was compared, a sharp decrease in the ester yield was found with a_w values higher than 0.07 (Fig. 3). With higher water contents, the equilibrium of the reaction was shifted toward the hydrolysis of the ester, resulting in low yields of products formed. Since a continuous removal of the water generated during the reaction is necessary, the effect of the addition of molecular sieves in various amounts to the reaction mixture was investigated. The highest product yield was obtained by the addition of 0.2 g ml^{-1} of molecular sieves, while no product could be detected in the absence of molecular sieves in the reaction mixture.

The reaction temperature also had a large effect on the product yield of PFAD glucose esters since the solubilization of the sugar and the reaction rate will be increased at higher temperatures [1]. However, at increased temperatures, the enzyme will become inactivated in the organic solvent. When the synthesis reaction was carried out in the range of 35–55°C, the highest yield of PFAD glucose ester (31.8 mg ml^{-1}) was obtained at 40°C (Fig. 4). A further increase in the reaction temperature resulted in lower yields indicating an inactivation of the enzyme in the presence of the organic solvent. In contrast, Rakmi et al. [17] found the highest yield of ester (17.7 mg ml^{-1}) formed from fructose and PFAD by *M. miehei* lipase at a reaction temperature of 55°C.

Adjusting the amount of immobilized enzyme added to the reaction mixture further optimized the synthesis reaction. Raising the enzyme concentration from 50 to 150 U ml^{-1} resulted in a concomitant increase in product yield while enzyme concentrations above 150 U ml^{-1} did not further increase the product yield.

After 72 h of incubation at 40°C, about 76% of the glucose contained in the reaction mixture was converted to the palmitate ester corresponding to 31.8 mg of product formed per ml of solvent. PFAD esters of other

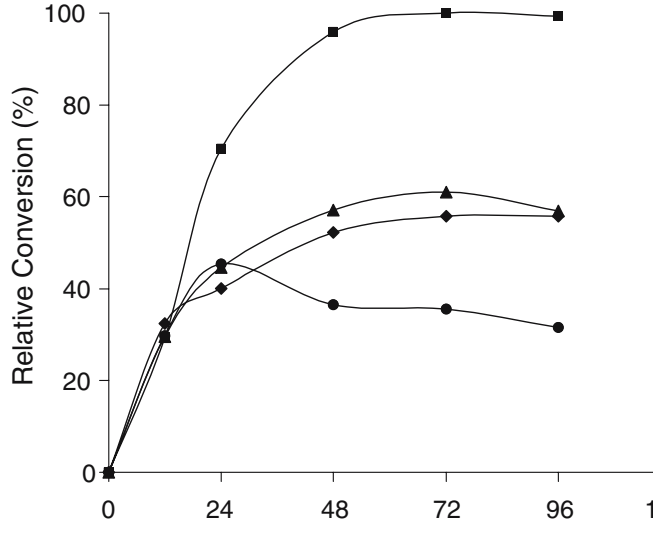

Fig. 2 Acylation of glucose with PFAD by *C. antarctica* lipase (10 U mg^{-1}) in *tert*-butanol (*filled diamond*), 2-methyl-2-butanol (*filled triangle*), and MTBE (*filled circle*) compared to the conversion in acetone (100%, *filled square*)

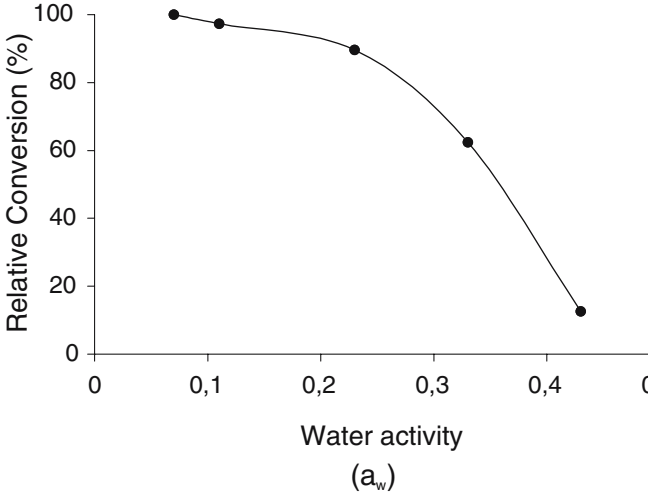

Fig. 3 Effect of the water activity (a_w) of the reaction mixture on the yield of PFAD glucose ester synthesized by *C. antarctica* lipase

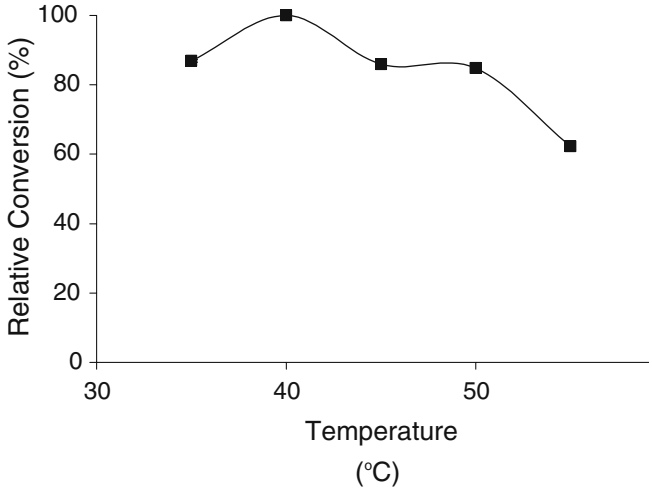

Fig. 4 Effect of the reaction temperature on the yield of PFAD glucose ester synthesized by *C. antarctica* lipase

monosaccharides, α-methyl-D-glucoside, sorbitol, and L-ascorbic acid could also be prepared (Table 1). Galactose and fructose appeared to be poor acyl acceptors compared to glucose, with only 9% (2.4 mg ml^{-1}) and 51% (15.9 mg ml^{-1}) converted to the palmitate esters, respectively. In contrast, higher yields of the fructose ester (17.0 mg ml^{-1}) compared to glucose ester (13.0 mg ml^{-1}) have been reported when *M. miehei* lipase was used as the biocatalyst [17]. These results indicate that the yield of a specific carbohydrate fatty acid ester can be controlled by the selection of a suitable biocatalyst for the production process.

Conclusion

Carbohydrate fatty acid ester could be prepared biocatalytically in high yield (31.8 mg ml^{-1} corresponding to 76.0% conversion) from PFAD and glucose using *C. antarctica* lipase B. The composition of the solvent, the control of its water content, the reaction temperature, and the enzyme concentration were major factors affecting the conversion yields. The process described is efficient and environmentally benign using readily available renewable resources and by-products for the preparation of biosurfactants with a commercial potential for PFAD-producing countries.

Table 1 Yields of PFAD ester with different acyl acceptors synthesized by *Candida antarctica* lipase

Acyl acceptors	Relative conversion (glucose = 100%)
L-Ascorbic acid	81
D-Fructose	51
D-Galactose	9
D-Glucose	100
D-Sorbitol	24
α-Methyl-D-glucose	103

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