Supplementary Information

From speciation to action: Cu(II) and Zn(II) tune histatins, but pH and enamel drive efficacy

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Stoichiometry

In the Cu(II)-histatin 1 spectra (Fig. S1A), the visable signals correspond to the free ligand (m/z = 970.42, z = 5+), its sodium (m/z = 975.07, z = 5+) and potassium (m/z = 979.40, z = 5+) adducts; the equimolar copper(II) complex (m/z = 982.84, z = 5+) and its sodium adduct (m/z = 987.22, z = 5+). In the Zn(II)-histatin 1 spectra (Fig. S1B), the most intense signal comes from the free ligand (m/z = 693.57, z = 7+). The second detected signal corresponds to zinc(II) complex (m/z = 701.62, z = 7+).

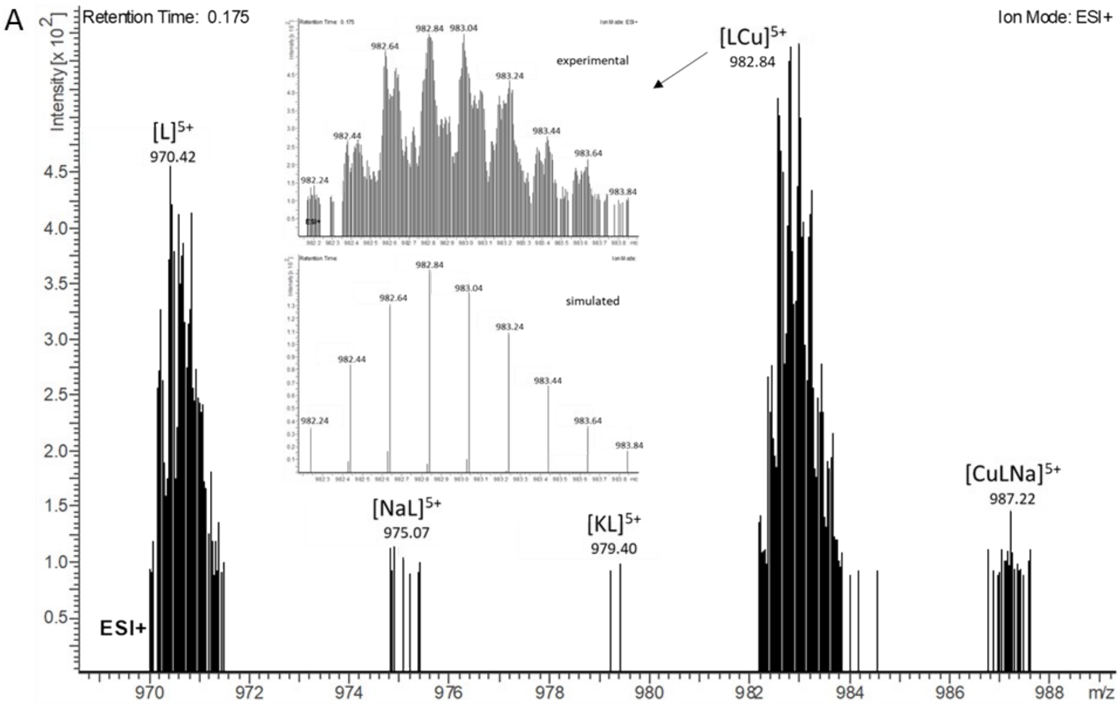
The mass spectrum obtained for Cu(II)-histatin 1-2 system (Fig. S1C) revealed the appearance of signals corresponding to the free ligand (m/z = 356.16, z = 4+), its sodium (m/z = 361.52, z = 4+) and potassium (m/z = 366.66, z = 4+) adducts, and the equimolar copper(II) complex (m/z = 371.40, z = 4+). In the Zn(II)-histatin 1-2 spectra (Fig. S1D), aside from the signal which comes from the free ligand (m/z = 474.56, z = 3+) signals from sodium adduct (m/z = 481.88, z = 3+), potassium adduct (m/z = 487.24, z = 3+) and zinc complex (m/z = 495.10, z = 3+) are visible.

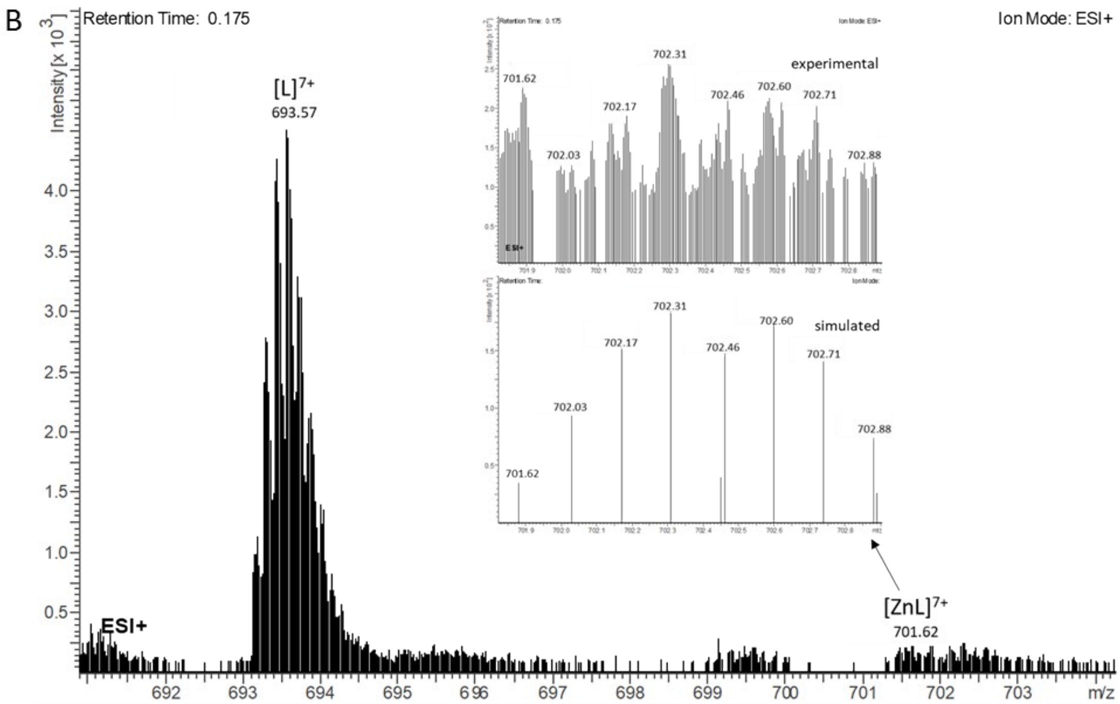
In the Cu(II)-histatin 2 spectra (Fig. S1E), the prevailing signals correspond to the free ligand (m/z = 862.13, z = 4+), its sodium (m/z = 867.38, z = 4+) and potassium adducts (m/z = 871.66, z = 4+); the Cu(II)-histatin 2 complex (m/z = 877.38, z = 4+) and its adducts with potassium atom (m/z = 887.16, z = 4+). In the Zn(II)-histatin 2 spectra (Fig. S1F) signals from free ligand (m/z = 862.13, z = 4+), its adducts with sodium (m/z = 867.65, z = 4+) and potassium (m/z = 871.64, z = 4+) and its Zn(II) complex (m/z = 878.13, z = 4+) were detected.

For the Cu(II)-histatin 7 sample, six peaks are observed (Fig. S1G). The first detected signal (m/z = 573.63, z = 4+) corresponds to the free peptide. The next two (m/z = 580.95 and 586.61, z = 3+) are peptide's sodium and potassium adducts, respectively. The most intense one (m/z = 593.93; z = 4+) is assigned to the Cu(II) complex. The other two signals correspond to the sodium (m/z = 601.23; z = 4+) and potassium (m/z = 607.23; z = 4+) adducts of Cu(II)-histatin 7 complex. For the Zn(II)-histatin 7 complex, the two most intense signals correspond to the free peptide and the Zn(II) complex (m/z = 573.99 and 594.27 respectively, z = 3+) (Fig. S1G). In the mass spectra also sodium (m/z = 601.58; z = 4+) and potassium (m/z = 607.23; z = 4+) adducts of the complex are visible.

In the Cu(II)-histatin 9 mass spectra (Fig. S1I) the prevailing four signals correspond to the free ligand (m/z = 469.75, z = 4+), its sodium adduct (m/z = 475.24, z = 4+), an equimolar Cu(II) complex

(m/z = 484.98, z = 4+) and its adduct with one sodium atom (m/z = 490.72, z = 4+). Similarly, four peaks are observed for the Zn(II)-histatin 9 sample (Fig. S1J). These signals correspond to the free peptide (m/z = 469.74, z = 4+), Zn(II) complex (m/z = 485.22, z = 4+) and its sodium and potassium adducts (m/z = 490.72 and 494.46 respectively, z = 4+).





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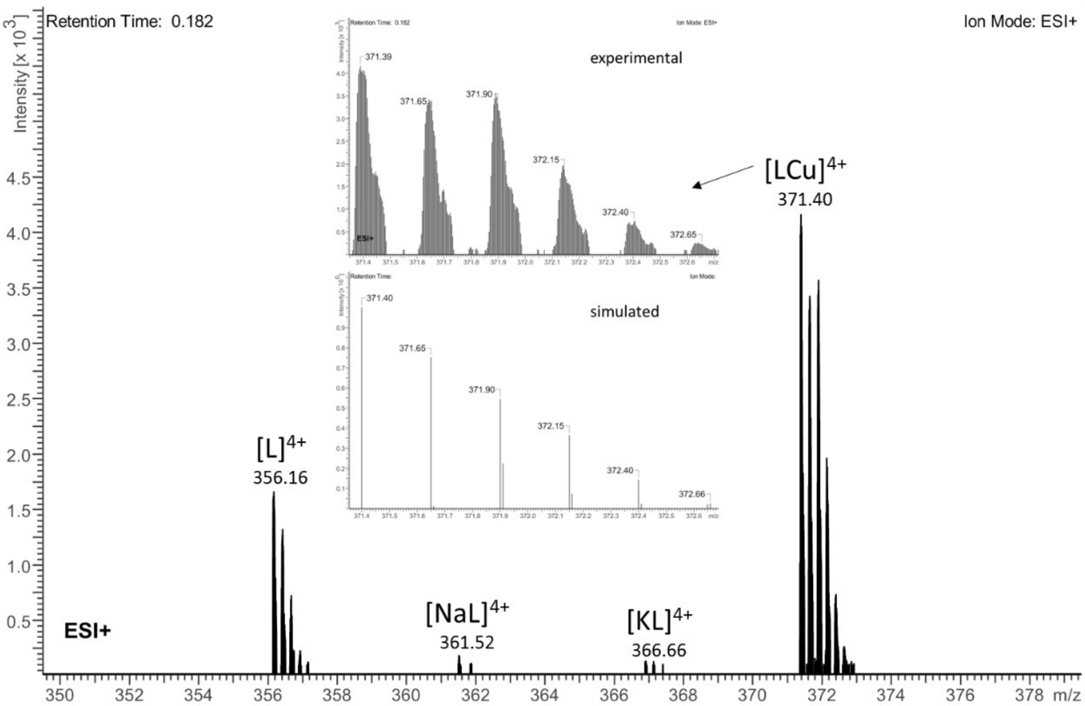
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361.52
[KL]4+
366.66
ESI+
m/z
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371.65
371.90
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372.40
372.65
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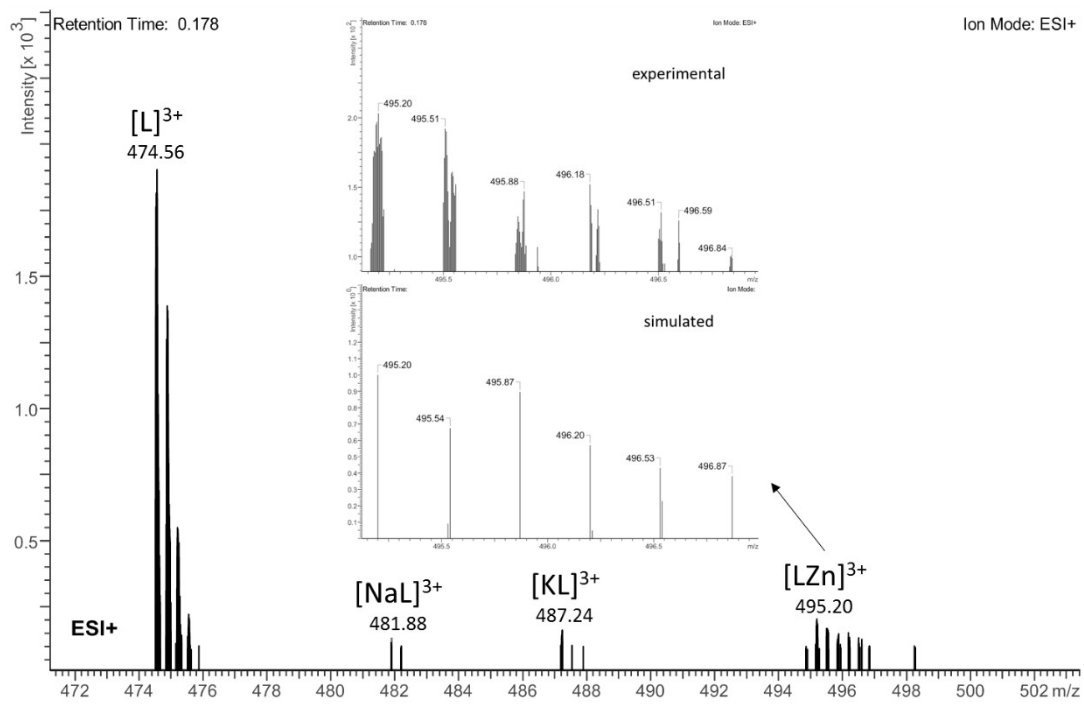
371.90
372.15
372.40
372.66

D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ESI+
m/z
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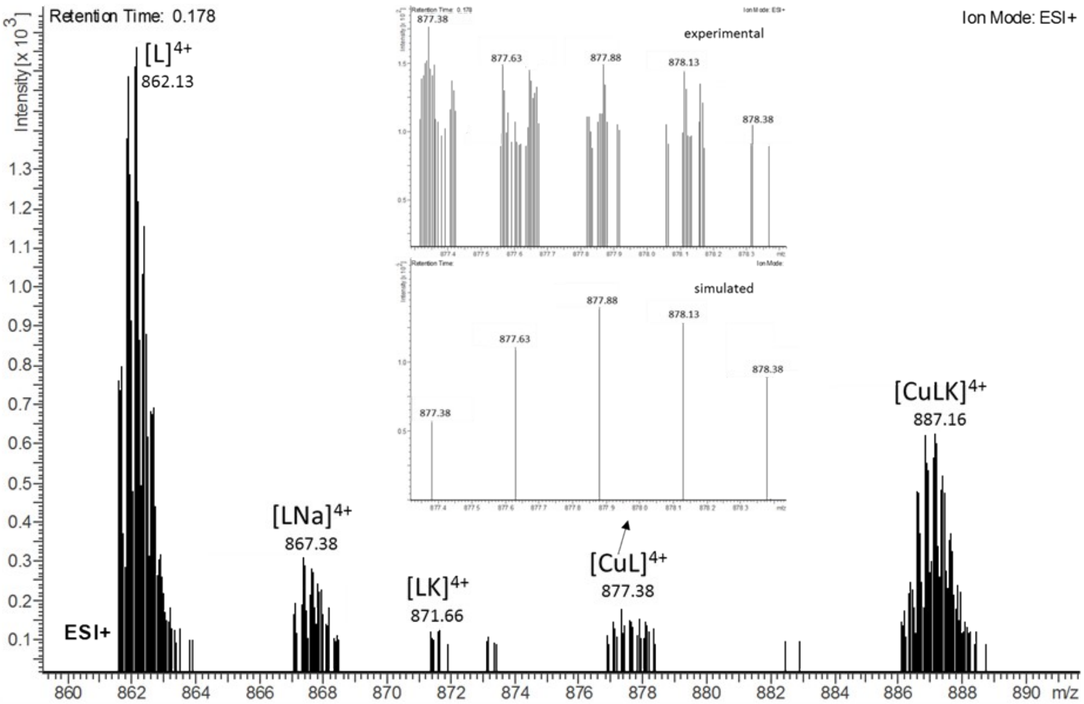
496.53
496.87

E



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877.38
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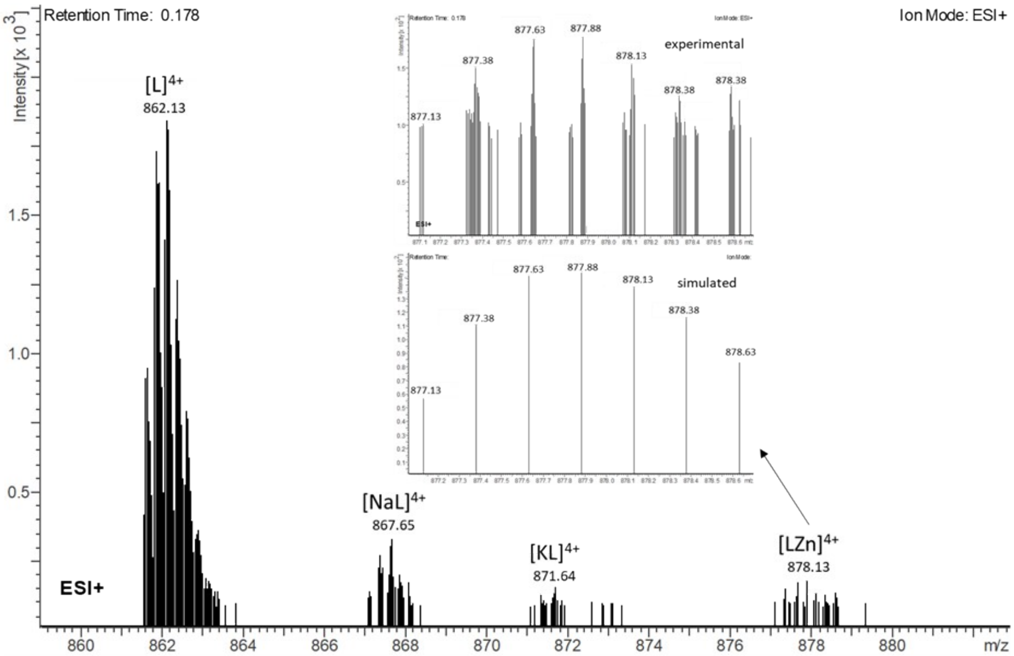
878.38
m/z

F



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871.64
[LZn]4+
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ESI+
experimental
simulated
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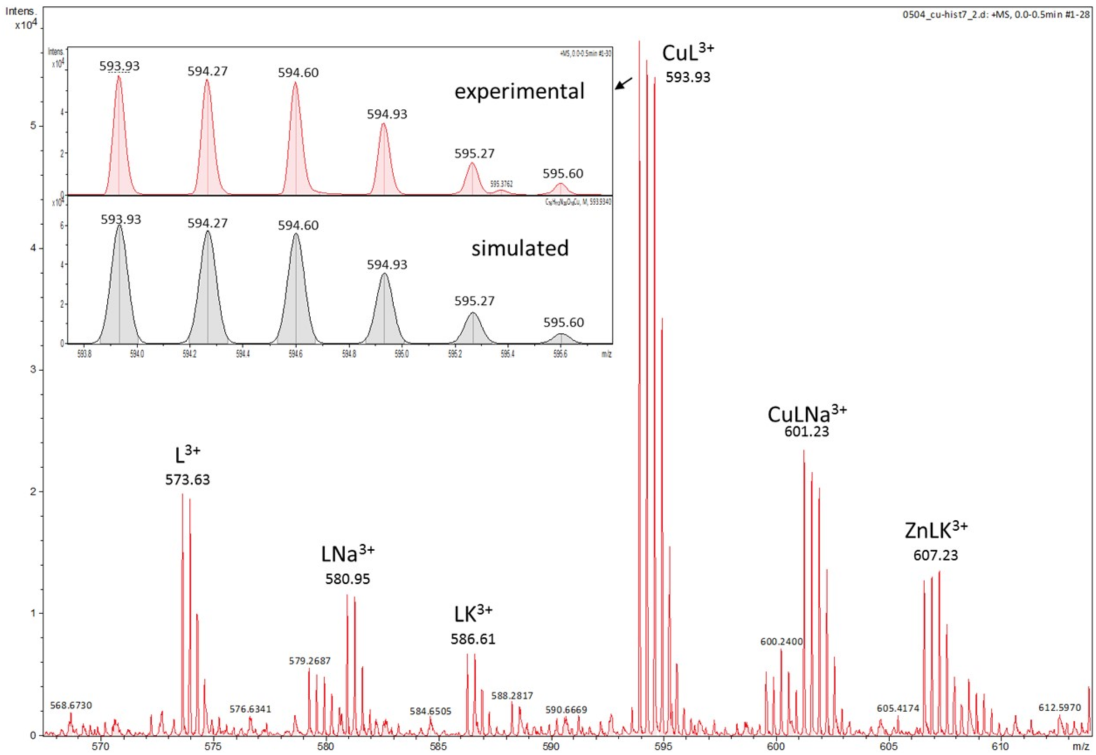
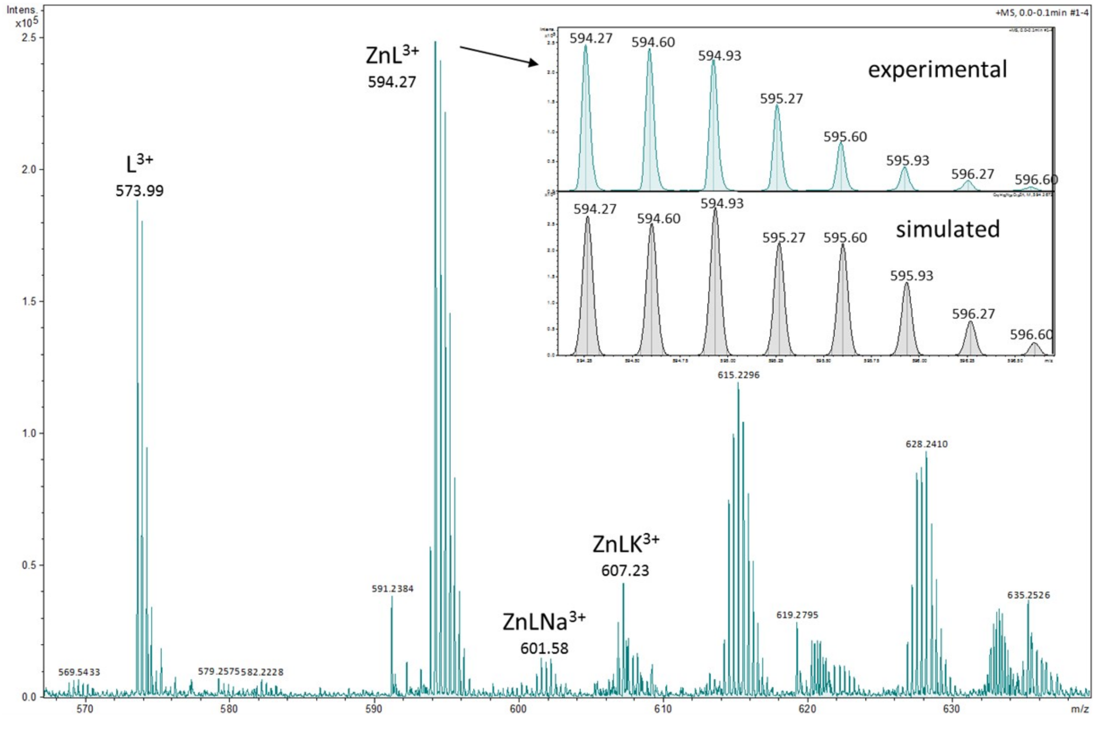
877.38
877.63
877.88
878.13
878.38
878.63
m/z

G



CuL3+
593.93
experimental
simulated
L3+
573.63
LNa3+
580.95
LK3+
586.61
CuLNa3+
601.23
ZnLK3+
607.23

H



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L3+
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experimental
simulated
ZnLK3+
607.23
ZnLNa3+
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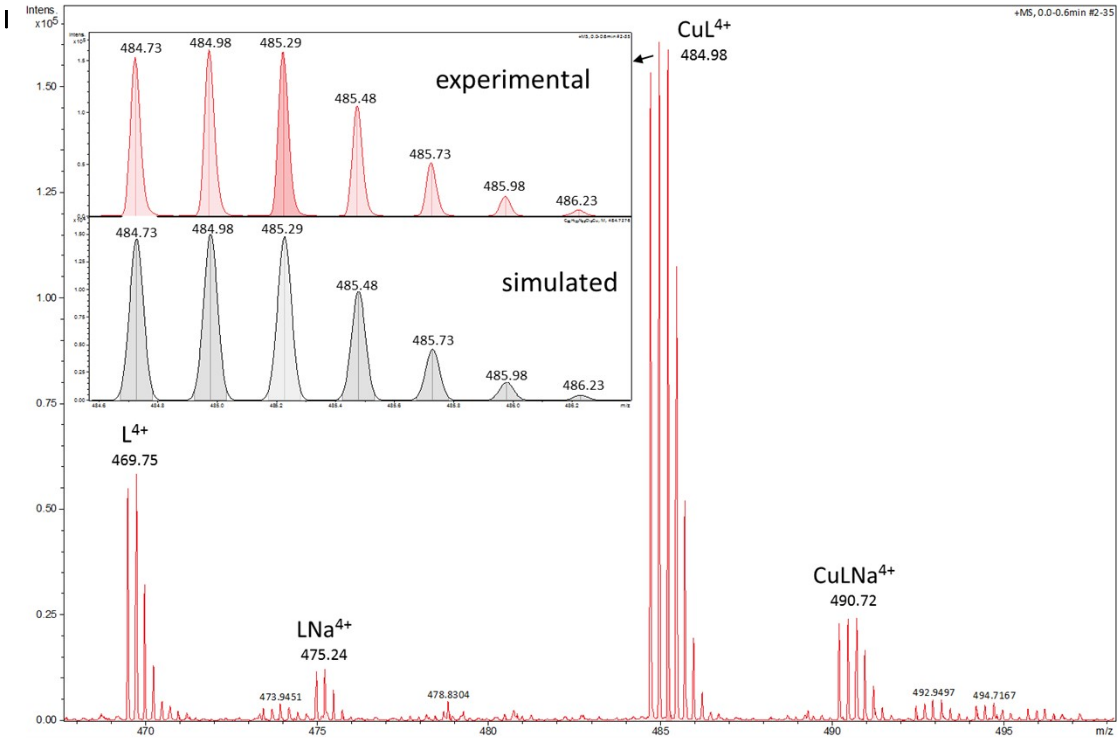


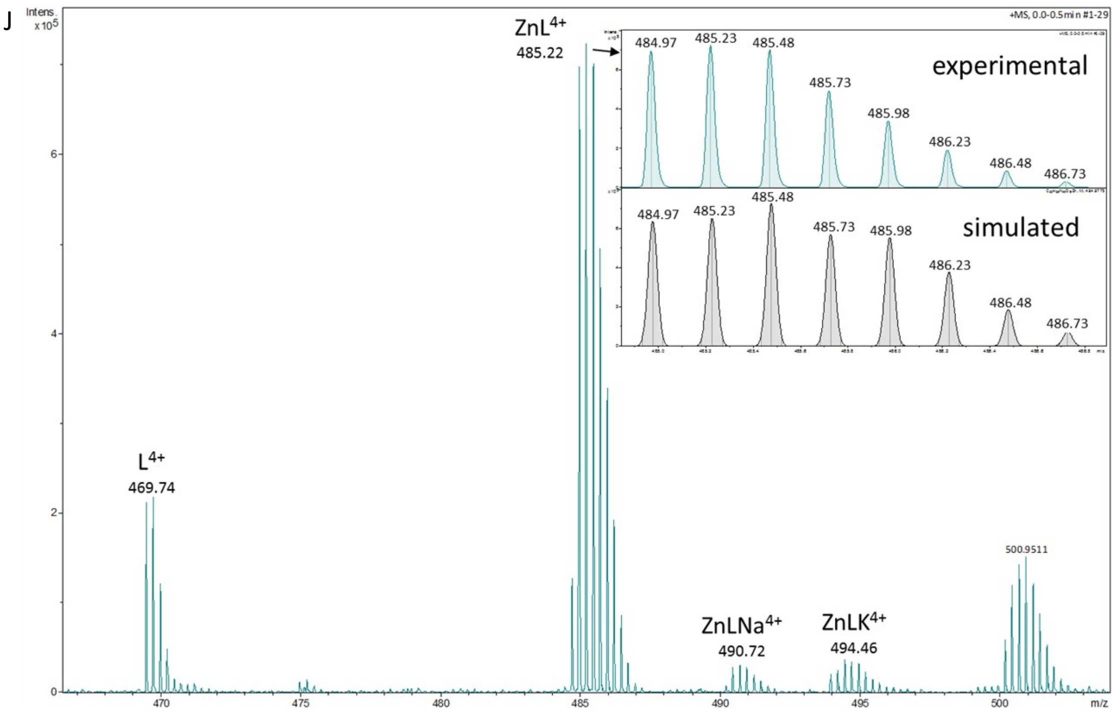


Figure S1. ESI-MS spectra of: (A) Cu(II)-histatin 1; (B) Zn(II)-histatin 1; (C) Cu(II)-histatin 1-2; (D) Zn(II)-histatin 1-2; (E) Cu(II)-histatin 2, (F) Zn(II)-histatin 2, (G) Cu(II)-histatin 7; (H) Zn(II)-histatin 7; (I) Cu(II)-histatin 9 and (J) Zn(II)-histatin 9. M:L molar ratio = 1:1, pH = 7.4 for Cu(II) complexes and 8.0 for Zn(II) complexes.

Ligand protonation

Potentiometric measurements detected nineteen deprotonation constants for histatin 1. The most acidic constants with pK_a values of 3.08, 3.60, 3.87, 4.17 and 4.78 come from the two Asp and three Glu side chains deprotonations, respectively. The next seven pK_a values (5.32, 5.38, 5.88, 6.55, 6.58, 7.59 and 8.00) arise from the deprotonation of the imidazole groups of seven His residues. The next constant (pK_a = 9.36) corresponds to N-terminal amino group. The following five pK_a values (9.46, 10.00, 10.02, 10.49 and 10.59) are related to the deprotonation of the five Tyr, and the last detected constant, pK_a = 10.70, comes from the deprotonation of Lys residue.

Histatin 1-2 behaves as an H_8L acid with the deprotonating groups corresponding at first to the Asp and Glu side chains (pK_a values of 2.57 and 3.78, respectively). The next three constants (pK_a = 5.76, 6.42 and 7.33) are related to the deprotonation of the three imidazole groups of His residues. The following pK_a (9.54) comes from the deprotonation of the N-terminal amino group. The last two and the highest pK_a constants (10.60 and 11.83) correspond to Tyr and Lys residues.

In the case of histatin 2, thirteen constants were detected. The first four acidic ones (pK_a of 2.86, 3.23, 3.96 and 4.41) correspond to two Asp and two Glu side chains. The next four constants, with pK_a values of 5.64, 6.22, 6.71 and 7.28, are related to the His imidazole groups, and the following one (pK_a = 9.22) corresponds to the N-terminal amino group. The four highest constants (9.69, 10.05, 10.51 and 10.53) come from the deprotonation of four Tyr.

Over the pH range 2-11, histatin 7 and histatin 9 show nine deprotonation constants. Both peptides deprotonate similarly, with deprotonation of the glutamic acid side chains (pK_a = 3.60 and 3.73, respectively). The following four pK_a values, in the range of 5.56-7.26 for histatin 7 and 5.57-7.22 for histatin 9, are related to the deprotonation of the four histidine residues. The next constant (pK_a = 9.54 and 9.51, respectively) corresponds to N-terminal amine group. The three highest constants (10.10, 11.02, 11.03 for histatin 7 and 10.00, 11.04, 11.15 for histatin 9) come from the deprotonation of tyrosine and two lysine residues.

Results from the potentiometric studies are presented in Table S1.

Table S1. Potentiometric data for proton of histatin 1, histatin 1-2, histatin 2, histatin 7 and histatin 9 at T = 298 K and I = 0.1 M $NaClO_4$. The standard deviations are reported in parentheses as uncertainties on the last significant figure. N-t refers to the N-terminal amine group.

	Histatin 1 (DSHEKRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYDN)		Histatin 1-2 (DSHEKRHHGYR)		Histatin 2 (RKFHEKHHSHREFPFYGDYGSNYLYDN)		Histatin 7 (RKFHEKHHSHRGY)		Histatin 9 (RKFHEKHHSHRGYR)	
species	logβ[a]	pK_a[b]	logβ[a]	pK_a[b]	logβ[a]	pK_a[b]	logβ[a]	pK_a[b]	logβ[a]	pK_a[b]
$H_{21}L$	158.20(5)	3.08(D)								
$H_{20}L$	155.12(6)	3.60(D)								
$H_{19}L$	151.52(6)	3.87(E)								
$H_{18}L$	147.65(6)	4.17(E)								
$H_{17}L$	143.48(5)	4.78(E)								
$H_{16}L$	138.70(4)	5.32(H)								
$H_{15}L$	133.38(4)	5.38(H)			112.67(5)	2.86(D)				
$H_{14}L$	127.55(6)	5.88(H)			109.81(5)	3.23(D)				
$H_{13}L$	121.67(5)	6.55(H)			106.58(6)	3.96(E)				
$H_{12}L$	115.12(4)	6.58(H)			102.62(6)	4.41(E)				
$H_{11}L$	108.54(3)	7.59(H)			98.21(4)	5.64(H)				
$H_{10}L$	100.95(3)	8.00(H)			92.57(5)	6.22(H)				
H_9L	92.95(2)	9.36(N-t)			86.35(4)	6.71(H)	70.83(2)	3.60(E)	70.79(1)	3.73(E)

H_8L	83.59(4)	9.46(Y)	57.83(2)	2.57(D)	79.64(5)	7.28(H)	67.23(1)	5.56(H)	67.06(1)	5.57(H)
H_7L	74.13(4)	10.00(Y)	55.26(2)	3.78(E)	72.36(3)	9.22(N-t)	61.67(2)	6.10(H)	61.49(1)	6.01(H)
H_6L	64.13(5)	10.02(Y)	51.48(1)	5.76(H)	63.14(4)	9.69(Y)	55.57(1)	6.62(H)	55.48(1)	6.56(H)
H_5L	54.11(2)	10.49(Y)	45.72(2)	6.42(H)	53.45(4)	10.05(Y)	48.95(2)	7.26(H)	48.92(1)	7.22(H)
H_4L	43.62(3)	10.59(Y)	39.30(2)	7.33(H)	43.40(6)	10.51(Y)	41.69(1)	9.54(N-t)	41.70(1)	9.51(N-t)
H_3L	33.03(3)	10.70(K)	31.97(1)	9.54(N-t)	32.89(5)	10.53(Y)	32.15(2)	10.10(Y)	32.19(1)	10.00(Y)
H_2L	22.33(4)	-	22.43(1)	10.60(Y)	22.36(3)	-	22.05(1)	11.02(K)	22.19(1)	11.04(K)
HL	-	-	11.83(2)	11.83(K)	-	-	11.03(3)	11.03(K)	11.15(2)	11.15(K)

[a] $\beta(H_jL_k) = [H_jL_k]/([H]^j[L]^k)$, in which [L] is the concentration of the fully deprotonated peptide.

[b] $\log\beta(H_jL_k) - \log\beta(H_{j-1}L_k) = pK_a$

Table 2. Potentiometric data for Cu(II) and Zn(II) complexes with histatin 1, histatin 1-2, histatin 2, histatin 7 and histatin 9 at T = 298 K and I = 0.1 M $NaClO_4$. The standard deviations are reported in parentheses as uncertainties on the last significant figure.

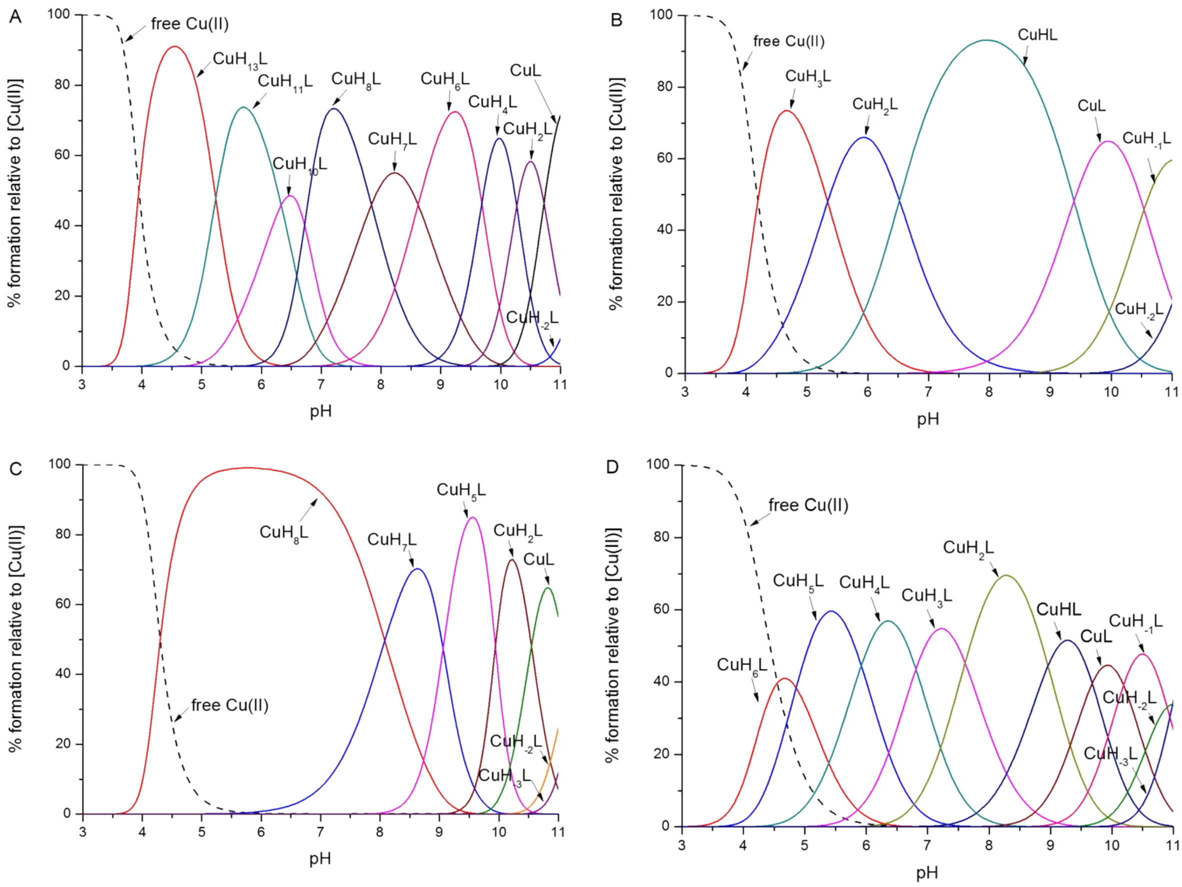
	Histatin 1 **(DSHEKRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYDN)**		**Histatin 1-2** **(DSHEKRHHGYR)**		**Histatin 2** **(RKFHEKHHSHREFPFYGDYGSNYLYDN)**		**Histatin 7** **(RKFHEKHHSHRGY)**		**Histatin 9** **(RKFHEKHHSHRGYR)**	
species	logβ[a]	pK_a[b]	logβ[a]	pK_a[b]	logβ[a]	pK_a[b]	logβ[a]	pK_a[b]	logβ[a]	pK_a[b]
Cu(II) complexes										
$CuH_{13}L$	129.48(2)	-								
$CuH_{12}L$	-	-								
$CuH_{11}L$	119.01(3)	-								
$CuH_{10}L$	112.68(5)	6.33								
CuH_9L	-	-								
CuH_8L	99.24(6)	-			89.08(7)	-				
CuH_7L	91.50(5)	7.74			81.00(5)	8.08				
CuH_6L	82.78(5)	8.72			-	-	61.61(1)	-	61.54(1)	-
CuH_5L	-	-			62.88(6)	-	56.75(1)	4.86	56.57(1)	4.97
CuH_4L	63.41(6)	-			-	-	50.83(1)	5.92	50.57(1)	6.00
CuH_3L	-	-	42.37(1)	-	-	-	44.02(1)	6.81	43.87(1)	6.70
CuH_2L	42.87(6)	-	37.03(1)	5.33	33.05(6)	-	36.41(1)	7.61	36.32(1)	7.55
CuHL	-	-	30.51(2)	6.53	-	-	27.45(1)	8.96	27.41(1)	8.91
CuL	21.42(5)	-	21.12(2)	9.39	11.95(5)	-	17.77(2)	9.68	17.83(2)	9.58
$CuH_{-1}L$	-	-	10.58(2)	10.54	-	-	7.58(2)	10.19	7.71(2)	10.12
$CuH_{-2}L$	-1.58(4)	-	-0.92(3)	11.49	-10.41(4)	-	-3.32(4)	10.90	-3.01(3)	10.72
$CuH_{-3}L$					-21.73(5)	11.32	-14.31(3)	10.99	-13.95(3)	10.94
Zn(II) complexes										
$ZnH_{13}L$	127.50(3)									
$ZnH_{12}L$	122.24(2)	5.26								
$ZnH_{11}L$	116.03(3)	6.21								
$ZnH_{10}L$	108.26(3)	7.77								
ZnH_9L	99.38(4)	8.88								
ZnH_8L					87.20(4)					
ZnH_7L					80.23(4)	6.97				
ZnH_6L					71.41(3)	8.82				

ZnH_5L					-	-	53.68(1)		53.51(1)	
ZnH_4L					51.60(3)	-	47.18(1)	6.50	47.00(1)	6.51
ZnH_3L			36.82(1)	-	41.07(4)	10.52	39.55(1)	7.63	39.35(1)	7.65
ZnH_2L			29.57(1)	7.25			30.85(1)	8.70	30.86(1)	8.49
ZnHL			21.13(1)	8.44			21.07(1)	9.78	21.43(1)	9.43
ZnL			11.18(1)	9.95			10.53(1)	10.54	11.19(1)	10.24
$ZnH_{-1}L$			0.12(2)	11.06					0.38(1)	10.81

Cu(II) and Zn(II) hydrolytic constants used to calculations:

form	pK_a
CuH_{-1}	-7.95
CuH_{-2}	-16.2
CuH_{-3}	-26.6
CuH_{-4}	-39.7

form	pK_a
ZnH_{-1}	-8.96
ZnH_{-2}	-16.9
ZnH_{-3}	-28.4
ZnH_{-4}	-41.2



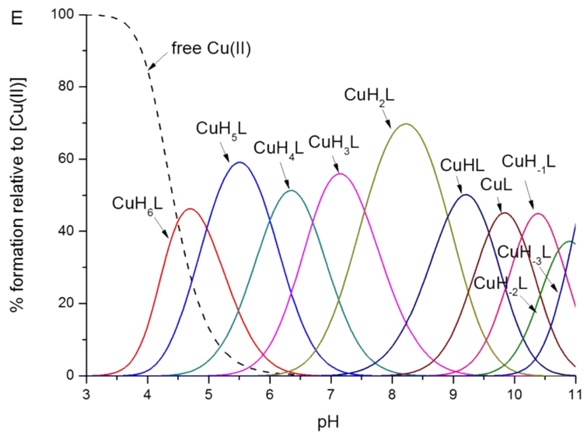


Figure S2. Distribution diagrams for the formation of Cu(II) complex with: A) histatin 1, (B) histatin 1-2, (C) histatin 2, (D) histatin 7 and (E) histatin 9. Conditions: T = 298 K, I = 0.1 M $NaClO_4$, [Cu(II)] = $0.4x10^{-3}$ M; M:L molar ratio = 0.8:1.

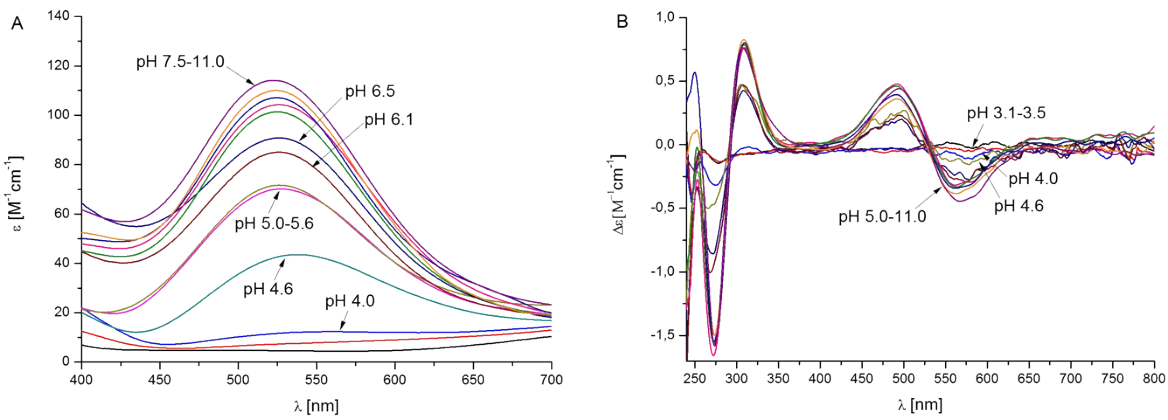


Figure S3. (A) UV-Vis and (B) CD spectra of Cu(II) complex with histatin 1 in pH range 2-11. Conditions: T = 298 K,

I = 0.1 M $NaClO_4$, [Cu(II)] = $0.4.10^{-3}$ M; M:L molar ratio = 0.8:1.

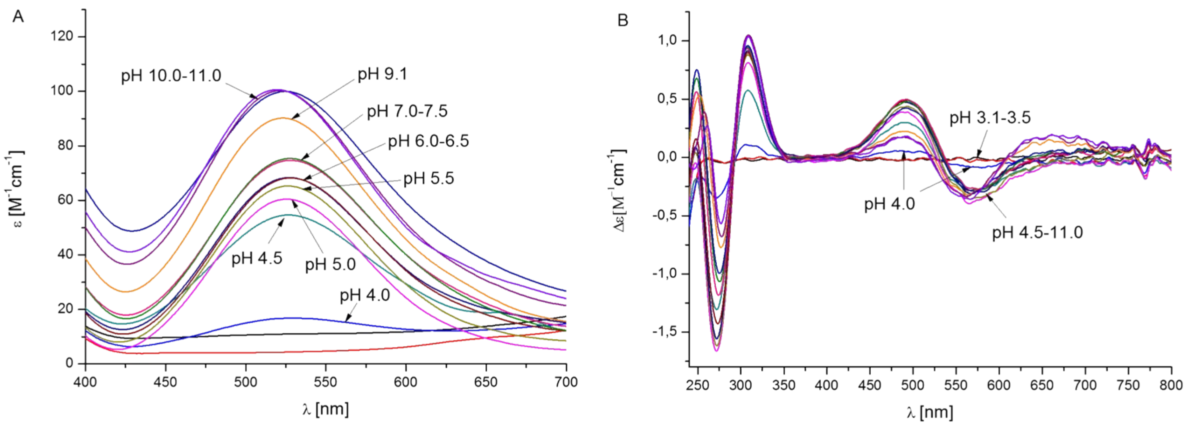


Figure S4. (A) UV-Vis and (B) CD spectra of Cu(II) complex with histatin 1-2 in pH range 2-11. Conditions: T = 298 K, I = 0.1 M NaClO4, [Cu(II)] = $0.4.10^{-3}$ M; M:L molar ratio = 0.8:1.

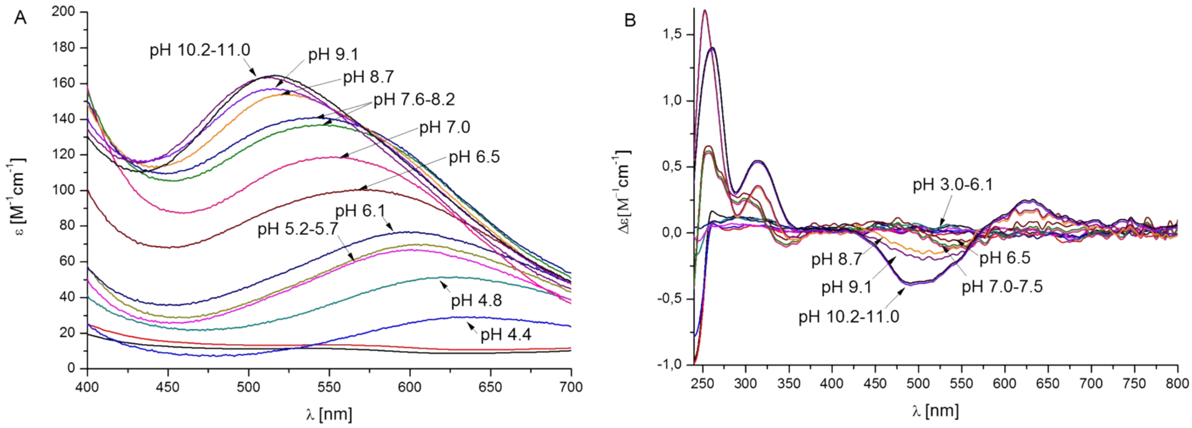


Figure S5. (A) UV-Vis and (B) CD spectra of Cu(II) complex with histatin 2 in pH range 2-11. Conditions: T = 298 K, I = 0.1 M NaClO4, [Cu(II)] = $0.4.10^{-3}$ M; M:L molar ratio = 0.8:1.

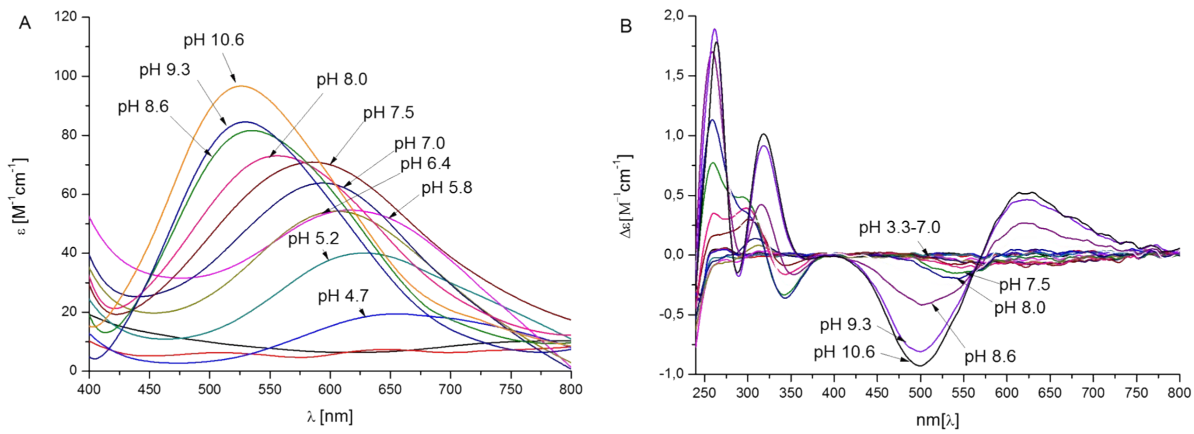


Figure S6. (A) UV-Vis and (B) CD spectra of Cu(II) complex with histatin 7 in pH range 2-11. Conditions: T = 298 K, I = 0.1 M $NaClO_4$, [Cu(II)] = 0.4×10^{-3} M; M:L molar ratio = 0.8:1.

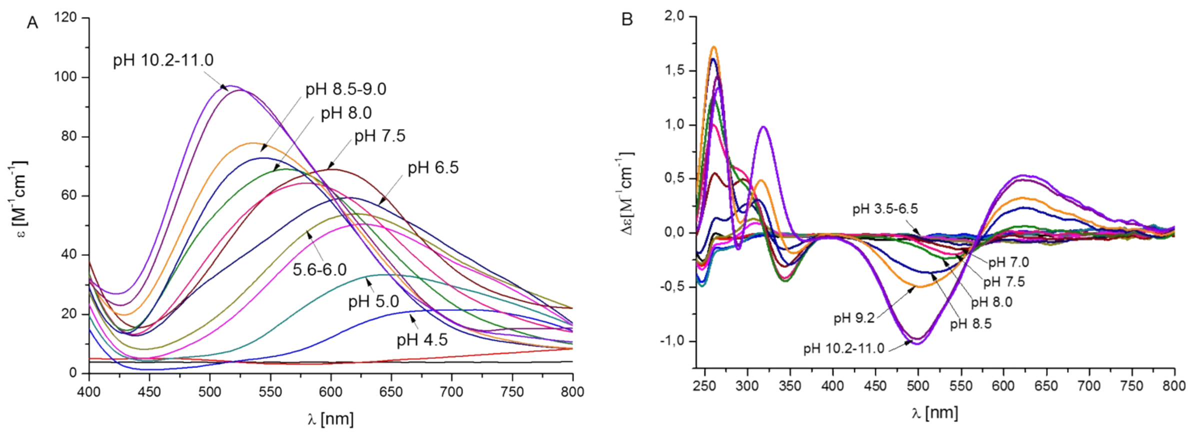


Figure S7. (A) UV-Vis and (B) CD spectra of Cu(II) complex with histatin 9 in pH range 2-11. Conditions: T = 298 K, I = 0.1 M $NaClO_4$, [Cu(II)] = 0.4×10^{-3} M; M:L molar ratio = 0.8:1.

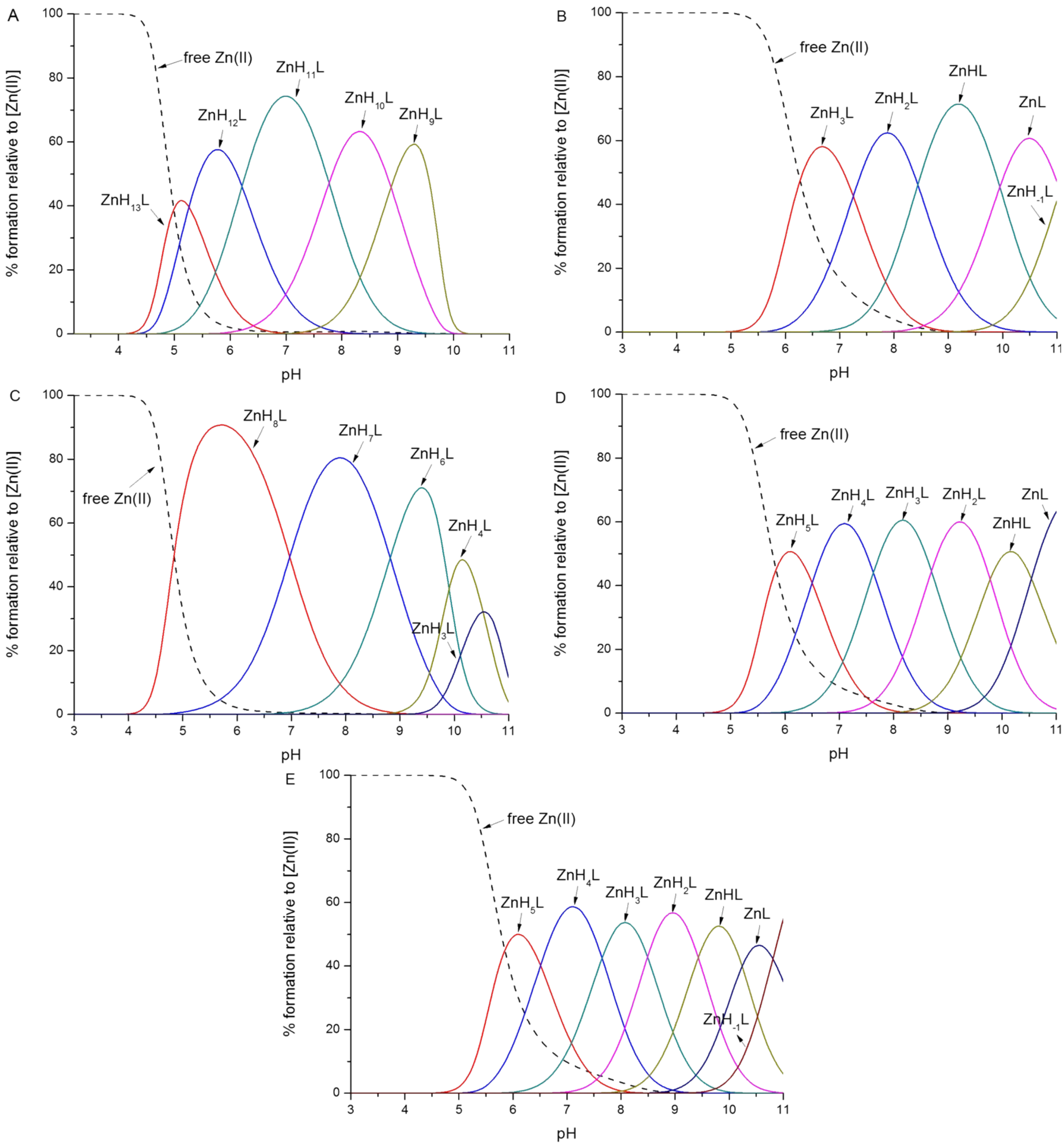


Figure S8. Distribution diagrams for the formation of Zn(II) complex with: A) histatin 1, (B) histatin 1-2, (C) histatin 2, (D) histatin 7 and (E) histatin 9. Conditions: T = 298 K, I = 0.1 M $NaClO_4$, [Cu(II)] = 0.4×10^{-3} M; M:L molar ratio = 0.8:1.