

Communication

## Lipase-catalysed sequential kinetic resolution of $\alpha$ -lipoic acid

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**Abstract:** Lipase from *Aspergillus* sp. WZ002 was employed to kinetically resolve racemic  $\alpha$ -lipoic acid by a sequential esterification process. Though the remoteness of this substrate's stereocentre from the reaction centre provided a significant challenge, the introduction of sequential kinetic resolution dramatically enhanced the lipase's enantioselectivity for esterification at the terminal carbonyl group, producing the desired (*R*)-enantiomer virtually enantiomerically pure. The enantiomeric excess of the (*R*)-enantiomer increased from 52% in the first step to 92% for in second step.

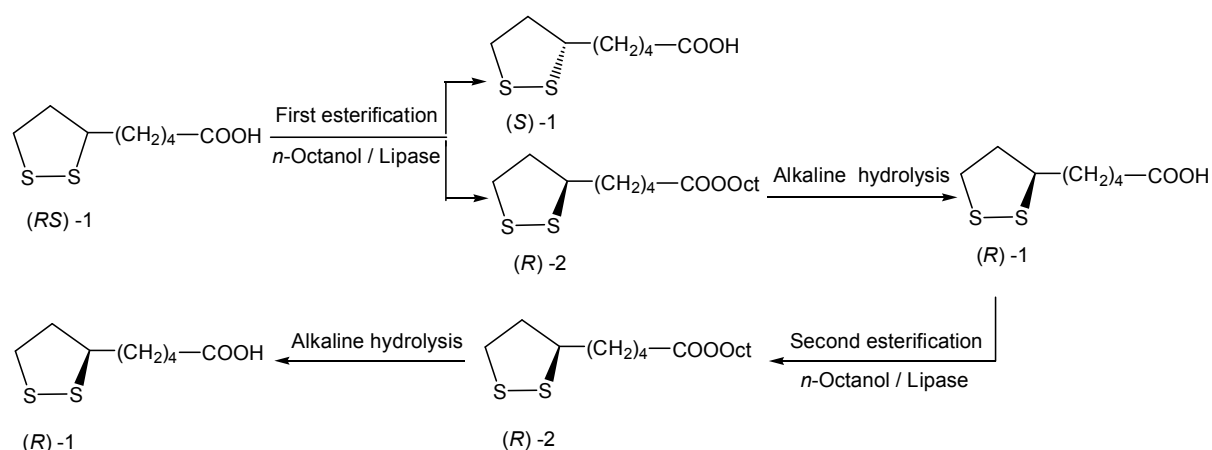
**Keywords:** esterification, lipase-catalysed reactions,  $\alpha$ -lipoic acid, sequential kinetic resolution

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### INTRODUCTION

(*R*)- $\alpha$ -Lipoic acid is a naturally occurring cofactor of several  $\alpha$ -keto acid dehydrogenases and a growth factor for a variety of microorganisms [1-3]. It has also been reported that  $\alpha$ -lipoic acid and its derivatives are highly active as an anti-oxidant [4], anti-inflammatory agent [5], anti-HIV [6] and anti-tumour [7]. Generally, the (*R*)-enantiomer is much more active than the (*S*)-enantiomer [8], which has fostered significant interest in stereoselective synthesis of the pure enantiomers. Chemical synthesis of (*R*)- and (*S*)- $\alpha$ -lipoic acid has been achieved either from a 'chiral pool' starting material [9] or by asymmetric synthesis [10-11]. Alternative methods involving enzyme catalysis include Bakers' yeast reduction [12], mono-oxygenase catalysis [13] and lipase-catalysed kinetic resolution [14-15].  $\alpha$ -Lipoic acid provides a significant challenge due to the remoteness of stereocentre located four carbon atoms away from the reaction centre (carboxylic group). Lipases from *Aspergillus oryzae* WZ007 [14] and

*Candida rugosa* [15] show enantioselectivity towards the (*S*)-enantiomer, leaving the target (*R*)- $\alpha$ -lipoic acid in unreacted form. In this study, the tested lipases show opposite enantioselectivity towards the (*R*)-enantiomer (Table 1) although with a low enantiomeric excess. In order to improve the enantiomeric purity, a sequential kinetic resolution was undertaken (Scheme 1). Herein we report on a successful application of sequential biocatalytic resolution of (*R*)- $\alpha$ -lipoic acid with high enantiomeric purity.



**Scheme 1.** Sequential kinetic resolution of  $\alpha$ -lipoic acid ((*RS*)-1) with lipase

## MATERIALS AND METHODS

### Chemicals and Reagents

$\alpha$ -Lipoic acid (purity  $\geq 98.0\%$ ) was purchased from Fluka BioChemika (Switzerland). (*R*)- $\alpha$ -lipoic acid (purity  $\geq 98.0\%$ ) was purchased from Aladdin Reagent (China). Porcine pancreas lipase (Type II) was purchased from Sigma (USA). Nov 435 (an immobilised lipase from *Candida antarctica*) and Lip $\square$  TL (an immobilised lipase from *Thermomyces lanuginosus*) were purchased from Novozymes (Denmark). Lipase from *Penicillium expansum* was purchased from Shenzhen Leveking Bio-engineering (China). All other chemicals were obtained from commercial sources and were of analytical reagent grade.

### Production of Lipase from *Aspergillus* sp. WZ002

The strain WZ002 of *Aspergillus* sp., isolated from carrion and conserved in our laboratory, was maintained on potato dextrose agar (PDA) medium. A sequence analysis revealed that the internal transcribed spacer (ITS) DNA sequence of the strain WZ002 (GenBank accession no. JQ670919) showed high similarity (100% homology) to 47 strains of *Aspergillus* sp. The strain WZ002 was therefore primarily identified as a strain of *Aspergillus* sp. The culture was grown aerobically at 30°C and 200 rpm for 48 hr in cell growth medium consisting of glucose (10 g/L), peptone (5 g/L),  $\text{KH}_2\text{PO}_4$  (1 g/L),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.5 g/L),  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (0.01 g/L), KCl (0.5 g/L) and olive oil (10 mL/L). After harvesting by filtration, cells were washed with 100 mM Tris-HCl buffer (pH 7.3) and then freeze-dried. The lyophilised microbial cells (intracellular lipase) were used to catalyse the esterification reaction.

### Screening of Lipase

Lipase from *Aspergillus* sp. WZ002 (ASL), porcine pancreas lipase (PPL), lipase from *Penicillium expansum* (PEL), lipase from *Candida antarctica* (Nov 435) and lipase from *Thermomyces lanuginosus* (Lip TL) were investigated in the catalysis of the esterification of  $\alpha$ -lipoic acid with *n*-octanol [14]. The reaction mixture was made of  $\alpha$ -lipoic acid (206 mg, 1 mmol), *n*-octanol (0.79 mL, 5 mmol), heptane (20 mL) and an appropriate amount of lipase. The reaction mixture was shaken at 200 rpm and after a specified time the reaction was quenched by removing enzyme particles through centrifugation. Unreacted  $\alpha$ -lipoic acid was extracted with 0.5% NaHCO<sub>3</sub> and recovered with dichloromethane after acidification with 20% HCl. Dichloromethane was removed by vacuum distillation and the recovered  $\alpha$ -lipoic acid was analysed by high performance liquid chromatography (HPLC).

### Effect of Time on ASL-catalysed Esterifying Reaction

To investigate the effect of time on the conversion ratio and enantiomeric excess of (*R*)-1 at the first step of the esterification reaction (Scheme 1), the reaction mixture consisting of  $\alpha$ -lipoic acid (206 mg, 1 mmol), *n*-octanol (0.79 mL, 5 mmol), heptane (20 mL) and ASL (400 mg) were shaken at 200 rpm and 40°C for 48, 60, 72 and 84 hr. Unreacted  $\alpha$ -lipoic acid was extracted with 0.5% NaHCO<sub>3</sub>, and the solvent of the resulting upper organic phase was removed by vacuum distillation to enrich ester (*R*)-2, which was hydrolysed to the corresponding (*R*)-1 by alkaline hydrolysis. The resulting enriched (*R*)-1 was subjected to HPLC analysis.

### Sequential Kinetic Resolution

The esterification reaction was carried out at 200 rpm and 40°C on a shaker. The reaction mixture was made up of  $\alpha$ -lipoic acid (206 mg, 1 mmol), *n*-octanol (0.79 mL, 5 mmol), heptane (20 mL) and ASL (400 mg). The reaction was quenched after 84 hr. Unreacted  $\alpha$ -lipoic acid was extracted with 0.5% NaHCO<sub>3</sub> and the solvent of the resulting upper organic phase was removed by vacuum distillation to enrich ester (*R*)-2. The ester was dissolved in 20 mL of 95% ethanol and mixed with 150 mg of NaOH. The mixture was stirred for 6 hr at room temperature. The solvent was removed by vacuum distillation and the residue was partitioned with 10 mL of heptane and 20 mL of distilled water. The resulting aqueous phase was acidified by 20% HCl. The enriched (*R*)-1 was obtained by extraction with dichloromethane from the acidified solution.

(*R*)-1 was then subjected to a second enzymatic resolution by the same procedure.

### Analysis

An Agilent 1100 HPLC with a Chiralpak AS-H column (250 mm×4.6 mm, 5  $\mu$ m, Daicel) was used to analyse the conversion ratio and enantiomeric excess of  $\alpha$ -lipoic acid. Hexane:2-propanol:trifluoroacetic acid (97:3:0.1) was used as eluent at a flow-rate of 0.8 mL/min. Absorbance of column effluent was monitored at 220 nm [14]. The two enantiomers of  $\alpha$ -lipoic acid were identified in the HPLC chromatogram by their different retention times using optically pure (*R*)- $\alpha$ -lipoic acid as reference compound. The enantiomeric ratios (*E*) were calculated using the following equations [16]:  $E = \ln[(1-c)(1-ee_S)]/\ln[(1-c)(1+ee_S)]$ ,  $c = [(c_0-c_e)/c_0] \times 100\%$ ,  $ee_S = [(S)-[R]]/([S]+[R]) \times 100\%$ ,  $ee_R =$

$[(R)-[S])/([S]+[R])]\times 100\%$ , where  $c$  is the conversion ratio of reaction,  $c_0$  is the initial amount of racemic  $\alpha$ -lipoic acid,  $c_e$  is the amount of residual  $\alpha$ -lipoic acid at the end of reaction,  $ee_S$  is the enantiomeric excess of the residual  $\alpha$ -lipoic acid, and  $[R]$  and  $[S]$  are the peak areas for (*R*)- $\alpha$ -lipoic acid and (*S*)- $\alpha$ -lipoic acid respectively.

## RESULTS AND DISCUSSION

### Screening of Lipase

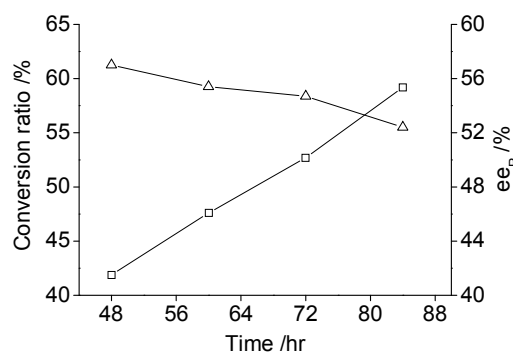
The results were summarised in Table 1. All lipases tested showed (*R*)-stereopreference and considerable conversion ratio, but the enantiomeric ratios (*E*) of the transformations were very low. After 84 hr of transformation with ASL (59.2% conversion), the ester (*R*)-2 was separated from the unreacted substrate (*S*)-1; then the ester (*R*)-2 was hydrolysed under alkaline conditions to yield the corresponding (*R*)-1 enantiomer ( $ee_R$  52.4%) (Scheme 1, Figure 1). PPL and PEL afforded 64.3% and 57%  $ee_S$  of the unreacted substrate ((*S*)-1) at 90.1% and 79.8% conversion respectively, and their *E*-values were close to 2.0. When immobilised Nov 435 and Lip TL were used, a high activity (>72% conversion after 1 hr) was observed, but the unreacted enantiomer ((*S*)-1) was obtained in poor enantiomeric excess, which was also shown in their *E*-values, suggesting that both enantiomers might easily enter into the catalytic active site of the enzymes, leading to little enantioselectivity by the enzymes [17]. Thus, ASL with the highest enantiomer selectivity ( $E = 3.4-3.6$ ) was chosen as the biocatalyst in sequential esterification to resolve  $\alpha$ -lipoic acid.

**Table 1.** Kinetic resolution of  $\alpha$ -lipoic acid by different lipases

Enzyme	Temp. (°C)	Time (hr)	$c$ (%)	$ee_S$ (%)	Preferred enantiomer	<i>E</i>
ASL (400mg)	40	60	47.6	38.0	<i>R</i>	3.5
	40	72	52.7	43.6		3.4
	40	84	59.2	54.4		3.6
PPL (400 mg)	37	4	63.6	32.8	<i>R</i>	1.9
	37	5	71.0	43.9		2.1
	37	6	90.1	64.3		1.8
PEL (1000 mg)	37	3	39.6	17.0	<i>R</i>	2.0
	37	4	59.2	33.1		2.1
	37	5	79.8	57.0		2.1
Nov 435 (50 mg)	26	0.5	54.8	9.3	<i>R</i>	1.3
	26	1	77.6	20.0		1.3
Lip TL (50 mg)	26	0.5	46.7	8.7	<i>R</i>	1.3
	26	1	72.5	17.2		1.3

### Effect of Time on ASL-catalysed Esterifying Reaction

Figure 1 plotted the time course for esterification of racemic  $\alpha$ -lipoic acid ((*RS*)-1) by ASL. The conversion ratio increased significantly with increase in reaction time. On the contrary, enantiomeric excess of the hydrolysate of ester (*R*)-2 ( $ee_R$ ), namely enriched (*R*)-1, decreased a little and ranged between 52.4-57%. So the reaction time of 84 hr, with 59.2% *c* and 52.4%  $ee_R$ , was selected in order to obtain a higher yield of the target (*R*)-enantiomer.



**Figure 1.** Time course for ASL-catalysed esterification of  $\alpha$ -lipoic acid ((*RS*)-1) at the first resolution step:  $\square$  = conversion ratio;  $\Delta$  =  $ee_R$  of (*R*)-1)

### Sequential Kinetic Resolution of $\alpha$ -Lipoic Acid

As shown in Scheme 1, racemic  $\alpha$ -lipoic acid ((*RS*)-1) was first subjected to enzymatic resolution to give ester (*R*)-2. After hydrolysis of the ester (*R*)-2, the resulting (*R*)-1 was then subjected to the second enzymatic resolution at about 65% conversion to furnish ester (*R*)-2. Subsequent hydrolysis of this ester (*R*)-2 furnished (*R*)-1 with an average  $ee$  of 92%, based on chiral chromatographic analysis (Table 2). Both the enantioselectivity of the lipase and the conversion ratio were clearly enhanced in the second resolution step, which could possibly be explained by the faster reacting (*R*)-enantiomer furnishing the major substrate in the second step.

**Table 2.** Second resolution of enriched (*R*)-1 by ASL

Reaction time (hr)	<i>c</i> (%)	$ee_R$ (%)
48	59.3	92.8
60	65.6	91.6
72	66.7	93.2

Note: Reaction condition: a mixture of enriched (*R*)-1 (52%  $ee$ , 1 mmol), *n*-octanol (5 mmol), heptane (20 mL) and ASL (400 mg) was shaken at 200 rpm and 40°C.

### CONCLUSIONS

A new and convenient way to prepare (*R*)- $\alpha$ -lipoic acid has been developed through a sequential kinetic resolution by *Aspergillus* sp. WZ002. Even though the substrate has a stereogenic centre four

carbon atoms away from the reaction site and a low *E*-value was exhibited in the first step, high enantiomeric purity (ee > 91%) of the target (*R*)-enantiomer was obtained at a high conversion ratio in the second step. The success of this method confirms the value of sequential kinetic resolution as an important approach in enzymatic resolution, especially in cases where a remote stereocentre is present.

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