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pH-imprinted lipase catalyzed synthesis of dextran fatty acid ester

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Abstract

The application of enzymatic catalysis for the synthesis of polysaccharide-based surfactants was investigated. The polysaccharide dextran, a neutral bacterial polysaccharide consisting of α-1,6 linked glucose units, was chemically modified by the attachment of hydrophobic groups through a transesterification reaction with a vinyl decanoate. A screening of commercially available lipases and protease for the synthesis of amphiphilic polysaccharides in DMSO suggested that lipase AY from *Candida rugosa* modified dextran T-40 with vinyl decanoate at the highest conversion. A pH-adjustment in a phosphate buffer at pH 7.5 prior to use is crucial to make this enzyme active in DMSO. The effect of enzyme concentration and mole ratio of fatty ester to dextran T-40 on the conversion and the rate of reaction were studied. Finally, investigation of the kinetics and regioselectivity of lipase AY-catalyzed modification offer a possibility to regulate the position and the extent of hydrophobic group attached to dextran. These two properties are fundamental for controlling the physico-chemical properties of the final polymeric surfactants.

Keywords: Amphiphilic polysaccharides; Lipases; Transesterification; Dextran; Vinyl decanoate; Regioselective modification

1. Introduction

Enzymatic catalysis has been applied for several decades to the synthesis of biosurfactants based on carbohydrates. Many examples involve lipase-catalyzed esterification or transesterification [1–3]. The advantages of enzymatic catalysis are mainly related to chemical selectivity of the reaction and to the mild conditions required.

Since the pioneering work of Landoll [4], several families of polymeric biosurfactants based on polysaccharides with well-defined structures have been developed. Polymeric biosurfactants exhibit valuable properties for aqueous formulations. At sufficiently high concentrations, they are efficient rheology modifiers. Moreover, they adsorb at oil–water interfaces and thus are emulsion stabilizers. For randomly modified polysaccharides, their multiple points of attachment lead to a quasi-irreversible adsorption which has a strong effect on emulsion properties like stability to dilution or surface rheological behaviour. Attempts have been made to prepare polysaccharide-based biosurfactants by microbial synthesis but this led only to poorly controlled structures in terms of number of hydrophobic groups, nature of the groups, distribution along the polysaccharide backbone, etc. This makes it difficult to understand and control the emulsifying properties.

Dextran derivatives have been shown to be efficient polymeric surfactants [5]. Their surface active properties have been correlated to their structural characteristics [5,6]. In addition to their biocompatible properties, these polymeric surfactant exhibit good surface tension lowering effects which make them very attractive in various applications such as emulsion stabilization, emulsion polymerisation and preparation of solid particles with controlled surface properties [6–9].

Enzymatic catalysis is known to be efficient for the modification of polymers and to provide high selectivity [10,11]. It has been applied to the modification of polysaccharides for several years [11–13]. Recently, lipase-catalyzed transesterifications involving polysaccharides were reported in the literature. Among the polysaccharides used were inulin [14], chitin [15]

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and dextran [16]. Nevertheless, no family of polymeric surfactants obtained by enzyme-catalyzed modification has been developed until now. An improved knowledge of the enzyme-catalyzed polysaccharide modification would lead to a better control of the extent of chemical modification which has direct effect on their emulsifying properties.

This work involves the application of enzymatic catalysis to the synthesis of polysaccharide-based surfactants. The polysaccharide will be chemically modified by the attachment of hydrophobic groups through a transesterification reaction with a fatty ester. The kinetics of enzyme-catalyzed modification will be investigated and related to the reaction conditions. The possibility of tuning the extent of polysaccharide modification will be also considered. This last point is fundamental for controlling the physico-chemical properties of the final polymeric surfactants. The outcome of this enzymatic process has tremendous applicability for the development of novel modified polysaccharides with valuable properties for the synthesis of nanoparticles used in drug delivery system and for aqueous formulations.

2. Experimental

2.1. Materials

The protease (EC 3.4.21.62) from *Bacillus subtilis* (Proleather FG-F) and the lipase (EC 3.1.1.3) from *Candida rugosa* (lipase AY) were generous gifts from Amano Enzyme Co. (Nagoya, Japan). The lipase L-1754 (EC 3.1.1.3) from *C. rugosa* was purchased from Sigma (St. Louis, USA). According to the specification sheets, the activities were 12,200 U/g for Proleather FG-F (Lot No. PRD0552709CFG), 32,800 U/g for lipase AY (Lot No. LAYE0151016) and 706 U/mg solid for lipase L-1754 (Lot No. 123K1334), respectively. Dextran T-40 (from *Leuconostoc mesenteriodes*) with average molecular weight (Mn) 26,000 g/mol and Mw 40,000 g/mol according to size exclusion chromatography measurements, was bought from Pharmacia (Uppsala, Sweden). Vinyl decanoate (VD) and dimethyl sulfoxide (DMSO, CROMASOV®) was purchased from Sigma–Aldrich (Buchs, Switzerland) and used without further purification. The membrane used for dialysis with MWCO 6-8000 was purchased from Spectrum Laboratories Inc. (CA, USA). All other chemicals and solvents used in this work were of analytical grade.

2.2. Methods

2.2.1. Preparation of pH-imprinted enzyme

One gram of enzymes (lipase AY, L-1754 or Proleather FG-F) was dissolved in 20 ml of 20 mM phosphate buffer pH 7.5 [16]. The enzymatic mixture was stirred at room temperature for 1 h. Then, the solution was flash-freezing in liquid nitrogen followed by lyophilization with a freeze drier (Labcongo Corp., USA) for 48 h. The water content of the lyophilized powder was determined by moisture analyzer (Sartorius MA 30, Medtel, Australia). The lyophilized pH-imprinted enzyme contained about 5–9% of water.

2.2.2. Stability of pH-imprinted lipase AY in DMSO

A modified assay was used [17]. Twenty millimolars of tributyrin was emulsified in 20 ml phosphate buffer at pH 7.5. Then 2% of Gum Arabic was added to the solution and an emulsion was made with a homogenizer (IKA Ultra Turrax T25, IKA Labortechnik, USA) for 20 s at 10,000 rpm. The operation was repeated three times. Then the temperature and pH of the solution were adjusted to 37 °C and 7.5, respectively. After adding 1 ml of the sample to be assayed (1 mg of enzyme incubated in 50 μl of DMSO at 37 °C or at 50 °C for 1–5 h), the enzyme activity was measured by titrating released fatty acid with 0.05N sodium hydroxide solution using a pH stat titrator (Mettler toledo DL50, Schwerzenbach, Switzerland). The initial velocity was determined from the slope of the graph of fatty acid concentration against time between 0 and 10 min. The same experiment was repeated two times and the average initial velocity was determined. The residual activity (%) was calculated from the percentage of ratio of the unit of activity of incubated enzyme solution at various times to the unit of activity of incubated enzyme solution at time zero.

The control reaction was performed without enzyme, which was replaced by an equal volume of DMSO. One enzyme unit hydrolyzes 1.0 μequiv. of fatty acid from a triglyceride in 1 h at pH 7.5 and at 37 °C (equivalent to approx. 10 μl of CO_2 in 30 min).

2.2.3. Preparation of deactivated enzyme

One gram of lipase AY was suspended in 20 ml of 20 mM phosphate buffer pH 7.5. The enzyme solution was refluxed for 5 h [16]. After which it was allowed to cool at room temperature, flash-freezing in liquid nitrogen and lyophilized in freeze drier for 48 h. The hydrolysis activities were determined using the method described in Section 2.2.2 and equal to 288.9 ± 10.4 and 2.39 ± 1.41 Unit/mg for active and thermally deactivated lipase AY, respectively.

2.2.4. Lipase-catalyzed transesterification of dextran T-40 with vinyl decanoate

A typical mixture used for enzyme screening contained 0.41 M of dextran T-40 (Dex T-40) and 0.20 M of vinyl decanoate (VD) ($[OH]_0/[ester]_0 = 2/1$) in 15 ml of DMSO, with the Dex T-40 being dissolved in DMSO prior to its mixing with the vinyl decanoate. The reaction mixture temperature was raised to 50 °C and kept constant for 5 min before addition of 300 mg of enzyme (lipase AY, L-1754 or Proleather FG-F). The enzymatic solution was left under stirring at 50 °C for the required reaction time. At selected reaction times, the reaction was stopped and the DMSO solution was dialyzed against ethanol–water mixture (85:15, % v/v) for 2 days followed by mixture of 60:40 (% v/v) for 2 days and then with distilled water for 2 days. Finally, the aqueous solution was lyophilized for 48 h (yield *ca.* 79–89%). Control experiments without enzyme and with deactivated enzyme were carried out with the same procedure (yield *ca.* 50%). The extent of dextran modification (%) was determined by ^{1}H NMR in DMSO-d_6. In addition, the effect of enzyme quantity and different molar ratio of Dex T-40 to VD were also investigated.

2.2.5. Analytical methods

^{1}H spectrum was recorded on a BRUKER 300 MHz spectrometer in DMSO-d_6 solution for Dex T-40–VD (62% conversion). ^{1}H NMR (δ, DMSO-d_6, ppm) peaks assigned were: 4.91 (O–*H*4), 4.86 (O–*H*3), 4.66 (H1), 4.51 (O–*H*2), 3.73 (H6), 3.61 (H5), 3.49 (H6), 3.41 (H3), 3.2 (H2), 3.15 (H4), 2.29 (–C*H*$_2$–C=O), 1.25 (–C*H*$_2$–CH$_2$–C=O), 1.23 (–CH$_2$–, 8H), 0.84 (–CH$_3$).

The signal of methylic proton of DMSO, used as reference signal, was set at δ 2.47 ppm. For the extent of modification (%) calculation, signal one (I_1) at 0.84 ppm corresponds to three methylic protons of the hydrocarbon chain of decanoate (I_1 = 3H) and signal two (I_2) of δ from 4.91 to 4.51 ppm (I_2 = 4H) corresponds to two esterified and one proton of anomeric carbon mixed with three non-esterified secondary hydroxyl protons at 2, 3 and 4 position and one proton of anomeric carbon of glucopyranoside unit of Dex T-40, respectively. The extent of modification (%) is given by the equation:

$$\%\text{modification} = \left(\frac{I_1/3}{(I_2 - I_1)/4}\right) \times 100$$

This value represents the percentage of glucopyranoside units of Dex T-40 modified by VD.

The ^{13}C NMR pattern was recorded on a BRUKER 500 MHz spectrometer in DMSO-d_6 solution for Dex T-40–VD (19% conversion). The denotation C*x*–S*y* means that the carbon *x* is adjacent to a substituted carbon *y*. ^{13}C NMR (δ, DMSO-d_6, ppm) signals were assigned as follows: 172.7 (C=O), 98.25 (C1), 95.49 (C1–S2), 74.99 (C3–S3), 73.35 (C3), 71.89 (C2), 70.39 (C5), 70.17 (C4), 66.17 (C6), 60.89 (C6), 34 (–*C*H$_2$–C=O), 31 (–*C*H$_2$–CH$_2$–CH$_3$), 28.72 (–*C*H$_2$–, 4C), 23 (–*C*H$_2$–CH$_2$–C=O), 22.09 (–*C*H$_2$–CH$_3$), 13.99 (–*C*H$_3$). The chemical shifts of C3–S2, C2–S2, C2–S3 and C4–S3 are not presented as interference occurred from the carbon peak of native Dex T-40.

The calculation of decanoate attachment ratio on the 2 position to 3 position of glucopyranosyl residues was done by dividing the intensity of two well separated signal corresponding to C1–S2 at 95.49 ppm by the one of C3–S3 at 74.99 ppm.

FTIR spectra were recorded with the BRUKER IFS 25 spectrometer. The lyophilized powder of samples was mixed with KBr and pressed into pellets under reduced pressure. FTIR (KBr, cm^{-1}) bands represented: 3426 ($\upsilon_{O–H}$), 2926 ($\upsilon_{C–H}$), 1733 ($\upsilon_{C=O}$), 1650($\upsilon_{O–H}$) bending or combination of O–H bond of alcohol and 1011 ($\upsilon_{C–O}$) stretching of C–O bond of 2° alcohol.

3. Results and discussion

3.1. Enzyme screening for the transesterification reaction of Dex T-40 with VD

The enzymatic derivatization of dextran is limited since this polysaccharide is soluble only in the most polar organic solvents such as DMSO, ethylene glycol and dimethyl formamide. Most lipases are readily inactivated because of the unfolding and destruction of hydration shell of enzyme whereas a protease of the subtilisin family was an unusual enzyme that was active in DMSO [18]. Recently, there was a surprising report of the activity of commercial lipase of yeast *C. rugosa* in this non-conventional solvent that makes this enzyme potentially useful for the lipase catalyzed-synthesis of polymeric surfactant derived from dextran [16]. Several lipase samples were screened in the transesterification of Dex T-40 with VD at 50 °C in DMSO. The extent of modification of Dex T-40 determined by ^{1}H NMR after 25 h of reaction is shown in Table 1.

Table 1
Conversion percentage for the transesterification of Dex T-40 with VD catalyzed by lipase (AY or L-1754) and protease (Proleather FG-F) in DMSO, at 50 °C and a reaction time of 25 h

Enzyme	Conversion (%)
pH-imprinted lipase AY	21.64
Non-pH-imprinted lipase AY	2.95
L-1754	2.09
pH-imprinted Proleather FG-F	8.54
Control (without enzyme)	1.93
Control (with deactivated lipase AY)	7.91

Lipase AY was the most efficient enzyme when pH was adjusted. Surprisingly, lipases of the same origin but from different suppliers possessed different transesterification activity. Lipase AY from Amano, for instance, gave 22% conversion while only 2% was obtained with pH-imprinted L-1754 from Sigma. There are three factors which control the activity of biocatalyst in organic solvents: (a) relative proportions of isoenzymes; (b) amount of water in the lyophilized crude enzyme and (c) amount of lipase proteins in the commercial powder [19]. Therefore, the consideration of biochemical composition of *C. rugosa* lipase might be useful to explain this difference in activity. As is commonly known, lipase of *C. rugosa* is composed of three different isoenzymes, Lip 1, Lip 2 and Lip 3. The proportion of isoenzyme varies depending on the origin of the enzymes and conditions used for fermentation. The specificity of Lip 1, Lip 2 and Lip 3 of lipases of *C. rugosa* in catalyzed transesterification of different alcohol with vinyl acetate showed that all three isoenzymes would be able to freely accept the primary alcohols [20]. In contrast, the acylation of cyclic alcohol with long chain fatty acid may be mainly catalyzed by Lip 3 isoenzyme [20]. The difference in specificity of these isoenzymes can be understood by taking into account that Lip 3 is described as a cholesterol esterase. Therefore, its ability to accept highly sterically hindered alcohols could explain why it is capable to fit cholesterol as substrate. Unfortunately, the proportions of Lip 1, Lip 2 and Lip 3 of the commercial lipases AY and L-1754 employed in this study is not known. However, according to the results obtained, it is possible that lipase AY has a higher proportion of Lip 3 than lipase L-1754. In addition, many applications of lipase AY for the modification of cyclic alcohol have been reported in the literature [16,21,22]. The pH-imprinted lipase AY was the best enzyme and it was used in all further experiments.

The pre-treatment of lipase before use can influence the activity of enzyme in DMSO. The activity in catalyzing transesterification was nearly zero when lipase AY was not pH-imprinted (Table 1). The measurement of residual activity of pH-imprinted lipase showed that the pH-adjustment had no effect on the stability of lipase AY. Assay of the residual lipase

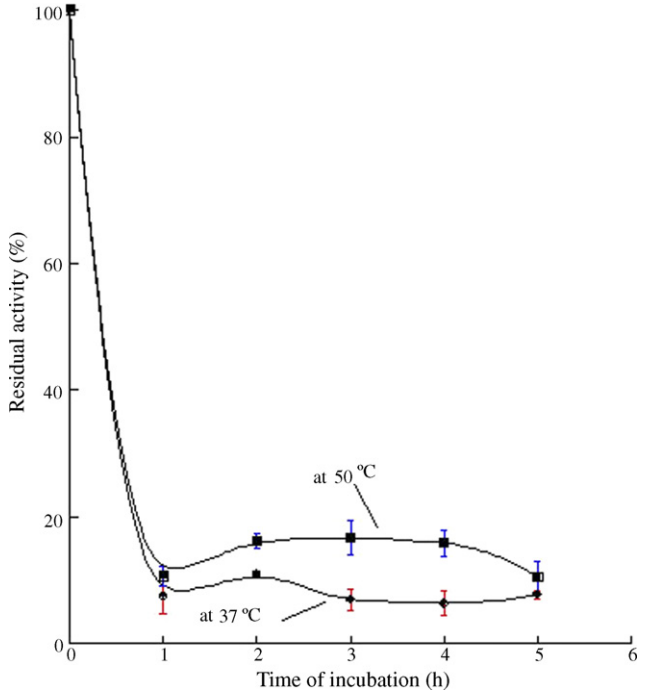


Fig. 1. Residual activity (%) of pH-imprinted lipase AY after 5 h of incubation in DMSO at 37 and 50 °C. Each value is the mean ± S.D. of triplicate measurement. The control reaction contained only DMSO (without enzyme).

activity with tributyrin showed that only *ca.* 7–10% of initial activity was maintained after 5 h of incubation in DMSO at 37 and 50 °C (Fig. 1).

Therefore, the difference in activity might result from the conformation change after pH-adjustment. However, careful control must be performed over the phosphate salt and filler used for enzyme preparation since it was demonstrated that these chemical compounds might catalyze the acylation of hydroxyl-containing compound as sucrose in DMSO when reactive vinyl ester was used [23]. Because the filler used for preparation of lipase AY was not known, we prepared the control reaction with the thermally deactivated lipase AY. In the presence of active lipase (Table 1), the modification percentage (22%) was higher than the results obtained in the absence of enzyme (2%) and in the presence of thermally deactivated lipase AY (8%). The higher modification percentage of the latter comparing with the reaction without enzyme suggests that there was side acylation reaction due to the commercial enzyme preparation. Nevertheless, this chemical acylation was far lower than the modification in the presence of active enzyme. Consequently, the results obtained strongly imply that Dex T-40 acylation by VD in DMSO was enzymatic. It is worth noting that the relative acidity of secondary hydroxyl group at C2 of glucopyranoside unit of dextran is lower than that those of sucrose due to the absence of intramolecular hydrogen bond [24].

In aqueous solution, lipase from *C. rugosa* shows interfacial activation [25]. This activation may be avoided by using organic solvent since all substrates are soluble in DMSO. However, it is necessary to prepare biocatalyst in an open conformation ready for binding with substrate and this conformation is subsequently fixed in DMSO. The incubation of lipase AY in phosphate buffer at pH 7.5 affected the charge density on the surface and might have fixed the conformation of the enzyme in the open form. It has been shown previously that surface charge and conformation were fixed in the organic solvent [26]. The obvious increase in activity of pH-imprinted lipase AY does seem to suggest that the isoenzyme structure of lipase AY was modified into a more favorable form to accept very high sterically hindered alcohols such as Dex T-40.

In this experiment, the conversion of the reaction catalyzed by Proleather FG-F was lower than the one catalyzed by lipase AY. These results contrast with those reported in the literature in which Proleather FG-F has been stated to exhibit higher activity in catalyzing transesterification of dextran with short chain fatty acid ester, i.e. vinyl acrylate [16] and vinyl adipate [22]. One explanation for these contradictory results might be due to the specificity of protease that is defined for short chain vinyl ester. For acylation of polysaccharides with long chain fatty acid like vinyl decanoate, lipases should be the most promising catalyst [27].

3.2. The effect of reaction condition

The effect of enzyme concentration on transesterification was studied by varying lipase AY concentration from 20 to 40 mg/ml of DMSO at fixed molar ratio of VD to dextran glucopyranosyl residues (1:2). The results are illustrated in Fig. 2. An increase in catalyst loading from 20 to 40 mg/ml of DMSO did not have any significant effect on the final conversion percentage. Therefore, the amount of biocatalyst used in this experiment is optimum at

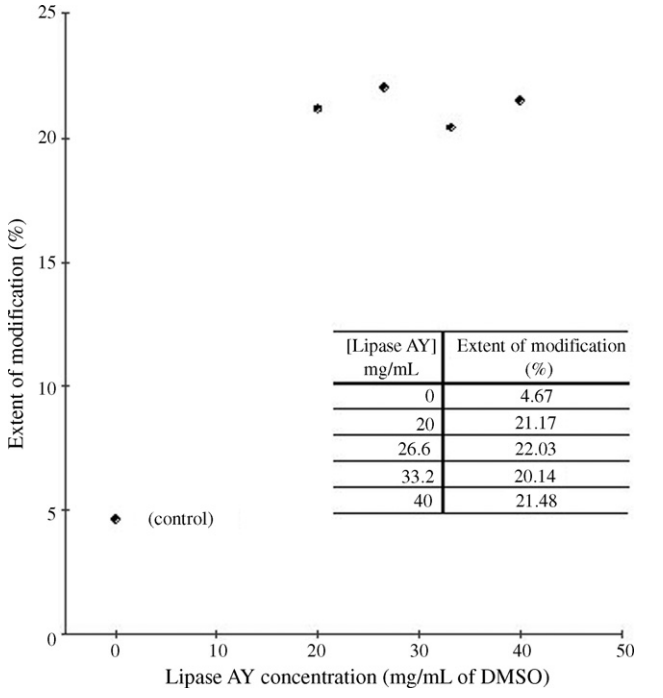
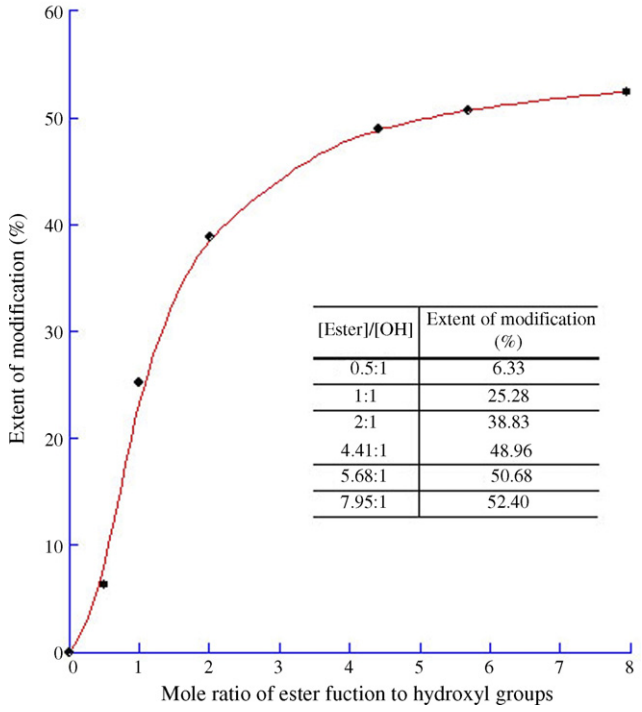


Fig. 2. Conversion as a function of concentration of lipase AY for the reaction of VD with Dex T-40 (1:2 molar ratio of VD to dextran glucopyranosyl residues, 125 h of reaction at 50 °C in DMSO).



[Ester]/[OH]	Extent of modification (%)
0.5:1	6.33
1:1	25.28
2:1	38.83
4.41:1	48.96
5.68:1	50.68
7.95:1	52.40

Fig. 3. Effect of mole ratio of VD to dextran glucopyranosyl residues (0.5:1, 1:1, 2:1, 4.4:1, 5.7:1 and 7.95:1) on % conversion of Dex T-40 by VD, reaction time 24 h at 50 °C in DMSO.

this particular ratio of Dex T-40 to VD. An amount of 26.6 mg lipase AY per milliliter of DMSO was used as a catalyst loading for further studies.

The effect of substrate molar ratio on the % conversion of transesterification was also investigated. In this study, we were only interested in the final conversion obtained after a reaction time of 24 h. The study of the excess of one substrate on the % conversion of Dex T-40 was performed at molar ratio's of VD to dextran glucopyranosyl residues ([ester]/[OH]) of 0.5:1, 1:1, 2:1, 4.4:1, 5.7:1 and 7.95:1.

The appropriate concentration of ester compound is important in lipase-catalyzed acylation since this reaction takes place via the formation of an acyl–enzyme intermediate. As can be seen from Fig. 3, conversion percentage increases according to the mole of ester function at fixed concentrations of alcohol and enzyme. This reaction is considered as a pseudo-single substrate reaction since the VD concentration is varied at a constant concentration of Dex T-40. Interestingly, there was a hyperbolic relationship between the extent of modification after 24 h reaction time and the initial VD concentration. Even the Michaelis–Menten model cannot applied fully here because the velocity is not the initial velocity and the stability of enzyme at 24 h is still questionable; however, the relation between the rate of reaction and the VD concentration is obvious. An excess of VD rapidly increased the conversion percentage of reaction. However, at the ratio of ([ester]/[OH]) higher than 4.4, the increase in molar ratio did not provoke a significant change in the extent of the chemical modification. This ([ester]/[OH]) ratio might be regarded as the concentration of substrate leading to maximum velocity when lipase AY was fixed at 26.6 mg/ml of DMSO. Therefore, the maximum velocity of enzyme is obtained when the VD concentration is *ca.* 0.14 mM.

It is surprising to note that an excess of ester function increased the conversion percentage rather than an excess of nucleophile. In general, the most frequently method of increasing the extent of transesterification is performing the reaction with an excess of nucleophile [28]. The increase of conversion percentage of Dex T-40 could be attributed simply to the mass effects since the tautomerisation of the product; vinyl alcohol, to acetaldehyde shifts the thermodynamic equilibrium of reaction towards the enzymatic modification of Dex T-40. Nevertheless, the limit of the conversion percentage of Dex T-40 at about 0.14 mM of VD may be due to the enzyme denaturation by high concentration of toxic acetaldehyde or just simply by the saturation of enzyme with VD as mentioned in the previous paragraph.

3.3. Rate of Dex T-40 transesterification with VD catalyzed by lipase AY

Despite the loss of enzyme activity in DMSO as mentioned previously (Fig. 1), the conversion of glucopyranosyl units reached *ca.* 40 and 47% after 11 and 26 h reaction time, respectively (Fig. 4). In contrast, the conversion in the absence of enzyme was *ca.* 2% after 11 h. This difference of conversion percentage observed after 11 h reaction time strongly suggested that the transesterification of Dex T-40 with VD in DMSO is enzymatic. Interestingly, a much lower conversion percentage of *ca.* 1.42 and 1.62% was determined in the presence of non-pH-imprinted lipase AY. It seems that the presence of non-pH-imprinted lipase AY protected hydroxyl group of glucopyranosyl unit of Dex T-40 from the chemical transesterification with VD. Broadening of resonance peak corresponding to the three protons of hydroxyl group of glucopyranoside unit was observed in ^{1}H NMR spectra (data not shown) and this may be due to their interaction with enzyme. Further study of this interaction was beyond the scope of this paper.

It is worth noting that after 11 h of reaction, the rate of conversion percentage of pH-imprinted lipase AY-catalyzed transesterification slowed down. The increase of conversion percentage at the interval of 26–103 h is very similar to that in the absence of enzyme calculated from 1 to 76 h as shown in Fig. 4 (inset). This might be taken as evidence that the enzyme is inactive after 11 h of reaction and the gentle rise of the conversion is due simply to the chemical modification of Dex T-40.

The use of vinyl ester to ensure a good irreversibility and the rate of reaction present a drawback on the stability of some lipases. During enzymatic transesterification, vinyl ester is converted into acetaldehyde that turns out to be toxic especially for *C. rugosa* lipase by interaction with ε-NH_2 lysine residues located on the surface of protein [29]. The improvement of lipase AY activity and stability in DMSO is actually in progress in our laboratory.

3.4. Chemical structure of modified polysaccharides

The synthesis of modified Dex T-40 was also confirmed by FTIR analysis and ^{1}H NMR (300 MHz) as mentioned in Sec-

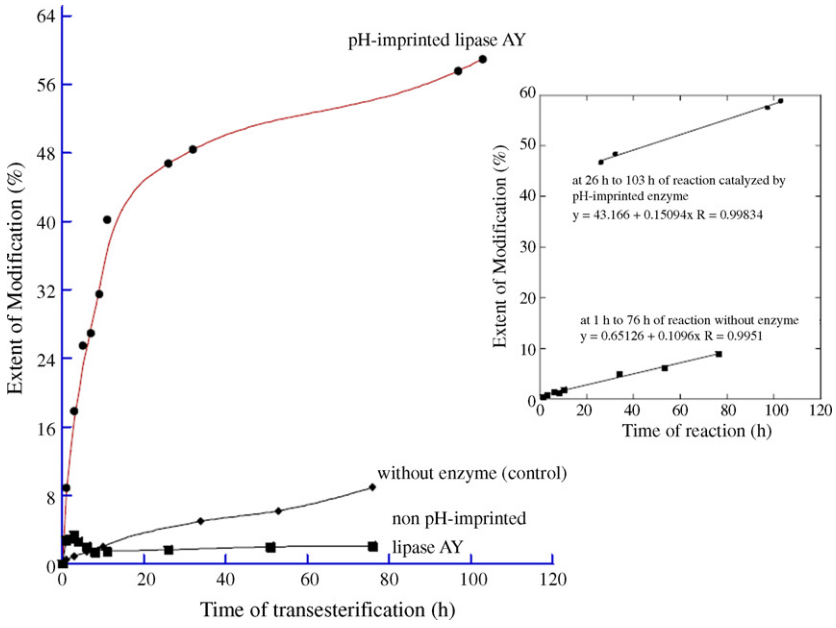


Fig. 4. Time course for the conversion percentage of transesterification of Dex T-40 with VD either in the absence or presence of pH-imprinted lipase AY, non-pH-imprinted lipase AY. Inset shows rate of transesterification catalyzed by pH-imprinted lipase AY during 26–103 h (0.151% conversion/h) and without enzyme during with a reaction time of 1–76 h (0.109% conversion/h).

tion 2.2.5. By FTIR, the absorption at 1721 cm^{-1} assigned to the stretching of the carbonyl group appeared as soon as the decanoate group was attached to the Dex T-40 backbone. The intensity of this band increases with the extent of modification. In contrast, the intensity of the band at 1625 cm^{-1} belonging to bending or combination of O–H bond of alcohol in the spectrum of Dex T-40 decreased with the rise of conversion percentage. By ^{1}H NMR, the signals from the decanoate groups attached to the Dex T-40 backbone were observed at δ 0.84, 1.23, 1.25 and 2.29 ppm.

The positional substitution was analyzed by ^{13}C NMR (500 MHz). According to the literature, acylation of a hydroxyl group of glucopyranoside units results in a downfield shift of the peak corresponding to the *O*-acylated carbon and an upfield shift of the peak corresponding to the neighboring carbon [16,30]. Based on this knowledge and a bi-dimensional NMR experiment (^{1}H–^{1}H COSY and ^{1}H–^{13}C HMQC, data not shown), it was found that the decanoates were substituted both at the 2- and 3-position of glucopyranoside units (Fig. 5). For modified Dex T-40 at various % of modification the signals of substituted C3; C3–S3 shifted downfield at 74.99 and the signal of neighboring carbons of modified C2; C1–S2 shifted upfield at 95.49 ppm (Fig. 5).

These two signals were well separated and were used for calculation of ratio of substitution on position 2 and position 3.

Two positional isomers were obtained: isomer 2 (substitution on the C2 in the glucopyranosyl residues) and isomer 3 (the C3 in the glucopyranosyl residues is substituted). The quantitative ^{13}C NMR was performed for chemical transesterification without enzyme (a, 10% conversion), transesterification catalyzed by pH-imprinted lipase AY for 8 h (b, 19% conversion) and for 103 h (c, 62% conversion). The ratio of isomer 2 to isomer 3 varied slightly at 47:52, 45:55 and 48:52 for a, b and c, respectively. The similar proportion obtained from these three experiments revealed that there was no difference in selectivity toward the secondary hydroxyl group at the 2- and 3-position. This result is in contrast to the chemical reactivity of secondary hydroxyl group in glucopyranosyl residues reported in the literature in which the order of reactivity ranked from high to low is hydroxyl group on C2 > C3 = C4 [31]. Concerning the transesterification catalyzed by pH-imprinted lipase AY for 8 h (Fig. 5b, 19% modification), the similar substitution pattern with the reaction without enzyme indicated that pH-imprinted lipase AY just accelerated the transesterification reaction, resulting in a faster rate without distinguishing between the secondary hydroxyl group on position 2 and position 3. This was confirmed by increasing the reaction time beyond 103 h in which the substitution ratio observed should be due to a combination of chemical and enzymatic reaction as explained formerly in the rate of reaction study. As expected, only a little change in the substitution pattern was observed and the proportion of isomer 2 to isomer 3 of 48:52 was obtained (Fig. 5c).

In this study, our results showed a preferential reactivity in lipase-catalyzed transesterification toward the C2 and C3 hydroxyls of the glucopyranosides unit similar to previous reports [16,32]. However, a discrepancy in C2:C3 ratio was observed. It is important to note that the ratio of isomer 2 to isomer 3 varies according to the type of vinyl ester used. When small and quite reactive vinyl esters such as vinyl acrylate was used, the substitution occurred mainly in the 3-position [16]. Using vinyl ester of medium size and less reactive as vinyl decanoate as in our study resulted in an increase in the percentage of substitution at position 2 whereas nearly the same ratio

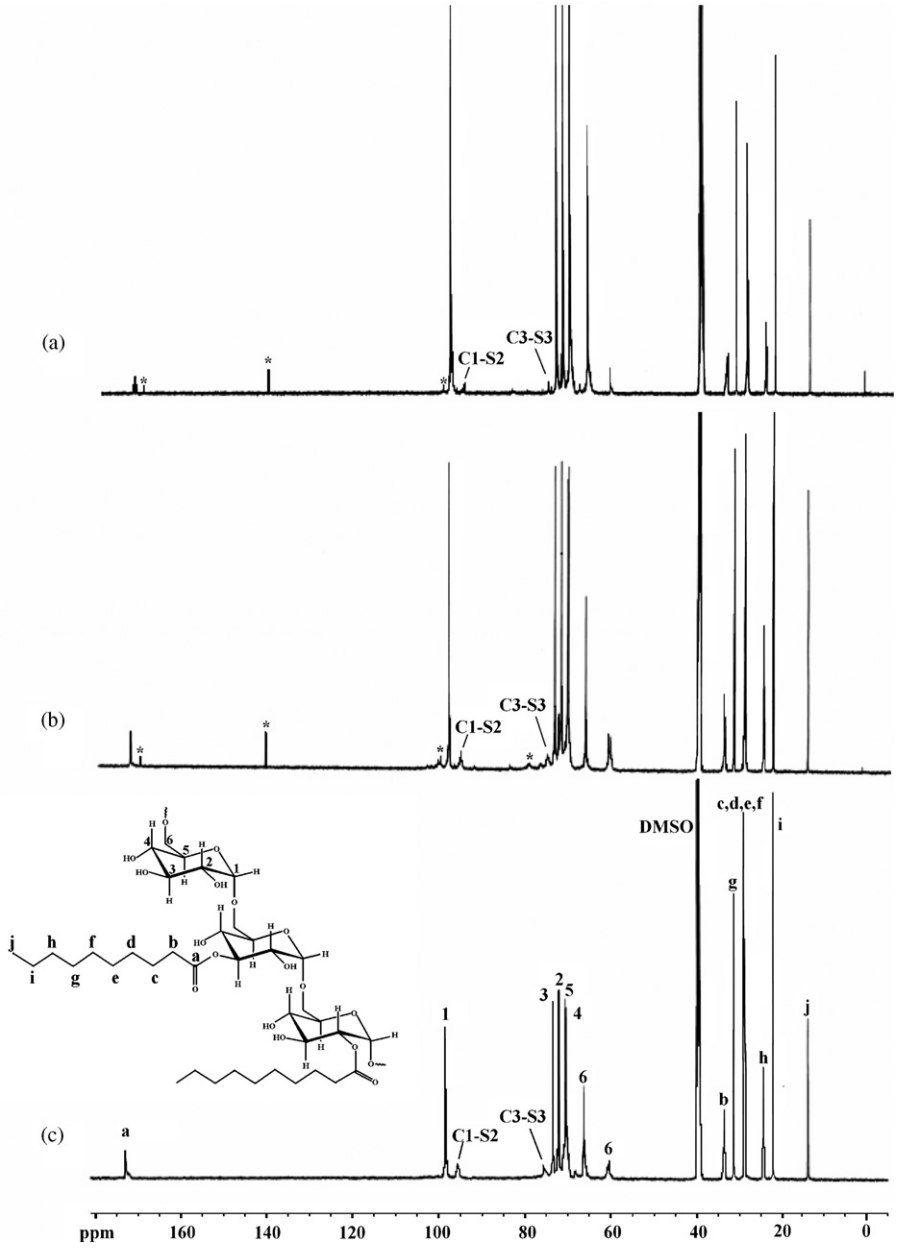


Fig. 5. ^{1}H NMR (500 MHz) spectra in DMSO-d_6 of Dex T-40 transesterified with VD; (a) without enzyme (103 h, 10% modification), (b) catalyzed by pH-imprinted lipase AY (8 h, 19% modification) and (c) catalyzed by pH-imprinted lipase AY (103 h, 62% conversion). * refers to the δ of carbons of residual VD.

of isomer 2 to isomer 3 was obtained. Nevertheless, it might be difficult at this stage to clearly demonstrate the selectivity of lipase AY in attaching the fatty acid chain of various sizes on Dex T-40 backbone if a long reaction time is used to achieve a high extent of conversion. At long reaction times, the substitution pattern obtained should be the combination of enzymatic and chemical substitutions. Considering all these problems, it is necessary to perform the reaction for a very short time with vinyl ester of different reactivity in order to suppress the chemical acylation and to see the relation between the type of acyl donor and the lipase AY regioselectivity. For this purpose, if a high conversion percentage is still required, the improvement of lipase AY stability in DMSO is of prime importance. This might be achieved by the pre-treatment of enzymes by lyophilization in the presence of lyoprotectants (such as sugar) [33] and crown ethers [34]. These experiments have been reported to preserve enzyme structure in organic solvent and enhance their stability. Another possibility is to reduce the amount of enzyme inactivating solvent (DMSO) by using a two-solvent mixture, *tert*-amyl alcohol and DMSO, as a medium of the reaction. This method represents a compromise between enzyme activity and sugar solubility [18]. These works are now in progress.

4. Conclusion

In this article we have shown an enzymatic methodology to prepare novel amphiphilic polysaccharides. pH-imprinted

lipase AY seems to have a specificity toward cyclic alcohol as dextran but works at only 15% of its performance in DMSO relative to the activity in aqueous solutions. DMSO is known as a potent denaturing solvent for most proteins. Changing enzyme concentration, substrate ratio and the rate of reaction leads to modified dextrans of different conversion percentage. The interfacial properties of amphiphilic dextrans obtained by enzyme-catalyzed transesterification in this study are under investigation. The position and extent of the hydrophobic group attached to dextran backbone are fundamental for controlling the physico-chemical properties of the final polymeric surfactants. The study of chemical structure of modified dextran obtained in this work reveals that the distinction between hydroxyl groups at 2- and 3-position of glucopyranosyl residues of lipase AY is not perfect. This is thought to be due to the reactive acyl donor used and the long reaction time needed to achieve a high extent of modification. More experiments will be carried out concerning the enhancement of the stability as well as the activity of enzyme in DMSO and the nature of the acyl donor agent. The outcome of this enzymatic process has tremendous applicability for the development of novel modified polysaccharides with valuable properties for the synthesis of nanoparticles used in drug delivery system and for aqueous formulations.

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