Supplementary Material (ESI) for New Journal of Chemistry

Supplementary Information

Hydrolysis of insulin chain B using zirconium(IV) at neutral pH

Sarah S. Cepeda and Kathryn B. Grant*

Department of Chemistry, Center for Biotechnology and Drug Design,

Georgia State University, P.O. Box 4098, Atlanta, Georgia 30302-4098

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Section 1: Additional Experimental Details.

Materials. Distilled, deionized water was utilized in the preparation of all buffers and all aqueous reactions. Chemicals were of the highest available purity and were used without further purification. L-glutamic acid, α-cyano-4-hydroxycinnamic acid (CHCA), human synthetic angiotensin I, and oxidized bovine insulin chain B were purchased from Sigma. Ethylenediaminetetraacetic acid disodium salt (EDTA) was from Fisher Scientific and HPLC grade acetonitrile was from Burdick & Jackson. All other reagents including 4,13-diaza-18-crown-6 (1,4,10,13-tetraoxa-7,16 diazacyclooctadecane), formic acid, and $ZrCl_4$ (purity > 99.99%) were obtained from the Aldrich Chemical Company.

Section 2: Representative HPLC-ESI Mass Spectra.

As described in the accompanying manuscript, a total of 500 µM of oxidized bovine insulin chain B was treated with 10 mM $ZrCl_4$ in 20 mM 4,13-diaza-18-crown-6 from t = 4 h to t = 8 h at pH 7.0 and 60 °C. Peptide hydrolysis products were then identified by HPLC-electrospray ionization mass spectrometry (HPLC-ESI MS). Shown in Figs. S1, S2, and S3 are representative ESI mass spectra at HPLC elution times of 18.36 min, 23.54 min, and 31.70 min, respectively.

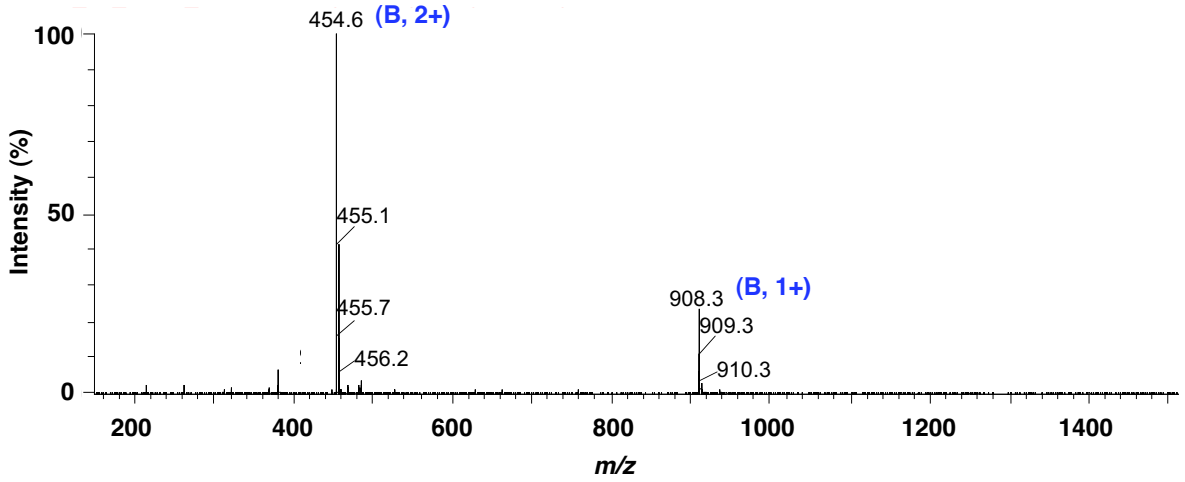

Fig. S1 HPLC-ESI mass spectrum at an HPLC elution time of 18.36 min. Oxidized bovine insulin chain B was treated with Zr^{IV}/4,13-diaza-18-crown-6 for t = 8 h. In the spectrum are the singly and doubly charged observed masses corresponding to insulin B chain hydrolysis fragment Phe1 to Cys(SO_3H)7 **(fragment B, Table S1)**. The spectrum was acquired in positive ion mode.

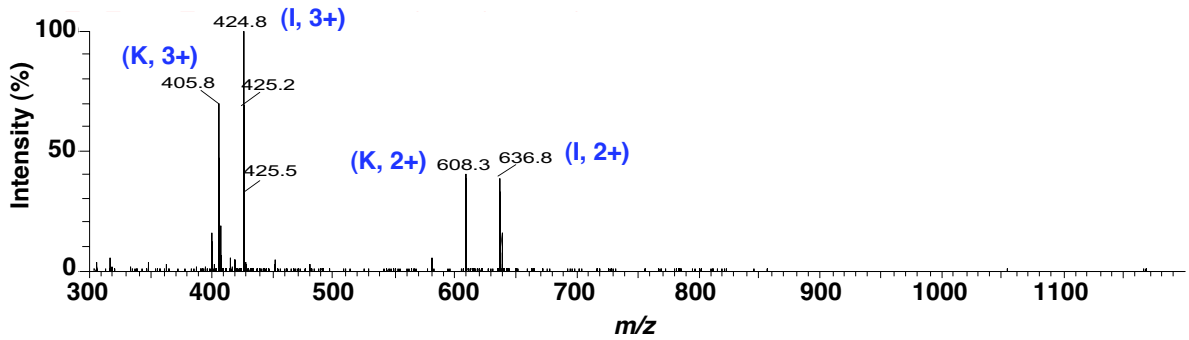

Fig. S2 HPLC-ESI mass spectrum at an HPLC elution time of 23.54 min. Oxidized bovine insulin chain B was treated with Zr^{IV}/4,13-diaza-18-crown-6 for t = 4. In the spectrum are the doubly and triply charged observed masses corresponding to insulin B chain hydrolysis fragments Gly20 to Ala30 **(fragment I, Table S1)** and Glu21 to Ala30 **(fragment K, Table S1)**. The spectrum was acquired in positive ion mode.

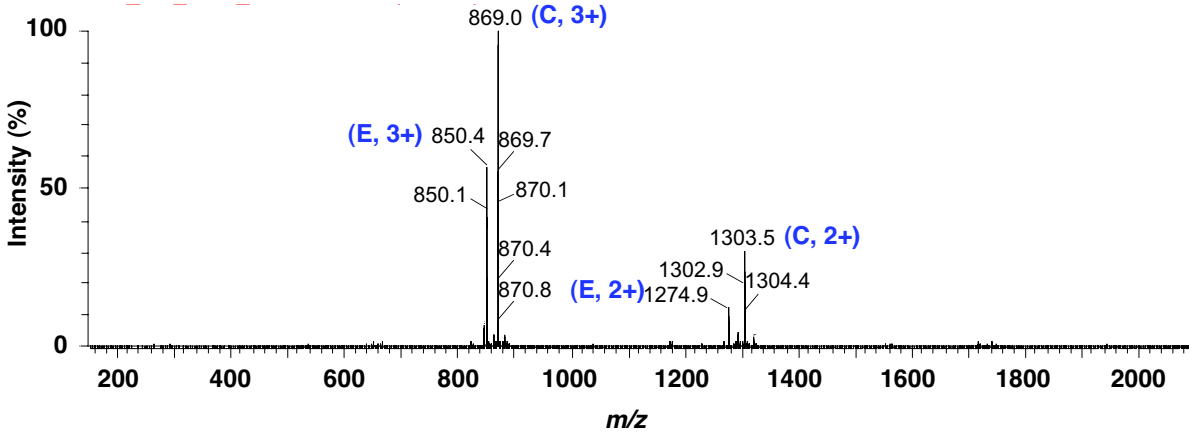

Fig. S3 Representative HPLC-ESI mass spectrum at an HPLC elution time of 31.70 min. Oxidized bovine insulin chain B was treated with Zr^{IV}/4,13-diaza-18-crown-6 for t = 4 h. In the spectrum are the doubly and triply charged observed masses corresponding to insulin B chain hydrolysis fragments Gly8 to Ala30 **(fragment C, Table S1)** and Ser9 to Ala30 **(fragment E, Table S1)**. The spectrum was acquired in positive ion mode.

Section 3: Zirconium(IV)-Assisted Deamidation and Lactamization Reactions.

Zirconium(IV)-Assisted Deamidation. HPLC-ESI analyses of the Zr^{IV} hydrolysis reactions detected the production of apparent trace quantities of the following three sets of chromatographically separable insulin chain B peptide fragments: B†(1) and B†(2); D†(1) and

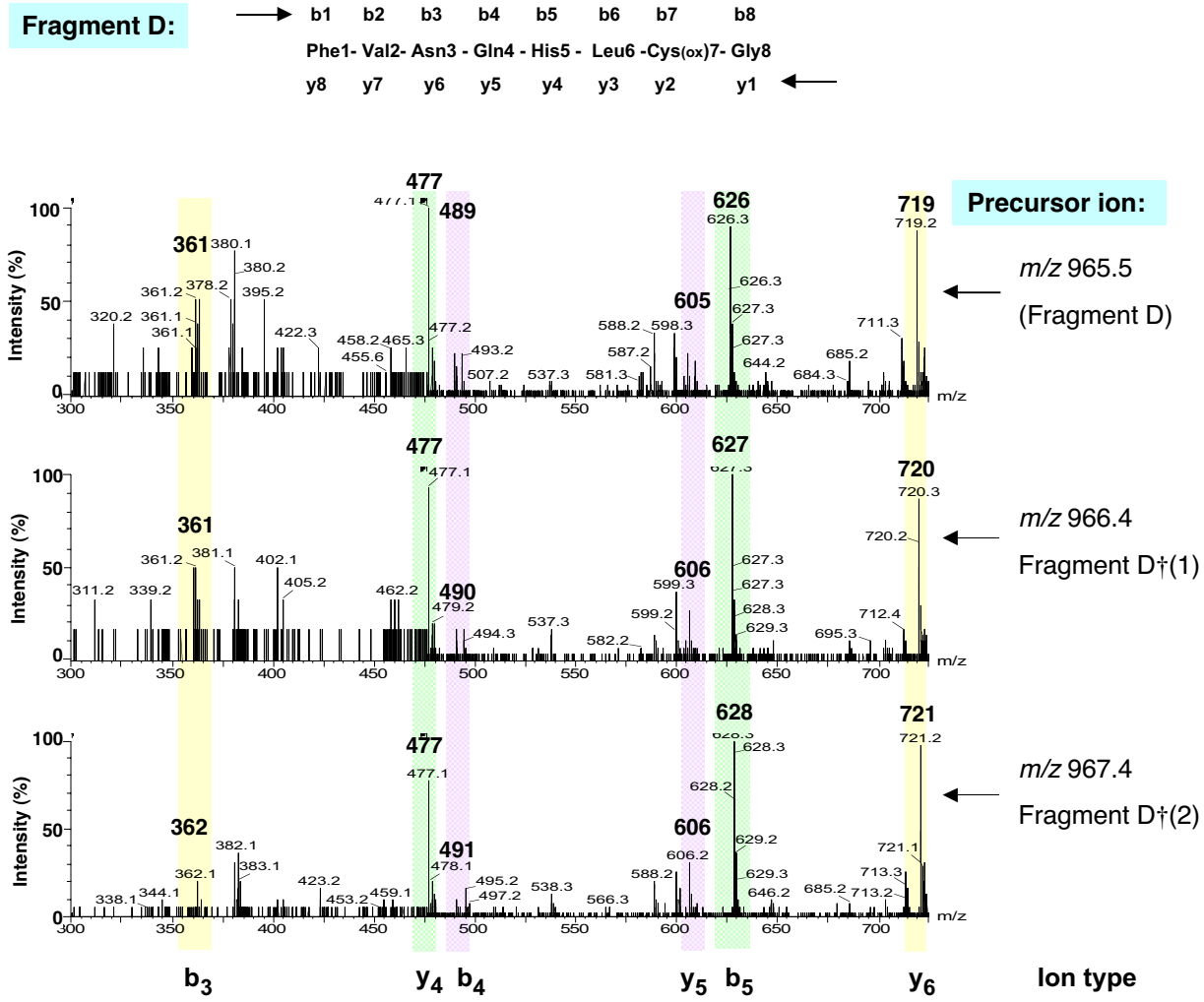

Fig. S4 A total of 500 µM of oxidized bovine insulin chain B was treated with 10 mM $ZrCl_4$ in 20 mM 4,13-diaza-18-crown-6 for t = 8 h at pH 7.0 and 60 °C. The MS/MS spectra show *N*-terminal b-ions and *C*-terminal y-ions generated from *m/z* 965.5 (Fragment D: Phe1-Gly8), from *m/z* 966.4 (Fragment D†(1): Gln4 in D converted to Glu4) and from *m/z* 967.4 (Fragment D†(2): Asn3 and Gln4 in D converted to Asp3 and Glu4). The *m/z* values in bold are rounded to the nearest Da. The spectra were acquired in positive ion mode.

D†(2); and F†(1) and F†(2) (Table S1). Fragments B†(1) at *m/z* 909.3, D†(1) at *m/z* 966.4, and F†(1) at *m/z* 1053.5 were all observed to have masses that were approximately one mass unit higher than the parent peptides B (Phe1 to Cys(SO_3H)7, at *m/z* 908.3), D (Phe1 to Gly8, at *m/z* 965.5), F (Phe1 to Ser9, at *m/z* 1052.5; Table S1). This result indicated that B†(1), D†(1), and F†(1) could be deamidated forms of the starting material with either Asn3 or Gln4 being converted to α-Asp3, α-Glu4, or to the geometric isomers β-Asp and γ-Glu.[1] Fragment B†(2) at *m/z* 910.3, D†(2) at *m/z* 967.4, and F†(2) at *m/z* 1054.4, were all approximately two mass units higher that the parent peptides B, D, and F. Therefore, B†(2), D†(2), and F†(2) were proposed to be doubly deamidated. We then employed ESI-MS/MS sequencing to demonstrate that the three sets of fragments were indeed produced by deamidation of parent peptides B, D, and F: in B†(1), D†(1), and F†(1), Gln4 in the corresponding parent was converted to Glu4; in B†(2), D†(2), and F†(2), Asn3 and Gln4 were deamidated to Asp3 and Glu4. Shown in Fig. S4 are representative HPLC-ESI-MS/MS spectra acquired from precursor ions at *m/z* 965.5 (Fragment D, HPLC elution time 18.50 min), at *m/z* 966.4 (Fragment D†(1), HPLC elution time 19.82 min), and at *m/z* 967.4 (Fragment D†(2), HPLC elution time 20.54 min). A side by side comparison of successive *N*-terminal fragment ions b_3 to b_5 and of successive *C*-terminal fragment ions y_4 to y_6 confirms the deamidation patterns described above. Because peptide deamidation at neutral pH is associated with the formation of α-Asp and β-Asp in a 1:3 ratio and α-Glu and γ-Glu in a 1:1.7 ratio,[1] we propose that insulin chain B fragments B†(1) and (2), D†(1) and (2), and F†(1) and (2) are likely to consist of mixtures of geometric isomers. With respect to parent fragments B, D, and F, the MS/MS data consistently revealed preferential deamidation of Gln4 over Asn3. This result was initially unexpected, in light of the fact that the majority of literature reports have maintained that asparaginyl residues are more susceptible to hydrolytic deamidation under neutral conditions.[2] Notwithstanding, deamidation rates are influenced by primary, secondary tertiary, and quaternary protein structures.[3] Consistent with our results, glutamine deamidation has been shown to be accelerated by flanking carboxyl side histidine residues (i.e., His5 in oxidized bovine insulin chain B).[4]

Zirconium(IV)-Assisted Lactamization. In order to further substantiate that Zr^{IV} is capable of promoting lactamization in the presence of 4,13-diaza-18-crown-6, a total of 2 mM of glutamate was reacted in the presence of 20 mM of the azacrown ether, with and without 10 mM of $ZrCl_4$. After 24 h of treatment at pH 7.0 and 60 °C, significant conversion of Glu (*m/z* 146.4) to *pyro*Glu (*m/z* 128.5) was observed in the reaction containing Zr^{IV}/4,13-diaza-18-crown-6, and not in the reaction in which Zr^{IV} was substituted by an equivalent volume of water (Fig. S5).

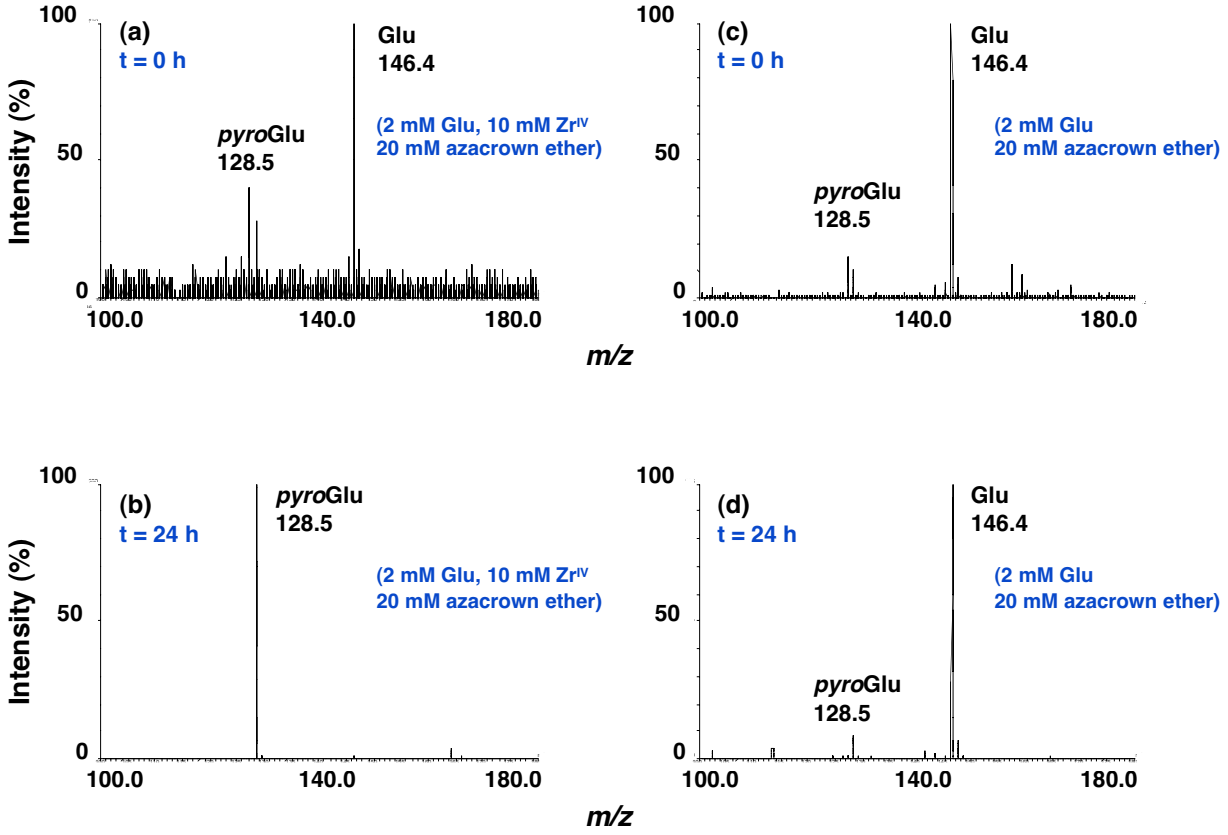

Fig. S5 ESI mass spectra of glutamate treated at pH 7.0 and 60 °C. (a) & (b): A total of 2 mM of Glu was reacted with 10 mM of $ZrCl_4$ in the presence of 20 mM 4,13-diaza-18-crown-6 for 0 h and 24 h, respectively. (c) & (d): A total of 2 mM of Glu in 20 mM 4,13-diaza-18-crown-6 was treated for 0 h and 24 h, respectively (no $ZrCl_4$). "Glu" identifies glutamate; "*pyro*Glu" identifies pyroglutamate. The spectra were acquired in negative ion mode.

Section 4: Tables.

Table S1 HPLC-ESI-MS analysis of peptides observed after oxidized bovine insulin chain B was reacted in the presence of $ZrCl_4$ and 4,13-diaza-18-crown-6 (pH 7.0 and 60 ºC)[a]

Peak assignment	elution time (min)	relative abundance HPLC (%)		*m/z* singly charged peptide		*m/z* doubly charged peptide		*m/z* triply charged peptide	
		t = 4 h	t = 8 h	obsd	calcd	obsd	calcd	obsd	calcd
A: Phe1 to Ala30	33.82	100	100	*no*	3494.7	1747.8	1747.8	1165.5*	1165.6
B: Phe1 to Cys(SO_3H)7	18.36	11	50	908.3*	908.4	454.6	454.7	*no*	303.5
B†(1): (Gln4→Glu4)	19.73	2	3	909.3*	909.4	455.1	455.2	*no*	303.8
B†(2): (Asn3→Asp3;Gln4→Glu4)	20.46	2	3	910.3*	910.4	455.6	455.7	*no*	304.1
C: Gly8 to Ala30	31.70	12	7	*no*	2605.3	1303.0	1303.1	869.1*	869.1
D: Phe1 to Gly8	18.50	11	50	965.5*	965.4	483.2*	483.2	322.7	322.5
D†(1): (Gln4→Glu4)	19.82	2	3	966.4*	966.4	483.6	483.7	*no*	322.5
D†(2): (Asn3→Asp3;Gln4→Glu4)	20.54	2	3	967.4*	967.4	484.2	484.2	*no*	323.1
E: Ser9 to Ala30	31.70	12	7	*no*	2548.3	1274.5	1274.6	850.0*	850.1
F: Phe1 to Ser9	18.74	11	50	1052.5*	1052.5	526.7	526.7	351.7	351.5
F†(1): (Gln4→Glu4)	19.99	2	3	1053.4*	1053.4	527.2	527.2	*no*	351.8
F†(2): (Asn3→Asp3;Gln4→Glu4)	20.67	2	3	1054.4*	1054.4	527.7	527.7	*no*	352.1
G: His10 to Ala30	31.70	12	7	*no*	2461.2	1231.0*	1231.1	821.0	821.1
H: Phe1 to Cys(SO_3H)19	33.79	5	5	*no*	2241.0	1121.0	1121.0	748.0	747.7
I: Gly20 to Ala30	23.54	9	44	1272.5	1272.6	636.8*	636.8	424.8	424.9
J: Phe1 to Gly20	33.79	5	5	2298.2	2298.1	1149.4	1149.5	766.7*	766.7
K: Glu21 to Ala30	23.54	9	44	1215.5	1215.6	608.3*	608.3	405.8	405.9
K‡: (Glu21→*pyro*Glu21)	26.48	3	16	1197.5*	1197.6	599.3*	599.3	400.1	399.9
L: Phe1 to Leu6	17.00	6	15	756.4*	757.4	378.6	379.2	*no*	253.1
M: Leu6 to Tyr16	28.11	1	4	*no*	1252.6	627.9	626.8	418.5	418.2

[a][oxidized bovine insulin chain B]$_0$ = 0.5 mM; [$ZrCl_4$]$_0$ = 10 mM; [4,13-diaza-18-crown-6]$_0$ = 20 mM. Data were acquired over multiple trials. Representative HPLC chromatograms are in Fig. 2 of the accompanying manuscript. Peak assignments highlighted in **blue** correspond to apparent **major** peptide hydrolysis products. Peak assignments in **red** correspond to apparent **intermediate** peptide hydrolysis products. Peak assignments in **green** correspond to apparent **trace** peptide hydrolysis products. Not observed = *no*. "*" indicates that peak assignments were confirmed by ESI-MS/MS sequencing. "†" indicates deamidation of peptide: Asn3 to Asp3 and/or Gln4 to Glu4. "‡" indicates lactamization of *N*-terminal Glu21 to pyroglutamate21 (*pyro* Glu21).

Continued: Table S1 HPLC-ESI-MS analysis of peptides observed after oxidized bovine insulin chain B was reacted in the presence of $ZrCl_4$ and 4,13-diaza-18-crown-6 (pH 7.0 and 60 ºC)[a]

Peak assignment	elution time (min)	relative abundance HPLC (%)		*m/z* singly charged peptide		*m/z* doubly charged peptide		*m/z* triply charged peptide	
		t = 4 h	t = 8 h	obsd	calcd	obsd	calcd	obsd	calcd
N: Gly8 to Cys(SO_3H)19	29.70	1	1	1351.8	1351.7	676.3*	676.3	*no*	451.2
O: Gly8 to Gly20	29.82	3	4	1408.6	1408.7	704.8	704.8	470.2	470.2
P: Ser9 to Cys(SO_3H)19	30.39	1	*no*	1294.5	1294.6	647.7	647.8	432.5	432.2
Q: Ser9 to Gly20	28.11	1	1	1351.8	1351.7	676.3*	676.3	451.5	451.2
R: His10 to Cys(SO_3H)19	33.40	2	2	1207.7	1207.6	604.73	604.3	403.5	403.2
S: His10 to Gly20	27.90	1	1	1264.5	1264.6	632.8	632.8	422.5	422.2
T: His10 to Lys29	29.61	1	1	*no*	2390.2	1195.4	1195.6	797.4	797.4
U: Val2 to Ala30	33.51	1	1	*no*	3347.6	1674.3	1674.3	1116.4	1116.5
V: Leu11 to Ala30	33.79	1	1	*no*	2324.2	1162.5*	1162.6	775.4	775.4
W: Gly23 to Ala30	25.82	6	25	930.5*	930.5	465.6	465.7	*no*	310.8
X: Phe24 to Ala30	24.19	3	13	873.3*	873.4	437.1	437.1	*no*	291.8

[a][oxidized bovine insulin chain B]$_0$ = 0.5 mM; [$ZrCl_4$]$_0$ = 10 mM; [4,13-diaza-18-crown-6]$_0$ = 20 mM. Data were acquired over multiple trials. Representative HPLC chromatograms are in Fig. 2 of the accompanying manuscript. Peak assignments highlighted in **blue** correspond to apparent **major** peptide hydrolysis products. Peak assignments in **red** correspond to apparent **intermediate** peptide hydrolysis products. Peak assignments in **green** correspond to apparent **trace** peptide hydrolysis products. Not observed = *no*. "*" indicates that peak assignments were confirmed by ESI-MS/MS sequencing. "†" indicates deamidation of peptide: Asn3 to Asp3 and/or Gln4 to Glu4. "‡" indicates lactamization of *N*-terminal Glu21 to pyroglutamate21 (*pyro* Glu21).

Table S2 MALDI-TOF MS analysis of peptides observed after oxidized bovine insulin chain B was reacted for 4.2 h in the presence of $ZrCl_4$ and 4,13-diaza-18-crown-6 (pH 7.2 and 60 ºC)[a]

Peak assignment	relative abundance MS (%)	*m/z* singly charged peptide		*m/z* doubly charged peptide	
	t = 4.2 h	obsd	calcd	obsd	calcd
A: Phe1 to Ala30	22	3494.65	3494.65	1747.81	1747.83
C: Gly8 to Ala30	18	2605.21	2605.28	*no*	1303.14
E: Ser9 to Ala30	54	2548.16	2548.26	*no*	1274.63
G: His10 to Ala30	21	2461.13	2461.22	*no*	1231.11
I: Gly20 to Ala30	30	1272.63	1272.64	*no*	636.82
K‡: (Glu21→*pyro*Glu21)	100	1197.60*	1197.61	*no*	599.31
M: Leu6 to Tyr16	1	1252.68	1252.59	*no*	626.80
T: His10 to Lys29	1	2390.12	2390.19	*no*	1195.60
U: Val2 to Ala30	3	3347.48	3347.58	*no*	1674.29
V: Leu11 to Ala30	1	2324.08	2324.16	*no*	1162.58

[a][oxidized bovine insulin chain B]$_0$ = 1 mM; $[ZrCl_4]_0$ = 10 mM; [4,13-diaza-18-crown-6]$_0$ = 20 mM. Data were acquired over multiple trials. Representative MALDI-TOF mass spectra are in Fig. 3 of the accompanying manuscript. Peak assignments highlighted in **blue** correspond to apparent **major** peptide hydrolysis products. Peak assignments in **red** correspond to apparent **intermediate** peptide hydrolysis products. Peak assignments in **green** correspond to apparent **trace** peptide hydrolysis products. Not observed = *no*. "*" indicates that peak assignments were confirmed by ESI-MS/MS sequencing. "‡" indicates lactamization of *N*-terminal Glu21 to pyroglutamate21 (*pyro*Glu21).

References:

1 (*a*) S. Capasso, L. Mazzarella, F. Sica and A. Zagari, A. *Peptide Res.,* 1989, **2,** 195; (*b*) S. Capasso, L. Mazzarella, F. Sica and A. Zagari, *J. Chem. Soc., Chem. Commun.,* 1991, 1667.

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