Kinetic resolution of (*R*,*S*)-1,2-isopropylidene glycerol (solketal) ester derivatives by lipases

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ARTICLE INFO

ABSTRACT

A study on the enantioselective hydrolysis of (*R*,*S*)-1,2-isopropylidene glycerol (4-hydroxymethyl-2,2-dimethyl-1,3-dioxolane, solketal) octanoate catalyzed by different lipases was carried out. Among them, *Pseudomonas sp.* lipase proved to be the most effective. It was shown that the ester bearing the longer octanoyl acyl chain is a more suitable substrate for this lipase compared to the acetate counterpart. By properly combining enzyme load, temperature and reaction time, either the (*S*)-alcohol or the remaining ester could be obtained in moderate to high selectivities. Ethyl acetate was found to be the best solvent for the kinetic resolutions effected by such lipase but our results show that toluene may prove useful.

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1. Introduction

The interest in industrial enzyme technology is increasing, especially in the areas of protein engineering and enzymology in unconventional media, which greatly expanded the potential application of enzymes as catalysts in industrial processes.

Lipases (EC 3.1.1.3) represent a group of affordable biocatalysts which generally have high stability in organic solvents, do not require co-factors, have high specificity for the substrate, exhibit high levels of enantioselectivity and operate under mild conditions of temperature and pH.

The use of enzymes, especially lipases in the resolution of racemates or synthesis of molecules for production of chiral drugs, agrochemicals and pesticides with high enantiomeric purity has become widespread, due to the advantages biocatalysts offer over traditional chemical routes [1–3].

Lipases commonly show high enantioselectivity towards secondary alcohols or ester derivatives, but in general exhibit low enantioselectivity towards primary substrates, the case of IPG and its derivatives in such cases, enantioselectivities usually range from low to moderate [4].

4-Hydroxymethyl-2,2-dimethyl-1,3-dioxolane or 1,2-*O*-isopropylidene glycerol (IPG), also called solketal, is a primary alcohol, prepared from glycerol by reaction with acetone. The formed acetal function blocks the central and one terminal hydroxyl groups. [5]. Solketal is an important chiral synthon in the synthesis of diglycerides, glyceryl phosphates, tetraoxaspiroundecanes and many biologically active compounds, such as glycerolphospholipids, β-blockers (aryloxypropanolamines such as (*S*)-propanolol), prostaglandins and leucotrienes [6–8].

Enantiomerically pure (*R*)-and (*S*)-IPG are commercially available, at high price, as their production by standard synthetic methods is costly and laborious [9,10]. The enantioselective hydrolysis of (*R*,*S*)-IPG esters is difficult to achieve with commercially available enzymes. In fact, the best results of enantioselective hydrolysis of ester derivatives of racemic IPG reported so far were attained by the use of purified esterases from yeast *Kluyveromyces marxianus* and *Bacillus* sp. In the former case, the first studies employed whole cells [5,7,11,12].

The aim of the present study was to investigate the resolution of ester derivatives of IPG catalyzed by commercial lipases (Fig. 1). Thus, the effects of reaction parameters such as temperature, concentration of enzyme and solvent, as well as the size of the acyl group, on conversion and enantiomeric excesses (*ee*) were taken into account.

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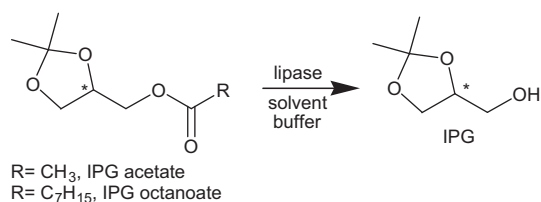
R= CH$_3$, IPG acetate
R= C$_7$H$_{15}$, IPG octanoate

Fig. 1. Lipase-catalyzed resolution of racemic IPG esters.

2. Experimental

2.1. General

Lipases from *Rhizopus orzae* (Amano F), *Pseudomonas fluorecens* (Amano AK, hydrolytic activity of 180U/g), *Candida rugosa* (Amano AY), *Aspergillus niger* (Amano A) and *Pseudomonas cepacia* (Amano PS) were purchased from Sigma Chemical Co. (St. Louis, USA). *Candida antarctica* lipase B (Novozyme 435) was a gift from NOVO (Bagsvaerd, Denmark). One unit (U) of enzyme activity was defined as the amount of enzyme which was able to catalyze the release of 1.0 μmol of *p*-nitrophenol per min and was expressed as specific activity (U/g of protein). The IPG esters were prepared in our laboratory as described below.

The solvents ethyl acetate, toluene and hexane were purchased from Vetec Química Fina (Rio de Janeiro, Brazil).

2.2. Esters of (R,S)-IPG

The chemicals used in this study were purchased from Sigma Chemical Co (St. Louis, USA). (R,S)-IPG-acetate [13] and octanoate [13,14] were synthesized by reaction of IPG with Ac$_2$O or octanoyl chloride according to literature procedures. The isolated products were purified by Flash Chromatography (Silica gel neutralized with 2% Et$_3$N, prior to elution with ethyl acetate–hexane mixtures).

2.3. Hydrolysis of racemic IPG ester derivatives

Hydrolysis of IPG esters was carried out in screw-capped tubes containing 3 mL of solvent, 3 mL of sodium phosphate buffer at pH = 7.0 and different enzyme concentrations. The reactions were initiated by adding 5 μl of IPG esters and the tubes were incubated in a thermostatized reactor. The control reaction (Blank) was carried out at the same condition in the absence of the enzymes.

2.4. Analytical methods

Samples (25 μl of each phase) were taken at determined intervals and mixed with 450 μl de acetonitrile. The enantiomeric composition and conversion were routinely determined by gas chromatographic (GC) analysis on a CHROMPACK CP 9000 equipped with a hydrogen flame ionization detector and a chiral capillary column (Hydrodex®-β-6TBDM, diameter 0.25 mm, length 25 m, thickness 0.25 μ).

The temperature of the injector and detector were maintained at 250 °C and 280 °C, respectively. The carrier gas was nitrogen and, after injection, the column temperature was kept at 100 °C for 1 min and then programmed to rise 2 °C/min reaching 106 °C. From 106 °C, the column temperature was raised to 160 °C at a rate of 40 °C/min and then to 165 °C at 1 °C/min, at which temperature it was maintained for 15 min.

The absolute configuration of produced IPG was determined by comparison to samples enantiopure commercially available samples (Sigma-Aldrich). The retention times for (R)-IPG, (S)-

Table 1
Hydrolysis of IPG esters using Novozyme 435 (T = 35 °C, 2 h).

Substrate	Organic solvent	X (%)	ee (P) (%)	ee (S) (%)
(R,S)-IPG acetate	Hexane	50	0.4	0.4
(R,S)-IPG octanoate	Hexane	30	5	3
(R,S)-IPG acetate	Ethyl acetate	9	30	4
(R,S)-IPG octanoate	Ethyl acetate	7	6	0.4

X (conversion,) P and S (refers to product and substrate, respectively).

Table 2
Hydrolysis of IPG esters using Amano AK (T = 35 °C, 2 h).

Substrate	Organic solvent	X (%)	ee (P) (%)	ee (S) (%)
(R,S)-IPG acetate	Hexane	11	23	4
(R,S)-IPG octanoate	Hexane	50	28	28
(R,S)-IPG acetate	Ethyl acetate	16	2	0.4
(R,S)-IPG octanoate	Ethyl acetate	6	99	6

X (conversion,) P and S (refers to product and substrate, respectively).

IPG, (R)-IPG acetate, (S)-IPG acetate, (R)-IPG octanoate and (S)-IPG-octanoate were 3.6, 3.8, 4.1, 4.6, 10.8 and 11.0 min, respectively.

Conversions (X) and enantiomeric excesses (ee) (P = hydrolysis product; S = ester substrate) were determined according to Chen et al. [15]. Enantioselectivity (E) was determined using conversions and ee by means of equations by Faber and Hoenig [16].

3. Results and discussion

3.1. Exploratory experiments of resolution of (R,S)-IPG esters

The acetyl and octanoyl derivatives of racemic IPG were screened against two lipases (Novozyme 435 and Amano AK) at 35 °C, pH = 7 and for 2 h in a biphasic medium (Fig. 1). The concentration of the substrates in this step was 4.1 mM and the load of enzymes, 50 mg. Tables 1 and 2 present the conversions (X) and enantiomeric excesses (ee) for these substrates obtained in 2 h-reactions.

The experiments with Novozym 435, a lipase widely employed for resolutions of racemates, with either hexane or ethyl acetate as co-solvents, did not afford good results. Despite the observed high conversions, especially in the reactions in hexane, the selectivities were very low. This result confirms that IPG is a challenging substrate.

Conversely, Amano AK displayed high selectivity (ee (P) = 99%, E > 200) in the hydrolysis of the octanoate derivative at a low conversion (with ethyl acetate), as shown in Table 2. It was found that, as it had occurred in the reactions of Novozyme 435 with hexane, Amano AK led to high conversions when IPG octanoate was the substrate. The activity of the enzyme is dependent on the amount of water associated with the enzyme, and to a lesser degree on the water content in the whole system. As long as a minimal amount of water is associated with the enzyme, its activity in organic media is retained. It is known that hydrophilic solvents (low Log P values) may deactivate these enzymes by removing H$_2$O molecules which are essential to their structure [17]. Accordingly, the more hydrophilic ethyl acetate promoted a lower hydrolysis rate what might have fostered more effective enantiomer discrimination by Amano AK in the reaction of IPG octanoate. Activity measurements did show a steep decrease for the catalyst in the reaction run with ethyl acetate (vide infra). On the other hand, we did not observe significant decrease in activity for the lipase which catalyzed the reaction in hexane (14%-decrease after 48 h), as expected [18]. Possibly, ethyl acetate might alternatively act as an inhibitor of the reactions.

These preliminary results also indicated a role for the acyl group in the reaction catalyzed by Amano AK. It is known that, in resolutions of ester derivatives of primary alcohols, change of the acyl group may increase the enantioselectivity of these processes, possibly by inducing a different transition state structure [4,5]. However, conclusions on this phenomenon, or the changes in the lipase structure caused by more hydrophilic solvents, are far beyond the scope of our study. Furthermore, Miyazawa et al. [19] reported that the *ee* of the acylation of 2-phenoxy-1-propanol catalyzed by lipase AL could be improved from 1.1 to 19 by replacing vinyl trifluoroacetate with vinyl butanoate as acylating agent. Liu et al. [20] had already shown that the enzyme from *Bacillus thermocatenulanatus* prefers longer acyl chains. Indeed, the use of a longer octanoyl chain proved beneficial in the reactions of IPG under study, more notably in the reaction in ethyl acetate (Table 2). At lower conversion, it was possible obtain (S)-IPG in high *ee*.

3.2. Enzyme screening

Having established IPG octanoate and ethyl acetate as a suitable substrate/solvent combination, for the intended resolution, a screening of enzymes (50 mg) for 2 h- and 24 h-reactions with ethyl acetate as co-solvent was undertaken (Fig. 2). All enzymes but Amano A led to (S)-IPG, preferentially. Amano AK lipase stood out in the assays as it displayed higher consistency. It was shown to be highly stereoselective in the formation of both IPG and unreacted IPG octanoate. Moreover, while Amano F also produced (S)-IPG in high *ee* at very low conversion, the *ee* of this compound dropped substantially after 24 h in the context of a very slow hydrolysis. Conversely, both Amano PS and Amano AY showed higher specificity in the formation of IPG octanoate in high *ee*.

The performance of Amano F is intriguing, as the produced IPG̈s *ee* sharply from >99% (2 h) to less than 40% (24 h), while conversion proceed to 16%, only. After 48 h, conversion increased little (reached 30%) and *ee* of both product and substrate remained virtually unchanged. The used catalysts consist of enzyme pools which might also contain substances, such as stabilizing agents. In fact, the enzyme fraction responsible for the desired stereoselectivity may be more susceptible to deactivation (by the medium or the mentioned agents). This could explain performances such as that of Amano F. Nevertheless, without knowing the purity of such preparations, the precise producing organism, it is not possible to draw valuable conclusions on the performance differences of the enzymes that showed high E. However, as this initial investigation only intended to identify enzyme preparations showing good potential, we did not carry out a detailed study on the supposed inactivation processes. Amano AY (93% conversion after 24 h) and Amano PS (87% idem) led to 100% conversion after 48 h. These enzyme reagents could possibly afford higher enantioselectivity (IPG octanoate) if the reactions were shortened, tough. Nevertheless, having secured better results with Amano AK, we chose to focus on such catalyst.
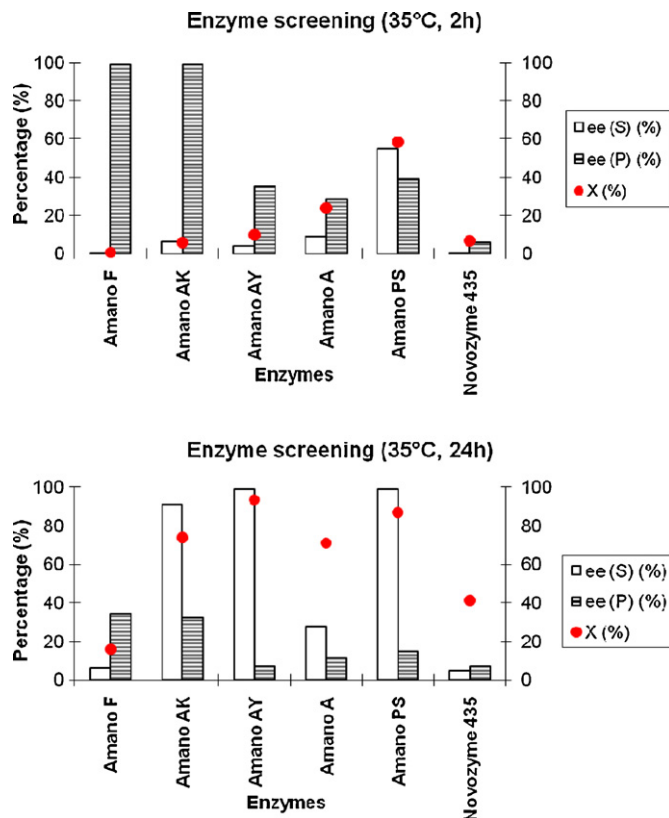

Fig. 2. Hydrolysis reactions of (R,S)-IPG octanoate with lipases in ethyl acetate at 2 h and 24 h.

3.3. Temperature and enzyme concentration evaluation for Amano AK

From a practical viewpoint, it is desirable to decrease the amount of biocatalyst in the resolution under study. Thus, with this in mind, further studies were performed by varying the mass of biocatalyst (Amano AK), reaction time and temperature (Table 3). Moreover, we decided to investigate the effect of medium, with a focus on solvent hydrophobicity. The partition coefficient (Log P) provides a useful measure of such property. Log P is very sensitive to even small polarity differences in a quite broad range. In addition, such parameter may be easily determined from the standard method or calculated from hydrophobic fragmental constants [17,21]. It is known that solvent polarity can affect the activity and enantioselectivity of lipases. Thus, toluene, a solvent of intermediate polarity compared to hexane and ethyl acetate, was included in this study.

Our data confirm the feasibility of decreasing the enzyme load (15 mg or 5 mg; 150 U/g). Moreover, as expected, the solvent nature
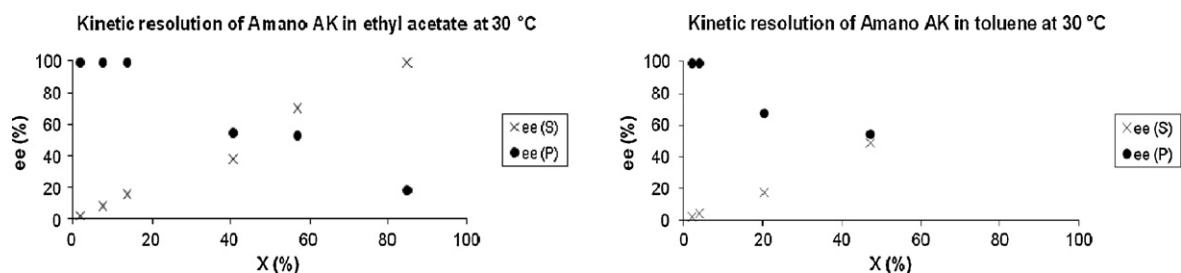
Table 3

Hydrolysis reactions of (R,S)-IPG octanoate in 5 h varying Amano AK concentrations, solvent and temperatures.

Solvent	Enzyme mass (mg)								Temperature (°C)
	5				15				
	X (%)	ee (P) (%)	ee (S) (%)	E	X (%)	ee (P) (%)	ee (S) (%)	E	
Ethyl acetate	7	99	8	>200	26	67	23	6.3	30
Toluene	4	99	4	>200	9	78	8	8.8	
Hexane	88	13	99	4,6	82	16	72	2.6	
Ethyl acetate	9	99	10	>200	16	99	18	15	10
Toluene	8	99	9	>200	21	99	27	200	
Hexane	72	39	99	3.1	96	4	99	3.1	

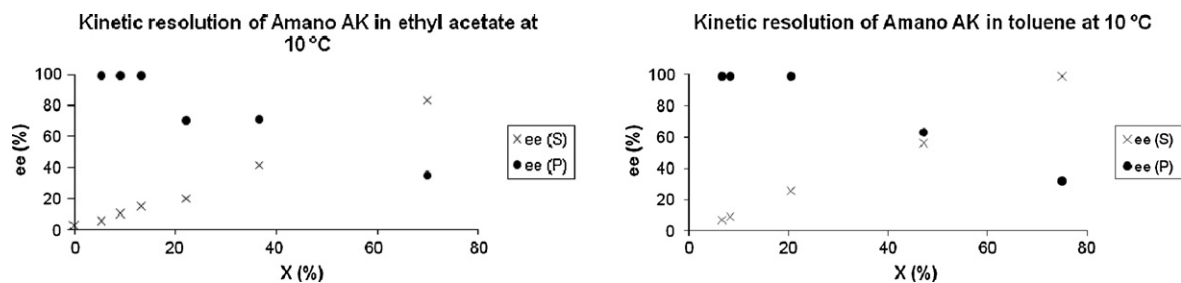**Fig. 3.** Kinetic resolution of (R,S)-IPG octanoate with 5 mg of enzyme Amano AK at 30 °C.

Fig. 4. Kinetic resolution of (R,S)-IPG octanoate with 5 mg of enzyme Amano AK at 10 °C.

affected the catalyst performance. Ethyl acetate ($\text{Log} P = 0.7$) and toluene ($\text{Log} P = 2.5$) stood out as co-solvents as they afforded moderate to high enantioselectivities ($E = 15$–200) in the 5 h-assays. The reactions with hexane ($\text{Log} P = 3.5$) did not perform well, confirming the results discussed before (Tables 1 and 2).

Moderate enantiomeric ratios ($E > 10$) enable efficient resolutions [22]. Under ideal conditions, reactions displaying $E \geq 100$ allow the formation of both product and residual substrate in high ee. On the other side, exceedingly high E values ($E > 200$) may result from deactivation (solvent, impurities, as mentioned before) or intrinsic uncertainties of the analyses. Small variations on ee cause significant change in E. Finally, one should note that the E values suppose that the reaction (first-order) proceeds at initial rate.

Decrease of temperature to 10 °C had a clearly positive effect on the resolutions with 15 mg of catalyst. Such combination provided satisfactory E values and higher conversions.

The beneficial effect of low temperatures in the resolution of solketal derivatives had been established by a previous study [23]. Nevertheless, for higher selectivities to be achieved, the reaction was carried out below −20 °C and a higher load of enzyme had to be employed. Naturally, not resorting to reaction cooling makes the process more practical. On the other hand, the stability of lipases increases at lower temperatures.

Our data show that IPG octanoate may be satisfactorily prepared as well by extending the reaction. For instance, the resolution at 10 °C with ethyl acetate and 5 mg of Amano AK provided IPG ocatanoate in 71% ee ($E = 9$) after 24 h. Moreover, a 48 h-quantitative experiment under essentially the same condition (30 °C) produced 22% yield of (R)-IPG octanoate (99%ee).

We observed that the catalyst activity in the reactions with ethyl acetate at 10 °C does not decrease after 48 h (about 14 U/mL and 40 U/mL for 5 mg and 15 mg of catalyst, respectively). Conversely, in the reactions at 30 °C with the same solvent, a 80%-decay occurs. However, for reactions with toluene, we observed 40%-decay, irrespective of the temperature, in the same time.

Figs. 3 and 4 show the results of the study of the resolution by Amano AK (5 mg) run in ethyl acetate and toluene at 30 °C and 10 °C, respectively. The information that these graphics convey confirms the discussions above. In particular, toluene (at 10 °C) appears to be promising concerning IPG formation. This would depend on a close follow-up of conversion. As for the enantioselective formation of IPG octanoate, these results and a time course study carried out for a 15 mg-load of lipase in ethyl acetate at 30 °C (not shown) altogether suggest that this process may be improved (higher yields and ee), possibly by employment of an intermediate load of enzyme.

Overall, we have established that combinations of reaction time, temperature and lipase load may be worked out to produce either IPG or IPG octanoate in moderate to high E values.

4. Conclusions

The lipase from *Pseudomonas fluorecens*, Amano AK, is effective in promoting the resolution of (R,S)-IPG esters. This enzyme shows a preference for longer acyl chains in the resolution of ester derivatives of IPG (Solketal). Our study established that it is feasible to obtain either IPG or unreacted IPG octanoate in moderate to high ee. Finally, ethyl acetate is the solvent of choice for these reactions, although our assays pointed out to toluene as an alternative. It is noteworthy that the established process is very simple and involves cheap solvents and an accessible glycerol derivative. Moreover, due to the fact that pure enzymes are employed, instead of whole cells, catalyst recovery through immobilization is possible. Through such technology, the use of more stable and active enzymes might lead to further improvements of this process.

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