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Molecular Mechanism of the Inhibitory Effect of Cobalt Ion on Thermolysin Activity and the Suppressive Effect of Calcium Ion on the Cobalt Ion-dependent Inactivation of Thermolysin

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Thermolysin activity in the hydrolysis of *N*-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide (FAGLA) and FA-L-leucyl-L-alanine amide (FALAA) was examined at various Co^{2+} and Ca^{2+} concentrations. It decreased to 28% with increasing [Co^{2+}] up to 18 mM. The Co^{2+}-dependent inactivation was in part suppressed by adding Ca^{2+} ion up to 0.5 mM, but 33% of the activity remained to be inactivated even with a sufficient concentration of Ca^{2+} (>0.5 mM). The Co^{2+}-dependent inactivation was shown to be composed of Ca^{2+}-sensitive and Ca^{2+}-insensitive parts. In the latter part which is observed at [Ca^{2+}] >0.5 mM, Co^{2+} plays as a competitive inhibitor. On the other hand, the Co^{2+}-dependent inactivation in the Ca^{2+}-sensitive part observed at [Ca^{2+}] <0.5 mM proceeds time-dependently following second-order kinetics, and the time-course is in good agreement with that of decrease in the thermolysin band due to autolysis in SDS–PAGE. This indicates that Co^{2+} accelerates the autolysis. Here, we describe the co-regulation of thermolysin activity by Co^{2+} and Ca^{2+} ions and propose a molecular mechanism for the inhibition of thermolysin by Co^{2+} and suppressive effect of Ca^{2+} on the Co^{2+}-dependent inhibition. Co^{2+} ion inhibits thermolysin activity not only as a competitive inhibitor but also promoting the autolysis.

Key words: autolysis, cobalt, inhibition, metalloproteinase, thermolysin.

Thermolysin [EC 3. 4. 24. 27] is a thermostable neutral metalloproteinase produced in the culture broth of *Bacillus thermoproteolyticus* (*1–4*). It requires one zinc ion for enzyme activity and four calcium ions for structural stability (*5–7*), and catalyses specifically the hydrolysis of peptide bonds containing hydrophobic amino acid residues (*8*). The amino acid sequence (*9*, *10*) and 3D structure (*11*) are available, and a reaction mechanism has been proposed (*12–14*).

We have reported that high concentrations (1–5 M) of neutral salts cause a remarkable activation of the thermolysin-catalysed hydrolysis and synthesis of *N*-carbobenzoxy-L-asparatyl-L-phenylalanine methyl ester (ZDFM), a precursor of a synthetic sweetener (*15*), and hydrolysis of *N*-[3-(2-furyl)acryloyl] (FA)-dipeptide amides with different amino acids at the scissile bond (*16*). The activation is brought about most effectively by NaCl and NaBr, and the activity increases in an exponential fashion with increasing salt concentration. The degree of activation at *x* M NaCl is expressed by 1.9^x at pH 7.5 (*16*). The molecular activity, k_{cat}, and Michaelis constant, K_m, can be evaluated separately in the cases of ZDFM, *N*-FA-L-leucyl-L-alanine amide (FALAA) and *N*-FA-L-phenylalanyl-L-alanine amide (FAFAA), and the activation has been demonstrated to be induced solely by an increase in k_{cat}, and K_m is not affected at all by the presence of salts (*15–17*). Recently, we have reported that the activation degree is dependent on the electrostatic charges on the substrate, and is higher with the substrate carrying positive charge than that carrying negative charge (*18*). We have observed a characteristic absorption difference spectrum on mixing thermolysin with NaCl and NaBr, suggesting changes in the states of tyrosyl and tryptophyl residues (*15*, *19*). The specific interaction between cations and thermolysin might be involved in the activation, and effectiveness is in the order of $Na^+ > K^+ > Li^+$ (*15*, *16*). We also demonstrated that a change in the ionization state on the surface of thermolysin affects the NaCl-dependent activation by means of nitration and amination of tyrosyl residues in the enzyme (*20*). Accordingly, the salt-dependent activation might be related to electrostatic interactions of thermolysin with ions in the medium. The solubility of thermolysin increases greatly in the presence of high concentrations of salts (*21*), and the thermal stability is also increased by the addition of NaCl (*22*). Unique interactions of the molecular surface of thermolysin with ions might change the solubility and thermal stability as well as the activity. To explore the mechanism of the salt-induced activation, we recently reported the preliminary X-ray crystallographic analysis of thermolysin in the presence of 4 M NaCl (*23*). Recently, we have established novel efficient methods for purification and expression of thermolysin, which enable us to prepare a wide range of thermolysin variants and to examine the reaction mechanism precisely (*24–27*).

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We have examined the effect of Co^{2+} on thermolysin activity (*28*). It has been found that thermolysin activity is greatly enhanced on the addition of Co^{2+} (<2 mM), but the enhanced activity decreases gradually with increasing [Co^{2+}] from 2 to 18 mM. It was shown that the activation of thermolysin observed with Co^{2+} of <2 mM was caused by replacing the catalytically essential Zn^{2+} ion in the active site with a Co^{2+} ion. However, the suppression mechanism of the enhanced activity with the higher concentration of Co^{2+} has not yet been studied well. It has been reported that many metalloproteases are inhibited by an excessive amount of metal ions. For example, Zn^{2+} inhibits thermolysin (*29*), carboxypeptidase A (*30*, *31*) and angiotensin converting enzyme (ACE) (*32*), and also Co^{2+} inhibits ACE (*31*). It has been shown that Zn^{2+} inhibits carboxypeptidase A in a competitive manner, suggesting that it binds to the active site of the enzyme (*30*, *31*), although the inhibition mechanism of other enzymes has not been investigated in detail.

In this study, we describe the inhibitory effect of Co^{2+} ions on thermolysin activity and the suppressive effect of Ca^{2+} ions on the Co^{2+}-dependent inhibition by kinetic analysis, and propose the molecular mechanism for the effects of Co^{2+} and Ca^{2+} ions on the activity and stability of thermolysin. We demonstrate that Co^{2+} inhibits thermolysin activity not only as a competitive inhibitor but also promoting the autolysis.

EXPERIMENTAL PROCEDURES

Materials—A three-times-crystallized and lyophilized preparation of thermolysin (Lot T5CB491; 8720 proteinase units/mg according to the supplier) was purchased from Daiwa Kasei (Osaka). This preparation was used without further purification. The solution of thermolysin was filtered through a Millipore membrane filter, Type HA (pore size: 0.45 μm) before use. The concentration of thermolysin was determined spectrophotometrically using an absorption coefficient, A (1 mg/ml), at 277 nm of 1.83 (*15*), and a molecular mass of 34.6 kDa (*9*, *15*). FAGLA (Lot 57H5800) and FALAA (Lot 460519) were purchased from Sigma (St Louis, MO, USA) and Peptide Institute (Osaka), respectively. The concentration of FAGLA and FALAA was determined spectrophotometrically using the molar absorption coefficient, $\varepsilon_{345} = 766\ M^{-1}\ cm^{-1}$ (*15*, *33*). All other chemicals were of reagent grade and purchased from Nacalai Tesque (Kyoto) and Wako Pure Chemical (Osaka).

Hydrolysis of FA-dipeptide Substrates—Hydrolysis of FAGLA and FALAA catalysed by thermolysin was performed in 40 mM HEPES–Na buffer at pH 7.0 and 25°C (buffer A). The enzyme solution (1.0 ml) preincubated with 0.01–18 mM $CoCl_2$ and 0.01–10 mM $CaCl_2$ for 30 min was added to the substrate solution (2.0 ml). The initial concentration of thermolysin at the start of the enzyme reaction in an optical cuvette was 120 nM, and those of FAGLA and FALAA were 0.3–0.4 and 0.2–1.1 mM, respectively. The hydrolysis of the FA-dipeptide substrates was followed by continuous monitoring of the decrease in absorbance at 345 nm with a Shimadzu UV-visible recording spectrophotometer UVmini-1240 (Kyoto). The amount of substrate hydrolysed was estimated using the molar absorptivity difference on the hydrolysis, $\Delta\varepsilon_{345} = -310\ M^{-1}\ cm^{-1}$ (*15*). Because of the high K_m value (~30 mM) and poor solubility of FAGLA, it was difficult to perform reactions at FAGLA concentrations large enough to separate the k_{cat} and K_m values. The reactions were performed under the conditions of $[FAGLA]_o \ll K_m$, where pseudo-first-order kinetics is valid, and the activity was expressed by the specificity constant, k_{cat}/K_m, throughout this study.

Preparation of Apo-thermolysin—Apo-thermolysin was prepared according to our previous method (*34*). Ten millilitres of thermolysin (25 μM) in buffer A containing 10 mM $CaCl_2$ and 5 mM 1,10-phenanthroline was applied to a Sephadex G-50 Fine column [size: 3.0 cm (inner diameter) × 10 cm] (Amersham Pharmacia, Uppsala, Sweden) and eluted with buffer A containing 10 mM $CaCl_2$ and 5 mM 1,10-phenanthroline. Fractions containing thermolysin were collected, and then applied to a PD-10 column (Sephadex G-25, Amersham Pharmacia) equilibrated with buffer A containing a certain concentration of Ca^{2+} ions to remove 1,10-phenanthroline and to exchange the solvent with the buffer containing the desired Ca^{2+} concentration. This process was repeated twice to complete the removal of 1,10-phenanthroline and exchange of the solvent.

Determination of Autolysis of Thermolysin—Apo-thermolysin (0.3 μM) was incubated with 0.01–17.5 mM $CoCl_2$ in buffer A containing 0.01 mM $CaCl_2$ at 25°C for an appropriate time (10–90 min). At every 10 min, 80 μl of the thermolysin solution was taken out and the autolysis was stopped by adding 1 μl of 1 mM phosphoramidon, a specific inhibitor of thermolysin. SDS–PAGE was performed according to the Laemmli's method (*35*) with a slight modification. The inactivated thermolysin solution was mixed with 20 μl of loading buffer [312.5 mM Tris–HCl, 10.75% (w/v) SDS, 50% (v/v) glycerol, 0.025% (w/v) bromophenol blue (BPB), pH 6.8] and incubated at 100°C for 5 min to denature thermolysin. The denatured sample (15 μl) was loaded to a 15–25% (w/v) gradient gel, PAG mini Daiichi 15/25 (Daiichi Pure Chemicals, Tokyo) in an electro-loaded buffer [0.025 M Tris–HCl, 0.192 M glycine, 0.1% (w/v) SDS, pH 8.4] at 40 mA constant current for 60 min. The gel was stained with a Coomassie Fluor Orange protein gel staining reagent (Molecular Probes, Eugene, OR, USA), and the image was documented with a BioDoc-It system (UVP, Cambridge, UK). The amount of undigested thermolysin was estimated from an intensity of the protein band quantified with a Scion Image image-analysis software (http://www.scioncorp.com).

RESULTS

Effects of Co^{2+} ion on Thermolysin Activity—The hydrolytic activity (expressed by k_{cat}/K_m) of FAGLA by the native thermolysin (ZnTLN) at 10 mM $CaCl_2$ was $(27.5 \pm 0.3)\ mM^{-1}\ s^{-1}$. The activity of an apo-thermolysin (apoTLN) preparation was 5% or less of that of ZnTLN. The reduced but resulting activity [$(1.81 \pm 0.26)\ mM^{-1}\ s^{-1}$] might be due to the remaining

Zn^{2+} ion not to be removed from the active site. It was confirmed that the activity was recovered to (27.5 ± 0.3) mM^{-1} s^{-1} by the addition of 0.10 μM $ZnCl_2$. The activity was also increased to $(1.00 \pm 0.04) \times 10^2$ mM^{-1} s^{-1} by the addition of 0.1 mM Co^{2+}. The change in the activity induced by the addition of $ZnCl_2$ and $CoCl_2$ are known to be raised by Zn^{2+} and Co^{2+} ions but not by Cl^- ions (*28*, *34*). Thus, the effect of chloride salts of Co^{2+} and Zn^{2+} ions are analysed by considering here-in-after to be the effect of Co^{2+} and Zn^{2+} ions solely (in this article, we assume that $CoCl_2$ and $ZnCl_2$ dissociate completely into Co^{2+} and Zn^{2+} and Cl^- in the aqueous solution, and the concentrations of Co^{2+} and Zn^{2+} are for the sake of convenience expressed with the dimension of mol/l or M). Figure 1 shows the effect of Co^{2+} concentration on the FAGLA hydrolysis activities of ZnTLN and apoTLN. The activity of ZnTLN increased with increasing [Co^{2+}] up to 2 mM, and then decreased gradually with increasing [Co^{2+}] from 2 to 18 mM. On the other hand, the activity of apoTLN increased drastically up to $(1.05 \pm 0.07) \times 10^2$ mM^{-1} s^{-1} by the addition of 0.01 mM Co^{2+}, and the activity was maintained at [Co^{2+}] up to 0.1 mM. It is noted that the activity observed when apoTLN was incubated with 0.01 mM Co^{2+} is in good agreement with that when ZnTLN was incubated with 0.1 mM Co^{2+}. However, the maximum activity obtained when apoTLN was incubated with 0.01 mM Co^{2+} decreased constantly with increasing [Co^{2+}] up to 18 mM. This indicates that apoTLN binds Co^{2+} ion at its active site to be converted to CoTLN when $CoCl_2$ as low as 0.01 mM was added. The activity-decreasing behaviors of ZnTLN and apoTLN observed when $CoCl_2$ was added higher than 2 mM are substantially the same, suggesting strongly that both ZnTLN and apoTLN are converted to the same thermolysin species. We have found that the activation of ZnTLN by Co^{2+} is due to substituting Co^{2+} for Zn^{2+} in the active site of ZnTLN to form Co-substituted thermolysin (CoTLN), which has a 3–4 times higher activity than that of ZnTLN (*28*, *34*). The same inactivation behaviours of ZnTLN and apoTLN observed at Co^{2+} concentration higher than 2 mM should be analysed as the inactivation of CoTLN by excessive Co^{2+} concentration.

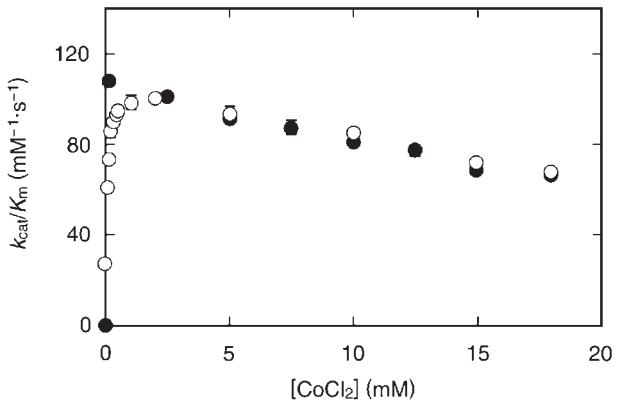

Fig. 1. **Effect of Co^{2+} ion on thermolysin activity.** The thermolysin-catalysed hydrolysis of FAGLA was measured using native-thermolysin or ZnTLN (open circle) and apo-thermolysin (filled circle). The reaction was performed in 40 mM HEPES-Na buffer (buffer A) containing 10 mM $CaCl_2$ and 0–18 mM $CoCl_2$, at pH 7.0 and 25°C. The initial concentrations of thermolysin and FAGLA in the reaction mixture were 0.1 μM and 400 μM, respectively.

Effect of Ca^{2+} on the Co^{2+}-dependent Inactivation of Thermolysin—The inhibitory effect of Co^{2+} on thermolysin activity in FAGLA hydrolysis was measured at various $CaCl_2$ concentrations in the range of 0–10 mM (Fig. 2). The activity (k_{cat}/K_m) observed by adding 0.01 mM $CoCl_2$ to apoTLN was set to 100% of the relative activity at respective $CaCl_2$ concentrations. The activity given by the addition of 0.01 mM $CoCl_2$ at 10 mM $CaCl_2$ was $(1.05 \pm 0.07) \times 10^2$ mM^{-1} s^{-1}. The inhibitory effect of Co^{2+} on CoTLN activity was suppressed with increasing [$CaCl_2$] from 0.01 to 10 mM. The IC_{50} value, the Co^{2+} concentration at which 50% of the enzyme activity is inhibited, is estimated as follows: 7 mM at 0.01 mM $CaCl_2$; 10 mM at 0.02 mM $CaCl_2$; 17 mM at 0.05 mM $CaCl_2$ and >20 mM at 10 mM $CaCl_2$. It is suggested that there might be three different metal-binding sites on the thermolysin surface: the primary binding site for Zn^{2+} or Co^{2+} existing at the active site of the enzyme and essential for the enzyme activity; the second Co^{2+}-binding site, at which the Co^{2+}-binding decreases the activity; and the Ca^{2+}-binding site, at which the Ca^{2+}-binding suppresses the inactivation induced when Co^{2+} binds to the second Co^{2+}-binding site.

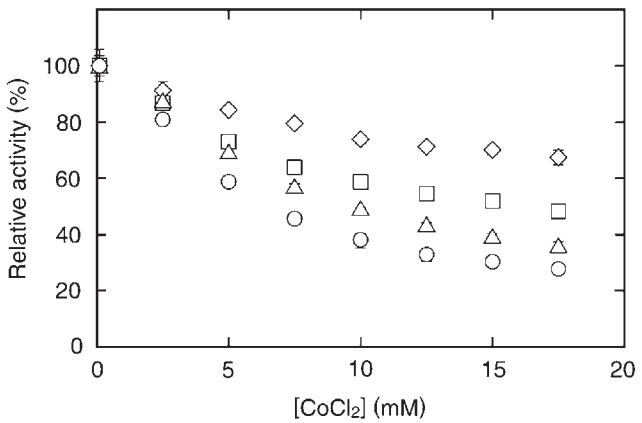

Fig. 2. **Suppressive effect of Ca^{2+} ion on the inactivation of thermolysin by Co^{2+} ion.** The thermolysin-catalysed hydrolysis of FAGLA at various Ca^{2+} ion concentrations was measured using apo-thermolysin in the presence of Co^{2+} ion concentrations indicated. The reaction was performed in buffer A containing 0.01–10 mM $CaCl_2$ and 0–17.5 mM $CoCl_2$, at pH 7.0 and 25°C. The initial concentrations of thermolysin and FAGLA in the reaction mixture were 0.12 and 300–400 μM, respectively. The concentration of $CaCl_2$ were 0.01 mM, open circle; 0.02 mM, open triangle; 0.05 mM, open square and 10 mM, open diamond. The activity was expressed by the specificity activity (k_{cat}/K_m). The activity (k_{cat}/K_m) observed by adding 0.01 mM $CoCl_2$ to apoTLN was set to 100% of the relative activity at respective $CaCl_2$ concentrations. The activity given by the addition of 0.01 mM $CoCl_2$ at 10 mM $CaCl_2$ was $(1.05 \pm 0.07) \times 10^2$ mM^{-1} s^{-1} (Fig. 1).

Figure 3 shows the dependence of thermolysin activity on the $CaCl_2$ concentration in the presence of 17.5 mM $CoCl_2$. When Ca^{2+} was not added to the thermolysin (0.3 μM apoTLN) solution, the relative activity observed at 17.5 mM Co^{2+} was decreased to 28% of that at 0.01 mM $CoCl_2$. However, it was recovered to 67% with increasing [Ca^{2+}] up to 0.5 mM, and stayed constant at 0.5–10 mM Ca^{2+}. This indicates that the Co^{2+}-dependent inactivation of thermolysin is suppressed by Ca^{2+}. The maximum suppressive effect was given at [Ca^{2+}] >0.5 mM, and the activity is recovered maximally from 28 to 67% of CoTLN activity. In other words, 33% of CoTLN activity cannot be recovered even with a sufficient amount of Ca^{2+} ion. It is suggested that the Co^{2+}-dependent inactivation of thermolysin is composed of at least two mechanisms: one of which is sensitive to Ca^{2+} and is recovered by the addition of Ca^{2+}, but the other is Ca^{2+}-insensitive. When CoTLN is treated with 17.5 mM Co^{2+}, 72% of the activity is lost, 39 and 33% of which are the degrees of the Ca^{2+}-sensitive inactivation and Ca^{2+}-insensitive one, respectively.

Effect of Co^{2+} on the Thermolysin-catalysed Hydrolysis of FALAA—FAGLA is the representative substrate for thermolysin, whereas the kinetic parameters, k_{cat} and K_m, could not be determined separately. Therefore, FALAA was used as the substrate to evaluate the effect of Co^{2+} ion on the kinetic parameters of thermolysin activity. The hydrolysis of FALAA was measured after incubating 0.14 μM apoTLN with 0.01, 5 or 10 mM $CoCl_2$ for 30 min in the presence of 10 mM $CaCl_2$. The Ca^{2+}-insensitive part of the Co^{2+}-dependent inactivation of CoTLN was observed under this condition. Lineweaver–Burk plot of the hydrolysis is shown in Fig. 4. The k_{cat} and K_m values of CoTLN for FALAA were estimated from the reaction at 0.01 mM $CoCl_2$ to be $(2.94 \pm 0.08) \times 10^{-3}\,s^{-1}$ and (0.599 ± 0.037) mM, respectively. All linear lines obtained with different Co^{2+} ion concentrations crossed on the vertical axis, indicating that Co^{2+} ion inhibits CoTLN activity competitively. Consequently, the Ca^{2+}-insensitive part of the Co^{2+}-dependent CoTLN inactivation is given by the competitive inhibition by Co^{2+} ion against CoTLN, and the inhibitor constant (K_i) was determined to be (22.2 ± 0.6) mM.

Progress Curves of CoTLN Activity in the Incubation with Co^{2+} ion—Progress curves of the thermolysin-catalysed hydrolysis of FAGLA was measured at 0.01 or 10 mM $CaCl_2$ after incubating 0.3 μM apoTLN with 0.01 or 17.5 mM $CoCl_2$ (Fig. 5). The activities (k_{cat}/K_m) obtained after incubating apoTLN with 0.01 mM $CoCl_2$ for 1 min in the presence of 0.01 or 10 mM $CaCl_2$ were set to 100% of the respective relative activities. Thermolysin activity incubated with 0.01 mM $CoCl_2$ at 10 mM $CaCl_2$ was hardly changed progressively, whereas that at 0.01 mM $CaCl_2$ decreased to 70% in 60 min. The activity incubated with 17.5 mM $CoCl_2$ at 10 mM $CaCl_2$ decreased rapidly to 70.4% within 1 min, and was kept constant till 60 min. On the other hand, at 0.01 mM $CaCl_2$, the activity incubated with 17.5 mM $CoCl_2$ decreased rapidly to 36.4%, and then decreased further gradually till 60 min. In the absence of Ca^{2+} ion added, the enzyme activity decreased progressively in the presence and absence of 17.5 mM Co^{2+} ion. On the other hand, in the presence of 10 mM Ca^{2+} ion, the enzyme activity was considerably kept in the time range up to 60 min regardless the presence or absence of 17.5 mM Co^{2+} ion. This result suggests that the Co^{2+}-dependent inactivation process could be divided into two steps. Namely, the rapid inactivation step was observed both at 0.01 and 10 mM $CaCl_2$, whereas the slow one was observed only at 0.01 mM $CaCl_2$. Accordingly, the rapid and slow inactivation steps might be related with

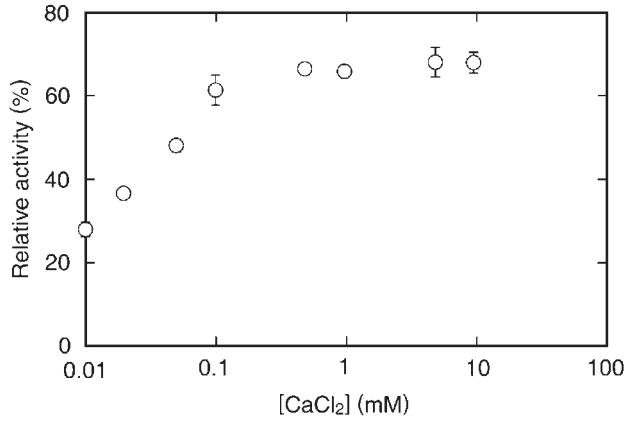

Fig. 3. **Suppressive effect of Ca^{2+} ion on the Co^{2+}-dependent inactivation of thermolysin.** The thermolysin-catalysed hydrolysis of FAGLA at various Ca^{2+} ion concentrations was measured using apo-thermolysin. Apo-thermolysin (0.3 μM) was incubated for 1 min in buffer A containing 0.01–10 mM $CaCl_2$ and 17.5 mM $CoCl_2$, at pH 7.0 and 25°C. The hydrolysis of FAGLA was performed under the same conditions, and the initial concentrations of thermolysin and FAGLA in the reaction mixture were 0.12 and 300–400 μM, respectively. The relative activity is defined as mentioned in Fig. 2.

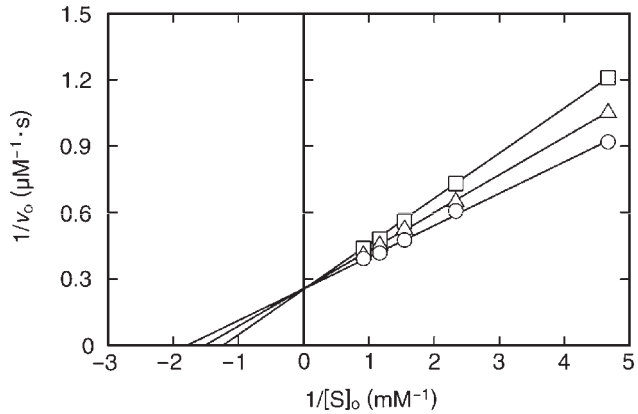

Fig. 4. **Lineweaver–Burk plot for the inhibition of cobalt ion against the thermolysin-catalysed hydrolysis of FALAA.** The hydrolysis was performed using apo-thermolysin in buffer A containing 10 mM $CaCl_2$ and 0.01–10 mM $CoCl_2$, at pH 7.0, 25°C. Thermolysin was incubated at 0.01 mM (open circle), 5 mM (open triangle) and 10 mM (open square) $CoCl_2$ for 30 min at pH 7.0 and 25°C. The initial concentrations of thermolysin and FALAA in the reaction mixture were 0.14 μM and 0.2–1.1 mM, respectively. v_o is the initial reaction rate, and $[S]_o$ is the initial concentration of FALAA.

the Ca^{2+}-insensitive and Ca^{2+}-sensitive parts of the Co^{2+}-dependent CoTLN inactivation, respectively.

Analysis of Time-dependent Inactivation Under Low $CaCl_2$ Concentration—The progress curves of the FAGLA hydrolysis by 0.3 μM apoTLN incubated with various concentrations (0.01, 5, 10 or 17.5 mM) of $CoCl_2$ in the presence of 0.01 mM $CaCl_2$ were observed (Fig. 6). Thermolysin activity obtained after the incubation with 0.01 mM $CoCl_2$ for 1 min at 0.01 mM $CaCl_2$ was set to 100% of the relative activity. The activity decreased progressively at every $CoCl_2$ concentration examined (Fig. 6A). The reciprocal of the activity was plotted against the incubation time. The plot showed a linear relationship (Fig. 6B), indicating that the Co^{2+}-dependent inactivation proceeds with second-order kinetics with respect to the enzyme concentration (Eq. 1).

$$d[E]/dt = -k_{obs}[E]^2 \quad (1)$$

where k_{obs} is an observed second-order rate constant of inactivation. Integrating of this equation gives the following equation (Eq. 2):

$$(k_{cat}/K_m)_o/(k_{cat}/K_m) = [E]_o/[E] = k_{obs}\,t[E]_o + 1 \quad (2)$$

where $(k_{cat}/K_m)_o$ and $[E]_o$ are the k_{cat}/K_m of CoTLN and CoTLN concentration, at time zero ($t = 0$), respectively. k_{obs} was estimated at each $CoCl_2$ concentration from the slope of Fig. 6B according to Eq. 2. The dependence of k_{obs} on $[CoCl_2]$ is shown in Fig. 7. The k_{obs} increased linearly from (0.454 ± 0.004) to $(1.56 \pm 0.01)\,mM^{-1}\,s^{-1}$ with increasing $[CoCl_2]$ from 0.01 to 17.5 mM, and was shown to be uniquely dependent on Co^{2+} ion added to the reaction mixture.

Autolysis of Thermolysin—It is known that the autolytic degradation of proteases obeys commonly second-order kinetics in enzyme concentration (*36*). In order to confirm whether the time-dependent inactivation of CoTLN (Fig. 6) is due to autolysis or not, the inactivation processes with 0.01 and 17.5 mM $CoCl_2$ in the presence of 0.01 mM $CaCl_2$ were followed by SDS–PAGE (Fig. 8). When thermolysin was incubated with 0.01 mM $CoCl_2$, the thermolysin band (34.6 kDa) was observed stably till 90 min. On the other hand, when it was incubated with 17.5 mM $CoCl_2$, the band disappeared gradually, and new bands (23.4, 21.5, 13.6 and 12.0 kDa) appeared.

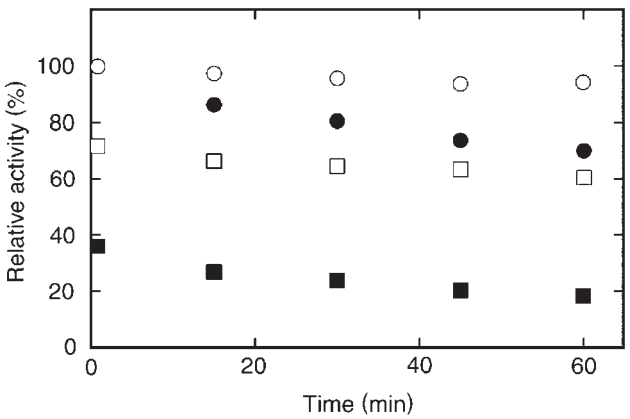

Fig. 5. **Time-dependent change in thermolysin activity incubated with Co^{2+} and Ca^{2+} ions.** The FAGLA hydrolysis activity of thermolysin incubated with $CoCl_2$ was examined. Apo-thermolysin (0.3 μM) was incubated for 1 min with 0.01 mM or 17.5 mM Co^{2+} at pH 7.0 and 25°C in the presence of either 0.01 or 10 mM Ca^{2+}. The initial concentrations of thermolysin and FAGLA in the reaction mixture were 0.12 and 300–400 μM, respectively. Symbols are; apo-thermolysin incubated with 0.01 mM $CoCl_2$ in the presence of 10 mM $CaCl_2$ (open circle) and 0.01 mM $CaCl_2$ (filled circle); incubated with 17.5 mM $CoCl_2$ in the presence of 10 mM $CaCl_2$ (open square) and 0.01 mM $CaCl_2$ (filled square). The activities (k_{cat}/K_m) obtained after incubating apo-thermolysin with 0.01 mM $CoCl_2$ in the presence of 0.01 mM or 10 mM $CaCl_2$ were set to 100% of the respective relative activities.

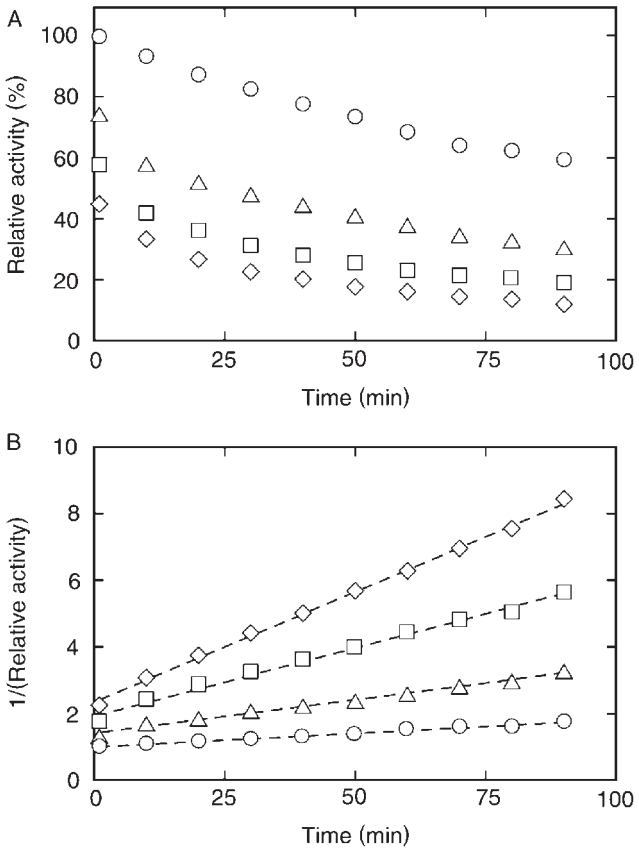

Fig. 6. **Effect of Co^{2+} ion on the time-dependent inactivation of thermolysin.** The progress curves of the FAGLA hydrolysis activity of thermolysin incubated with $CoCl_2$ were examined. Apo-thermolysin (0.3 μM) was incubated with 0.01 mM (open circle), 5 mM (open triangle), 10 mM (open square) and 17.5 mM (open diamond) $CoCl_2$ in buffer A at pH 7.0 and 25°C for 1 min in the presence of 0.01 mM $CaCl_2$. The initial concentrations of thermolysin and FAGLA in the reaction mixture were 0.12 and 300–400 μM, respectively. The activity (k_{cat}/K_m) obtained after incubating apo-thermolysin with 0.01 mM $CoCl_2$ in the presence of 0.01 mM $CaCl_2$ was set to 100% of the relative activity. Panel A: time-dependence of the relative activity. Panel B: time-dependence of the reciprocal relative activity. Dashed linear lines are the second-order reaction curves fitted to Eq. 1 using the data collected from the reaction after 30 min, and the second-order rate constant (k_{obs}) of the time-dependent inactivation of thermolysin by Co^{2+} was estimated from the slope of the linear line.

This degrading pattern is in good agreement with that of autolysis observed in the presence of EDTA (*37–40*). Because all Ca^{2+} ions bound to the thermolysin molecules are removed under the condition with EDTA, there might be a possibility that the degradation of thermolysin observed with 17.5 mM $CoCl_2$ (Fig. 8B) is derived from autolyis of CoTLN in parallel with losing Ca^{2+} ions from the Ca^{2+}-binding sites. In order to confirm this possibility, the 34.6-kDa band of CoTLN was quantified (Fig. 9A). The reciprocal of the CoTLN quantity showed a linear relationship with the incubation time (Fig. 9B). This relationship is similar to that observed with thermolysin activity for FAGLA hydrolysis (Fig. 7). The second-order rate constant (k_{obs}) for the CoTLN-autolysis detected by SDS–PAGE was estimated from the slope of the linear plot in Fig. 9B according to Eq. 2. The k_{obs} values were estimated to be (41.4 ± 5.8) and (1.37 ± 0.12) $mM^{-1}\ s^{-1}$, respectively, at 0.01 and 17.5 mM $CoCl_2$. The k_{obs} value estimated from the autolysis at 0.01 mM $CoCl_2$ is considerably different from that estimated from inactivation, whereas that at 17.5 mM is in good agreement with that from inactivation.

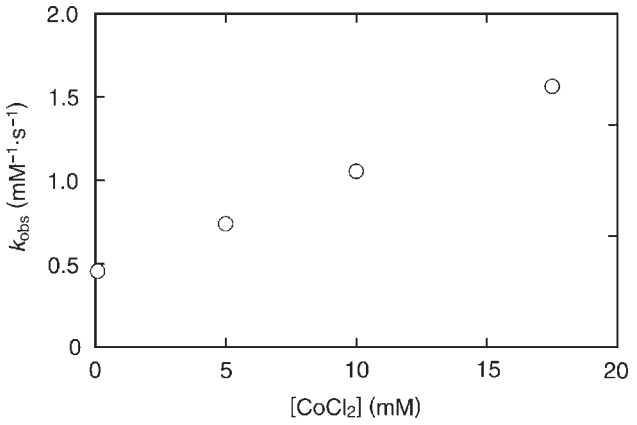

Fig. 7. **Effect of Co^{2+} ion on the second-order rate constant (k_{obs}) of the time-dependent inactivation of thermolysin.** The rate constant, k_{obs}, was determined from Fig. 6B.

DISCUSSION

Co^{2+}-binding Sites in the Active Site of Thermolysin— It is known that an excessive amount of metal ions inhibits metalloproteases. Thermolysin is inhibited by high concentration of Zn^{2+} ion (*29*), although its mechanism is not clarified. The inhibitory mechanism of carboxypeptidase A by Zn^{2+} ion was reported to be brought about the competitive binding of a Zn^{2+} ion to

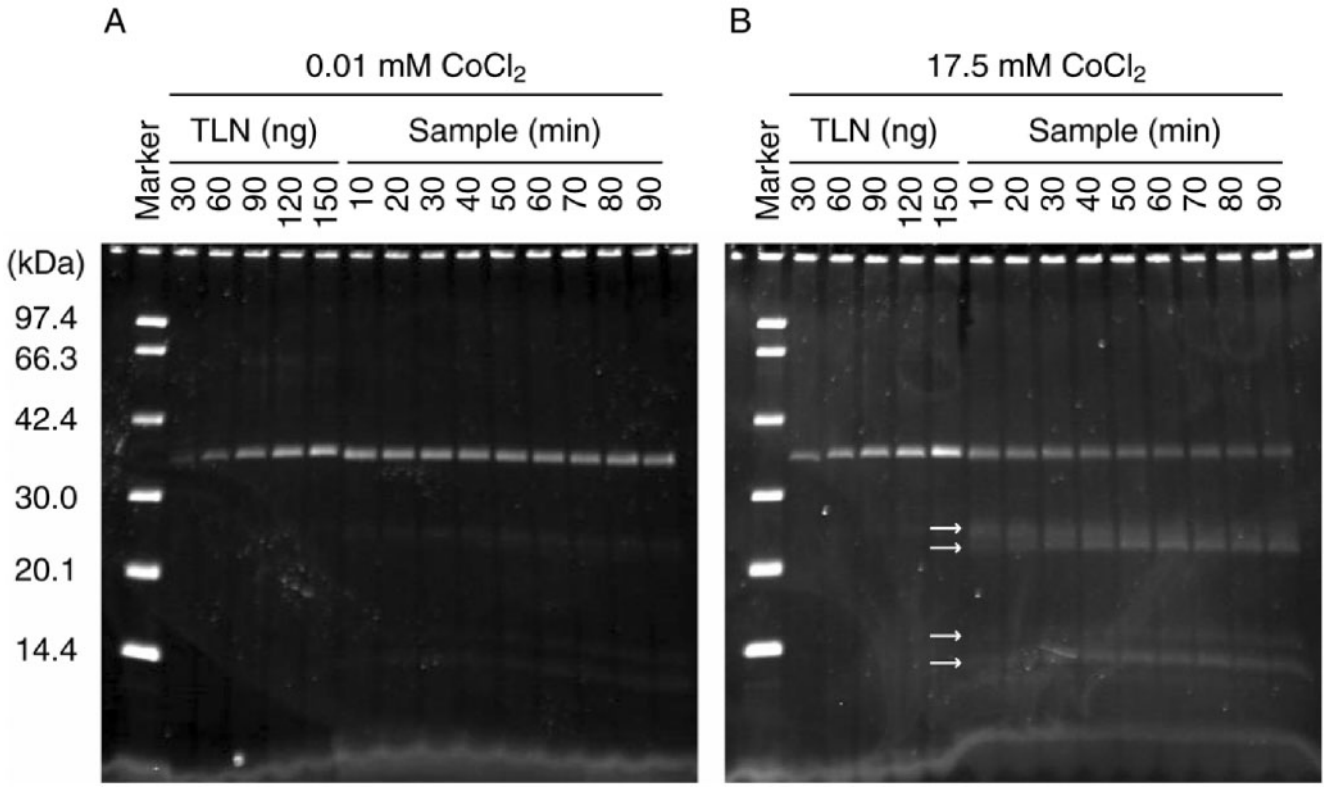

Fig. 8. **SDS–PAGE of thermolysin incubated with Co^{2+} ion.** Various quantities (30, 60, 90, 120 and 150 ng) of thermolysin (TLN) were applied to SDS–PAGE to make a calibration curve for quantitative determination of thermolysin. Apo-thermolysin (3 μM) was incubated with either 0.01 mM $CoCl_2$ (A) or 17.5 mM (B) $CoCl_2$ for 10–90 min in buffer A (pH 7.0 and 25°C) containing 0.01 mM $CaCl_2$, and 20 μl of the thermolysin solution was applied to SDS–PAGE. The signals of digestive products are indicated by white arrows in Panel B, and their sizes were estimated to be 23.4, 21.5, 13.6 and 12.0 kDa from their mobility.

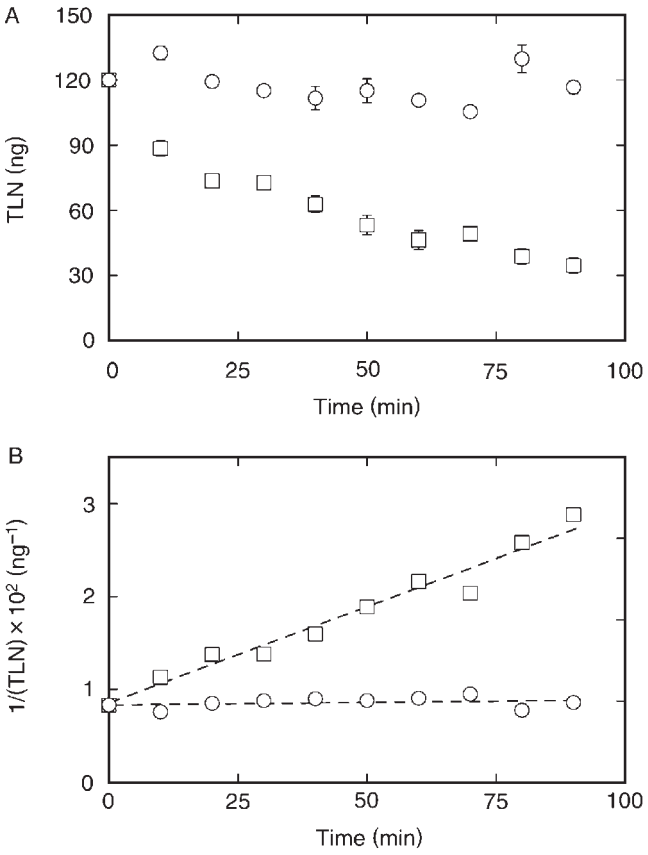

Fig. 9. **Analysis of autolysis of thermolysin incubated with Co^{2+}.** The quantity of residual thermolysin (TLN) (Panel A) estimated from the intensity of the band in Fig. 8 and its reciprocal (Panel B) are plotted against the incubation time of thermolysin with Co^{2+}. Dashed linear lines are the second-order reaction curves fitted to Eq. 1.

the active site of the enzyme (*30*, *31*). The structure of carboxypeptidase A, obtained from the monoclinic crystal soaked with excess Zn^{2+} (*41*), confirmed the model of Zn^{2+}-inhibited carboxypeptidase A deduced from kinetic data (*31*). The carboxypeptidase A structure solved in a different crystal form postulated inhibitory Zn^{2+} site in close proximity to the catalytic Zn^{2+} ion (*42*). A hydroxo bridge is found between the catalytic Zn^{2+} and the inhibitory Zn^{2+}, and the inhibitory Zn^{2+} has Glu270 as a monodentate ligand. A crystallographic study of thermolysin soaked with zinc ions showed that thermolysin has a second Zn^{2+} ion in its active site in addition to the catalytic Zn^{2+} ion (*43*). The second Zn^{2+} interacts with the side chains of His231 and Tyr157 in thermolysin. Both residues are considered important for stabilizing the transition-state intermediate, and thus the binding of the second Zn^{2+} ion to the active site might cause the competitive inhibition.

In the present study, we have found that CoTLN is inactivated by an excessive amount of Co^{2+} ion. A part of this Co^{2+}-dependent inactivation is Ca^{2+}-sensitive and the other part is Ca^{2+}-insensitive. The latter part is characterized by that Co^{2+} ion plays as a competitive inhibitor toward the substrate (Fig. 4), suggesting that an extra Co^{2+} ion binds to the active site of CoTLN in addition to the catalytic Co^{2+} ion to inhibit the activity. Because Co^{2+} is similar to Zn^{2+} in coordination chemistry and ligand selectivity, it is reasonable to consider that the inhibitory Co^{2+}-binding site located in the active site of thermolysin should be the secondary Zn^{2+}-binding site constructed with His231 and Tyr157. However, Co^{2+} ion was not observed in the active site as revealed in the crystallographic structure of thermolysin soaked with Co^{2+} (*44*). This might be attributed to that the affinity of Co^{2+} ion to this site is too low to be observed in the X-ray crystallographic analysis. In fact, the inhibition of thermolysin by Co^{2+} is caused by mM-order of Co^{2+}, whereas the inhibition by Zn^{2+} is caused by μM-order of Zn^{2+} (*30*).

The Ca^{2+}-sensitive part of the Co^{2+}-dependent Inactivation of Thermolysin—It is considered that the substitution of Ca^{2+} ion for the inhibitory Co^{2+} ion is involved in the Ca^{2+}-sensitive part of the Co^{2+}-dependent inactivation of thermolysin. According to the crystallographic study of the thermolysin soaked with Co^{2+} (*43*), one of the four Ca^{2+} ions bound to the thermolysin molecule is replaced by Co^{2+}. This replaced Ca^{2+} ion is Ca3 which is located most far from the active site among the four Ca^{2+} ions bound. However, Ca3 is considered to be crucial for the thermal stability of thermolysin (*44*). The thermal inactivation of thermolysin might be initiated by unfolding or conformational change followed by autolysis, and the initiation can be caused by the release of Ca3. The relationship between the autolysis and Ca4 was also studied in the presence of EDTA (*37*). When the Ca^{2+} ions bound to thermolysin were depleted by incubating thermolysin in the presence of EDTA, the autolytic degrading pattern of thermolysin was different from that observed in the absence of EDTA. In the presence of EDTA, one of the autolytically cleaved points was near the Ca4 binding site. Therefore, it was proposed that the autolysis of thermolysin in the presence of EDTA is caused by the release of Ca4. In the present study, it was shown that the autolytic fragments of thermolysin formed in the presence of Co^{2+} were similar to those formed by depleting Ca^{2+} with EDTA (*44*). Additionally, the autolysis was shown to be promoted further with increasing Co^{2+} concentration. Therefore, the autolysis is considered to be induced by replacing Ca^{2+} in thermolysin with Co^{2+} or by eliminating the Ca^{2+} ion by a conformational change induced by the interaction of Co^{2+} with thermolysin. The replaced Ca^{2+} ion might be most plausibly Ca4 from the similarity in the degrading pattern to that observed in the presence of EDTA (*44*) where the autolytically cleaved position is near Ca4. It is noteworthy that there has been no reports that Ca4 was replaced with any other metals, Zn^{2+}, Co^{2+}, Cd^{2+}, Mn^{2+} and Fe^{2+}, in the crystallographic study (*43*), and this suggests a possibility that Co^{2+} ion induces a conformational change in thermolysin to release the Ca4 followed by autolysis. The Ca^{2+}-sensitive part of the Co^{2+}-dependent inactivation is not sufficiently explained by solely autolysis. Thermolysin inactivates progressively in the presence of 0.01 and 17.5 mM Co^{2+} (Figs 5 and 6), although the autolytic fragmentation of thermolysin molecules was

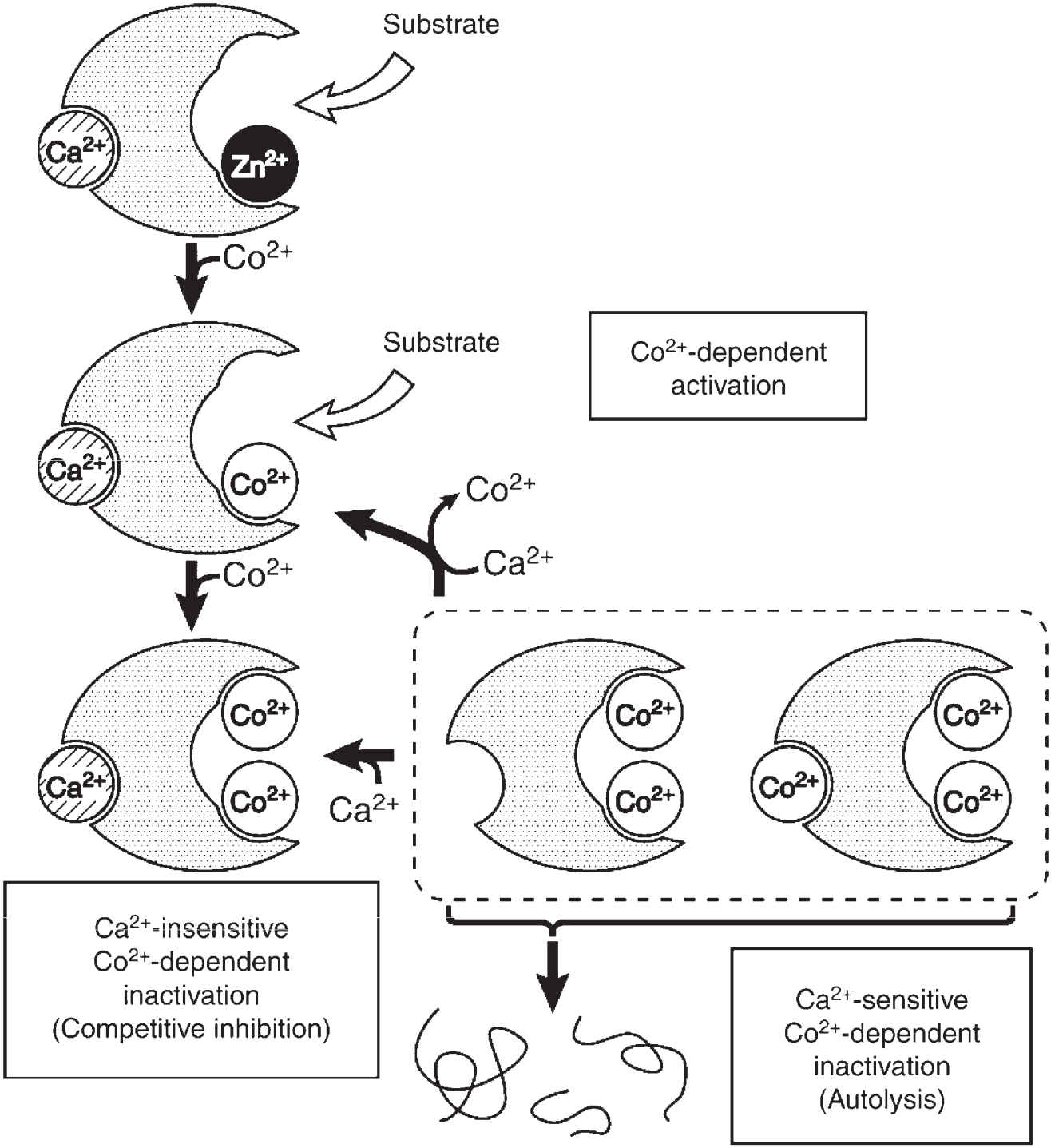

Fig. 10. **Illustration of the effects of Ca^{2+} and Co^{2+} ions on the activity and stability of thermolysin.**

observed only in the presence of 17.5 mM Co^{2+} but not with 0.01 mM Co^{2+} (Fig. 8). The inactivation process must include not only autolysis, but also another mechanism such as conformational change or denaturation. It is of note that the inactivation process without accompanying autolysis at 0.01 mM Co^{2+} observed in Fig. 5 is too slow to judge if the process follows first-order or second-order kinetics by semilogarithmic or reciprocal plots. Actually, it can be analysed even with first-order kinetics, suggesting that a slow denaturation process of the enzyme might be rate-limiting in a possibility.

Figure 10 shows an illustration of the effects of Co^{2+} and Ca^{2+} ions on the activity and stability of thermolysin drawn by the evidence obtained in this study. Co^{2+} ion is

catalytically essential when it binds to the Zn^{2+}-binding site in the active site of thermolysin instead of Zn^{2+} to form CoTLN. However, we have found two types of inhibitory effects of Co^{2+} ion against thermolysin activity in the presence of higher concentration of Co^{2+}. Co^{2+} ion binds to the second Co^{2+}-binding site in/near the active site in the presence of 10 mM Ca^{2+}, and plays as a competitive inhibitor. Co^{2+} ion also binds to the third binding site, which may be the Ca4-binding site or another, in the presence of 0.01 mM Ca^{2+}, to induce autolysis. It is suggested that higher Ca^{2+} concentration is needed to suppress the autolysis. It should be studied further why CoTLN is subjected to autolysis under lower Ca^{2+} concentration, whereas ZnTLN is substantially not subjected to it. There might be a differential effect between Co^{2+} and Zn^{2+} ions on the conformation of the autolysis-susceptible site. Mechanism of Co^{2+}-dependent inactivation of thermolysin especially in the presence of lower Ca^{2+} concentration remains to be revealed. To reveal it further, effects of conformational change and autolysis on the inactivation should be analysed separately. The Ca^{2+} ion which is replaced by Co^{2+} ion should be determined, and the relationship between the Ca^{2+}-replacement and the inactivation and autolysis of thermolysin. These points are currently under investigation.

Recently, we reported molecular engineering of thermal stability of *Bacillus* α-amylases (*45*, *46*), and demonstrated that modification of Ca^{2+}-binding to the enzyme is effective for regulating its stability. This result as well as that reported in the present study provides us with insights into strategies on engineering the activity and stability of enzymes.

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