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1. Introduction

Enzymes are very effective and biodegradable catalysts, and act under mild conditions such as room temperature, atmospheric pressure, and around pH 7. Consequently, the applications of enzymes to the organic synthesis have extensively been studied from the standpoint of the development of sustainable synthetic processes (Truesdell, 2005). Enzymes exhibit the high activity and specificity not only in conventional aqueous solutions but also in non-aqueous reaction media (Klibanov, 2001; Noritomi et al., 2007a). Enzymes in non-aqueous media have especially been applied to numerous synthetic processes because of the following benefits, although the enzyme has the low activity in organic solvents compared with that in water: (i) the solubilities of non-polar reactants and products are improved; (ii) the thermostability of enzymes is highly improved; (iii) the stereoselectivity of enzymes is markedly altered; (iv) the thermodynamic equilibria of many processes such the formation of peptide bond by protease are favorable; (v) the immobilization of enzymes is not necessary, and enzymes are easily recycled by recovering them with the filtration or the centrifugation, since enzymes are insoluble in organic solvents; (vi) the product is easily recovered with the evaporation when using the volatile organic solvent as the reaction medium; (vii) the contamination due to the growth of microorganisms is inhibited by organic solvents. Furthermore, it has been found that the activity and selectivity of enzymes can be manipulated by the choice of solvents or enzyme preparation, although they were changed only by protein engineering or enzyme screening prior to the advent of nonaqueous enzymology (Koskinen & Klibanov, 1996; Noritomi et al., 1996; Wescott et al., 1996).

Proteases are stable hydrolytic enzymes with high selectivity, do not need expensive cofactors, and are used as useful synthetic tools for side-directed peptide cleavages, regiospecific ester hydrolyses, or the kinetic resolution of racemates (Bordusa, 2002). In order to improve undesired hydrolysis and substrate solubility in aqueous media, proteases have been used in organic solvents for organic synthesis. Proteases in organic solvents can catalyze water sensitive reactions, such as esterification, transesterification, and peptide synthesis, which are usually impossible in aqueous media.

Ionic solvent that is liquid at room temperature has attracted increasing attention as a green solvent for the chemical processes because of the lack of vapor pressure, the thermal stability, and the high polarity (Welton, 1999; Greaves & Drummond, 2008). Chemical and physical properties of ionic liquids can be changed by the appropriate modification of...
organic cations and anions, which are constituents of ionic liquids. Biotransformation in ionic liquids has increasingly been studied (Moniruzzaman et al., 2010; Yang & Pan, 2005). However, despite such potential capability of proteases, there has been only a limited number of works on protease-catalyzed reactions in ionic liquids.

In this chapter, the effects of ionic liquids on esterification of amino acid with alcohol and peptide synthesis of amino acid ester with amino acid amide catalyzed by proteases are discussed (Noritomi et al., 2007b; Noritomi et al., 2009). The former is the reverse reaction of protease-catalyzed amino acid ester hydrolysis, whereas the latter is the aminolysis of amino acid ester.

### 2. Protease-catalyzed esterification of amino acid in ionic liquids

#### 2.1 Activity of protease in ionic liquids

Figure 1 shows structures and properties of ionic liquids introduced in this chapter. 1-Ethyl-3-methylimidazolium bis(fluorosulfonyl)imide, 1-ethyl-3-methylimidazolium trifluoromethanesulfonate, 1-ethyl-3-methylimidazolium tetrafluoroborate, and 1-ethyl-3-methylimidazolium hexafluorophosphate are abbreviated to [emim][FSI], [emim][Tf], [emim][BF₄], and [emim][PF₆], respectively. Their properties such as melting point and water-miscibility alter by switching from one anion to another.

In order to synthesize amino acid esters by protease in non-aqueous media, there are two reaction processes: the reverse reaction process of protease-catalyzed amino acid ester hydrolysis via thermodynamical control and the transesterification process of amino acid ester with alcohol via kinetical control. The protease activity of the transesterification is much higher than that of the esterification in organic solvents, since esterified amino acids are activated substrates (Kise et al., 1990). On the other hand, acyl enzyme intermediate is formed from neutral carboxylic acid substrate in the esterification process. Consequently, the formation of N-acetyl-L-phenylalanine ethyl ester (N-Ac-L-Phe-OEt) via protease-catalyzed esterification of N-acetyl-L-phenylalanine (N-Ac-L-Phe-OH) with ethanol (equation (1)) is enhanced in water-miscible organic solvents, since the apparent pK value of carboxylic acid in N-Ac-L-Phe-OH is shifted higher.

<table>
<thead>
<tr>
<th>Ionic liquid</th>
<th>Structure</th>
<th>m.p. (°C)</th>
<th>Water miscibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>[emim][FSI]</td>
<td><img src="image1" alt="Structure" /></td>
<td>-12.9</td>
<td>Partially miscible</td>
</tr>
<tr>
<td>[emim][Tf]</td>
<td><img src="image2" alt="Structure" /></td>
<td>-9</td>
<td>Miscible</td>
</tr>
<tr>
<td>[emim][BF₄]</td>
<td><img src="image3" alt="Structure" /></td>
<td>14.6</td>
<td>Miscible</td>
</tr>
<tr>
<td>[emim][PF₆]</td>
<td><img src="image4" alt="Structure" /></td>
<td>62</td>
<td>Partially miscible</td>
</tr>
</tbody>
</table>

Fig. 1. Structures of ionic liquids.
Moreover, enzymatic reactions in hydrophilic solvents have the advantage of the solubility of a variety of substrates, including amino acid derivatives, which are poorly soluble in hydrophobic solvents. [emim][Tf] is a water-miscible ionic liquid and suitable for the solubilities of N-acetylated amino acids, similar to a hydrophilic organic solvent, such as acetonitrile. Figure 2 shows the time courses of subtilisin-catalyzed esterification of N-acetyl-L-phenylalanine (N-Ac-L-Phe-OH) with ethanol in [emim][Tf] and acetonitrile containing 0.2% (v/v) water at 30 °C. The esterification in [emim][Tf] proceeded with the reaction time, and the concentration of N-acetyl-L-phenylalanine ethyl ester (N-Ac-L-Phe-OEt) in [emim][Tf] exhibited 14-fold compared to that in acetonitrile when the reaction time reached 90 min. When a hydrophilic solvent is used as a reaction medium, the enzyme molecule directly contacts with the solvent, and thereby its activity is strongly influenced by the nature of the solvent (Klibanov, 2001; Noritomi et al., 2007). From this point of view, subtilisin seems to be stable to [emim][Tf].

Figure 3 shows the initial rates of esterification of N-Ac-L-Phe-OH with ethanol catalyzed by free subtilisin and α-chymotrypsin in [emim][Tf] and organic solvents containing 0.2% (v/v) water at 30 °C. In organic solvent systems using subtilisin, the more hydrophobic solvent was used, the greater initial rate was obtained. The hydrophobicity is attributable to the partition of water between enzyme molecules and the bulk organic phase in reaction system (Klibanov, 2001; Zaks & Klibanov, 1988). When the same amount of water is added in different organic solvents, the more hydrophobic the reaction medium is, the more amount of water is located around the enzyme molecule. Consequently, the flexibility of the enzyme molecule, which is essential for catalytic activity, increases in hydrophobic solvents, and thereby high activity is exhibited. The initial rate in [emim][Tf] is superior to the initial rates in all organic solvents, and, for example, is about three times greater than that in octane. On the other hand, the initial rate of α-chymotrypsin in octane is also highest in the organic

\[
N\text{-Ac-L-Phe-OH} + \text{EtOH} \rightleftharpoons N\text{-Ac-L-Phe-OEt} + \text{H}_2\text{O}
\]
solvent system. Furthermore, the initial rate of α-chymotrypsin in [emim][Tf] is about fourteen times greater than that in octane. Adding salts to an aqueous solution containing enzymes is a useful method for improving enzyme stability (Troller & Christian, 1978). Before using the enzyme powder as a suspension in an organic solvent, the inclusion of simple salts during lyophillization or freeze-drying of the enzyme is one of the most effective activation methods. For instance, lyophilized enzyme powder prepared from enzyme solution including excess KCl increases the catalytic efficiency of subtilisin-catalyzed transesterification by 3750-fold (Khmelnisky et al., 1994). Addition of buffer salts or KCl also increases the catalytic activity of lyophilized *Candida Antarctica* lipase 4-fold over that without added salt (Triantafyllou et al., 1997). Moreover, the lyophilizate including KCl enhances thermolysin-catalyzed peptide synthesis in tert-amyl alcohol (Bedell et al., 1998). These reports indicate that salts tend to induce activation of enzymes. Furthermore, an amino acid interacts with [emim], and [emim][amino acid] is formed as an ionic liquid (Fukumoto et al., 2005). In the present reaction system, the enhancement of enzymatic activity might be attributable to the interaction between [emim][Tf] and N-Ac-L-Phe-OH. As seen in Figs.3, the initial rate of α-chymotrypsin is greater than that of subtilisin in [emim][Tf] system, while the initial rate of α-chymotrypsin is smaller than that of subtilisin in organic solvent system. α-Chymotrypsin tends to prefer the atmosphere of high salt concentration (Martin & Niemann, 1958). As a result, the initial rate of α-chymotrypsin is effectively enhanced compared with that of subtilisin by changing from the organic solvent to [emim][Tf].

![Fig. 3. Solvent-dependence of esterification of amino acid catalyzed by subtilisin and α-chymotrypsin.](image)

**2.2 Influence of water content on activity of protease in ionic liquids**

A common thread in all studies of enzymes in organic solvents is that the amount of water associated with the enzyme is a key determinant of the properties (e.g. activity, stability, and specificity) that the enzyme exhibits (Klibanov, 2001). Moreover, water can act as a substrate in reactions using hydrolytic enzymes. On the other hand, the esterification of N-Ac-L-Phe-OH with ethanol is a reverse reaction of hydrolysis and is therefore thermodynamically...
controlled. These result in lower product yields in reaction media containing higher water content. However, the initial rate of subtilisin-catalyzed esterification of N-Ac-L-Phe-OH with ethanol in [emim][Tf] system at 5 % (v/v) water is enhanced 2.4-fold compared to that at 0.2 % (v/v) water. Similarly, the initial rate in acetonitrile system at 5 % (v/v) water is about seven times greater than that at 0.2 % (v/v) water. When a certain amount of water is added into the non-aqueous enzymatic reaction system, some water is bound to the enzyme, and thereby has a large influence on the enzymatic activity, while the other amount of water is dissolved in the solvent (Klibanov, 2001). Water associated with the enzyme activates the enzyme by increasing the internal flexibility of the enzyme molecule, since water acts as a plasticizer to increase the flexibility (Zaks & Klibanov, 1988). The water content is an influential parameter for the activity in [emim][Tf] system, similar to the case in acetonitrile system.

3. Protease-catalyzed peptide synthesis in ionic liquids
3.1 Protease-catalyzed peptide synthesis

Peptides are molecules of great importance in the pharmaceutical and food fields (Guzman et al., 2007; Gill et al., 1996). Several technologies for peptide synthesis are available: a) chemical synthesis; b) enzymatic synthesis; c) recombinant DNA technology. However, the application of recombinant DNA technology requires a long and expensive research and development phase. The chemical peptide synthesis has some problems such as racemization during peptide bond formation, the requirement of extensive protection of the side chain functionalities of amino acids, and the use of a large excess of coupling reagents and acyl donors. Proteases have been used as a biocatalyst in order to carry out peptide synthesis since 1938 (Bordusa, 2002). The advantages of protease-catalyzed peptide synthesis are freedom from racemization, minimal requirements for carboxyl activation and side-chain protection, mild reaction conditions, and high region- and stereoselectivity. For example, α-chymotrypsin-catalyzed peptide synthesis of N-acetyl-L-tryptophan ethyl ester (N-Ac-Trp-OEt) with glycyl glycinamide (Gly-Gly-NH$_2$) is shown in Fig. 4. When using

$$
\begin{align*}
E + Ac-Trp-OEt & \underset{k_1}{\overset{k_2}{\rightleftharpoons}} E \cdot Ac-Trp-OEt \\
& \overset{k_{h, H_2O}}{\longrightarrow} Ac-Trp-Gly-Gly-NH_2 \\
& \overset{k_{p, Gly-Gly-NH_2}}{\longrightarrow} Ac-Trp-OH
\end{align*}
$$

Fig. 4. α-Chymotrypsin-catalyzed peptide synthesis of N-acetyl-L-tryptophan ethyl ester (N-Ac-Trp-OEt) with glycyl glycinamide (Gly-Gly-NH$_2$) to N-acetyl-L-tryptophan glycyl glycinamide (N-Ac-Trp-Gly-Gly-NH$_2$) and the competing hydrolysis (N-Ac-Trp-OH).
amino acid esters as a substrate, the peptide synthesis is a kinetically controlled reaction process. This process involves the competitive distribution of the rapidly formed acyl enzyme intermediate between water (hydrolysis) and another nucleophilic reagent such as an amino acid derivative (peptide synthesis). Since the nucleophilic reaction is rate-determining step, and $k_1$, $k_{-1}$, and $k_2$ are much greater than $k_p$ and $k_h$ (Fersht, 1999), the initial rates are shown as

$$V_p = k_p[\text{Ac-L-Trp-E][Gly-Gly-NH}_2]$$

(2)

$$V_h = k_h[\text{Ac-L-Trp-E][H}_2\text{O}]$$

(3)

where $V_p$ is the initial rate of peptide synthesis, $V_h$ the initial rate of hydrolysis, $k_p$ the rate constant of peptide synthesis, and $k_h$ the rate constant of hydrolysis. From equations (2) and (3), the selectivity ($k_p/k_h$) is derivated as the following equation.

$$k_p/k_h = V_p[H_2O] / V_h[\text{Gly-Gly-NH}_2]$$

(4)

3.2 Dependence of $\alpha$-chymotrypsin-catalyzed peptide synthesis on water content in [emim][FSI]

Figure 5 shows the plot of the initial rates of N-acetyl-L-tryptophan glycyglycinamide (N-Ac-Trp-Gly-Gly-NH$_2$) and N-acetyl-L-tryptophan (N-Ac-Trp-OH) and the selectivity against the water content in [emim][FSI] at 25 °C. The inherent enzymatic hydrolytic reaction is

Fig. 5. Effect of water content on $\alpha$-chymotrypsin-catalyzed peptide synthesis in [emim][FSI].
inhibited by low water content, while the enzymatic peptide synthesis is promoted. The initial rates of N-Ac-Trp-Gly-Gly-NH₂ and N-Ac-Trp-OH vs. the water content display a bell-shaped curve below 10% (v/v) water. Below 10% (v/v) water, the peptide and hydrolysis profiles are similar to those in acetonitrile and acetonitrile/supercritical carbon dioxide systems (Kise et al., 1988; Noritomi et al., 1995). The relationship between the activity and water content tends to exhibit bell-shaped curve. The optimal water content is due to the balance between kinetic rigidity and thermodynamic stability of enzyme structures, and is called essential water (Klibanov, 1986). The kinetic rigidity is relaxed by increasing water content, while native enzyme structure gradually changes through thermodynamic stability. Thus, the activity increases with an increase in the flexibility of rigid enzyme, and it decreases with an increase in disturbance of enzyme structure. On the other hand, above 10% (v/v) water the hydrolysis increased with an increase in water content. It is suggested that above 10% (v/v) water the amount of water exceeds the solubility of water in the system, the distribution of water is enhanced around the enzyme, and the hydrolysis is enhanced. As the optimum initial rate and selectivity of peptide synthesis were observed at 5% (v/v) water, the enzyme reaction experiments shown below were carried at 5% (v/v) water.

### 3.3 Peptide synthesis catalyzed by α-chymotrypsin in ionic liquids

Peptide synthesis is advantageous not in hydrophobic organic solvents but in hydrophilic organic solvents due to the solubility of amino acid derivatives (Kise et al., 1990). As seen in Table 1, both the activity and selectivity of peptide synthesis by free α-chymotrypsin in [emim][FSI] were much superior to those in [emim][BF₄] and [emim][ Tf], although [emim][BF₄] and [emim][Tf] are more hydrophilic than [emim][FSI] as shown in Fig.1. [emim][FSI] has a good solubility of amino acid derivatives. It has been well known that ionic liquids can dissolve many organic and inorganic compounds, since they exhibit a wide range of intermolecular interactions by the design of ionic liquids (Moniruzzaman et al., 2010). On the other hand, enzymes are generally more stable in hydrophobic solvents than in hydrophilic solvents. Moreover, it has been reported that the activity of Candida antarctica lipase B in a transesterification reaction is strongly dependent upon a sort of anion consisting of the same cation when using an ionic liquid as a solvent (Lau et al., 2004).

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Initial rate (μM/min • mg of enzyme)</th>
<th>Selectivity (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peptide</td>
<td>Hydrolysate</td>
</tr>
<tr>
<td>[emim][FSI]</td>
<td>280</td>
<td>180</td>
</tr>
<tr>
<td>[emim][BF₄]</td>
<td>0.031</td>
<td>0.093</td>
</tr>
<tr>
<td>[emim][Tf]</td>
<td>0</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Table 1. Initial rates of N-Ac-Trp-Gly-Gly-NH₂ and N-Ac-Trp-OH in the peptide synthesis of N-Ac-Trp- OEt with Gly-Gly-NH₂ catalyzed by free α-chymotrypsin in several kinds of ionic liquids containing 5% (v/v) water at 25°C

### 3.4 The solvent dependence of peptide synthesis catalyzed by α-chymotrypsin

As seen in Fig. 3, enzymatic activity of esterification of amino acid markedly depends upon the nature of solvents. Figure 6 shows the initial rates of peptide synthesis and hydrolysis
and the selectivity catalyzed by free α-chymotrypsin in ionic liquid, [emim][FSI] and conventional organic solvents at 5% (v/v) water and 25 °C. The initial rates of peptide synthesis and hydrolysis tended to be dramatically dependent upon a kind of solvents. The reactivity in hydrophilic organic solvents was observed as shown in Fig.6, while that in octane was hardly observed due to low solubility of substrates. Enzymatic reactions in hydrophilic organic solvents have the advantage of the solubility of amino acid derivatives, which are poorly soluble in hydrophobic solvents. The initial rate of peptide synthesis in [emim][FSI] was superior to that in organic solvents, and, for example, was about six times greater than that in tetrahydrofuran. The conformation of enzymes in hydrophilic solvents is remarkably rigid, and disturbs the induced-fit process of enzyme reaction, since essential water is stripped off from enzymes by hydrophilic solvents. As a result, the enzymatic activity in hydrophilic organic solvents is low, compared to that in [emim][FSI]. Moreover, the powder of α-chymotrypsin was finely dispersed in [emim][FSI], and the reaction system was visually transparent, while the enzyme powder was suspended in organic solvent systems. In the heterogeneous catalytic system the apparent rate corresponds upon the rate per unit area of catalysts multiplied by the surface area. The small surface area of enzyme powder resulted in the low initial rate in organic solvents. The sufficient reactivity observed in [emim][FSI] might be attributable to the improvement of the surface area of an enzyme powder due to the fine dispersion.

Fig. 6. Solvent-dependence of α-chymotrypsin-catalyzed peptide synthesis.

3.5 The temperature dependence of peptide synthesis catalyzed by α-chymotrypsin in [emim][FSI] and organic solvents
Enzymatic reactions, as well as chemical reactions, obey the Arrhenius correlation between reaction rate constant and temperature, although the temperature range is quite limited. Figure 7 shows the plots of initial rates of peptide synthesis in [emim][FSI] and organic solvents containing 5% (v/v) water against temperature. The initial rate of peptide synthesis in [emim][FSI] exhibited a maximum around 30 °C, and then decreased with an increase in
temperature. Likewise, the initial rates in tetrahydrofuran and tetrahydropyran showed a maximum around 40 °C. According to the Arrhenius equation, the initial rate becomes high at a high temperature. However, the thermal denaturation of enzymes proceeds reversibly and/or irreversibly. Consequently, the profile of activity has an optimal temperature. Comparing [emim][FSI] system with organic solvent systems, the initial rate of peptide synthesis in [emim][FSI] was 6-fold superior to that in tetrahydrofuran at 60 °C, and was seventeenth times greater than that in acetonitrile and tetrahydropyran at 60 °C. On the other hand, thermal denaturation of enzymes in aqueous solutions begins at 45 to 50 °C and is severe. The activity of α-chymotrypsin in aqueous solutions was not observed at 60 °C.

As the temperature increases, the atoms in the enzyme molecule have greater energies and a greater tendency to move, acquire sufficient energy to overcome the weak interactions keeping the tertiary structure of the enzyme molecule, and denaturation follows. The thermostability of enzymes in anhydrous media is highly improved, compared with that in aqueous solutions, since the thermal denaturation of enzymes is due to water (Klibanov, 2001). In other words, the disturbance of the atoms in the enzyme molecule tends to be relaxed, since the enzyme molecule in limited water content is rigid. Furthermore, it has been reported that Candida antarctica lipase B is stabilized by ionic liquids in ester synthesis, since ionic liquids maintain the enzyme conformation into the ionic net, and work as both immobilization support and reaction media (Lozano et al., 2001). As shown in Table 2, the activity of α-chymotrypsin in peptide synthesis in [emim][FSI] and [emim][PF₆] was observed, although the initial rates of peptide and hydrolysate at 80 °C were about one-tenth smaller than those at 25 °C in [emim][FSI]. On the other hand, the production of peptides or hydrolysates by enzyme reaction was not shown in [emim][BF₄] and [emim][Tf].
Table 2. Initial rates of N-Ac-Trp-Gly-Gly-NH$_2$ and N-Ac-Trp-OH in the peptide synthesis of N-Ac-Trp-OEt with Gly-Gly-NH$_2$ catalyzed by free α-chymotrypsin in several kinds of ionic liquids containing 5% (v/v) water at 80°C.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Peptide Initial rate (μM/min • mg of enzyme)</th>
<th>Hydrolysate Initial rate (μM/min • mg of enzyme)</th>
<th>Selectivity (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[emim][FSI]</td>
<td>25</td>
<td>20</td>
<td>290</td>
</tr>
<tr>
<td>[emim][PF$_6$]</td>
<td>22</td>
<td>61</td>
<td>83</td>
</tr>
<tr>
<td>[emim][BF$_4$]</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>[emim][Tf]</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

4. Conclusion

In this chapter the effects of ionic liquids on the catalytic behavior and stability of protease have been described. The activities of protease in both esterification of amino acid and peptide synthesis in ionic liquids were superior to those in conventional organic solvents. The activity in ionic liquid systems was dependent upon the nature of solvent, similar to the case of organic solvent systems, and the tendency in ionic liquid systems seemed to be more sensitive than that in organic solvent systems, since the activity markedly altered by switching from one anion to another of an ionic liquid consisting of the same cation. Concerning the thermostability, the same tendency was exhibited. These indicate that the constituents of ionic liquids directly affect protease, compared to the case of organic solvent systems. For instance, it is apparent that [emim][FSI] functions as a thermal stabilizing agent. When using protease in non-aqueous reaction media, reaction media having the properties of hydrophobicity, good solubility of amino acid derivatives, and the fine dispersion of enzyme are desirable. The conventional organic solvents such as acetonitrile lack these properties, while [emim][FSI] has these properties. Moreover, it is expected that the ionic liquid, which is more suitable for the catalytic behavior and stability of protease, is prepared by tailoring the constituents of ionic liquids.

5. References


Protease-Catalyzed Synthetic Reactions in Ionic Liquids


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This book is the second in the series of publications in this field by this publisher, and contains a number of latest research developments on ionic liquids (ILs). This promising new area has received a lot of attention during the last 20 years. Readers will find 30 chapters collected in 6 sections on recent applications of ILs in polymer sciences, material chemistry, catalysis, nanotechnology, biotechnology and electrochemical applications. The authors of each chapter are scientists and technologists from different countries with strong expertise in their respective fields. You will be able to perceive a trend analysis and examine recent developments in different areas of ILs chemistry and technologies. The book should help in systematization of knowledges in ILs science, creation of new approaches in this field and further promotion of ILs technologies for the future.

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