ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 6 (1999) 279–285

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Lipase-catalyzed solid-phase synthesis of sugar esters. Influence of immobilization on productivity and stability of the enzyme [1]

Linqiu Cao, Uwe T. Bornscheuer, Rolf D. Schmid *

Institut für Technische Biochemie, Universität Stuttgart, Allmandring 31, D-70569 Stuttgart, Germany

Received 26 January 1998; revised 16 March 1998; accepted 17 March 1998

Abstract

The lipase-catalyzed synthesis of 6-*O*-glucose palmitate in a mainly solid-phase system was investigated. Lipase from *Candida antarctica* B was immobilized on various carriers by physical absorption or covalent binding. Highest conversion (84%, 24 h) and productivity (0.69 mmol product per gram lipase and hour) were achieved with lipase immobilized on polypropylene (EP 100), whereas other carriers gave at maximum 46% conversion. A good agreement between aquaphilicity of the carrier and conversion was found. The lipase was reused six times and conversion decreased by only approximately 25%. The influence of temperature, organic solvent and fatty acid chain length on lipase stability was also investigated. For the latter, a correlation between the log *P* of the fatty acid (determined from hydrophobic fragment constants) and lipase stability was observed. Palmitic acid and the corresponding vinyl ester gave highest conversion in the acylation of β-D(+)-glucose, whereas tripalmitin, palmitic acid anhydride and the methyl ester allowed only low conversion. Beside β-D(+)-glucose, also other monosaccharides and trehalose were acylated.

Keywords: Lipase; Sugar ester; *Candida antarctica*; Solid phase

1. Introduction

Sugar fatty acid esters are widely used as non-ionic surfactants in cosmetic and food applications. Current chemical production is based on a high-temperature esterification between sugars and fatty acids, using an alkaline catalyst leading to a mixture of products. Alternatively, sugar fatty acid esters can be obtained by fermentation as so-called biosurfactants [1]. The direct esterification of sugar and fatty acid using isolated enzymes—mainly lipases—is hampered by the low solubility of sugars in most organic solvents. Good conversions can be achieved in pyridine [2], but this solvent is incompatible with food applications. Other solutions are based on the use of alkylglycosides [3] or protected sugars like isopropylidene [4] or phenylboronic acid [5] derivatives, which require additional synthesis steps.

* Corresponding author. Tel.: +49-711-685-3193; fax: +49-711-685-3196; e-mail: rolf.d.schmid@rus.uni-stuttgart.de.

[1] Dedicated to Professor Hideaki Yamada in honor of his 70th birthday.

1381-1177/99/$ - see front matter
PII: S1381-1177(98)00083-6

We have shown recently [6,7], that the lipase-catalyzed synthesis of a wide variety of sugar fatty acid esters can be performed in a mainly solid phase system containing a small amount of solvent. This solvent—'adjuvant'—is necessary to create a catalytic liquid phase in which the reaction occurs. Furthermore, the sugar fatty acid ester precipitates from the reaction mixture shifting the equilibrium towards product formation. Best results were found in acetone or *t*-butanol and up to 87% conversion in the esterification between β-D(+)-glucose and palmitic acid were achieved using the commercial immobilized lipase preparation from *Candida antarctica* B (Novo). However, at least 48 h reaction time were necessary. In the present paper, we have performed further optimizations to increase productivity and reduce reaction times by immobilization and a detailed investigation of further reaction parameters.

2. Materials and methods

2.1. Lipases and chemicals

Lipase from *C. antarctica* B (CAL-B) was used as free (SP525) and immobilized (SP435) preparation and was a gift from Novo Nordisk, Bagsvaerd, Denmark. Amberlite XAD-7, β-D(+)-glucose, sorbitol, mannitol, lauric acid, and myristic acid were obtained from Sigma, Deisenhofen, Germany. Celite 545, Duolite 568, Hyflo Super Cel, *t*-butanol, dioxane, methanol, dichloromethane, ethylacetate, acetic acid, sulphuric acid, *t*-amyl alcohol, D(+)-galactose, fructose and D(+)-mannose were purchased from Fluka, Buchs, Switzerland. Acetone, acetonitrile and tetrahydrofuran were purchased from Riedel de Haën, Seelze, Germany. Inositol and palmitic acid were obtained from Merck, Darmstadt, Germany. Caprylic acid and caproic acid were gifts from Henkel, Düsseldorf, Germany. Polypropylene (EP 100) was a gift from Akzo Nobel Faser, Obernburg, Germany. Vinyl palmitate was from TCI, Tokyo, Japan.

2.2. General procedure for enzymatic reactions

The reaction mixture consisted of equimolar (typically 0.5 mmol) free carbohydrate and fatty acid, 2 eq. (w/w of substrates) organic solvent (usually *t*-butanol) serving as adjuvant and 0.2 eq. (w/w of substrates) of activated molecular sieve (4 Å, 10 mesh) for the adsorption of water generated during esterification. The reaction mixture was incubated in a capped vial, placed in an oil bath, thermostated to 60°C and stirred by a magnetic bar (250 rpm). The reaction was started by the addition of lipase preparation. Product isolation and sample analysis by thin layer chromatography and HPLC were performed as described previously [6,7].

2.3. Immobilization

2.3.1. Celite, Hyflo Super Cel or Amberlite XAD-7

200 mg free lipase from *C. antarctica* B was dissolved in 10 ml phosphate buffer (20 mM, pH 7.0) on ice and 2 ml chilled acetone was added. After addition of 2 g carrier, the mixture was stirred for 2 h. Upon addition of 10 ml chilled acetone the mixture was filtrated and washed several times with chilled acetone. The immobilized enzyme was dried in a desiccator for 2 h.

2.3.2. Polypropylene (EP 100)

One gram free lipase from *C. antarctica* B was dissolved in 100 ml phosphate buffer (20 mM, pH 6.0), 5 g EP 100 (pre-wet with 25 ml acetone) was added to the solution and stirred at room temperature for 5 h. The immobilized lipase was collected by filtration and washed with distilled water and phosphate buffer (50 mM, pH 7.0) followed by drying in a desiccator.

2.3.3. Silica gel or PEG

Immobilization was performed following a modified protocol from Cho and Rhee [8]. Acti-

vation: to 250 ml *p*-aminopropyl-triethoxysilane in acetone (1%) 10 g silica (80–800 mesh) or PEG 2000 monomethyl ether was added. After evaporation of acetone the mixture was heated overnight at 110°C. Immobilization: to 4 g of this activated carrier, 40 ml 2.5% glutaraldehyde was added and the reaction mixture was stirred at room temperature for 1 h. After thorough washing with water, 40 ml 1% (w/v) lipase solution (free CAL-B in phosphate buffer, 50 mM, pH 7.0) was added and the mixture was stirred for 2 h. The immobilized enzyme was collected by filtration and washed several times with distilled water and phosphate buffer (20 mM, pH 7.0) and dried overnight in a desiccator. PEG modified CAL-B was further immobilized on EP 100 by adsorption as described above and was abbreviated as CAL-B-PEG-EP.

2.3.4. Magnetic polypropylene

Four grams of polypropylene EP 100 was prewetted with 15 ml ethanol and 60 ml distilled water was added. To this mixture 20 ml $FeSO_4$ solution (1.5%) was added under bubbling with nitrogen gas and 4 ml hydrogen peroxide (0.06%) was slowly added to the solution while stirring at 50°C. The pH was adjusted to 10.0 by addition of 3 N sodium hydroxide solution. When the color of the mixture became dark, the polymer was collected by filtration and washed with distilled water and dried under vacuum. To 2 g of this polymer 6 ml ethanol and 40 ml 1% (w/v) lipase solution (free CAL-B in phosphate buffer, 20 mM, pH 6.0) were slowly added and the mixture was stirred at room temperature overnight. The immobilized enzyme was collected by filtration and washed several times with distilled water and phosphate buffer (20 mM, pH 7.0) and dried overnight in a desiccator.

2.4. Enzyme reuse

Immobilized lipase was separated from the reaction mixture after addition of *n*-hexane to dissolve sugar and sugar fatty acid ester. The lipase was collected by filtration, washed with *n*-hexane and dried in a desiccator before reuse. The conversion achieved in the first cycle was set to 100%. In a similar manner, the influence of temperature or organic solvent on the residual activity of CAL-B-EP was determined.

2.5. Determination of hydrolytic activity of lipase

Hydrolytic activity of lipases was assayed in a pH-stat system (Metrohm, Herisau, Switzerland) with 5% (w/v) olive oil emulsion (pH 8.0) containing 2% (w/v) gum arabic and 22% (w/v) $CaCl_2$ at 37°C. After addition of a known amount of lipase (dissolved or solid) liberated fatty acids were titrated automatically with 0.1 N NaOH. One unit (U) of lipase activity was defined as the amount of enzyme which liberates 1 μmol fatty acid per min under assay conditions.

3. Results and discussion

3.1. Choice of lipase and immobilization on different carriers

According to our previous studies [7], lipase from *C. antarctica* (CAL-B) was selected and used as immobilized preparation (SP435) or the free enzyme (SP525) was immobilized onto several carriers by absorption as well as covalent binding. After immobilization, the hydrolytic activity of all lipases was determined in the pH-stat assay using olive oil as substrate (Table 1). Highest conversions in the synthesis of 6-*O*-glucose palmitate were found for CAL-B immobilized on polypropylene (84%, CAL-B-EP, magnetic CAL-B-EP (82%) and CAL-B-PEG-EP (80%)), whereas the commercial CAL-B-SP435 gave only 41% conversion after 24 h reaction time in accordance to our previous results. From all other supports investigated, only Amberlite XAD-7 led to an acceptable conversion of 46%. No correlation between the

Table 1
Influence of support on the CAL-B-catalyzed solid phase synthesis of 6-*O*-glucose palmitate

Support	Aquaphilicity[a]	Hydrolytic activity (U/g)	Conversion (%)
Duolite 568	3.33	239	15
Activated silica	1.74	23	< 2
Celite545	1.52	157	3
Hyflo Super Cel	0.62	230	13
Activated PEG	n.d.	399	25
Amberlite XAD-7	0	25	46
CAL-B-PEG-EP	0.22	378	80
Polypropylene (EP)	0.22	418	84
Magnetic EP	0.22	420	82
CAL-B-SP435	n.d.	12	41

[a]Aquaphilicity was measured according to the method described by Reslow et al. [9].
n.d., not determined.

hydrolytic and the synthetic activity could be derived. For instance, Celite-immobilized CAL-B shows a moderate hydrolytic activity of 157 U/g (13 times higher than CAL-B-SP435) but gave only 3% conversion in the synthesis of 6-*O*-glucose palmitate. On the other hand, immobilization on Amberlite XAD 7 gave a hydrolytic activity of only 25 U/g, but high synthetic activity (46%) was observed. In order to find a rationale for the large differences found between these carriers, the supports were arranged according to their aquaphilicity [9]. The hydrolytic activity was totally independent of the aquaphilicity, but the conversion in the synthesis of 6-*O*-glucose palmitate increased with decreasing aquaphilicity of the support; the more hydrophobic the carrier material, the higher the conversion (Table 1). We suggest that the aquaphilicity of the support leads to an unfavored partition of water between enzyme and support, thus stripping essential water from enzyme molecules and reducing the lipase activity. In contrast, with hydrophobic support materials the partition of the lipophilic fatty acids in the pore of the support might be favored leading to the observed increase in synthetic activity. Other factors such as pore size, specific surface area and the immobilisation method might also play a role.

Immobilization of CAL-B on EP 100 in the presence of $FeSO_4$ results in a magnetic carrier, which allows similar conversions as CAL-B-EP, but in addition facilitates an easy separation of the immobilized lipase from the reaction mixture.

Table 2
Acylation of glucose using different palmitic acid derivatives as acyl donors using commercial CAL-B-SP435 (reaction time 48 h)

Acyl donor	Conversion (%)
Palmitic acid	76
Palmitic acid methylester	4
Palmitic acid vinylester	80
Tripalmitin	17
Palmitic acid anhydride[a]	16

[a]Diacylation was observed.

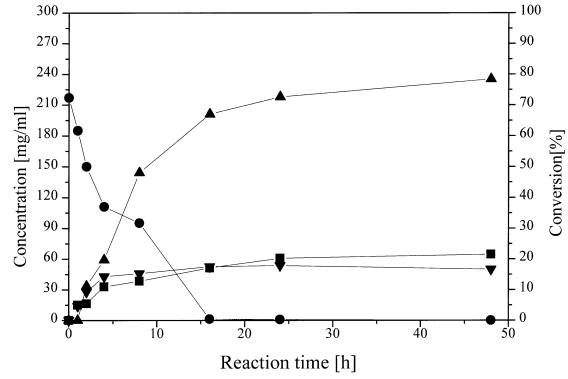

Fig. 1. Time-course of the solid-phase synthesis of 6-*O*-glucose palmitate using vinyl palmitate as acyl donor and lipase B from *C. antarctica* (SP435); ▲, conversion of sugar; ■, concentration of product in the liquid phase; ●, concentration of vinyl palmitate in the liquid phase; ▼, concentration of fatty acid in the liquid phase.

Table 3
CAL-B-EP-catalysed solid phase synthesis using different sugars as acyl acceptors

Sugar	Conversion (%)	Productivity (mmol/g h)
β-D(+)-glucose	84 (87)	0.69 (0.30)
D(+)-mannose	74 (62)	0.59 (0.22)
D(+)-galactose	46 (13)	0.37 (0.05)
D(−)-fructose	80 (n.d.)	0.64 (n.d.)
D(+)-maltose[a]	4.3 (n.d.)	0.036 (n.d.)
D(+)-sucrose[a]	2.6 (n.d.)	0.021 (n.d.)
D(+)-lactose[a]	0 (0)	0 (0)
D(+)-trehalose[a]	100 (n.d.)	0.52 (n.d.)
D(+)-mannitol	79 (35)	0.63 (0.12)
D(+)-sorbitol	90	0.72 (0.34)
inositol	0 (0)	0 (0)

[a] 0.5 mmol disaccharide was used.
Values in brackets refer to reactions with CAL-B-SP435 under similar conditions, but reaction time was 72 h instead of 24 h.
n.d., not determined.

3.2. Choice of acyl donor

Beside palmitic acid, a range of palmitic acid derivatives were used as acyl donors in the reaction with β-D(+)-glucose using CAL-B-SP435. It is obvious from Table 2 that highest conversions were achieved with palmitic acid (76%) or the corresponding vinyl ester (80%). For the latter, the high conversion was due to the introduction of an irreversible step by tautomerization of the released vinyl alcohol to acetaldehyde. The only disadvantage of vinyl esters is their relatively high price compared to free fatty acids. A time course of this reaction is shown in Fig. 1, after approximately 16 h more than 70% conversion were achieved. All other substrates gave considerably lower conversions and palmitic acid methylester was found to be the worst acyl donor (4%).

Table 4
Log *P* values of different fatty acids, their solubility in water and residual activity of CAL-B-SP435 after 72 hours reaction time

Fatty acid	Log *P*[a]	Solubility in water[b] (g/l)	Residual activity (%)
Stearic acid	8.1	0	100
Oleic acid	7.7	0	100
Palmitic acid	7.1	0	96
Myristic acid	6.1	0	95
Lauric acid	5.1	0	90
Capric acid	4.1	0.15	70
Caprylic acid	3.0	0.68	n.d.
Caproic acid	1.9	9.7	n.d.

[a] Calculated from the hydrophobic fragment constant [11].
[b] At 20°C as given by the supplier.
n.d., not determined.

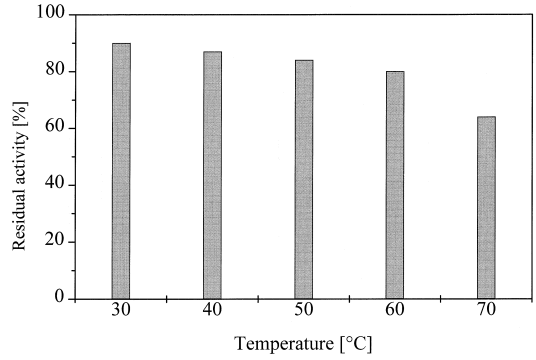

Fig. 2. Influence of temperature on the stability of CAL-B-EP after 24 h in the acylation of β-D(+)-glucose with octanoic acid.

3.3. Acylation of other sugars using CAL-B-EP

In our previous publications, we have shown that the lipase-catalyzed solid-phase synthesis also occurs with sugars other than β-D(+)-glucose [6,7]. It was found that all monosaccharides with primary hydroxyl groups were acylated, but the activity of CAL-B-SP435 was quite diverse. Immobilization of CAL-B on EP

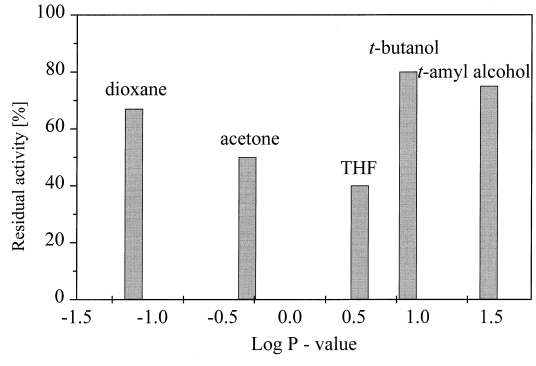

Fig. 3. Influence of organic solvent on the stability of CAL-B-EP after 24 h in the acylation of β-D(+)-glucose with octanoic acid.

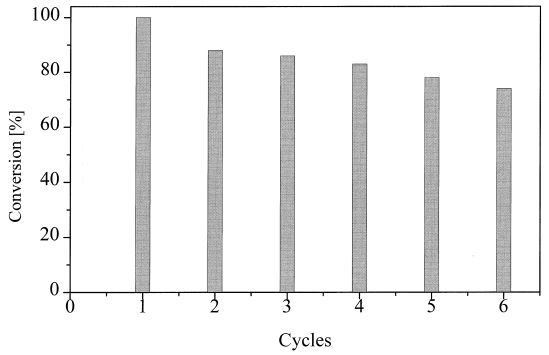

Fig. 4. Long-term stability of CAL-B-EP in the acylation of β-D(+)-glucose with palmitic acid at 60°C. One cycle corresponds to 24 h reaction time.

100 led to a further increase in conversion and usually a two-fold higher productivity compared to SP435 using a variety of monosaccharides was achieved (Table 3), although the reaction time was only one third. The activity of CAL-B-EP towards most disaccharides such as sucrose, lactose and maltose, was very low, but with trehalose complete conversion was possible (Table 3).

3.4. Stability of lipase

For technical applications, the reusability of the enzyme is of considerable importance. Thus, we investigated the long-term stability of CAL-B and CAL-B-EP in the solid-phase system. Decreased residual activity of CAL-B took place with decreased fatty acid chain lengths. With palmitic or stearic acid no change in the activity was observed, but with shorter fatty acids, approximately 30% activity (capric acid) was lost after 24 h (Table 4). In the solid-phase system, the polarity of the reaction phase is influenced not only by the organic solvent, but also by the fatty acid, which significantly contributes to the liquid phase. The influence of the sugar or sugar fatty acid ester on lipase stability can be neglected due to their low solubility (e.g., 3 mg β-D(+)-glucose per ml *t*-butanol). The log *P*-concept is often used to explain the catalytic behavior of enzymes in organic solvents and it was observed that in hydrophobic solvents usually better results can be achieved [10]. To take into account the polarity of the fatty acid, log *P* values were calculated from the hydrophobic fragment constant according to Rekker [11]. Indeed, log *P* values decreased with decreasing chain length of fatty acids and this might explain the lower conversions and lipase stabilities observed using short chain fatty acids (Table 4).

CAL-B-EP exhibits highest activity around 60°C, but at temperatures above 40°C enzyme deactivation also takes places (Fig. 2). At 70°C almost 35% loss in activity was observed. Beside *t*-butanol other organic solvents can also serve as adjuvants to maintain a catalytic phase in the solid-phase system [7]. Acetone, especially, allows high conversions as *t*-butanol, but the enzyme looses almost half of its activity in acetone, whereas the stability in *t*-butanol and *t*-amyl alcohol is considerably higher (Fig. 3).

Finally, the long-term stability of CAL-B-EP during six reaction cycles (corresponding to six days reaction time) was investigated. It was found that even without addition of fresh lipases only approximately 25% reduction of the conversion occurred (Fig. 4).

Acknowledgements

We would like to thank Novo Nordisk, Bagsvaerd, Denmark and Amano, Nagoya, Japan for their generous gift of lipases, and the Konrad Adenauer Foundation, Bonn, Germany, for their financial support to Linqiu Cao.

References

[1] A. Fiechter, Trends Biotechnol. 10 (1992) 208.
[2] M. Therisod, A.M. Klibanov, J. Am. Chem. Soc. 108 (1986) 5638.
[3] K. Adelhorst, F. Björkling, S.E. Godtfredsen, O. Kirk, Synthesis, 1990, p. 112.
[4] G. Fregapane, D.B. Sarney, E.N. Vulfson, Enzyme Microb. Technol. 13 (1991) 796.
[5] I. Ikeda, A.M. Klibanov, Biotechnol. Bioeng. 42 (1993) 788.

[6] L. Cao, U.T. Bornscheuer, R.D. Schmid, Fett/Lipid 98 (1996) 332.
[7] L. Cao, A. Fischer, U.T. Bornscheuer, R.D. Schmid, Biocatal. Biotransform. 14 (1997) 269.
[8] S.W. Cho, J.S. Rhee, Biotechnol. Bioeng. 41 (1993) 204.
[9] M. Reslow, P. Adlercreutz, B. Mattiasson, Eur. J. Biochem. 172 (1988) 573.
[10] C. Laane, J. Tramper, Chemtech. 20 (1990) 502.
[11] R.F. Rekker, The Hydrophobic Fragment Constant, Elsevier, Amsterdam, 1977.