$ZnCl_2$ 与氧化胰岛素 B 链结合位点和切割位点的质谱指认

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摘要:采用电喷雾质谱和串联质谱研究了氧化胰岛素 B 链与 $ZnCl_2$ 的键合作用并成功地确定了切割位点。质谱研究显示在 pH 值 2.5 及 40 ℃条件下,Zn^{2+}通过与氧化胰岛素 B 链的氨基酸侧链 His5,His10 和 Arg22 结合,选择性地水解了肽键 Asn3-Gln4, His5-Leu6, Gly8-Ser9 和 Glu21-Arg22。

关键词:氧化胰岛素 B 链;质谱;$ZnCl_2$;水解切割
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Site-specific Hydrolytic Cleavage of Oxidized Insulin B Chain with Zn(Ⅱ) Ion Studied by Electrospray Ionization Mass Spectrometry and MS/MS Mass Spectrometry

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Abstract: The bonding interaction and hydrolytic cleavage of oxidized insulin B chain with $ZnCl_2$ in molar ratios of 1∶1 1∶2 and 1∶3 at pH value of 2.5 and 40 ℃ were investigated by electrospray ionization mass spectrometry and tandem mass spectrometry. The results show that the binding sites of Zn^{2+} with oxidized insulin B chain are Arg22 and imidazole groups of His5, His10, which leads to the selective cleavages of the peptide bonds at Asn3-Gln4, His5-Leu6, Gly8-Ser9 and Glu21-Arg22 of oxidized insulin B chain.

Key words: oxidized insulin B; mass spectrometry; $ZnCl_2$; hydrolytic cleavage

Selective cleavage of peptides and proteins is an important procedure in biochemistry and molecular biology. At room temperature and pH value of 4~8, the half-life for the hydrolysis of amide bonds is 350~500 years[1]. Because the amide bond in peptides and proteins is remarkably inert towards hydrolysis under usual conditions, new methods for efficient and selective cleavage in mild conditions are needed.

In recent years, a great deal of interest has focused on the role of metal ions and/or complexes of Ce(Ⅳ)[2],Cu(Ⅱ)[3,4],Fe(Ⅱ,Ⅲ)[5],Ni(Ⅱ)[6],Zr(Ⅱ)[7],Zn(Ⅱ)[8],Pd(Ⅱ)[9,10],Pt(Ⅱ)[9c,9f,9g,9j,9k,11,12], and Co(Ⅱ,Ⅲ)[4h,13], for the effect hydrolytic cleavage of unactivated amide bonds in small peptides and proteins at particular sites. These artificial proteases can overcome thermal, chemical, and mechanical instability of natural proteases and hy-

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drolytically cleave short peptides and proteins selectively and quickly.

However, among all these metalloenzymes, the peptide bond cleavage induced by Zn(Ⅱ) enzymes has rarely been observed[14]. It has been reported that due to the low tendency of Zn(Ⅱ) to hydrolyze the amide bonds, the cleavage occurs only at high temperature in aqueous inhibit the cleavage of the peptide bond[15]. By contraries, latest research shows that hydrolysis of dipeptides having a serine residue can be accelerated by a metal ion, in particular by $ZnCl_2$. Bazzicalupi[8b] also indicated that Zn(Ⅱ) complex as a receptor for amino acid and dipeptides could lead to hydrolysis of an activated peptide bond. Even now, the reports concerning the ability of Zinc compounds to promote the selective hydrolytic cleavage are still limited on a very few number of short peptides. So in order to develop more zinc-based cleaving agents, systematic experiments with various proteins are needed to determine their selectivity, efficiency and other properties.

Electrospray ionization mass spectrometry (ESI-MS), pioneered by Dole et al.[16] and recently introduced by Fenn and co-workers[17~19], has been widely used to study the non-covalent interactions of metal ions with oligonucleotides and DNA, as well as peptides and proteins. Furthermore, tandem mass spectrometry (MS/MS) analysis has been used in determining the sequence of peptides or proteins by cleaving them into fragments (typically via Collision Induced Dissociation). In our previous studies, ESI-MS combined with MS/MS has been proven to be a powerful technique to provide some valuable structure information about the species formed in solution and determine the binding sites of metal complexes with peptides and proteins[9j,11b,12b, 20,21]. Because the cleavage takes place at peptide bonds in the vicinity of the binding sites, identifying the binding sites of metal ions with peptides and proteins is helpful for us to understand their cleavage activity.

Insulin is one of the most widely studied peptide hormones, and continues to play a central role in the treatment of diabetes. It is composed of two chains, A and B linked by two intermolecular disulfide bonds. Currently, the site specific cleavages of Oxidized Insulin B in the presence of $CuCl_2$, Copper(Ⅱ), palladium(Ⅱ) and platinum(Ⅱ) complexes have been investigated in some detail[3a,20]. The results of these studies reveal that the binding sites of them with oxidized insulin B chain are terminal NH_2, imidazole groups of His5 and His10. In oxidized insulin B chain, only one cleavage site was observed to be located at Gly8-Ser9 cleaved by $CuCl_2$ reagents, whereas the cleavage promoted by the pendant hydroxyl group in $[CuL(H_2O)]^{2+}$ complexes occurred at Phe1-Val2, Asn3-Gln4 and Gly8-Ser9. For the palladium(Ⅱ) complexes, the hydrolytic cleavages were achieved at Leu6-Csy7 and Gly8-Ser9, while the *cis*-$[Pt(en)(H_2O)_2]^{2+}$ promoted cleavage only happened at His10-Leu11. In the present work, we proposed a simple $ZnCl_2$ as a cleave reagent and oxidized insulin B chain was taken as a target peptide to study the binding sites and cleavage activity in weakly acidic solutions via a hydrolytic pathway. So far, the main studies of Zn-mediated hydrolytic cleavage are focused only on some serine-containing peptides. To the best of our knowledge, there is no report about the peptide cleavage upon anchoring of Zn^{2+} to histidine and arginine residues in peptides. In addition, we consider that there might be significant differences in the rate, specificity and efficiency compared with $CuCl_2$, Copper(Ⅱ), palladium(Ⅱ) and platinum(Ⅱ) complexes. Above all, we ascertain precisely the binding sites in Zn^{2+} bound oxidized insulin B using LC-ESI-MS and MS/MS through observation of the cleaved fragments.

1　Materials and methods

1.1　Chemicals and sample preparation

Doubly distilled water (dd H_2O) was used for preparation of solutions. Oxidized insulin B chain was purchased from Sigma (Scheme 1). Trifluoroacetic acid (TFA) was purchased from Sigma-Aldrich and acetonitrile of HPLC grade was obtained from Fisher. The other chemicals were of reagent grade.

Oxidized insulin B chain was stored in aqueous solution of H_3PO_4 at pH value of 2.5 with concentration of 3.0 $mmol \cdot dm^{-3}$. As shown in Fig.1, the molecular mass of oxidized insulin B chain determined

Phe-Val-Asn-Gln-His-Leu-Cys(-SO_3H)-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr
-Leu-Val-Cys(-SO_3H)-Gly-Glu-Arg-Gly -Phe-Phe-Tyr-Thr-Pro-Lys-AlaOH

Oxidized insulin B chain

Scheme 1

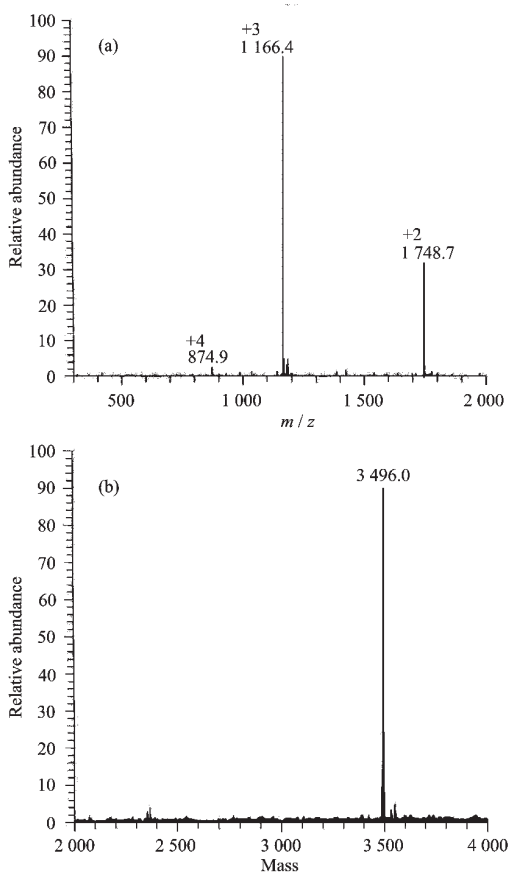


Fig.1 ESI mass spectra of oxidized insulin B chain after purification on HPLC with a Hypersil 5 C8 column (a)raw spectra (b)transformed spectra calculated by Bioexplore.

by LC-ESI-MS is 3496.0 Da, precisely equals to 3495.9 calculated by IsoPro3.0 program for $C_{157}H_{232}N_{40}O_{47}S_2$.

1.2 Interaction of $ZnCl_2$ with oxidized insulin B

40 μL (3.0 mmol·dm^{-3}) of oxidized insulin B chain was mixed with 1.2, 2.4, and 3.6 μL of $ZnCl_2$ (100 mmol·dm^{-3}) in molar ratios of 1:1, 1:2, and 1:3, respectively, HBF_4 or NaOH and extra dd H_2O was then added to make the total volume of the mixed solution up to 60 μL and pH value of 2.5. The solutions were incubated at 37 ℃ for 1 h, 2 h, 3 h, 7 h and 24 h, respectively and then measured by ESI-MS.

1.3 Hydrolytic cleavage of oxidized insulin B chain by $ZnCl_2$

40 μL of 3.0 mmol·dm^{-3} oxidized insulin B chain was mixed with 6.0 μL of 100 mmol·dm^{-3} Zn(Ⅱ) reagents and 14.0 μL of aqueous solution of H_3PO_4 at pH value of 2.5. The mixed solutions were incubated at pH value of 2.5 and 40 ℃ for 2 days, and then quenched by addition of ethylenediamine tetraacetic acid (EDTA, 0.5 mol·dm^{-3}) with a 10-fold excess over $ZnCl_2$ followed by incubation again at 40 ℃ for 6 h to remove the coordinated Zn(Ⅱ) ion. The obtained clear digestion solution was analyzed by LC-ESI-MS.

In the control experiments for possible "background" cleavage, the conditions were the same, except that the Zn(Ⅱ) reagent was absent.

1.4 Mass spectrometry

ESI mass spectra and tandem mass spectra were recorded using a Finnigan MAT LCQ ion-trap mass spectrometer in the positive ionization mode by loading 1.0 μL of solution into the injection valve of the LCQ unit and then injecting into the mobile phase solution (50% of aqueous methanol) which was carried through the electrospray interface into the mass analyzer at a rate of 200 μL·min^{-1}. The voltage employed at the electrospray needles was 4.5 kV, N_2 sheath gas flow was 35 units (arbitrary for the LCQ-Deca system) and the capillary was heated to 200 ℃. Helium gas was admitted directly into the ion trap, and was used as the damping gas to improve trapping efficiency and as collision gas in the Collision-Induced Dissociation (CID) experiments. A maximum ion injection time of 200 ms along with 10 scans was set. The m/z values of metal-anchored species shown in the text represent the most intense peaks in the isotopic distribution. Zoom scan was used in these experiments. The predicted isotope distribution patterns for each of the complexes were calculated using the IsoPro 3.0 program.

LC-ESI-MS, with a chromatographic column directly connected to the injection valve of mass spectrometer, was used for analysis of oxidized insulin B chain and its fragments-containing samples. Typically, 1.5 μL of pre-treated reaction solution was first injected into HPLC with a Hypersil 5 C8 column of size 2.1 mm×100 mm using a gradient of water/acetonitrile, both containing 0.1% CF_3COOH. The gradient was made by increasing the amount of acetonitrile from 0 to 60% in 60 min, at a flow rate of 0.2 mL·min^{-1}. The isolated solution was then directly transported into ESI-MS instrument. The molecular mass was determined by transformation of ESI-MS raw data into a true molecular mass scale using Bioexplore software. The measured molecular mass of a given fragment was compared with the value calculated by PAWS software, obtained from ProteMetrics, LLC.

For MS/MS analysis, the interesting ions in the spectrum were selected as precursor ions, and a collision-induced dissociation scan with an isolation width of 3 amu was performed with the relative collision energy of 30% to optimize the fragmentation of the metal-bound peptides.

The pH value was measured with a pHS-3C pH instrument and a phoenix Ag-AgCl reference electrode.

2 Results and discussion

2.1 Interaction of the oxidized insulin B chain with Zn^{2+} ion

ESI mass spectra were measured after incubation of mixed solution of oxidized insulin B chain (abbreviated as B) and Zn^{2+} in molar ratios of 1:1, 1:2, and 1:3 at pH value of 2.5 and 37 ℃, labeled as samples a, b, and c. The peak at *m/z* 1 166.3 is attributed to $[B+3H^+]^{3+}$. After 1 h of incubation of sample a, besides free B, the singly Zn(Ⅱ)-coordinated species $[Zn·B]^{2+}$ and $[Zn·B+H^+]^{3+}$ were detected very weakly, corresponding to *m/z* 1 780.2 (Calcd:1 780.7) and 1 187.2 (Calcd:1 187.1), respectively. After 2 h of incubation, the peak of the two singly Zn(Ⅱ)-coordinated species $[Zn·B]^{2+}$ and $[Zn·B+H^+]^{3+}$ increased without new peaks. Prolonging the incubating time to 7 h, no new peaks appeared. As to sample b, after 1 h of incubation, in addition to the species observed in sample a, a new peak was observed at *m/z* 1 812.2 assigned as $[(Zn)_2·B-2H^+]^{2+}$ with the calculated *m/z* value of 1 812.4, in which B is anchored by two Zn(Ⅱ) ions. After 2 h of incubation, the peak of the doubly-coordinated $[(Zn)_2·B-2H^+]^{2+}$ was detected strongly while the two peaks of singly species were decreased and no new peaks appeared. As the incubation time was prolonged to 3 h, a new peak at *m/z* 1 208.2 comes out which is assigned as B anchored by two Zn (Ⅱ) ions, $[(Zn)_2·B-H^+]^{3+}$ (Calcd: 1 208.6), belonging to the same species with different charges formed from B and Zn(Ⅱ) ion with a stoichiometric ratio of 1:2. The two doubly-coordinated adducts were always observed, as the molar ratio of Zn^{2+} to B was varied from 2:1 to 3:1 under the same experimental conditions. The sample c was similar to samples a and b, just these species were easily observed and more intensive as early as 1 h of incubation, in addition, a new triply Zn(Ⅱ)-coordinated species of $[(Zn)_3·B-3H^+]^{3+}$ appeared weakly at *m/z* 1229.8 (Calcd:1 229.7). Prolonging the incubating time, no new peaks appeared. Fig.2 shows a spectrum of sample c after 3 h of incubation. The zoom scans of the singly, doubly, and triply-coordinated species were also presented. It is evident that only three binding sites in B are preferred by the Zn(Ⅱ) ion during the incubation time. Any species of B anchored by more than three Zn(Ⅱ) ion was not detected after extra 24 h of incubation.

2.2 $ZnCl_2$-promoted hydrolytic cleavage of oxidized insulin B chain

A mixture solution of B and Zn^{2+} in molar ratio of 1:5 was incubated at 40 ℃ and pH value of 2.5 for 2 days. The digestion solution quenched by EDTA was separated by HPLC and measured by ESI-MS. As shown in Table 1, the molecular mass of the fragments produced by the cleavage was determined to be 378.2, 964.4, 1 800.8, 3 135.0 and 2 547.3 Da, which correspond to cleavages of the Asn3-Gln4 (site 1), the His5-Leu6 (site 2), the Gly8-Ser9 (site 3), and the Glu21-Arg22 (site 4) peptide bonds, respectively, as shown in Scheme 2.

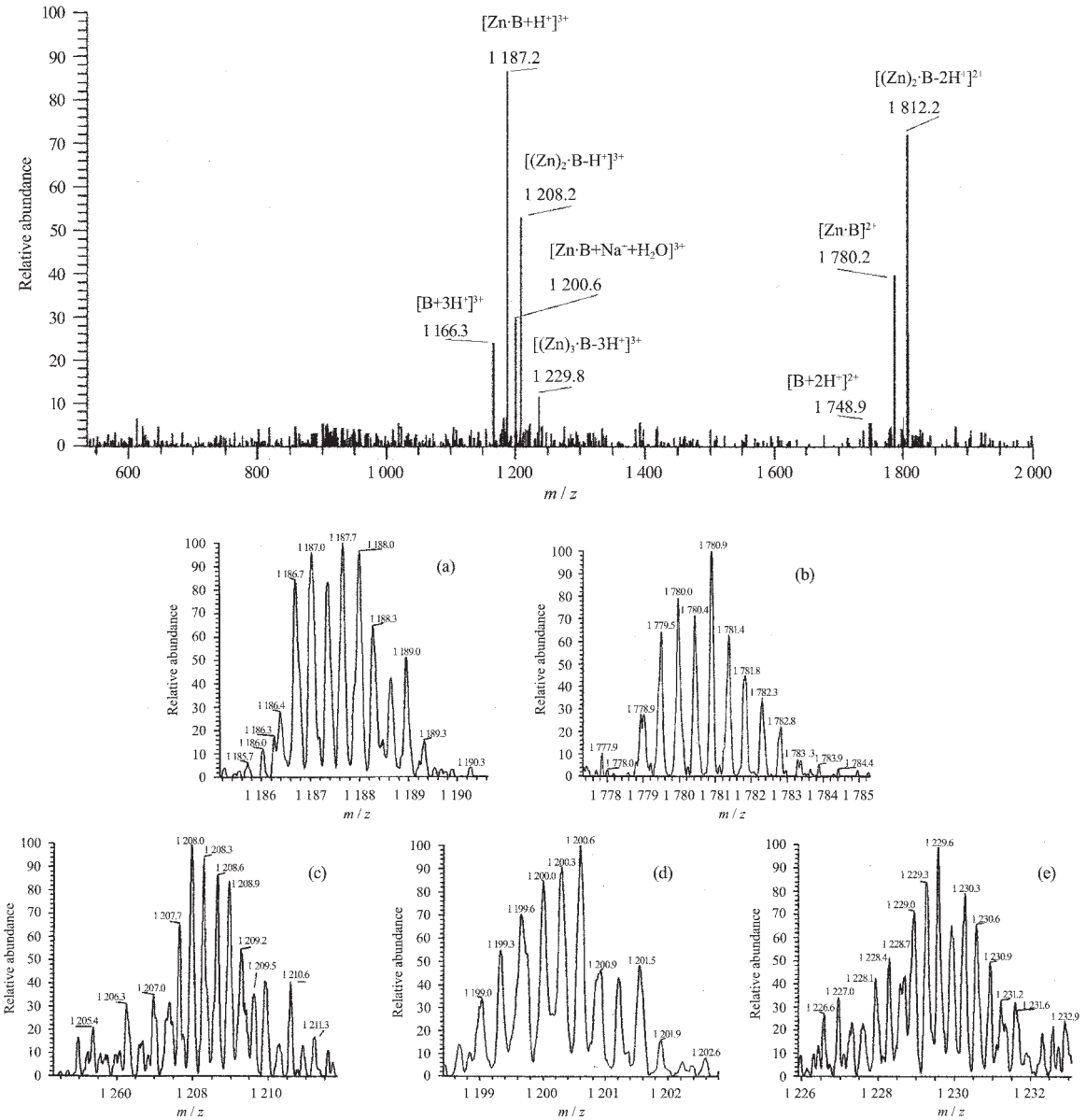
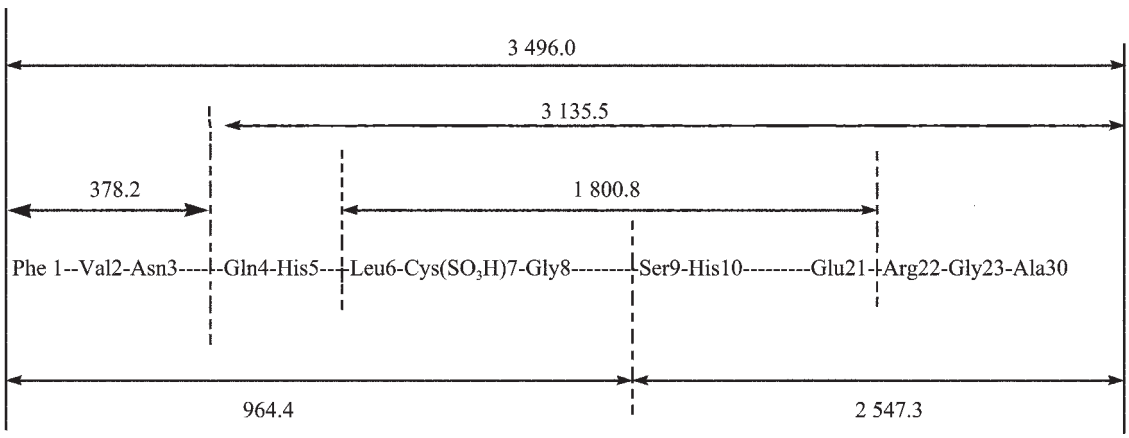


Fig.2 ESI mass spectrum of a mixed solution of B with $ZnCl_2$ in a molar ratio of 1:3 after 3 h of incubation at 37 °C (a) (*m/z* 1 187.2) (b) (1 780.2 *m/z*) (c) (*m/z* 1 208.2) (d) (*m/z* 1 200.6) and (e) (*m/z* 1 229.8) are zoom scan spectra for peaks at *m/z* 1 187.2, 1 780.2, 1 208.2, 1 200.6 and 1 229.8, respectively

The cleaved sites of 1 and 3 were the same as $[CuL(H_2O)]^{2+}$-promoted cleavage of oxidized insulin B chain [20], and the site 3 was also observed in the cleavage of oxidized insulin B chain with $CuCl_2$[20] and *cis*-$[Pd(en)(H_2O)_2]^{2+}$[3a]. Therefore, the mechanism of cleavage seems to be similar. The cleaved site 2 and 4 were observed for the first time. As is well known from some previous studies [9g,9h,9k], the hydrolysis of peptides and proteins promoted by $[CuL(H_2O)]^{2+}$ proceeds in two steps. Firstly, Cu(Ⅱ) complexes anchor to the side chains of peptides and proteins, and then followed by cleavage of the first or second peptide bond in the vicinity of the binding sites. So it is reasonable to believe that the binding sites of Zn^{2+} are associated with cleaved sites of 1, 2 and 3 are His5 and His10. This is consistent with the binding sites of $[CuL(H_2O)]^{2+}$ with oxidized insulin B chain in our previous studies[20]. As for the cleavage site 4 of Glu21-Arg22, the cleavage seems to occur at the first peptide upstream from arginine22 residue, that is the X-Arg bond in the se-

Table 1　ESI mass spectra for fragments of oxidized insulin B chain cleaved by $ZnCl_2$

Fragment	Multiply charged ions (m/z)	Averaged molecular mass / Da	
		Calculated	Observed
(Phe1~Asn3)+H^+	–	379.2	378.8±0.2
(Phe1~Gly8)+H^+	–	965.4	965.4±0.1
[(Leu6~Glu21)+Zn^{2+}+H^+]$^{3+}$	–	622.4	622.1±0.2
[(Leu6~Glu21)+2H^+]$^{2+}$	–	901.4	900.5±0.2
(Ser9~Ala30)	850.1(+3),1274.6 (+2)	2547.3	2547.0±0.3
(Gln4~Ala30)	1046.1(+3),1568.4(+2)	3135.5	3135.0±0.3



Scheme 2

quence segments X-Arg-Y. This new finding is of interest. Latest research shows, a Cu(Ⅱ) complex of cyclen acting as a protein-cleaving catalyst, can selectively cleave lysozome at peptide bond of Arg114-Cys115 and Arg125-Gly126, which is near arginine residue [4a,13b]. Shields [22] proposed that the Cu (Ⅰ) ion could bind to the guanidine group of arginine residue and fragmentation occurred remote from the Cu (Ⅰ) ionattached site. Lavanant [23] also indicated that the sites of cleavage were the same in the [MCu(Ⅰ)]$^+$ and [M-H+Cu(Ⅱ)]$^+$ complexes, in which M represents the dipeptides containing arginine or lysine. Therefore, it is reasonable to believe that the cleavage site 4 is relative to arginine22. The Zn^{2+} anchors to the side chain of Arg22, resulting in cleavage of the first peptide bond upstream from the binding site.

The control experiment of oxidized insulin B chain incubated at 40 ℃ and pH value of 2.5 for 2 days was monitored by HPLC and ESI-MS. No appreciable peaks were observed, indicating the negligible background cleavage.

2.3　MS/MS Analysis

In order to confirm the binding sites of Zn^{2+} with B, MS/MS analysis for the peaks containing Zn(Ⅱ) ion was performed. Because MS/MS analysis for the singly-, doubly- and triply-coordinated species was unsuccessful, we chose another strategy previously used in identification of the binding sites in carboplatin-bound cytochrome c[12b]. A mixture solution of B and Zn^{2+} in molar ratio of 1∶5 was incubated at 40 ℃ and pH value of 2.5 for 2 days. The digestion solution after quenched by EDTA was separated by HPLC and measured by ESI-MS. As shown in Fig.3, a peak at m/z 622.1 observed was attributed to a cleaved fragment of [(Leu6-Glu21)+Zn^{2+}+H^+]$^{3+}$, zoom scan of [(Leu6-Glu21)+Zn^{2+}+H^+]$^{3+}$ is shown as inset (a). The spectrum simulation shown in inset (b) fits inset (a) fairly well. In order to further confirm the composition, MS/MS analysis was performed. The fragment ions according to the conventional notation[9k,12a] are assigned a_n, b_n, c_n when the charges are retained by N-terminal fragments, and assigned as x_n, y_n, z_n when the charges are

retained by C-terminal fragments. Only b_n and y_n were characterized because they are the most common fragments with greater stability towards further dissociaton. MS/MS fragmentation was repeated for several times for averaging.

As shown in Table 2, whether from N-terminal fragments or from C-terminal fragments, successive fragmental ions were found to be precisely equal to calculated values. It is revealed that the binding site of Zn(Ⅱ) ion with B is the imidazole groups of His10. This means that some removed Zn^{2+} is able to coordinate to N-terminal amino group of the fragment again.

Another peak at *m/z* 900.5 shown in Fig.4 was identified as the same fragment $[(Leu6\sim Glu21)+2H^+]^{2+}$ without a coordinated Zn ion. Table 3 shows the successive N-terminal and C-terminal fragment ions that exactly match the sequence of Leu6 ~Glu21 in oxidized insulin B chain. These results further confirm that the sites of cleavage are at Asn3-Gln4, His5-Leu6, Gly8-Ser9, and Glu21-Arg22.

In conclusion, oxidized insulin B chain was remarkably cleaved by Zn^{2+} with explicable specificity, and only four cleaved sites were detected. The binding sites of Zn^{2+} with B, determined by the MS/MS analysis of the cleaved fragments, are the histidine residues of His5 and His10 and Arg22. This study proves again that ESI-MS combined with MS/MS analysis is a powerful technique for determining the binding sites and cleavage sites of metal complex-bound peptides. This study revealed that $ZnCl_2$ was able to selectively hydrolytic cleavage of oxidized insulin B chain.

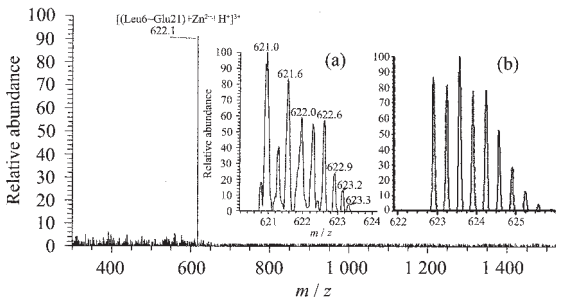


Fig.3 ESI mass spectrum of the fragment [(Leu6-Glu21) + Zn^{2+}+H^+]$^{3+}$. Inset (a) and (b) are zoom scan and isotope simulation of [(Leu6-Glu21) + Zn^{2+}+H^+]$^{3+}$, respectively

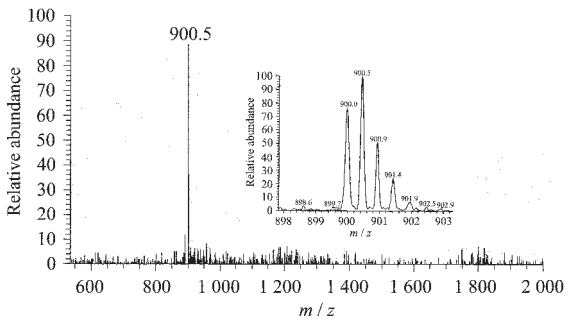


Fig.4 ESI mass spectrum of the fragment $[(Leu6\sim Glu21)+2H^+]^{2+}$. Inset are zoom scan of $[(Leu6\sim Glu21)+2H^+]^{2+}$

Table 2 MS/MS analysis for [Leu6-Glu21 + Zn+H^+]$^{3+}$ (*m* / *z* 622.1)

Assignment	*m* / *z* value		Assignment	*m* / *z* value	
	Calculated	Observed		Calculated	Observed
b_3^+	322.1	–	y_4^+	407.1	–
b_4^+	409.2	–	y_5^+	520.2	–
$(b_5+Zn)^+$	610.6	610.4	y_6^+	683.3	683.0
$(b_6+Zn)^+$	723.7	723.8	y_7^+	796.4	796.2
$(b_7+Zn)^+$	822.8	822.5	y_8^+	867.4	–
$(b_8+Zn)^+$	951.8	–	y_9^+	996.5	996.3
$(b_9+Zn)^+$	1022.8	1022.6	y_{10}^+	1095.5	1095.8
$(b_{10}+Zn)^+$	1135.9	1135.9	y_{11}^+	1208.6	1208.6
$(b_{11}+Zn)^+$	1298.9	1298.8	$(y_{12}+Zn)^+$	1410.1	1410.3
$(b_{12}+Zn)^+$	1412.0	1411.8	$(y_{13}+Zn)^+$	1497.1	1497.0
$(b_{13}+Zn)^+$	1511.1	1511.4	$(y_{14}+Zn)^+$	1554.1	1554.3
$(b_{14}+Zn)^+$	1662.1	1662.0	$(y_{15}+Zn)^+$	1657.1	1657.0
$(b_{15}+Zn)^+$	1719.2	–			

Table 3　MS/MS analysis for [Leu6-Glu21+2H$^+$]$^{2+}$ (m / z 900.5)

Assignment	m / z value		Assignment	m / z value	
	Calculated	Observed		Calculated	Observed
b_3^+	322.1	–	y_4^+	455.1	–
b_4^+	409.2	–	y_5^+	568.2	–
b_5^+	546.2	–	y_6^+	731.3	731.3
b_6^+	659.3	659.0	y_7^+	844.4	844.6
b_7^+	758.4	758.2	y_8^+	915.4	915.3
b_8^+	887.4	887.4	y_9^+	1044.5	1044.7
b_{92}^+	958.4	958.2	y_{10}^+	1143.5	1143.8
b_{10}^+	1071.5	1071.3	y_{11}^+	1256.6	1256.6
b_{11}^+	1234.6	1234.5	y_{12}^+	1393.7	1393.4
b_{12}^+	1347.7	1347.8	y_{13}^+	1480.7	–
b_{13}^+	1446.7	–	y_{14}^+	1537.7	1537.9
b_{14}^+	1597.8	1597.6	y_{15}^+	1688.7	–
b_{15}^+	1654.8	1654.9			

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