Salt-activation of thermolysin was examined using a positively charged fluorescent substrate, (7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly; MOCAc-PLG, (7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly-L-Leu-[N\(^{3}\)-2,4-dinitrophenyl]-L-2,3-diaminopropionyl]-L-Ala-L-Arg-NH\(_2\) [MOCAC-PLGL(Dpa)AR]. Thermolysin activity increased in a biphasic exponential fashion and was 40 times higher in the presence of 4 M NaCl than in its absence. The degree of activation at x M NaCl was shown by the log v vs. [NaCl]\(_o\) plots. The v values at 0 M and 4 M NaCl were 0.40 ± 0.02 and 15.9 ± 0.9 nm/s\(^{-1}\) respectively. Activation behavior was analyzed by the method introduced previously. The degree of activation in the presence of x M NaCl is expressed as \(v_x/v_0\), where \(v_x\) and \(v_0\) are reaction rates at x M and 0 M NaCl, determined to be 4.7\(^{a}\) at x < 0.5 and 2.3\(^{a}\) at x > 0.5 respectively. The parameters of salt-activation of thermolysin obtained with FAGLA and ZDFM, and that of human matrix metalloproteinase 7 (MMP-7), a collagenase closely related to thermolysin, are summarized in Table 1. Salt-activation of the hydrolysis of a neutral substrate, FAGLA, is characterized by a single exponential curve, whereas that of a negatively charged substrate, ZDFM, is characterized by a biphasic exponential one. It should be noted that the \(v_x/v_0\) value with FAGLA is almost the same and the value with ZDFM lower than those at x > 0.5, probably due to the repulsive and attractive electrostatic interactions between thermolysin and the substrates respectively. It is suggested that thermolysin prefers negatively charged substrates at low concentrations of salts. The \(v_x/v_0\) value with FAGLA is almost the same as that as with ZDFM at x > 0.5, suggesting that salt-activation with these dipeptide substrates is substantially the same when the electrostatic interaction is negligible. On the other hand, the \(v_x/v_0\) value obtained with MOCAC-PLGL(Dpa)AR at x > 0.5 is much higher than those with FAGLA and ZDFM, suggesting that activation at x > 0.5 depends on the chain length or hydrophobicity of the substrate. The hydrolysis of MOCAC-PLGL(Dpa)AR by MMP-7 is activated by NaCl, and this activation is brought about solely through an increase in \(K_m\). The exponential behavior of salt-

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**Key words:** metalloproteinase; thermolysin; salt-activation; halophilicity
activation of MMP-7 is similar to that of thermolysin, but the magnitude of the activation is much lower and the activation is attributable to stabilization of the ES complex. These observations suggest that halophilic properties are different depending on the species of enzyme and substrate.

The pH-dependence of the thermolysin-catalyzed hydrolysis of MOCAc-PLGL(Dpa)AR was also examined. A narrow bell-shaped pH-dependence of thermolysin activity was obtained in the absence and the presence of 4 M NaCl. The maximum activities were 0.59 and 22.7 nM s$^{-1}$ respectively (Fig. 2A). The pH-dependence of $v$ follows the ionization of free thermolysin under the pseudo-first-order conditions. The $pK_{e1}$ and $pK_{e2}$ values were determined by the log $v$ vs. pH plots (Dixon plots) to be 5.56 and 7.52 in the absence of NaCl, and 6.05 and 7.54 in the presence of 4 M NaCl respectively (Fig. 2B). These values were separated by less than 2 pH units, and thus the $pK_e$ values were evaluated also according to the following equation using the non-linear least-squares method: $v = \frac{k_{cat}}{[S]} + \frac{K_m}{[S]}$.

**Table 1. Degree of Activation of Thermolysin and MMP-7 by NaCl**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Thermolysin</th>
<th>MMP-7$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAGLA$^a$</td>
<td>1.9$^b$</td>
<td>n.d.$^d$</td>
</tr>
<tr>
<td>ZDFM$^c$</td>
<td>1.2$^c$ (x &lt; 0.5)</td>
<td>n.d.</td>
</tr>
<tr>
<td>MOCAc-PLGL</td>
<td>4.7$^d$ (x &gt; 0.5)</td>
<td>2.1$^e$ (x &lt; 0.5)</td>
</tr>
<tr>
<td>-(Dpa)AR</td>
<td>2.3$^f$ (x &lt; 0.5)</td>
<td>1.4$^f$ (x &gt; 0.5)</td>
</tr>
</tbody>
</table>

$^a$, Ref. 7; $^b$, Ref. 4; $^c$, Degree of activation at x M NaCl at pH 7.5, 25°C; $^d$, not detected; $^e$, Ref. 3.

Fig. 1. Effect of NaCl on the Thermolysin-catalyzed Hydrolysis of MOCAc-PLGL(Dpa)AR.
The reaction was performed in 50 mM Tris–HCl buffer (pH 7.5) plus 10 mM CaCl$_2$, 0.6% dimethyl sulfoxide at 25°C. The initial concentrations of thermolysin and MOCAc-PLGL(Dpa)AR were 7.2 nM and 750 nM respectively. A, effect of NaCl on the reaction rate ($v$) and the degree of activation; B, logarithmic relationship of the reaction rate with [NaCl].

Fig. 2. pH-Dependence of the Thermolysin-catalyzed Hydrolysis of MOCAc-PLGL(Dpa)AR.
The reaction was performed in 50 mM acetate (pH 4.0–5.8), MES (pH 5.8–7.0), Tris–HCl (pH 7.0–9.0) plus 10 mM CaCl$_2$, 0.6% dimethyl sulfoxide at 25°C. NaCl concentrations added to the buffers: 4 M, ○; 0 M, ●. The initial concentrations of thermolysin and MOCAc-PLGL(Dpa)AR were 7.2 nM and 750 nM respectively. A, effect of pH on the reaction rate ($v$); B, logarithmic relationship of the reaction rate with pH.
\[ v = \frac{v_{\text{max}}}{1 + ([H^+] / K_{e1}) + (K_{e2} / [H^+] )} \]  

(1)

where \( v_{\text{max}} \) is the theoretical maximal activity, \( v_{\text{max}} \), \( pK_{e1} \), and \( pK_{e2} \) were determined to be 0.80 nM s\(^{-1}\), 5.60, and 7.45 at 0 M NaCl, and 32.9 nM s\(^{-1}\), 6.12, and 7.40 at 4 M NaCl, respectively. There is no significant difference in \( pK_e \) values between the two methods, and it is noted that \( pK_{e1} \) shifts from 5.6 to 6.1 with an increase in [NaCl] from 0 to 4 M, while \( pK_{e2} \) is unaltered. This shift can be attributed to a microenvironmental change around the \( pK_{e1} \) group, suggesting that high concentrations of NaCl may induce a conformational change in thermolysin. These observations might provide a clue to understand salt-activation of thermolysin, MMP-7, and other halophilic enzymes.

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**References**


