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Regioselectivity and Fatty Acid Specificity of *Chromobacterium viscosum* Lipase

Raúl J. Barros, Pedro G. V. Moura-Pinto, Maurice C. R. Franssen,* Anja E. M. Janssen†‡ and Aede de Groot*

Department of Organic Chemistry, Wageningen Agricultural University, Dreijenplein 8, 6703 HB Wageningen, The Netherlands

‡*Food and Bioprocess Engineering Group, Department of Food Science, Wageningen Agricultural University, Bomenweg 2, 6703 HD Wageningen, The Netherlands*

Abstract—The fatty acid specificity of *Chromobacterium viscosum* lipase was studied by comparing the pseudo-first-order rate constants for the transesterification of different fatty acid methyl esters with 1-propanol in dry acetonitrile as solvent. It was found that this enzyme shows a significant preference towards long chain fatty acids and, for chains with the same length, towards saturated ones. The same enzyme was used to study the esterification of sorbitol and decanoic acid. A mixture of mono-, di-, tri- and tetraesters was obtained. The concentration of esters was strongly increased upon raising the temperature from 35 to 70 °C. The structures of the di-, tri- and tetraesters were determined using ^{13}C NMR spectrometry. The diester appeared to be sorbitol 1,6-didecanoate, the triester was sorbitol 1,5,6-tridecanoate and the tetraester was the 1,2,5,6-tetradecanoate, which indicates that the *C. viscosum* lipase acylates sorbitol in a regioselective manner.

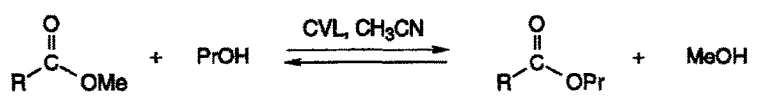
Introduction

Lipases, or acylglycerolhydrolases (EC 3.1.1.3), have been defined as enzymes which catalyze the hydrolysis of long-chain aliphatic acids from acylglycerols at an oil/water interface.[1] Large-scale applications, making use of this ability, are found in food-processing, sewage treatment and the detergent industry.

Lipases catalyze the hydrolysis of esters other than acylglycerols. Furthermore, they have been shown to be active and stable in organic solvents which, among other advantages, make them adequate for synthetic reactions, such as esterifications, transesterifications, aminolysis, thiotransesterifications and oximolysis.[2–7] These properties, together with the commercial availability of a wide range of lipases from different sources (microbial, yeast, fungal, mammal) at low cost, make them interesting as catalysts for organic synthesis.

The lipase from *Chromobacterium viscosum* (CVL), which is very stable and active in organic solvents, has been used in the synthesis of highly valuable polyunsaturated fatty acid glycerides,[8] and so-called natural surfactants. Of these natural surfactants, it is of special interest to mention monoglycerides and sugar esters, which can be used as surfactants in the food, detergent and cosmetic industries. The main advantages of these surfactants are their good biological degradability and low toxicity, which make them environmentally friendly.[9–12]

Furthermore, CVL has been shown to possess marked regioselectivity and stereoselectivity in a wide range of organic reactions.[13–25] It is therefore especially useful in site-selective synthesis steps, resolution of racemic mixtures or in the synthesis of chiral compounds. This selectivity, allied with its high activity in mild reaction conditions, are characteristics considered highly valuable for the pharmaceutical and fine-chemical industries.
In previous work on the synthesis of sugar esters using CVL as catalyst, it was found that unsaturated fatty acids were not incorporated in the products, even though they were available in high concentrations.[12] This suggests a preference towards saturated fatty acids, which has led us to a further study of the fatty acid specificity of CVL. For this study, the transesterification of fatty acid methyl esters with 1-propanol, carried out in dry acetonitrile, was employed as a model reaction (see Scheme I).



Scheme I.

†Present address: Dept of Bioscience and Biotechnology, University of Strathclyde, 204 George St, Glasgow G1 1XW, U.K.

We here report on the fatty acid specificity of CVL, which has been deduced from pseudo-first-order rate constants of this model reaction using substrates with different fatty acid chain lengths. The regioselectivity of CVL has been determined by elucidating the structure of sorbitol tri- and tetraesters, which are formed during the incubation of sorbitol with decanoic acid at elevated temperature.

Results

Fatty acid specificity

When CVL is incubated with a fatty acid methyl ester and a large excess of 1-propanol, the initial rate of transesterification is almost identical to the pseudo-first-order rate constant (hereafter called the rate constant). Two effects of the fatty acid chain on the rate constant of this reaction were investigated: fatty acid chain length and fatty acid degree of unsaturation. These effects are presented in Figures 1 and 2, respectively.

Figure 1 clearly shows a general trend in the rate constant: an increase of the fatty acid chain length results in an increase of the rate constant parameter. It reaches a maximum for methyl stearate (octadecanoate, $C_{18:0}$), a value of 0.38 h^{-1}, being about 32-fold higher than the value for methyl caproate (hexanoate, $C_{6:0}$). The variation in between is rather smooth, except for methyl margarate (heptadecanoate, $C_{17:0}$), which is considerably lower than methyl stearate and methyl palmitate (hexadecanoate, $C_{16:0}$). The value for methyl behenate (docosanoate, $C_{22:0}$) does not fit in the general trend because of solubility problems under the conditions used.

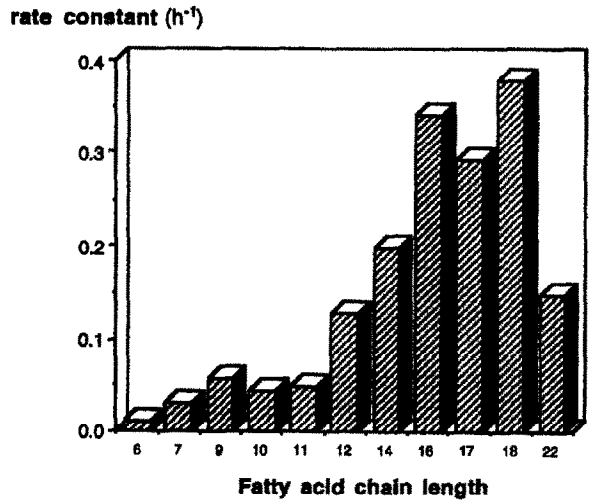


Figure. 1. The effect of fatty acid chain length on the pseudo-first-order rate constant of CVL-catalyzed transesterification of fatty acid methyl esters with 1-propanol. CVL shows a preference towards long chain fatty acids.

The effect of the degree of unsaturation was studied in the following series: methyl stearate (octadecanoate, $C_{18:0}$), oleate (*cis*-9-octadecenoate, $C_{18:1}$), linoleate (*cis,cis*-9,12-octadecadienoate, $C_{18:2}$) and linolenate (*cis,cis,cis*-9,12,15-octadecatrienoate, $C_{18:3}$). Figure 2 shows that the rate constant drops to less than half, from 0.38 to 0.18 h^{-1}, when a double bond is introduced at the 9-position of the fatty acid chain. The introduction of a second double bond at the 12-position does not have a significant effect on that parameter, but a third double bond at the 15-position reduces again by half the efficiency of the catalysis, changing the rate constant from 0.18 to 0.084 h^{-1}.

We tried to confirm these results regarding the influence of double bonds for a different chain length, but a comparison between methyl behenate (docosanoate, $C_{22:0}$) and erucate (cis-13-docosenoate, $C_{22:1}$) could not be made, due to the solubility problems of methyl behenate. It is, however, interesting to note that the rate constant for $C_{22:1}$, 0.20 h^{-1}, is about the same as for $C_{18:1}$ and $C_{18:2}$.

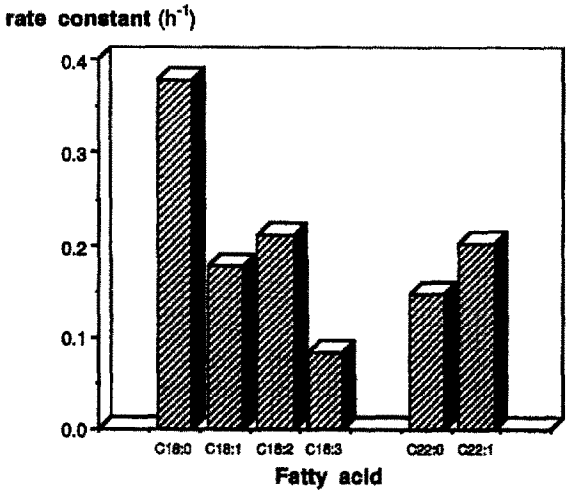


Figure. 2. The effect of fatty acid degree of unsaturation on the pseudo-first-order rate constant of CVL-catalyzed transesterification of fatty acid methyl esters with 1-propanol. CVL shows a preference towards saturated fatty acids. The inverse behaviour for $C_{22:0}$ is observed due to the insolubility of this compound under the conditions used.

Esterification of sorbitol

During our studies on the esterification of sorbitol with fatty acids, catalyzed by the lipases from *C. viscosum* and *Candida rugosa*, we not only found preferential incorporation of saturated fatty acids in the product, but also higher esters of sorbitol.[12] In order to investigate the possible regioselective character of these conversions, the structures of these higher esters were determined.

The esterification of sorbitol with fatty acids can be done in a water/fatty acid two-phase system in the presence of an organic solvent (2-pyrrolidone)[12] or in its absence.[26] The latter system is preferable, because it is simpler and does not involve a potentially toxic cosolvent. In this system, the aqueous phase is composed of sorbitol and enzyme, dissolved in water; the organic phase is composed of fatty acid. Water is necessary to dissolve sorbitol and to ensure enzyme activity. On the other hand, water activity in the aqueous phase should remain as low as possible, in order to drive the reaction equilibrium to the ester side. This can be accomplished by taking a high sorbitol concentration.[26]

However, the sorbitol concentration is limited by the solubility of sorbitol in water. At a reaction temperature of 35 °C, the maximum sorbitol mole fraction in the aqueous phase is 0.22.

CVL catalyzes the conversion of sorbitol into a mixture of 1- and 6-monoester when incubated with crude oleic acid.[12] However, di-, tri- and tetraesters are also observed.[27] The total concentration of esters which can be obtained at equilibrium is low. This can be improved by reducing the water activity via an increase of the concentration of dissolved sorbitol by raising the temperature. This hypothesis was proven by experiments in which a constant amount of solid sorbitol was added to the reaction mixture at different temperatures. Upon raising the temperature, less water was necessary to dissolve the sorbitol. Hence, the mole fraction of sorbitol increases and the water activity in the system decreases by raising the temperature. This effect indeed proved to be dramatic, as the total mole fraction of esters in the organic phase rose from 0.07 at 35 °C to 0.26 at 70 °C (see Figure 3). The ratio between mono-, di- tri- and tetraester mole fractions is hardly affected by temperature and initial sorbitol mole fraction (results not shown).

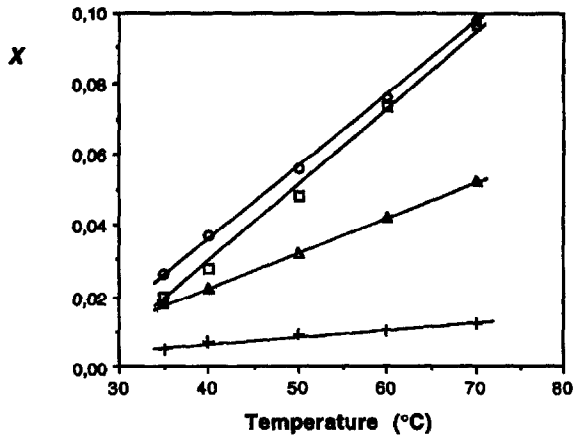


Figure 3. Sorbitol mono- (□), di- (○), tri- (Δ) and tetraester (+) mole fractions at equilibrium as a function of the temperature at increasing mole fraction of sorbitol. Decanoic acid was used as the fatty acid.

Knowledge about the structure of the sorbitol triester and tetraester which are formed in this reaction will give information on the possible regioselectivity of CVL. It is clear from Figure 3 that the most convenient way to isolate the higher esters is to perform the reaction at elevated temperature, since their concentrations are highest under those conditions. In this way, relatively pure sorbitol di-, tri- and tetradecanoate(s) were obtained by column chromatography of the organic phase obtained after incubation of sorbitol and decanoic acid in the presence of CVL and a small amount of water. The structures of the esters were proven by NMR, IR and FD - MS.

The use of ^{13}C NMR spectrometry was instrumental in the assignment of the substitution pattern of the esters. The interpretation of the ^{13}C NMR spectra is based on the changes in chemical shifts of the carbon atoms of the sorbitol backbone, which are induced by acylation of hydroxyl groups. The acylation effect was estimated as a +3.0 ppm downfield shift for Cα and a −2.5 ppm upfield shift for Cβ.[28,29] The effect on more distant carbon atoms is negligible. With this rule of thumb in mind, comparisons were made between the measured chemical shifts of the sorbitol part of the esters, and some calculated chemical shifts. The results for the sorbitol di- and triester are depicted below.

Table 1 shows that the observed chemical shift values for sorbitol didecanoate are compatible with the values calculated for the 1,6-ester. This is not surprising, since the monoester is already a mixture of the 1- and the 6-ester.[12] The isolated triester appears to be sorbitol 1,5,6-tridecanoate rather than the 1,2,6-isomer, according to Table 2. All other possibilities (e.g. 1,3,6-ester) gave large deviations from the observed chemical shift values. However, some minor peaks were visible in the ^{13}C NMR spectrum, which might be derived from another tridecanoate. The intensity of the peaks was too small to allow identification of this compound; separation of the isomers was not possible either. Hence, the CVL-catalyzed formation of sorbitol 1,2,6-tridecanoate cannot be excluded.

Table 1. Comparison of the ^{13}C chemical shifts of the isolated sorbitol didecanoate with calculated values for the 1,6-diester. Chemical shifts are in ppm

Carbon Atom	Chem. Shifts Diester (exp.)	Chem. Shifts 1,6-Didecanoate (calcd.)
C_1	66.6	66.6
C_2	72.5	72.5
C_3	70.8	70.5
C_4	73.6	73.0
C_5	71.4	70.7
C_6	67.8	67.2

Table 2. Comparison of the ^{13}C chemical shifts of the isolated sorbitol tridecanoate with calculated values for the 1,2,6- and 1,5,6-triester. Chemical shifts are in ppm

Carbon Atom	Chem. Shifts Triester (exp.)	Chem. Shifts 1,2,6-Tridecanoate (calcd.)	Chem. Shifts 1,5,6-Tridecanoate (calcd.)
C_1	66.4	64.6	67.1
C_2	72.5	75.5	72.5
C_3	70.5	68.3	70.8
C_4	70.8	73.6	71.1
C_5	73.0	70.4	73.4
C_6	64.0	67.8	65.3

Table 3. Comparison of the ^{13}C-chemical shifts of the isolated sorbitol tetradecanoate with calculated values for the 1,2,5,6-triester. Chemical shifts are in ppm

Carbon Atom	Chem. Shifts Tetraester (exp.)	Chem. Shifts 1,2,5,6-Tetradecanoate (calcd.)
C_1	63.3	64.6
C_2	74.4	75.5
C_3	66.2	68.3
C_4	69.4	71.1
C_5	72.4	73.4
C_6	63.8	65.3

When the same theory was applied to the obtained tetraester, complete matching of the experimental and the calculated chemical shifts was impossible. The calculated values for the 1,2,5,6-tetraester predict the correct trend, but the difference with the observed values is around 1.4 ppm. However, all other possibilities give much larger deviations (results not shown). This phenomenon was also observed by Yoshimoto *et al.*[29] Working with *O*-acylglucoses these authors found that the additivity of the shift parameter rule was not valid for molecules with more than three acylated positions. They attributed this to a change of conformation of the acyl groups resulting from their accumulation on the molecule. An average difference between experimental and calculated chemical shifts of 1.7 ppm was estimated for a penta-acylated molecule. This value is quite close to the $\Delta\delta$ value found for the 1,2,5,6-tetradecanoate, indicating that a similar explanation may hold for these sorbitol esters.

On determining the structure of polyol esters one should always be aware of the fact that these compounds are prone to acyl migration.[30] In principle, the sorbitol esters formed could be derived from regioselective enzymatic acylation reactions, or from (non)selective acylation followed by acyl migration until the thermodynamically most stable isomer is obtained. To address this question, molecular mechanics calculations were performed with sorbitol triester and tetraester. The minimum energy was determined for all possible triesters and tetraesters which can be formed from the 1,6-didecanoate, using the CHARMm 21.3 force field.[31] The 1,5,6-tridecanoate was found to be the most stable triester (85.2 %), followed by the 1,3,6-triester (14.1 %). Remarkably, 1,4,5,6-tetradecanoate is by far the most stable tetraester (99.99+ %). This indicates that the observed esterification reaction is regioselective and enzyme-mediated.

Discussion

Figure 1 clearly shows that CVL has a marked preference towards long-chain fatty acids. This suggests that the active site of CVL, where the substrate has to fit and establish favourable interactions, consists of a rather large hydrophobic pocket. The smaller fatty acids are probably too short to establish strong interactions with it, and do not bind to the active site as tightly as the longer fatty acids. Also possible effects of the solvent used must be considered. Acetonitrile is a rather polar solvent (Log P = −0.33), so it will not solvate the more hydrophobic substrates as efficiently as the shorter fatty acids, which facilitates the interaction of hydrophobic substrates with the enzyme. If this is the case then the specificity could be attenuated, or even reversed, in less polar solvents.

Figure 2 shows that CVL significantly prefers a saturated fatty acid to three other fatty acids of the same chain length but with increasing degrees of unsaturation. Considering that the double bonds are positioned at a large distance from the reactive part of the molecules (eight C–C bonds and more), and that the differences in hydrophobicity between these compounds are too small to account for such a big difference, this effect can only be attributed to a more hindered binding of the unsaturated substrates to the active site of CVL. In fact, the existence of *cis* double bonds confers some rigidity to the fatty acid chains and causes them to bend, making it more difficult to fit the hydrophobic pocket of CVL. The small difference between the results of methyl oleate and methyl linoleate suggests that the second *cis* double bond of this last compound does not introduce additional conformational strain or steric hindrance to the interaction between enzyme and substrate. It should be noted that, since we only measure pseudo-first-order rate constants in these studies, our results do not differentiate between changes in k_{cat} and changes in K_m on going from one substrate to another.

The preference of CVL for long-chain saturated fatty acids is similar to the fatty acid specificities reported for *Candida antarctica*, *Mucor miehei* and *Rhizopus arrhizus* lipases.[17,19,20,32] This suggests that there may be an analogy between the structures of the active centers of these enzymes, or at least a similarity among the interactions between enzyme and substrate. Other kinds of fatty acid specificity have been reported. *Aspergillus* lipase and porcine pancreatic lipase show preference towards short fatty acids,[19,20] *Candida rugosa* lipase towards $C_{4:0}$[17,19] and *Geotrichum candidum* lipase towards *cis*-9 or *cis,cis*-9,12 unsaturated fatty acids.[17,18]

The results presented here indicate that CVL not only possesses fatty acid selectivity, but also some regioselectivity in the esterification of sorbitol. The enzyme first esterifies the primary hydroxyl groups of sorbitol, followed by the adjacent secondary hydroxyls. This is not unexpected since primary hydroxyl groups are more reactive towards acylation then secondary.[33] Attachment of the third and fourth acyl chain also seems to be regioselective, since 1,2,5,6-tetradecanoate was formed instead of the thermodynamically more stable 1,4,5,6-isomer. It was shown that the 1,5,6-tridecanoate is the preferentially formed triester, although some 1,2,6-tridecanoate seems to be formed as well.

It should be mentioned here that the molecular mechanics calculations should be treated with caution, because of the simplifications that are assumed in the calculations. For instance, solvent interactions and entropy effects were not taken into account. However, in the reaction mixture, solvent effects cannot be neglected because ester molecules coexist with decanoic acid molecules in the organic phase.

Thus, hydrophobic interactions may also occur between the free fatty acid and the ester molecules side chains, and will eventually result in the change of the number of degrees of freedom of a given conformation, in which case entropy effects have to be considered.

CVL-catalyzed regioselective reactions have been observed before: acylation of 5α-androstane-3β,17β-diol gave only the 3β-ester; acylation of glucose 6-monobutanoate yielded only the 3,6-diester.[34] On the other hand, the data available on CVL specificity for primary or secondary hydroxyl groups seem to be somewhat contradictory. Although said to be non-specific in the hydrolysis of triglycerides,[30] CVL has demonstrated a striking preference for the primary positions of sugar alcohols[11,12] and alkanediols.[21]

The reason why CVL preferably esterifies hydroxyl groups next to already existing ester bonds is not clear. It might be that favourable hydrophobic interactions exist between one fatty acid chain of the sorbitol diester/triester and the active site of the enzyme. This would lower the energy of the transition state, and maybe lead to a conformation in which the adjacent hydroxyl group is orientated close to the acylated serine moiety of the enzyme.

Further studies have to be done to test these hypotheses about the active center of CVL and its interaction with different substrates. The determination of the 3D structure of the active site of this enzyme, together with molecular dynamics calculations, could give the confirmation, or dismissal of these hypotheses. However, lipases are difficult to crystallize. Only recently the first 3D structures of enzymes of this kind have been determined by X-ray crystallography, and CVL has not yet been studied.

Clearly, the enzymatic production of a biosurfactant like acylated sorbitol is preferable to chemical synthesis. The latter has to be carried out at 180 °C, using calcium and barium acetates, carbonates, oxides or hydroxides as catalysts.[33] In these conditions, polymerization of esters occurs, which results in higher viscosity of the reaction medium. Furthermore, free hydroxyl groups in the sugar are reactive, leading towards the formation of epoxides and ethers with the concomitant release of water. The CVL-catalyzed process has three distinct advantages: it is specific with respect to the fatty acid, it is regioselective and it is performed under mild conditions. An additional advantage in large scale processes will be the reduced costs of waste treatment.[35]

Experimental Section

Materials

Enzyme. CVL was obtained from Biocatalysts Ltd. For the esterification of sorbitol, the enzyme was used 'straight from the bottle'. For the transesterification reactions, it was subjected to 'pH-adjustment' before use, as follows: 3.0 g of enzyme was dissolved in 100 mL of double-distilled water, the pH was adjusted to 7.0 using diluted KOH and the resulting solution was freeze-dried. The resulting powder showed a five-fold higher activity compared to the untreated enzyme, regarding the transesterification of methyl stearate with 1-propanol.

Chemicals. Sorbitol, capric acid and all fatty acid methyl esters, with exception of methyl enantoate (heptanoate, $C_{7:0}$) and methyl pelargonate (nonanoate, $C_{9:0}$), were obtained from Merck and were of p.a. grade. Methyl enantoate and methyl pelargonate were prepared by chemical esterification of the respective fatty acids with methanol, and were pure according to GC. Acetonitrile and 1-propanol, both p.a. quality, were obtained from Janssen Chimica and Merck, respectively, and dried over 4 Å molecular sieves. P.a. methanol and chloroform were from Merck and Janssen Chimica, respectively.

Analytical procedures. The transesterification reactions were monitored by injecting, at regular time intervals, 1 mL samples of the reaction mixture in a Varian 3300 GC equipped with a flame ionization detector. The separations were carried out on a 300 × 1/8" nickel column packed with 3.7 g of Chromosorb 100-120 plus 3.1 % OV-17 as stationary phase. The injector port and column temperatures were set according to the substrate studied and ranged, respectively, between 120–270 °C and 90–255 °C. Chromatograms were recorded on a Hewlett-Packard HP 3396A integrator.

The esterification of sorbitol was followed by HPLC using two size exclusion columns (PLgel 30 cm, Polymer Laboratories) placed in series. The organic phase of the sampled reaction mixture was separated by centrifugation and diluted in THF in a 9/1 volumetric ratio. The same solvent was used as a mobile phase, with a flow rate of 1.0 mL/min. The effluent was monitored using a refractive index detector. Chromatograms were recorded on a Spectra-Physics SP4290 Integrator.

The preparative separation of sorbitol esters was performed on silica gel 60 (particle size 0.040–0.063 mm); fractions were examined by TLC on silica plates (60 F_{254}, Merck), using 5 % methanol in chloroform as the solvent. Spots were detected by iodine vapour or by spraying with phosphomolybdic acid/acetic acid/sulfuric acid (22.5 g/415 mL/22.5 mL, resp.).

IR spectra were recorded on a Philips PU9700 apparatus, using chloroform as a solvent. ^1H and ^{13}C NMR spectra were taken on a Bruker AC 200E (200 MHz) spectrometer, using CD_3OD as a solvent and TMS as a reference; mass spectra obtained by Field Desorption-Ionisation Mode (FD) were recorded on a AEI MS-902 mass spectrometer equipped with a VG ZAB console.

Transesterification reactions

The transesterification reactions were carried out at 45 °C and 250 rpm in 4 mL screw cap vials (15 × 45 mm) from Chrompack, placed inside a G24 Environmental Incubator Shaker from New Brunswick Scientific Co. The reaction mixtures were prepared by mixing 500 μL of 20 mM fatty acid and 500 μL of 1 M 1-propanol containing the adequate internal standard for GC analysis. The enzyme, 10.0 mg,

was added only after the correct temperature was attained. In all cases, the solvent was acetonitrile.

Kinetic data processing

The concentration of fatty acid methyl ester for each sample was obtained by the internal standard method, through the ratio of the peak areas of this compound and of the internal standard. In order to work under pseudo-first-order conditions, the concentration of the second substrate (1-propanol) was taken as high as possible (50-fold excess). The data obtained for concentration of methyl ester versus time were indeed found to correlate with a first-order rate equation, which is expected for Michaelis–Menten kinetics when the concentration of substrate is much lower than the Michaelis constant. Since the effects of product inhibition and diffusional limitation were not studied, these values will be referred to as the pseudo-first-order rate constants. All the reactions were run in duplicate, and the results showed an agreement within 5 % precision.

Esterification of sorbitol

The effect of temperature on the yield of esters (Figure 3) was studied by incubating 20 mmol of decanoic acid, 20 mmol of sorbitol, a variable amount of water and 25 mg of CVL in a stoppered bottle which was placed in an end-over-end incubator in a temperature-controlled cupboard. Samples of the organic phase were analyzed by HPLC as described above. Decanoic acid was chosen in these studies rather than hexadecanoic and octadecanoic acid, because the latter two are solids at the lower temperatures of this study.

The large-scale esterification of sorbitol was carried out as follows. Sorbitol (55.0 g, 302 mmol) together with 5.00 mL of water was placed in a temperature-controlled cylindrical glass vessel with an approximate volume of 200 mL. The temperature of the vessel was raised to 70 °C under vigorous magnetic stirring; when all the sorbitol was dissolved, 18.0 g (104.5 mmol) of decanoic acid was added, followed by 250 mg of CVL in 2.00 mL of water. The vessel was covered in order to avoid losses of water during the reaction. The reaction was stopped after 20 days when the composition of the product mixture did not change anymore. As was observed earlier,[26] the final reaction mixture was still a biphasic system consisting of an aqueous layer composed of unreacted sorbitol, water and lipase, and an organic phase, composed of unreacted acid and sorbitol esters. The phases were separated by centrifugation and the organic layer was collected.

Separation of sorbitol esters

Initial attempts to extract the organic phase with saturated sodium carbonate, in order to remove the remaining decanoic acid, resulted in precipitation of decanoic acid and sodium decanoate with the subsequent formation of an interfering interphase. Therefore, direct column chromatography of the organic phase was chosen to separate and purify the sorbitol esters formed. Best separation was obtained when a stepwise gradient of 1–10 % methanol in chloroform was used as the eluent (steps of 1 % methanol, volume per step 200–400 mL). The elution order was: sorbitol tetraester, decanoic acid, sorbitol triester, diester and monoester.

Spectral data for the sorbitol esters

Data for the 1- and the 6-ester were given earlier.[12] The IR spectra of all sorbitol esters showed peaks at 3500 (br, OH), 2950 and 2890 (C-H) and 1730 cm^{-1} (C=O).

Sorbitol. ^{13}C NMR: 75.0 (C_2), 73.6 (C_4), 72.9 (C_5), 70.8 (C_3), 64.8 (C_6), 64.1 (C_1).

Sorbitol 1,6-didecanoate. Mass spectrum (FD): m/e = 490 ($M^{+\cdot}$); ^{13}C NMR: 175.8, 175.5 (ester carbonyls), 73.6 (C_4), 72.5 (C_2), 71.4 (C_5), 70.8 (C_3), 67.8 (C_6), 66.6 (C_1), 35.0, 33.0, 30.6, 30.4, 30.2, 26.0, 23.7 (fatty acid methylenes), 14.5 (CH_3).

Sorbitol 1,5,6-tridecanoate. ^{13}C NMR: 175.2, 175.1, 174.3 (ester carbonyls), 73.0 (C_5), 72.5 (C_2), 70.8 (C_4), 70.7 (C_3), 66.4 (C_1), 64.0 (C_6), 35.1, 33.1, 30.6, 30.5, 30.3, 26.1, 23.8 (fatty acid methylenes), 14.5 (CH_3).

Sorbitol 1,2,5,6-tetradecanoate. Mass spectrum (FD): m/e = 800 $(M+1)^{+\cdot}$. ^{13}C NMR: 175.0, 174.8, 174.2, 174.0 (ester carbonyls), 74.4 (C_2), 72.4 (C_5), 69.4 (C_4), 66.2 (C_3), 63.8 (C_6), 63.3 (C_1), 35.0, 33.1, 30.7, 30.5, 30.3, 26.1, 23.8 (fatty acid methylenes), 14.5 (CH_3).

Molecular mechanics calculations of sorbitol tri- and tetradecanoate

The different structures of these compounds were constructed from the linear molecule of sorbitol by attaching linear decanoic acid chains to it. The torsion angles over the sugar bonds C_1–C_2, C_2–C_3, C_3–C_4, C_4–C_5 and C_5–C_6 were initially set to –60 ° and then varied to 60 and 180 °, respectively. The starting conformations obtained in this way were energy minimized using the CHARMm 21.3 force field.[31] The following simplifications were made: solvent interactions were not taken into account during the minimizations and entropy effects were neglected in the statistical calculations. Side-chains were taken as linear chains. The minimization was performed by a 50 steps steepest descend method, followed by conjugate gradient minimization until the gradient was smaller than 0.01 kcal/mol/Å. Hydrogen bonds were taken into account using the Coulomb interaction function. This method gives the relative energies of the regio-isomers, which can be transformed into relative abundances using Boltzmann's equation.

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