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Metal-ion-assisted hydrolysis of dipeptides involving a serine residue in a neutral aqueous solution †

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Dipeptides having a serine residue at the *C*-terminus, X-Ser, where X is an appropriate amino acid residue, were efficiently hydrolyzed in the presence of $ZnCl_2$ at pH 7.0. The rapid hydrolysis of X-Ser is due to an autocatalysis of the hydroxy group in the serine residue, and is found to be accelerated by a metal ion, in particular by $ZnCl_2$. Roles of the metal ion in the hydrolysis of peptides involving a serine residue, in relation to the recently reported protein cleavages, are discussed.

Introduction

The non-enzymatic hydrolysis of peptides has been attracting much attention.[1–7] Recently, the peptide hydrolyses promoted by Cu^{2+},[2] Pd^{2+},[3] Pt^{2+},[3a,b] $Fe^{2+/3+}$,[4] Ni^{2+} [5] and Ce^{4+} [6] under either near neutral or acidic conditions have been reported. The studies indicate that catalysts involving these transition metal ions are promising as non-enzymatic cleavage reagents under near physiological conditions.[1] The site specific cleavages of proteins in the presence of Pd^{2+},[3d] Cu^{2+} [2d] and Ni^{2+} [5] have been reported.

It has been known that proteins involving a serine or a threonine residue undergo hydrolysis *via* an N⟶O acyl rearrangement under non-physiological extreme acidic or basic conditions.[8,9] The reaction proceeds by an intramolecular attack of the hydroxy group in the serine or the threonine residue on the carbonyl carbon in the preceding amide bond to form an ester intermediate. Under the conditions where the rapid hydrolysis of the ester intermediate occurs, the reaction results in the peptide hydrolysis by a two-step-reaction.[8]

Recently, the N⟶O acyl rearrangement has been attracting much attention in relation to important biological events; some post translational modifications of proteins, such as protein splicing, autoproteolysis and autoprocessing, have been known to proceed self-catalytically, and the first step of these reactions is the N⟶O acyl rearrangement.[10] Many promising applications of the protein splicing have been demonstrated by using parts of naturally occurring proteins that can promote protein splicing.[10,11] Thus, finding a way to accelerate reactions involving the N⟶O acyl rearrangement, and, accordingly, to regulate them will open an avenue for the site-specific cleavage or the modification of a protein.

This study deals with the effect of a metal ion on the hydrolysis of peptides involving a serine residue. We have focused on the hydrolysis of dipeptides, because the coordination behaviour of the metal ion and the functional groups in the peptide are relatively simple compared with longer peptides.

† Electronic supplementary information (ESI) available: Kinetic studies involving pH and concentration profiles of the rate constant. See http://www.rsc.org/suppdata/ob/b2/b209565c/

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Table 1 Results of the hydrolysis of dipeptides in the presence of $ZnCl_2$ at pH 7.0 (0.1 M HEPES), 70 °C and 24 h[a]

Entry	Dipeptide	Conv (%)
1	Gly-Ser	83
2	Gly-Ser	22[b]
3	Gly-Ser	6[c]
4	Ser-Gly	4
5	Phe-Ser	91
6	Leu-Ser	81
7	Ile-Ser	10
8	Pro-Ser	80
9	His-Ser	49
10	Val-Ser	14
11	Gly-Thr	35
12	Thr-Gly	2
13	Gly-Asp	5
14	Gly-Asn	2
15	Gly-Gly	6
16	Gly-Ala	6

[a] [dipeptide] = 10 mM, [$ZnCl_2$] = 10 mM. [b] At 50 °C. [c] Without $ZnCl_2$.

Results and discussion

Hydrolysis of dipeptides with $ZnCl_2$

The hydrolysis of dipeptides (10 mM) were conducted in the presence of $ZnCl_2$ (10 mM) at pH 7.0 and 70 °C. The starting peptide and the resulting amino acids were separated and determined by HPLC.[2b,6b,12] With the decrease of the starting dipeptide, corresponding α-amino acids involved in the starting peptide were formed. No other amino acid or peptide was detected. The mass balance before and after the reaction was sufficiently good (at least 90%), indicating that the hydrolysis of the peptide bond was the main reaction under the present experimental conditions. The conversions estimated from the formation of amino acids are summarized in Table 1.

Obviously, the conversion was largely dependent on the sequence of dipeptides. The conversion after the 24 h reaction at pH 7.0 and 70 °C ranged from 2% (Thr-Gly, Gly-Asn) to 91% (Phe-Ser). Among dipeptides examined, Gly-Ser, Phe-Ser, Leu-Ser, Pro-Ser and His-Ser were the most readily hydrolyzed.

Ile-Ser, Val-Ser and Gly-Thr were moderately active for the hydrolysis. All dipeptides that were readily hydrolyzed have a serine residue at the *C*-terminus, indicating the important role of the Ser hydroxy group on the peptide hydrolysis as an intramolecular nucleophile, as expected. The interpretation that the hydrolysis of X-Ser is due to the N⟶O acyl rearrangement is strongly supported by the marked difference between the conversion of Gly-Ser (83%) and that of Ser-Gly (4%). The intramolecular attack of the Ser hydroxy group on the amide carbonyl carbon is possible in Gly-Ser, while it is impossible in Ser-Gly, because in the former case the intramolecular attack of the Ser hydroxy group results in a five-membered ring transition state, while in the latter case the intramolecular attack should form an unfavorable four-membered ring transition state (Fig. 1). The corresponding ester intermediate was not detected in any reaction of X-Ser, but only corresponding amino acids were detected.

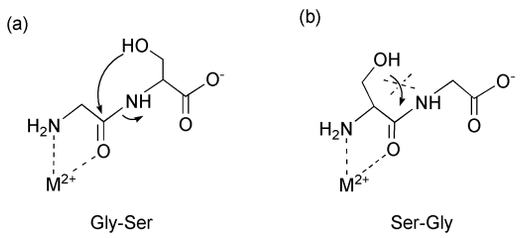


Fig. 1 A comparison of Gly-Ser and Ser-Gly.

Hydrolysis of Gly-Ser with various metal salts

Effects of various metal ions on the hydrolysis of Gly-Ser were examined at pH 7.0 and 70 °C. Results are summarized in Table 2. All transition metal salts examined, except $CuCl_2$, showed considerable effects. $ZnCl_2$ was the most effective among metal salts examined.

The low conversion with $CuCl_2$ is reasonably explained as the following: Cu^{2+} is known to bind to a dipeptide through NH_2 (*N*-terminal), NH (amide) and COO^-(*C*-terminal) groups to form a stable tridentate chelate (Fig. 2b). Because the amide proton in such a chelate readily dissociates under neutral or weakly basic conditions, the dipeptide is inactive for the hydrolysis under such conditions.[2b,13] When the formation of the tridentate chelate of a dipeptide is not favored upon using $[CuCl_2(tach)]$[2b] or $[CuCl_2([9]aneN_3)]$[2c] (tach = *cis*,*cis*-1,3,5-triaminocyclohexane, $[9]aneN_3$ = 1,4,7-triazacyclononane), the dipeptide hydrolysis is efficiently promoted by the Cu^{2+} complex under nearly neutral conditions.[2b,c] Contrary to this, in the case of Zn^{2+} and other transition metal ions except Cu^{2+}, the tridentate chelate of a dipeptide would be formed only marginally, and a high hydrolysis activity would be observed (Fig. 2a).

Table 2 Effects of metal ions on the hydrolysis of Gly-Ser at pH 7.0 (0.1 M HEPES) and 70 °C[a]

Entry	Metal chloride	Conv (%)[b]
1	none	6
2	$MgCl_2$	8
3	$CaCl_2$	6
4	$NiCl_2$	52
5	$CuCl_2$	6
6	$ZnCl_2$	83
7	$CdCl_2$	43
8	$LaCl_3$	28
9	$CeCl_3$	49
10	$PrCl_3$	66
11	$EuCl_3$	58
12	$ErCl_3$	30
13	$LuCl_3$	13

[a] [Gly-Ser] = 10 mM, [metal chloride] = 10 mM. [b] After 24 h reaction.

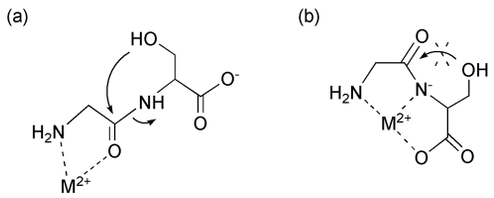


Fig. 2 Two types of coordination of a metal ion to a dipeptide. (a) The amide carbonyl group is activated for the hydrolysis. (b) The amide carbonyl group is inactivated for the hydrolysis.

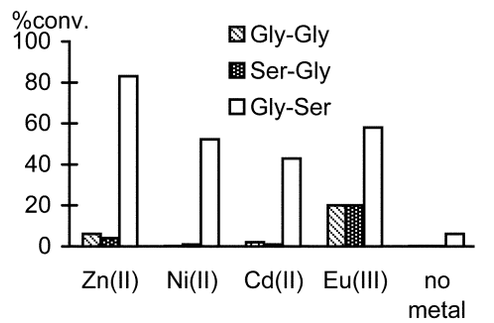


Fig. 3 A comparison of the sequence dependence of the metal assisted hydrolysis of dipeptides at 70 °C, 24 h and pH 7.0 (0.1 M HEPES). [Dipeptide] = 10 mM, [metal chloride] = 10 mM.

Sequence dependence of the metal-ion-assisted hydrolysis of the dipeptide

Fig. 3 shows the comparison of the hydrolyses of three dipeptides, Gly-Gly, Ser-Gly and Gly-Ser, in the presence of $ZnCl_2$, $NiCl_2$, $CdCl_2$ and $EuCl_3$. When $ZnCl_2$, $NiCl_2$ and $CdCl_2$ were used, Gly-Ser was hydrolyzed selectively. In the presence of $EuCl_3$, however, not only Gly-Ser, but also Gly-Gly and Ser-Gly were hydrolyzed considerably. We have previously reported that lanthanide salts promote the hydrolysis of dipeptides very efficiently.[6] The present results indicate that, compared with the strong catalytic effect of $EuCl_3$ on the peptide hydrolysis due to an external nucleophile, the effect of the Ser hydroxy group is relatively small.

Thus, a highly preferential hydrolysis of Gly-Ser over Gly-Gly and Ser-Gly was achieved in the presence of $ZnCl_2$, $NiCl_2$ and $CdCl_2$.

Rate constants for the hydrolysis of X-Ser

In order to study the hydrolysis of dipeptides due to the combination of the Ser hydroxy group and $ZnCl_2$ in more detail, kinetic studies of the reactions of X-Ser were conducted with or without $ZnCl_2$ at pH 7.0 (0.1 M HEPES) and 50 °C. The reactions were followed by the formation of the corresponding amino acids, *i.e.* X and Ser. The pseudo-first-order rate constants are summarized in Table 3.

The rate constant was significantly dependent on X. When X of the dipeptide was Ala, Leu or Phe, the rate was greater

Table 3 Pseudo-first-order rate constants for the hydrolysis of X-Ser at pH 7.0 (0.1 M HEPES) and 50 °C

	$k_{obs}/10^{-3}$ h^{-1}	
Dipeptide[a]	With $ZnCl_2$[b]	Without $ZnCl_2$
Ala-Ser	24 ± 4	2.0 ± 0.2
Phe-Ser	32 ± 8	1.4 ± 0.4
Leu-Ser	22 ± 3	1.6 ± 0.4
Gly-Ser	12 ± 2	0.58 ± 0.05
Val-Ser	2.0 ± 0.2	0.26 ± 0.04
Ile-Ser	1.22 ± 0.04	0.26 ± 0.05

[a] [Dipeptide] = 10 mM. [b] [$ZnCl_2$] = 10 mM.

than that of Gly-Ser. In contrast, when X was Val or Ile, the rate was smaller than that of Gly-Ser.

In the case of Val and Ile, the side chain has a branched structure at the β-position, while the side chain of Ala, Leu or Phe has no branched structure at the β-position (Fig. 4). The

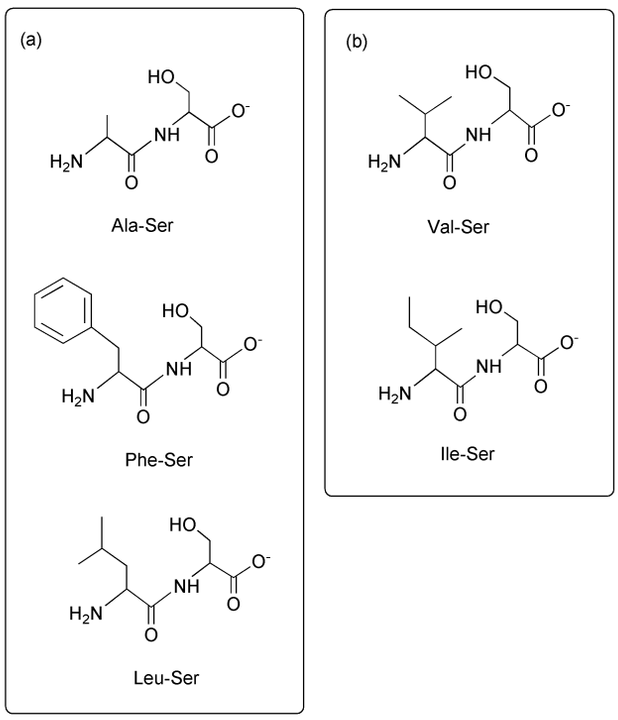


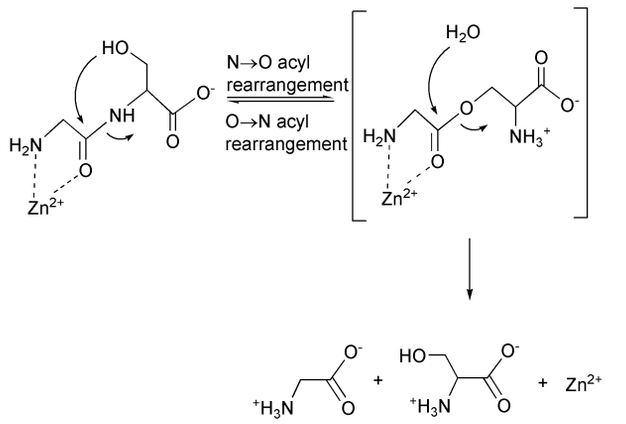
Fig. 4 Structures of dipeptides whose hydrolysis rates were greater than Gly-Ser (a), and those whose hydrolysis rates were smaller than Gly-Ser (b).

alkyl side chain with the β-branched structure would cause a steric hindrance therefore inhibiting a nucleophilic attack, resulting in a relatively slow peptide hydrolysis. In contrast, quite interestingly, when the alkyl side chain in X does not have a β-branched structure, the hydrolysis is accelerated. Such an effect of X is almost independent of Zn^{2+}, since the rate constants for the reactions both with and without $ZnCl_2$ showed similar tendencies.

Role of the metal ion

The present results indicate that the metal ion, in addition to the hydroxy group within the peptide, plays an important role in the rapid hydrolysis of a dipeptide. In the Cu^{2+}-complex-assisted hydrolysis of Gly-Ser, the coordination of Cu^{2+} to the carbonyl oxygen of the peptide was found to be an essential factor.[2a,b] Moreover, it has been known that bovine lens leucine aminopeptidase catalyzes the peptide hydrolysis by using two Zn^{2+} ions in the catalytic center. One of roles of Zn^{2+} is suggested to be the ability to polarize the carbonyl group by coordination to facilitate the attack of the nucleophile.[14] Thus, a possible role of Zn^{2+} in the present reaction is to coordinate to the carbonyl oxygen and to assist the attack of the nucleophile. The coordination of Zn^{2+} with the dipeptide through NH_2 and C=O is the most probable (Fig. 2a).[13] Such coordination of Zn^{2+} with a dipeptide has been confirmed by X-ray crystallography.[15] The overall reaction scheme is shown in Scheme 1.

The coordination of a metal ion to the NH_2 group is important only in assisting coordination to the C=O group. Therefore, in the case of a longer peptide or a protein, when the coordination of a metal ion to the C=O group is facilitated by the assistance of appropriate functional group(s) in the amino acid residue(s) in the stereochemical vicinity of the target amide bond, the peptide bond scission *via* the N⟶O rearrangement



Scheme 1

would be accelerated. Recently, highly site-specific cleavage of proteins assisted by a metal ion, *i.e.* Pd^{2+},[3d] Cu^{2+},[2d] or Ni^{2+},[5] have been reported. Since these reactions resulted in X-Ser or X-Thr bond cleavage, their reaction mechanisms could be similar to those in the present reactions.[1c]

The reaction mechanism for Zn^{2+}-assisted peptide hydrolysis could be better illustrated on the basis of kinetic studies involving the pH profile or the concentration profile of the rate constant (see Electronic supplementary information †). The kinetic data in the pH region above pH 7 are of special interest with respect to discussing the nature of the nucleophile. However, such experiments could not be conducted because of the solubility of $ZnCl_2$; insoluble Zn compounds were formed in the pH region higher than 7. Instead, kinetic studies using a Zn^{2+} complex with an appropriate ligand would be useful.

Conclusion

A rapid hydrolysis of a dipeptide under neutral conditions was achieved by the combination of an internal hydroxy group and $ZnCl_2$. The reaction of X-Ser proceeds self-catalytically, and is accelerated by a transition metal ion, in particular by Zn^{2+}.

It has been suggested that the protein degradation *via* the N⟶O acyl rearrangement in vivo can be assisted by Cu^{2+} or Ni^{2+}.[1c] The present results suggest that Zn^{2+} could also accelerate the self-catalytic reactions of proteins *via* the N⟶O acyl rearrangement.[10]

Experimental

Materials

Dipeptides were purchased from Sigma-Aldrich Co. All other reagents were purchased from commercial chemical sources and used as received.

Hydrolysis of dipeptides in the presence of metal salts. In a typical experiment, to a 10 ml aqueous buffer (pH 7.0, 100 mM HEPES) were added Gly-Ser (0.1 mmol) and $ZnCl_2$ (0.1 mmol). The pH of the resulting solution was adjusted to 7.0 at rt (20 °C) with 0.1 M NaOH, and heated at 70 °C for 24 h. The reaction temperature was controlled using a Lauda thermostat M3T. The reaction mixture was analysed by HPLC [Column, TOSOH TSKgel Aminopak; eluent, citrate buffer (67 mM, pH 3.41 or 4.25); flow rate, 0.6 ml min^{-1}] and was fluorometrically detected (ex 345 nm, em 455 nm) after the on-line post-column derivatizing reaction with *o*-phthalaldehyde–2-mercaptoethanol at 60 °C.[2b,6b,12] The detected species were identified by comparing their elution times with those of standard samples. The quantification of each detected species was carried out by comparing the peak area with that of the corresponding standard sample.

Kinetic constant measurement of the hydrolysis of X-Ser in the presence of $ZnCl_2$. In a typical experiment, to 10 ml aqueous buffer (pH 7.0, 100 mM HEPES) were added Gly-Ser (0.1 mmol) and $ZnCl_2$ (0.1 mmol). The pH of the resulting solution was adjusted to 7.0 (20 °C) with 0.1 M NaOH, and heated at 50 °C. After appropriate reaction periods, the reaction mixture was subjected to the analysis as described above. The reactions were followed for at least 30 h. Within this time period, all reactions showed pseudo-first-order kinetics.[2b]

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