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Substrate-dependent Activation of Thermolysin by Salt

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Note

Substrate-dependent Activation of Thermolysin by Salt

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Salt-activation of thermolysin was examined using a positively charged fluorescent substrate, (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 expressed as 4.7^x when $[NaCl]_o < 0.5$ M and 2.3^x when $[NaCl]_o > 0.5$ M respectively.

Key words: metalloproteinase; thermolysin; salt-activation; halophilicity

Thermolysin [EC 3.4.24.27] is a thermostable neutral metalloproteinase isolated from *Bacillus thermoproteolyticus*.[1] It requires essentially one zinc ion for enzyme activity and four calcium ions for structural stability.[2] We have previously reported remarkable activation of thermolysin by high concentrations (1–5 M) of neutral salts in the hydrolysis and synthesis of ZDFM, a precursor of a synthetic sweetener, aspartame,[3] and in the hydrolysis of FAGLA.[4,5] Activity increases apparently in an exponential fashion with increases in salt concentration, and activation is brought about solely through an increase in k_{cat}.[3,4] This suggests that activation is attributable to stabilization of the transition state of the enzymatic reaction. Little is known, however, about the cause of salt-activation of thermolysin at present.

In this study, we describe salt-activation of thermolysin using a positively charged fluorescent substrate, MOCAc-PLGL(Dpa)AR. The peptide bond between the Gly and Leu residues was cleaved by thermolysin,[6] and the amount of the product MOCAc-PLG was estimated by fluorescence intensity at 393 nm with excitation at 328 nm by comparing it with that of the authentic MOCAc-PLG solution. The reaction was carried out under pseudo-first-order conditions, where the substrate concentration [S] is much lower than the K_m value, in order to avoid absorptive quenching effects.[7] Thus the reaction rate (v) was expressed as $k_{cat}\,[E]_o[S]/K_m$, and thermolysin showed k_{cat}/K_m value of 75.0 $mM^{-1}\,s^{-1}$ at pH 7.5, 25 °C.

Figure 1 shows activation of thermolysin by NaCl in the hydrolysis of MOCAc-PLGL(Dpa)AR. Thermolysin activity (v) increased in a biphasic exponential fashion with increases in NaCl concentration, as is clearly 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.[4] The degree of activation in the presence of x M NaCl is expressed as v_x/v_o, where v_x and v_o are reaction rates at x M and 0 M NaCl, determined to be 4.7^x at $x < 0.5$ and 2.3^x 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_o value at $x < 0.5$ obtained with MOCAc-PLGL(Dpa)AR is higher 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_o value with FAGLA is almost the same as that 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_o 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 decrease in K_m.[7] The exponential behavior of salt-

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Abbreviations: FAGLA, *N*-[3-(2-furyl)acryloyl]-Gly-L-Leu-NH_2; MES, 2-(*N*-morpholino)ethanesulfonic acid; MMP-7, matrix metalloproteinase 7; MOCAc-PLG, (7-methoxycoumarin-4-yl)acetyl-L-Pro-L-Leu-Gly; MOCAc-PLGL(Dpa)-AR, (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; ZDFM, *N*-carbozoxy-L-Asp-L-Phe-methyl ester

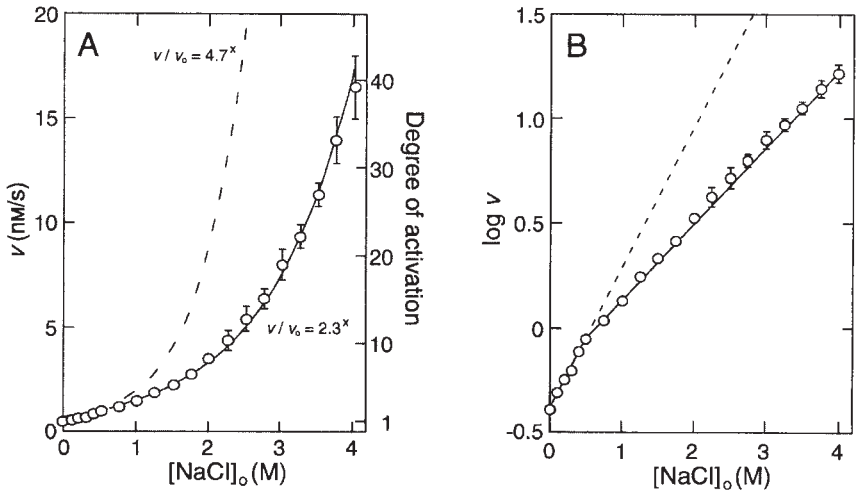

Fig. 1. Effect of NaCl on the Thermolysin-catalyzed Hydrolysis of MOCAc-PLGL(Dpa)AR.
The reaction was performed in 50 mM Tris–HCl buffer (pH 7.5) plus 10 mM $CaCl_2$, 0.6% dimethyl sulfoxide at 25 °C. The initial concentrations of thermolysin and MOCAc-PLGL(Dpa)AR were 7.2 nM and 750 nM respectively. A, effect of NaCl on the reaction rate (v) and the degree of activation; B, logarithmic relationship of the reaction rate with [NaCl].

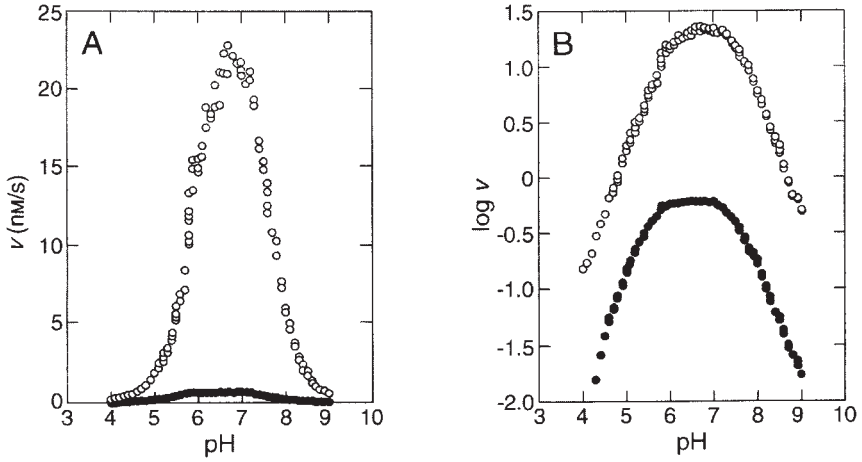

Fig. 2. pH-Dependence of the Thermolysin-catalyzed Hydrolysis of MOCAc-PLGL(Dpa)AR.
The reaction was performed in 50 mM acetate (pH 4.0–5.8), MES (pH 5.8–7.0), Tris–HCl (pH 7.0–9.0) plus 10 mM $CaCl_2$, 0.6% dimethyl sulfoxide at 25 °C. NaCl concentrations added to the buffers: 4 M, ○; 0 M, ●. The initial 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.

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

Substrate	Thermolysin	MMP-7[a]
FAGLA[b]	1.9^{xc}	n.d.[d]
ZDFM[e]	1.2^x ($x < 0.5$)	n.d.
	1.8^x ($x > 0.5$)	
MOCAc-PLGL	4.7^x ($x < 0.5$)	2.1^x ($x < 0.5$)
-(Dpa)AR	2.3^x ($x > 0.5$)	1.4^x ($x > 0.5$)

[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.

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:[8)]

$$v = v_{max}/[1 + ([H^+]/K_{e1}) + (K_{e2}/[H^+])] \quad (1)$$

where v_{max} is the theoretical maximal activity. v_{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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