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Study on enzyme’s 1,3-positional specificity during Lipozyme TL-mediated biodiesel production

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Abstract

The 1,3-positional specificity of lipases plays an important role in obtaining high yield of products especially in the methanolysis for biodiesel production. In this paper, effect of solvent and water activity on enzyme’s 1,3-positional specificity during Lipozyme TL-catalyzed methanolysis of triglyceride (TAG) for biodiesel production was explored. And then the effect of organic solvent and water activity on the methanolysis of monoglyceride (MAG) was further carried out from the aspect of kinetics study. It was found that either in the methanolysis of TAG or MAG, the 1,3-positional specificity of Lipozyme TL was correlated well with the logP of organic solvents. With the increase of LogP of the organic solvent, the enzyme’s 1,3-positional specificity decreased. Interestingly, in each group of solvents (ketones, alkanes and chlorinated hydrocarbon), the 1,3-positional specificity of the lipase improved with water activity increased from 0.11 to 0.53, while it decreased with water activity further increased from 0.53 to 0.97. Further exploration on the related mechanism with molecular dynamics simulation revealed that organic solvent influenced the dehydration state of the lipase, which might subsequently influenced the lipase’s 1,3-positional specificity.

Key words: Biodiesel; Lipase; Organic solvent; Water activity; Positional specificity; Methanolysis
1. Introduction

Lipases with 1,3-positional specificity play an important role in the production of biodiesel as well as in the synthesis of 1,3-diacylglycerol [1]. During lipase-mediated biodiesel preparation, it is necessary to weaken the 1,3-positional specificity for obtaining a high yield of biodiesel, while during lipase-mediated synthesis of 1,3-diacylglycerol (DAG), it is very important to strengthen the lipase’s 1,3-positional specificity to promote the production of DAG [2]. Herein, the investigation on the 1,3-positional specificity of lipases is very significant for promoting the application of lipase-mediated reactions for the production of biodiesel or some important biochemical such as 1,3-DAG.

It was found that organic solvents had profound influence on enzyme’s enantiospecificity as well as substrate specificity to a varied extent [3-6]. In some cases, the enantiospecificity as well as substrate specificity was found to be correlated with the LogP of the organic solvents [3-6]. While in some cases, it was reported that the structure of the organic solvent had pronounced influence on the enzyme’s enantiospecificity as well as substrate specificity [6]. However, regarding their influence on the 1,3-positional specificity of lipases, little exploration was carried out, which was mostly due to the complicated process especially during lipase-mediated methanolysis of oils for biodiesel production [7]. Taking lipase-mediated methanolysis of triglyceride (TAG) for biodiesel production as the example (Scheme 1), acyl migration existing in the process of alcoholsysis makes the reaction process rather complicated [7-8]. As can be seen from Scheme 1, during lipase-mediated methanolysis of TAG for biodiesel production, k1/k15 and k3/k19 can be used to describe the lipase’s 1,3-positional specificity from the kinetics aspect. However, there are so many steps involved in the process, making it hard to justify the
suitability of the kinetic models. Monoglyceride (MAG), as one of intermediates during the methanolysis of TAG for biodiesel production and the lipase’s 1,3-positional specificity also influences biodiesel yield to a great extent. Herewith, in this paper, the influence of organic solvent and water activity on lipase’s 1,3-positional specificity was firstly studied during Lipozyme TL-mediated methanolysis of TAG for biodiesel preparation. And then from kinetic aspects, further study was conducted with MAG as the starting substrate to explore the related influence. Finally, molecular modeling was adopted to illustrate the related mechanism.

2. Material and methods

2.1 Material

Lipozyme TL (immobilized lipase from Thermomyces lanuginosus) was a gift from Novozymes (Denmark). 1-monoolein, 2-monoolein and methyl ester of oleic acid were purchased from Sigma-Aldrich (St. Louis, MO) and were chromatographically pure. All other chemicals and reagents were obtained commercially and were of analytical grade.

2.2 Control of water activity

The method of maintaining a constant water activity was adopted according to reference [9]. Prior to reaction, the reaction solution and Lipozyme TL were separately equilibrated with saturated salt solution of known $a_w$ for 16 h in two air tight desiccators (fitted with a rubber septum on the lid). The following salts were used: LiCl ($a_w=0.11$), Mg(NO$_3$)$_2$ ($a_w=0.53$) and K$_2$SO$_4$ ($a_w=0.97$).

2.3 General procedure for methanolysis of MAG and TAG

The enzymatic methanolysis was performed in a 50 mL flask on a rotary shaker at 150 rpm, 35°C. The compositions of the reaction mixtures were as follows: MAG
0.112 M (including 1-MAG 0.104 M and 2-MAG 0.008 M), methanol 0.112 M and lipase 50 mg. Samples of 50 μL were taken from the reaction mixture, centrifuged to obtain the upper layer for MAG analysis by HPLC.

The enzymatic methanolysis was performed in a 50 mL flask on a rotary shaker at 150 rpm, 35 °C. The compositions of the reaction mixtures were as follows: TAG 0.83M, methanol 0.83M and lipase 500 mg. Samples of 50 μL were taken from the reaction mixture, centrifuged to obtain the upper layer for MAG and DAG analysis by HPLC.

2.4 Analysis of the samples

The contents of MAG and DAG were analyzed by a Shimadzu 20A HPLC system (Shimadzu Corp., Kyoto, Japan) with an ELSD-LT II low temperature-evaporative light scattering detector. The resultant sample of 2 μl and acetone of 1 ml were precisely measured and mixed thoroughly. The aforementioned mixture of 20 μl of was injected for analysis. The stationary and mobile phases were C18 column (5μm, 250mm×4.6 mm, Dikma Technology, PLATISIL ODS, China) and a gradient elution program (table 1) by acetonitrile and dichloromethane at 1.5 ml min⁻¹. The column temperature and the drift pipe temperature were controlled at 40 °C. and 70 °C respectively. The nitrogen pressure was controlled at 320 kPa.

The fatty acid methyl esters (FAME) was detected by Agilent 7890A GC system (Agilent Technologies, Santa Clara, USA) equipped with a CP-FFAP CB capillary column (25 m×0.32 mm×0.30μm, Agilent Technologies, USA). Heptadecanoic acid methyl ester was served as the internal standard. 50mg of the upper layer and 0.6 ml of 0.7mg/ml heptadecanoic acid methyl ester (ethanol as the solvent) were mixed thoroughly. The resultant mixture of 1μl was injected for analysis. The initial column
temperature was set at 180°C and held for 0.5 min, then heated to 250°C at the rate of 10°C/min and maintained for 6 min. Injector and detector temperatures were set at 245°C and 250°C, respectively.

2.5 Molecular dynamics simulation

The structure of native Lipozyme TL was obtained from the Brookhaven Protein Data Bank (PDB code: 1DTE). To be in consistency with the experimental conditions, neutral pH was selected. The GROMACS 4.01 package was used to perform molecular dynamics (MD) simulation. This package is a collection of programs and libraries for MD simulation and the subsequent analysis of trajectory data. Pressure and temperature coupling was implemented for all types of simulation cells. The Berendsen’s weak coupling algorithm scheme was used for both pressure and temperature.

During the simulation, Lipozyme TL was put into the centre of a dodecahedron box using periodic boundary conditions with either water or organic solvent molecules. The system containing one Lipozyme TL molecule and a certain number of water molecules or organic solvent molecules was submitted to 500 steps of steepest descent minimization converging to a value of 2,000 kJ•mol⁻¹•nm⁻¹, applying the Particle-Mesh Ewald method at 308 K (35 °C). Then a 10 ps position-restrained MD simulation was performed by keeping the protein coordinate fixed, and allowing the water and acrylamide molecules to equilibrate themselves. Then a 1,000 ps MD was performed at 308 K and a leap-frog algorithm was used for integrating the Newtonian equations of motion for 500,000 simulation steps, with a time step of
0.002 ps. All bonds are constrained by using LINear Constraint Solver denoted by LINCS in Gromacs.

Two order parameters, *i.e.*, the root mean square distance (RMSD) and the number of hydrogen bonds (H-bond), were used to evaluate the simulations. RMSD reflects the similarity of the specific conformation to the native one, which can be calculated by least-square fitting the structure to the reference native structure in the following way:

\[
RMSD(t, t_0) = \left[ \frac{1}{M} \sum_{i=1}^{N} m_i \left\| \mathbf{r}_i(t) - \mathbf{r}_i(t_0) \right\| \right]^{1/2}
\]

where \( m_i \) is the mass of atom \( i \) in whole Lipozyme TL or its active site and lid, \( r_i(t) \) is the internal coordinate of atom \( i \) of conformation at time \( t \) and \( r_i(t_0) \) is the internal coordinate of atom \( i \) at initial state, that is, native conformation.

\( M = \sum_{i=1}^{N} m_i \) is the mass of Lipozyme TL. A lower RMSD value of Lipozyme TL indicates that its conformation is closer to that of native Lipozyme TL. Hydrogen bonds were determined based on cutoffs for the angle (30°) Acceptor-Donor-Hydrogen and the distance Hydrogen-Acceptor (0.35 nm).

The radial distribution function (RDF) or pair correlation function \( g_{ab}(r) \) was also used to illustrate the distribution of solvents around Lipozyme TL, which is defined by the following equation:

\[
g_{ab}(r) = \frac{\langle \rho_a(r) \rangle}{\langle \rho_a \rangle_{\text{local}}} = \frac{1}{\langle \rho_b \rangle_{\text{local}}} \frac{1}{N_A} \sum_{i=A}^{N_A} \sum_{j=B}^{N_B} \delta(\mathbf{r}_i - \mathbf{r}_j)
\]

with \( \langle \rho_a(r) \rangle \) the particle density of type \( B \) at a distance \( r \) around particles \( A \), and \( \langle \rho_a \rangle_{\text{local}} \) the particle density of type \( B \) averaged over all
spheres around particles $A$ with radius $r_{\text{max}}$. In the present study, $r_{\text{max}}$ is half of the box length.

3. Results and discussion

3.1 Influence of organic solvent and water activity on lipase’s 1,3-positional specificity during the methanolysis of TAG for biodiesel production

It was demonstrated that organic solvents might influence enzymes’ specificity in non-aqueous solution [10]. The influence of organic solvent and water activity on lipase’s 1,3-positional specificity during Lipozyme TL-catalyzed methanolysis of TAG for biodiesel production was firstly investigated. Considering the complexity of the process, the concentration ratios of 1,2-DAG to 1,3-DAG and 2-MAG to 1-MAG were used to characterize the 1,3-positional specificity.

It was noticed that the ratio of 1,2-DAG to 1,3-DAG as well as the ratio of 2-MAG to 1-MAG was both the highest in acetone, and both the lowest in hexane, indicating enzyme shows the highest 1,3-positional specificity in acetone and the lowest 1,3-positional specificity in hexane (Fig.1). While no obvious correlation between the enzyme’s 1,3-positional specificity with other parameters such as $\mu$, $E_T^N$, $DN^N$ and $E_T^N + DN^N$ was observed.

Three different water activities (from 0.11 to 0.97) were selected to investigate their effects on the 1,3-positional specificity of Lipozyme TL-mediated methanolysis of TAG (Fig 2). It was noticed that when water activity was 0.53, the ratio of 1,2-DAG to 1,3-DAG as well as the ratio of MAG to 1-MAG was the highest, indicating the highest 1,3-positional specificity achieved with water activity of 0.53.

The above study showed that during lipase-mediated biodiesel production, hydrophobic solvent such as hexane is recommended for achieving high yield of biodiesel, while the water activity of 0.53 should be avoided since the lipase had
highest 1,3-positional specificity at this water activity. As also could be seen from Fig.1, with the increase of the transformation rate of methanol, the ratio of 1,2-DAG to 1,3-DAG as well as the ratio of 2-MAG to 1-MAG decreased (the formation of FAME is almost linear to the consumption of methanol, data not shown). Herewith, It is very significant to study the lipase’s 1,3-positional specificity from a kinetic point of view.

3.2 The solvent effect on Lipozyme TL-mediated methanolysis of MAG

Further study was carried out to investigate the influence of organic solvent and water activity on lipase’s 1,3-positional specificity during Lipozyme TL-mediated methanolysis of MAG for biodiesel production from the kinetic aspect. Here three groups of solvents (ketones, alkanes and chlorinated hydrocarbon) with LogP ranging from -0.23 to 8.8 were adopted for the study of 1,3-positional specificity of Lipozyme TL during the methanolysis of MAG. The concentration change of 1-MAG and 2-MAG in different solvents was shown in Fig.3, Fig.4 and Fig.5 respectively.

It was found that in all cases, the concentration of 1-MAG decreased gradually with time proceeding while the concentration of 2-MAG increased. This phenomenon should be due to the enzyme’s 1,3-positional specificity combined with acyl migration from 1-MAG to 2-MAG occurred in the methanolysis process. The reaction rates were found to be varied in different solvents. To further explore the exact influence of organic solvent on the enzyme’s 1,3-positional specificity, a kinetic model of LipozymeTL-mediated methanolysis of MAG was proposed, as indicated in Scheme 2.

As shown in Scheme 2, lipase-mediated methanolysis of MAG belongs to second-order reversible reactions and the acyl migration reactions are one-order reversible reactions. The differential equations characterizing the whole reaction process were listed as follows.
\[ \frac{d[2\text{MAG}]}{dt} = -k_1[2\text{MAG}]+k_3[1\text{MAG}]-k_3[1\text{MAG}] [\text{CH}_3\text{OH}] + k_6[G][\text{FAME}] \]

\[ \frac{d[1\text{MAG}]}{dt} = k_2[2\text{MAG}]-k_1[1\text{MAG}]+k_3[1\text{MAG}] [\text{CH}_3\text{OH}] + k_6[G][\text{FAME}] \]

\[ \frac{d[\text{CH}_3\text{OH}]}{dt} = -k_5[2\text{MAG}][\text{CH}_3\text{OH}]+k_6[G][\text{FAME}]-k_3[1\text{MAG}] [\text{CH}_3\text{OH}] + k_6[G][\text{FAME}] \]

\[ \frac{d[\text{FAME}]}{dt} = k_2[2\text{MAG}][\text{CH}_3\text{OH}]-k_6[G][\text{FAME}]+k_3[1\text{MAG}] [\text{CH}_3\text{OH}] - k_5[G][\text{FAME}] \]

\[ \frac{d[G]}{dt} = k_2[2\text{MAG}][\text{CH}_3\text{OH}]-k_5[G][\text{FAME}]+k_3[1\text{MAG}][\text{CH}_3\text{OH}]-k_5[G][\text{FAME}] \]

[1\text{MAG}], [2\text{MAG}], [\text{CH}_3\text{OH}], [G], [\text{FAME}] referred to the concentration of 1-monoacylglycerol, 2-monoacylglycerol, methanol, glycerol and Fatty Acid Methyl Ester, respectively. The rate constants were identified by solving the rate equations within a nonlinear regression procedure [11]. The corresponding rate constants were listed in Table 2 and the ratio of \( k_3 \) to \( k_5 \) was used to characterize the 1,3-positional specificity of Lipozyme TL from the aspects of kinetics as indicated in Table 3.

It could be seen that in each group of solvents (ketones, alkanes and chlorinated hydrocarbons), the acyl-migration rate constant decreased as Log P enhanced. It was found in our previous study that the acyl migration would take place spontaneously and solvent polarity influenced the acyl migration rate through the influence of the charge dispersion of the transition state [12]. High polarity of the solvent was unfavorable to the transition state charge dispersion, which would increase its energy state, and thus decreased the acyl migration rate.

The methanolysis rate constant increased as LogP enhanced, which was in agreement with other enzymatic catalysis in non-aqueous medium [13]. The Log P of organic solvent was further correlated with the enzyme’s 1,3-positional specificity (\( k_3/k_5 \)), just as shown in Fig. 6.

It could be seen that the 1,3-positional specificity of the lipase was correlated well with the log P of the solvent despite of their differences in structure. Organic solvent with higher logP gave a lower 1,3-positional specificity, which had the same trend as the influence of organic solvent on enzyme’s other specificity such as
enantiospecificity Error! Reference source not found.

There was also no obvious correlation existed between the enzyme’s 1,3-positional specificity with other parameters such as $\mu$, $E_T^N$, $DN^N$ and $E_T^N + DN^N$ (Table 4).

3.3 The influence of water activity on Lipozyme TL-mediated methanolysis of MAG

There were many reports indicating that water activity would influence the specificity of enzymes to a varied extent [15]. While in some other cases, it was found that water activity did not influence the enzyme’s specificity at all [14]. Here different water activities (from 0.11 to 0.97) were selected to investigate their effects on the 1,3-positional specificity of Lipozyme TL during its-catalyzed methanolysis of MAG. The comparative studies were performed at four different solvents (acetone, cyclohexanone, hexane and hexadecane) and the related results were presented in Table 3.

It was noticed that in all organic solvents (acetone, cyclohexanone, hexane and hexadecane), the methanolysis rate increased when water activity enhanced from 0.11 to 0.53. With the increase of the water activity, more water molecular would bind near the active site of the enzyme and consequently the enzyme became more flexible, which resulted in the improved enzymatic activity as well as the improved methanolysis reaction rate [16-17].

Interestingly, it was found that with the water activity increased from 0.11 to 0.53, the 1,3-positional specificity of the lipase ($k_3/k_5$) increased to a varied extent in different solvents. The solvent with lower log P gave higher 1,3-positional specificity in terms of maintaining the same water activity ranging from 0.11 to 0.53. In acetone, $k_3/k_5$ improved from 361 at water activity 0.11 to 497 at water activity 0.53; in cyclohexanone, $k_3/k_5$ improved from 238 at water activity 0.11 to 356 at water activity
0.53; in hexane, $k_3/k_5$ improved from 164 at water activity 0.11 to 211 at water activity 0.53; and in hexadecane, $k_3/k_5$ increased from 104 at water activity 0.11 to 135 at water activity 0.53. Jonsson A et al also reported the similar phenomenon during the stereoselective reduction of ketones catalyzed by alcohol dehydrogenase, where the enantiospecificity ($E$ value) increased from 2.6 at water activity 0.53 to 4.6 at water activity 0.97 [16]. So far, the exact mechanism regarding to the improved specificity of enzyme with the increase of water activity still remains unknown.

Further increase in water activity from 0.53 to 0.97 led to an obvious decrease in enzyme’s 1,3-positional specificity ($k_3/k_5$), just as presented in Table 3. With increase of the water activity, more water molecule would bind to the active site of the enzyme making the enzyme molecule is more flexible and consequently, the binding to 2-MAG was promoted, which finally resulted in the reduced enzyme’s 1,3-positional specificity [16]. It was also noticed that the methanolysis reaction rate decreased with the increase of water activity from 0.53 to 0.97, which might be due to the enhanced competitive hydrolysis reaction when much water was present in the system.

3.5 Mechanism exploration using molecular dynamics simulation

It has been found that the enzyme surface and the active site region are well hydrated in aqueous medium, whereas with increasing polarity of the organic solvent, the hydration water is stripped from the enzyme surface resulting in some changed properties and organic solvent might influence the hydration state of the enzyme subsequently resulting in the change of enzymes’ some properties [18].

Molecular dynamics simulation was adopted further to explore the related mechanism of how organic solvent influenced the lipase’s 1,3-positional specificity. Based on the description given in the Methods section, the radial distributions of solvent molecules were studied firstly with molecular modeling. As could be seen
from Fig.7 and Fig.8, acetone and cyclohexanone showed a clear affinity for the active site and the lid of the lipase, since there were more solvent molecules in the final state than that in the initial state. While in hexane and hexadecane, there were no differences between the initial states and final states. The phenomenon indicated that the hydrophilic solvents would stay near the active site and lid of the lipase, which subsequently forced the water molecules to fall off from the lipase and changed the dehydration state of the lipase. The dehydration change caused by organic solvent might be responsible for the variation of lipase’s 1,3-positional specificity, which accorded with our previous experimental results that the 1,3-positional specificity of Lipozyme TL was correlated well with the logP of organic solvents either in the methanolysis of MAG or TAG for biodiesel production.

From the above study, it could be seen that the weakened 1,3-positional specificity of lipase could be achieved in hydrophobic solvent like hexane and hexadecane and such reaction medium is beneficial for achieving high yield of biodiesel [19].

4. Conclusion

Both organic solvent and water activity had pronounced influence on enzyme’s 1,3-positional specificity either in Lipozyme TL-mediated methanolysis of MAG or TAG for biodiesel production. The 1,3-positional specificity of Lipozyme TL was correlated well with the logP of organic solvents and with the increase of logP of the organic solvent, the enzyme’s 1,3-positional specificity decreased. The water activity showed varied influence: The 1,3-positional specificity of the lipase improved with water activity increased from 0.11 to 0.53, while it decreased with water activity further increased from 0.53 to 0.97. Further study showed that even with TAG as the substrate for biodiesel production, the organic solvent and water activity had similar
influence on enzyme’s 1,3-positional specificity as those obtained in the methanolysis of MAG. The exploration on the related mechanism with molecular dynamics simulation revealed that the dehydration change caused by organic solvent might be responsible for the variation of lipase’s 1,3-positional specificity.

Acknowledgement

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Reference

Scheme 1. Lipase-mediated methanolysis of TAG for biodiesel production

Scheme 2 Lipase-mediated methanolysis of MAG for biodiesel preparation

Table 1 Gradient elution program

Table 2 Rate constants in eight solvents ($a_w=0.53$)

Table 3 Effect of water activity on the 1,3-positional specificity of the enzyme

Table 4 Effect of solvent on the 1,3-positional specificity of the enzyme

Figure 1 The ratio changes of 1,2-DAG,1,3-MAG as 2-MAG to 1-MAG in different solvent

Figure 2 The ratio change of 1,2-DAG to 1,3-MAG as 2-MAG to 1-MAG in different water activity

Figure 3 Content change of 1-MAG and 2-MAG in alkanes ($a_w=0.53$)

Figure 4 Content change of 1-MAG and 2-MAG in chlorinated hydrocarbons ($a_w=0.53$)

Figure 5 Content change of 1-MAG and 2-MAG in acetones ($a_w=0.53$)

Figure 6 Effect of physical parameters of organic solvent on $k_3/k_5$

Figure 7 The radial distributions of solvent molecules in the active site

Figure 8 The radial distributions of solvent molecules in the Lid
Scheme 1
Scheme 2
Fig. 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Table 1

<table>
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<tr>
<th>Time (min)</th>
<th>Flow rate (ml/min)</th>
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<th>Dichloromethane (v/v, %)</th>
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### Table 3

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<td>$a_w=0.53$</td>
<td>0.00372</td>
<td>0.00198</td>
<td>1.51</td>
<td>0.00715</td>
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<td>$a_w=0.97$</td>
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<td>0.00156</td>
<td>0.81</td>
<td>0.00577</td>
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<td>$a_w=0.11$</td>
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<td>0.00166</td>
<td>1.243</td>
<td>0.0119</td>
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<td>1.69</td>
<td>0.0123</td>
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<td>0.000990</td>
<td>1.01</td>
<td>0.0114</td>
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<td>Solvent</td>
<td>$k_3/k_5$</td>
<td>Log P</td>
<td>$\mu$</td>
<td>$E_T^N$</td>
<td>$DN^N$</td>
<td>$E_T^N + DN^N$</td>
</tr>
<tr>
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<tr>
<td>hexane</td>
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<td>3.5</td>
<td>0</td>
<td>0.074</td>
<td>(0.000)</td>
<td>0.074</td>
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<td>8.8</td>
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<td>1.1</td>
<td>0.090</td>
<td>(0.000)</td>
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