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Journal of Molecular Catalysis B: Enzymatic 48 (2007) 64–69
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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Resolution of 2-octanol by SBA-15 immobilized *Pseudomonas* sp. lipase

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Received 8 January 2007; received in revised form 7 June 2007; accepted 7 June 2007
Available online 14 June 2007

Abstract

Lipase from *Pseudomonas* sp. (PSL) was immobilized on SBA-15 (a highly ordered hexagonal array mesoporous silica molecular sieve) through physical adsorption and the immobilized PSL was used in resolution of (*R*,*S*)-2-octanol with vinyl acetate as acyl donor. Enhanced activity and enantioselectivity were observed for the immobilized PSL compared with those of the free one. The effects of reaction conditions, such as solvents, temperature, water activity and substrate ratio were investigated. Under the optimum conditions, the residual (*S*)-2-octanol was recovered with 99% enantiomeric excess at 52% conversion. The results also indicated that the immobilized PSL maintained 90% of its initial activity even after reusing it five times.

Keywords: Lipase; SBA-15; Immobilization; Resolution; 2-Octanol; Enantioselectivity

1. Introduction

In 1998, Zhao et al. [1] reported the synthesis of SBA-15, a new kind of mesoporous molecular sieve. It possesses large surface area, high pore volume and abundant surface silanol groups [2], which makes it highly suitable for immobilization. Since then it has received much attention in the field of catalysis, especially for their use as supports. Crosman and Hoelderich [3] immobilized chiral rhodium diphosphine complexes on Al-SBA-15 and high activities and reusability were observed with these supported organometallic complexes in hydrogenation of dimethyl itaconate. Xiang et al. [4,5] reported that the enantioselectivity of chiral Mn (salen) complex immobilized on SBA-15 was notably increased because of the unique spatial environment imposed by the mesopores. Recently, the use of SBA-15 as support for enzyme immobilization have been of particular interest because the pore size of SBA-15 (6–30 nm) which is approximately the same as those of the enzymes molecules [6,7] should allow the bulky enzyme molecules to diffuse into the pore. On the other hand, the terminal silanol groups present on the surface of SBA-15 may facilitate immobilization of enzymes via hydrogen bonding and enclosure of the protein in a well-defined space which may also help prevent denaturing of the protein and enhance enzyme stability. Vinu et al. [8] and Deere et al. [9] have observed adsorption loadings as high as 500 mg/g for cytochrome *c* on SBA-15. Adsorption of lysozyme and bovine serium albumin (BSA) on SBA-15 has also been studied by Katiyar et al. [10] and a very high affinity and capacity for proteins is observed. In many cases, enzymes immobilized on SBA-15 exhibit not only improved stability and reusability, but also enhanced activity. He et al. [11] have observed that lipase from *Porcine pancreatic* immobilized on SBA-15 exhibits high activity in hydrolysis of olive oil and Zheng et al. [12] have described the immobilization of lipase from *Pseudomonas cepacia* on SBA-15, and find that the activity, stability and reusability of immobilized enzyme are superior to those obtained from free enzyme. While the advantages of using SBA-15 as enzyme supports have been widely demonstrated, however, there remains relatively little research so far on the applications of these immobilized enzymes in resolution reactions, and demonstration of their utility remains a pressing concern.

In the present study, lipase from *Pseudomonas* sp. is immobilized on SBA-15 through physical adsorption and the

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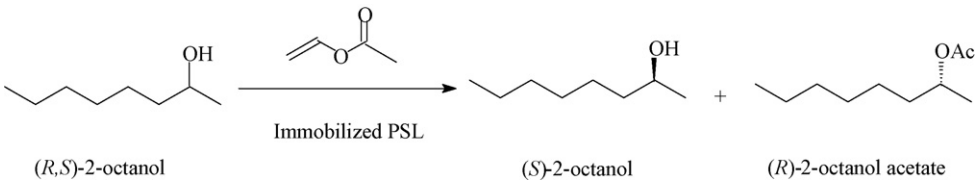
1381-1177/$ – see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.molcatb.2007.06.002



Scheme 1. Resolution of 2-octanol catalyzed by the immobilized PSL.

immobilized PSL is used in resolution of (*R*,*S*)-2-octanol with vinyl acetate as acyl donor (Scheme 1). The effects of various reaction conditions were also investigated.

2. Materials and methods

2.1. Materials

SBA-15 was kindly donated by College of Chemistry, Jilin University (Changchun, China) and was dried at 200 °C in an oven for 2 h before use. Lipase from *Pseudomonas* sp. (PSL) and *Candida cylindracea* A.Y. lipase (AYL) was purchased from Amano Pharmaceutical Co. Ltd. (Japan). *Mucor miehei* lipase (MML) was purchased from Novo (Bagsvaerd, Denmark). *Porcine pancreatic* lipase (PPL) was purchased from Shanghai Dongfeng Biochemical Reagent Co. Ltd. (China). *Candida lipolytic* lipase (CLL) was provided by Wuxi enzyme preparation plant (China). The lipase powder of BSL was over expressed from *Bacillus subtilis* strain IFFI10210 in our laboratory according to the method we have reported [13]. The enzyme was lyophilized and used without further purification. Folin substrate, 2-octanol, vinyl acetate and other organic solvents of analytical grade were purchased from Shanghai Chemical Reagent Company (China). *R*-(+)-1-Phenylethyl isocyanate (*R*-(+)-PELC) was purchased from Fluka (USA, 97%).

2.2. Preparation the enzyme solution

PSL powder (1 g) was dispersed in phosphate buffer (100 ml, pH 8.0, 0.1 M) at 4 °C for 2 h under stirring, and the insoluble impurity was removed by centrifugation (8000 rpm, 5 min). Finally the supernatant was lyophilized. Enzyme solution (10 mg/ml) was prepared by dissolving the lyophilized PSL (1 g) in phosphate buffer (100 ml, pH 8.0, 0.1 M).

2.3. Immobilization of PSL

SBA-15 (1 g) was suspended in enzyme solution (30 ml) at 20 °C for 24 h under stirring. Then the immobilized PSL were separated from the supernatant by centrifugation (8000 rpm) and washed with the phosphate buffer (pH 8.0, 0.1 M) over three times. The immobilized PSL was dried overnight under vacuum. The amount of PSL immobilized on the solid support was measured by the Lowry method with BSA as a standard for protein concentration [14].

2.4. Resolution of (R,S)-2-octanol catalyzed by immobilized enzyme

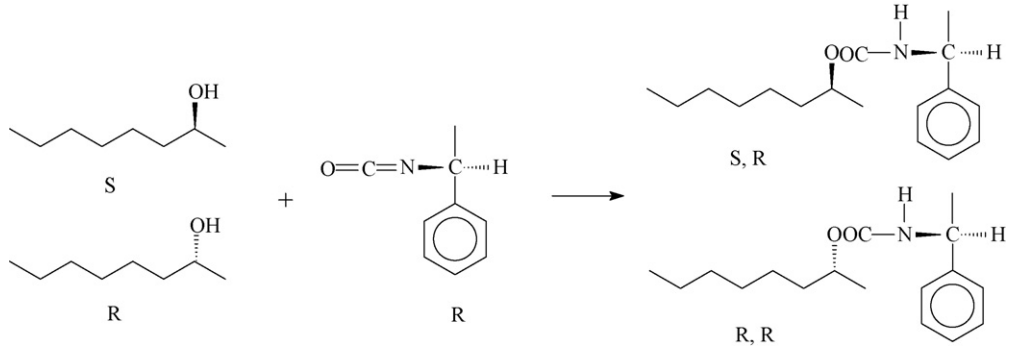
The reaction was performed in a round bottom flask contained (*R*,*S*)-2-octanol (1 mmol), vinyl acetate (2 mmol), *n*-hexane (10 ml), water activity (a_w = 0.56) and immobilized PSL (50 mg) at 50 °C for 30 h. One unit (U) of enzymatic activity was defined as the amount of enzyme required to produce 1 μmol of 2-octanol acetate/min in the first 0.5 h.

2.5. Reusability

To test the stability of the immobilized enzyme in repeated use, batch resolution of (*R*,*S*)-2-octanol (1 mmol) and vinyl acetate (2 mmol) was conducted by the addition of immobilized PSL (50 mg) in *n*-hexane (10 ml) at 50 °C for 30 h with water activity of 0.56. The immobilized PSL was recovered by centrifugation after each batch and was reused for the next batch reaction under the same conditions.

2.6. Determination of enantiomeric excess and E value

The samples were withdrawn from the vials and analyzed directly by gas chromatograph on a Shimadzu gas chromato-



Scheme 2. The formation of isomer for pre-column derivation.

graph (GC-14B) equipped with a FID detector and a column (EC-1000, 30 m × 0.25 mm × 0.25 μm, Alltech). The temperatures of the injector and the detector were 200 and 290 °C, respectively. Nitrogen was used as the carrier gas at a flow rate of 60 ml/min. Temperature programming between 110 and 210 °C with the increment of 15 °C/min was used to determine the concentration of 2-octanol. (*S*)-2-Octanol or (*R*)-2-octanol was derived from *R*-(+)-PELC (Scheme 2). The distinction of *R* from *S* enantiomers was achieved with temperature programming between 110 and 222 °C with the increment of 10 °C/min. The retention time for *S* and *R* diastereomer was 12.36 and 12.84 min, respectively.

The degree of conversion (*C*) was determined from the ratio of the peak areas of the produced 2-octanol acetate to the total peak areas of the residuary 2-octanol and the produced 2-octanol acetate. The enantiomeric excess of the 2-octanol (ee_S) was determined by calculating the peak areas of the two derivatives. The enantiomeric ratio (*E* value) was determined from *C* and ee_S by using Eq. (1) [15]:

$$\text{enantiomeric excesses,}\quad ee_s\,(\%) = \frac{[S-R]}{[S+R]} \times 100$$
$$\text{enantioselectivity,}\quad E = \frac{\ln[(1-C)(1-ee_S)]}{\ln[(1-C)(1+ee_S)]} \qquad (1)$$

where *S* and *R* represent the concentrations of the (*S*,*R*)-diastereomer and (*R*,*R*)-diastereomer, respectively.

3. Results and discussion

3.1. Lipase screening

The most important factor for production of (*S*)-2-octanol with high enantiomeric purity was the catalyst (lipase). In order to find the most suitable lipase, lipases from different resources were immobilized on SBA-15 and the free or the immobilized enzymes were screened for resolution of (*R*,*S*)-2-octanol. As shown in Table 1, it was found that the activity and enantioselectivity of all the selected lipases were improved by immobilization on SBA-15. Among the immobilized lipases, the activity of SBA-15-MML was the highest and 55% convention was obtained only after 4 h, but it displayed poor enantioselectivity (*E* = 10). On the contrast, SBA-15-BSL displayed the highest enantioselectivity (122) whereas it exhibited low activity. As far as the higher activity and enantioselectivity were concerned, the lipase PSL immobilized on SBA-15 was selected for further investigation.

Table 1
Resolution of (*R*,*S*)-2-octanol catalyzed by various free and immobilized enzymes

Enzyme	Time (h)	Conversion (%)	ee_s (%)	*E* value
MML	6	54.1	62	*6*
SBA-15-MML	4	55.0	78	10
BSL	150	50.2	92	71
SBA-15-BSL	100	51.8	99	122
PSL	72	51.6	94	56
SBA-15-PSL	30	52.0	99	114
PPL	72	7.3	11	7
SBA-15-PPL	72	10.5	15	10
CLL	72	2.7	1	2
SBA-15-CLL	72	5.3	3	4
AYL	72	–	–	–
SBA-15-AYL	72	1.2	2	4

Reactions were carried out in *n*-hexane (10 ml) with (*R*,*S*)-2-octanol (1 mmol), vinyl acetate (2 mmol), free or immobilized enzyme that had the same protein content (10 mg) and water activity (0.56) at 50 °C.

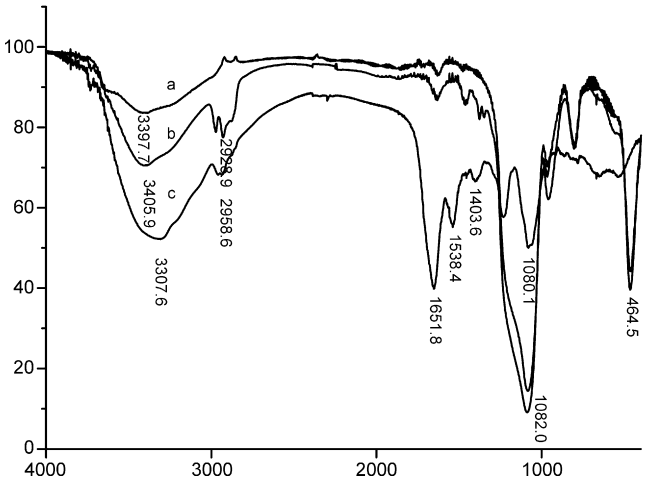


Fig. 1. FT-IR spectra of (a) SBA-15, (b) SBA-15-PSL and (c) free PSL.

3.2. Immobilization of PSL

In order to confirm that PSL was immobilized on SBA-15, the FT-IR spectra of SBA-15, PSL and immobilized PSL was studied. As shown in Fig. 1, the presence of an intense absorption band at 3720 cm^{-1} indicated that SBA-15 had an abundance of free Si–OH groups [16]. After adsorption of PSL, the intensity of this band decreased dramatically, suggesting an hydrogen bonding interaction between free Si–OH groups and the N–H groups of PSL. Furthermore, absorption bands associated with C–H stretching (between 2900 and 3000 cm^{-1}), C=O stretching (1651 cm^{-1}) and C–H deformations (around 1453 cm^{-1}) were observed in the spectra of PSL and immobilized PSL but not in the spectra of SBA-15. These results confirmed that the PSL had been successfully immobilized on the SBA-15.

3.3. Optimization of the reaction conditions

In order to optimize the reaction conditions, the effect of several aspects, including organic solvents, temperature, water activity and substrate ratio on the reaction were studied.

3.3.1. Organic solvents

Many reports demonstrated that organic media not only influenced the enzymatic activity but also the enantioselectivity [17]. In this study, effects of the solvents with different log *P* (logarithm of the partition coefficient of a given solvent between *n*-octanol and water and was widely used to denote the polarity or hydrophobicity of a solvent) were investigated (Table 2). When polar solvents such as 1,4-dioxane or DMF were used,

Table 2
Effect of solvents on the activity and enantioselectivity of the immobilized PSL in resolution of (*R*,*S*)-2-octanol

Solvent	log *P*[a]	ee_s (%)	Enzyme activity (μmol/g min)	*E* value
n-Heptane	4.6	99	172	102
n-Hexane	3.5	99	185	114
Toluene	2.5	97	156	95
Cyclohexane	1.2	95	121	90
Acetone	−0.23	80	68	67
Acetonitrile	−0.33	65	44	42
DMF	−1.0	18	19	19
1,4-Dioxane	−1.1	12	15	11

Reactions were carried out in different organic solvents (10 ml) with (*R*,*S*)-2-octanol (1 mmol), vinyl acetate (2 mmol), immobilized PSL (50 mg) and water activity (0.56) at 50 °C for 30.
[a] Source of date: Refs. [19,20].

the enzyme activity and *E* value were very low. This may be explained as follows: when the reaction was performed under polar solvents, water exhibited higher affinity in solvent rather than bound to the enzyme, so the hydrophilic solvent may disrupt the functional structure of enzyme by stripping off the essential water from the enzyme whose function were to preserve the correct conformation of enzyme molecular [18]. In addition, the strong interaction of polar solvent with enzyme may result in the variation of the conformation of the enzyme active site and decrease the *E* values. As shown in Table 2, there was an increase in both of the enzyme activity and *E* value as the hydrophobicity of solvents increasing. The highest enzyme activity and *E* value was achieved when *n*-hexane was used as solvent.

3.3.2. Temperature

The effect of temperature on the immobilized PSL in resolution of (*R*,*S*)-2-octanol was examined in the range of 20–70 °C. As shown in Fig. 2, the activity increased as temperature increased from 20 to 50 °C, followed by a decrease at higher temperature. When the reaction temperature elevated, the collision chance between enzyme and substrate molecules increased which might help to form enzyme–substrate complexes and then the reaction rate was improved. On the other hand, the protein can fluctuate to relieve the steric repulsion by elevating the temperature, and this fluctuation may also have contributed to the rate acceleration at elevated temperatures [21]. In contrast, an increased *E*-value was observed with a decrease in the temperature, the result was in accordance with Phillips who found that enzymes exhibited their highest enantioselectivity at low temperatures [22]. Since enzyme activity was found to be greatest at 50 °C while maintaining a higher *E* value (114), 50 °C was selected as the optimal temperature for this reaction. Further increasing of temperature may destroy the conformation of enzyme by heat-induced destruction of noncovalent interactions [23] and resulted in decreasing of enzyme activity and enantioselectivity.

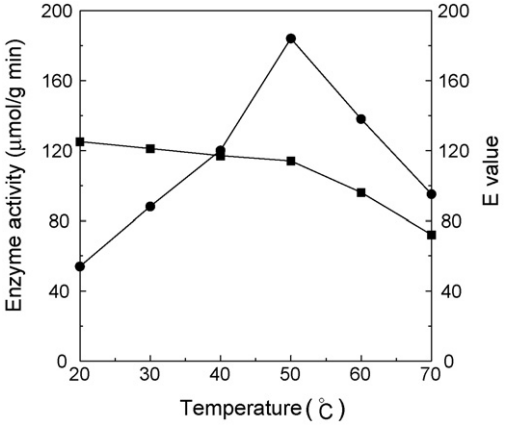


Fig. 2. Effect of temperature on the activity (●) and enantioselectivity (■) of immobilized PSL in resolution of (*R*,*S*)-2-octanol. Reactions were carried out in *n*-hexane (10 ml) with (*R*,*S*)-2-octanol (1 mmol), vinyl acetate (2 mmol), immobilized PSL (50 mg) and water activity (0.56) at various temperatures (20–70 °C) for 30 h.

3.3.3. Water activity

Water activity (a_w) was a crucial parameter in nonaqueous enzymology [24]. In the present study, the a_w was controlled by addition of salts or salt hydrates in the organic solvent or substrate as described by Halling [25]. The reaction catalyzed by immobilized PSL was conducted at fixed water activity ranging from 0.06 to 0.97, and the results were shown in Fig. 3. The enzyme activity exhibited a bell shaped curve with the water activity changing (Fig. 3). At low a_w values (0.06–0.11), low enzyme activity were observed. Especially, no transesterification was detected with dried enzymes. In nonaqueous media, a certain amount of water was necessary for the enzyme to maintain its proper conformation so as to keep its catalytic activity. At low water activity, the conformation of PSL was excessively rigid which disturbed the "induced-fit" process of PSL and decrease the enzyme activity [26]. The immobilized PSL exhibited highest activity when $a_w = 0.56$. However, a further increase in the initial a_w value to 0.97 resulted in an obvious decrease in enzyme activity. The decrease in enzyme activity at higher initial a_w values can be attributed to the observed enzyme particle aggregation that may, in turn, had limited access of the substrate to the enzyme active site. Another explanation may be that, the

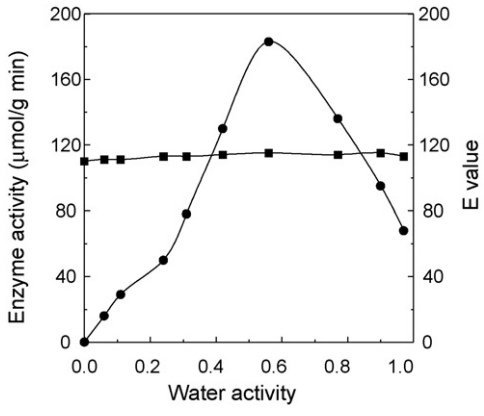


Fig. 3. Effect of water activity on the activity (●) and enantioselectivity (■) of immobilized PSL in resolution of (*R*,*S*)-2-octanol. Reactions were carried out in *n*-hexane (10 ml) with (*R*,*S*)-2-octanol (1 mmol), vinyl acetate (2 mmol), immobilized PSL (50 mg) and water activity (0.06–0.97) at 50 °C for 30 h.

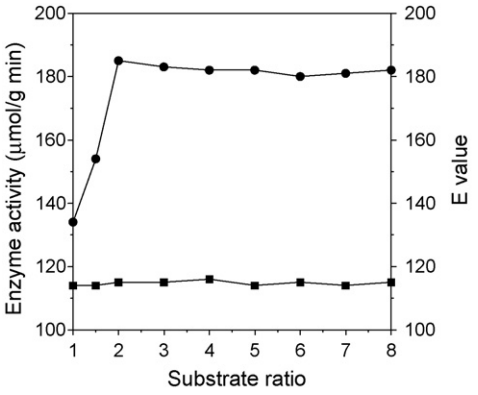


Fig. 4. Effect of substrate ratio on the activity (●) and enantioselectivity (■) of immobilized PSL in resolution of (*R*,*S*)-2-octanol. Reactions were carried out in *n*-hexane (10 ml) with immobilized PSL (50 mg) and water activity (0.56) at 50 °C for 30 h. The concentration of 2-octanol was kept constant (1 mmol), whereas the concentration of vinyl acetate was varied from 1 to 8 mmol.

conformation of PSL was excessively flexible at higher initial a_w values, and the water in the reaction mixture may act as competing nucleophile for acyl-enzyme, thus suppressing the expected acyl transfer and cause unfavourable equilibrium position in reversed hydrolysis. Overall, these results suggest that water activity strongly influenced the hydration level of the enzyme that in turn affected the transesterification activity. Concerning the effect of a_w on enantioselectivity, data reported in the literature are contradictory, alternately showing increases, decreases or no dependence [27,28]. The results in this study indicated that the *E* value remained almost the same with the variation of a_w values. The possible explanation may be that, water would only participate in the enantioselective step of reaction when the acyl part of the substrate is chiral [29]. In this reaction, the acyl part was not chiral, water may not act as a competitive nucleophile for the acyl enzyme intermediate, and so the enantioselectivity was not affected by water activity.

3.3.4. Substrate ratio

The rate of an enzyme-catalyzed reaction depended on the concentrations of enzyme and substrate. The effect of mole ratio of vinyl acetate to 2-octanol from 1:1 to 8:1 on transesterification was studied in detail (Fig. 4). It had been shown experimentally that if the amount of the enzyme was kept constant and the substrate ratio was then gradually increased, the enzyme activity increased until it reached a maximum. After this point (2:1), the enzyme activity could not be improved as the substrate ratio increased. On the other hand, it was observed that the enantioselectivity was not affected by the increase of substrate ratios.

3.4. Reusability of enzyme

The results in Table 3 showed that 90% activity of the immobilized PSL was remained after five cycles while the *E* value did not change expressly. The good reusability of the immobilized PSL may account for the large pore of SBA-15. After the immobilization, PSL was wrapped in the pore and prevented from injuring due to direct exposure to environmental change, and so it kept very stable and exhibited high activity. In addition, the average enzyme activity and enantioselectivity of five batches in succession were much higher than that of the reported in literature about the resolution of (*R*,*S*)-2-octanol [30–32] which may mainly be due to the predominant properties of SBA-15 used for lipase immobilization.

Table 3
Resolution of (*R*,*S*)-2-octanol in repeated batch process by PSL immobilized on SBA-15

Batch	Enzyme activity (μmol/g min)	ee_s (%)	Conversion (%)	*E* value
1	184	99.0	52.0	114
2	180	98.4	51.3	110
3	174	97.9	50.8	105
4	170	97.2	50.2	99
5	166	95.6	49.5	92

4. Conclusion

The lipase from *Pseudomonas* sp. was successfully immobilized on the mesoporous molecular sieves SBA-15 through physical adsorption. The strong interaction of the enzyme with hydrophobic groups from the support contributed to decrease the mobility of the immobilized protein and thus to increase its stability. The immobilized PSL gave a higher activity and enantioselectivity for transesterification of 2-octanol. Under the optimum conditions, the residual 2-octanol catalyzed by immobilized PSL was recovered with 99% enantiomeric excess at 52% conversion rate. The immobilized PSL also proved to be stable and lost little activity when was subjected to repeated uses.

Acknowledgements

The authors are grateful for the financial support from National Natural Science Foundation of China (nos. 20432010, 30570405, and 20672045) and the Foundation of Research Program of Jilin University, China.

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