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Evaluation of Binding Affinity of N-Terminally Truncated Forms of Cystatin for Papain with Electrospray Ionization Mass Spectrometry†

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This paper presents the results of electrospray ionization mass spectrometry (ESIMS) applied to an enzyme-inhibitor complex, using papain and cystatin with partly lagged N-terminus. It has been reported that the inhibitory activity of cystatin, a thiol protease inhibitor, toward papain decreases in several orders of magnitude when the N-terminal seven or eight residues are lost. In the absence of papain, multiply charged full-length cystatin was mainly observed accompanied by the signals of minor components, N-terminally truncated forms. When cystatin was mixed with equimolar quantity of papain, the relative intensity of the free full-length cystatin dramatically decreased. It might be caused by the higher binding affinity of the intact cystatin for papain than those of the truncated forms. The present study indicates the potential of ESIMS for the investigation of the structure-activity relationship without isolation of each inhibitor species carrying different level of inhibitory activity.

1. Introduction

Electrospray ionization mass spectrometry (ESI-MS)[1),2)] has enabled to observe various molecular interactions,[3)] such as protein-ligand,[4)-7)] protein-nucleotide,[8),9)] protein-protein interactions.[3),9),10)] It provides a direct figure of the binding stoichiometry. Multiply charged non-covalent complexes can be observed if the experimental condition is gentle enough to avoid the dissociation of the complex. If a harsh experimental condition is applied, only components of the dissociated complexes are observed. In ESIMS, low capillary-skimmer voltage generates a mild electrospray while high potential leads to a severe condition. The condition suitable for the observation of multiply protonated non-covalent complexes sometimes causes adduct attachment from the low desolvation efficiency,[10)] resulting in poor signal-to-noise ratio. Proteins with high molecular weight in aqueous solution without any organic solvent usually require high capillary-skimmer potential, leading to the dissociation of the complex. Therefore, it is not so easy to detect all protein-protein interactions by ESIMS that reflect the real biological affinity.

Egg cystatin[11),12)] is a thiol protease inhibitor that forms a tight, reversible 1 : 1 complex with a thiol protease.[13)] The interaction between cystatin and papain, a thiol protease, has been investigated by several groups, and the inhibition mechanism has also been extensively studied.[14)-17)] Binding mechanism of cystatin with papain was proposed based on the crystal structure of the complex of papain-stefin B, another thiol protease inhibitor.[18)] Three parts of the inhibitor are in close contact with the active site cleft of papain: the N-terminal region, the first (53-57) and second (102-107) hairpin loops. Among these three parts, the N-terminal region of cystatin is involved in main chain-main chain hydrogen bonding. The two hairpin loops play a role in hydrophobic contacts with papain. The importance of the seventh or eighth residue from the N-terminus has been confirmed with the inhibition assay of N-terminally truncated forms of cystatin toward papain.[14),15)] Binding affinity of the N-terminally truncated forms is lower than that of the intact cystatin in several orders of magnitude as indicated by their dissociation constants (K_d).[15)] For the determination of the K_d values, binding affinity assays were carried out individually on each isolated N-terminally truncated form of cystatin.

In the present study, ESIMS was applied to characterize the interaction between papain and cystatin with partly lagged N-terminal region. ESI mass spectra were obtained at low and high capillary-skimmer potential for solutions in different molar ratio of cystatin to papain. FTICR MS was used for the detection of ions generated by ESI to determine the molecular mass of each component with high accuracy. The results indicated that ESIMS analysis is effective for the study of binding specificity of inhibitor for the target enzyme, without any isolation process of each inhibitor form with different level of affinity to papain.

2. Experimental

2.1 Materials and sample preparation

Egg cystatin (Lot# 78H40891) and papain were purchased from Sigma (St. Louis, MO) and Elastin Products Co., Inc. (Owensville, MO), respectively. Cystatin was used without further purification. Cystatin was weighed, dissolved in water at 100 pmol/μL, and used as a stock solution.

Papain was purified by ion exchange chromatogra-

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phy using a TSK-gel SP-5PW (7.6 mm×50 mm, Tosoh, Japan). The solvents consisted of (A) 20 mM sodium acetate (pH 5.0) with 10% (v/v) acetonitrile and (B) 20 mM sodium acetate (pH 5.0) containing 10% (v/v) acetonitrile and 1 M sodium chloride. A linear gradient elution of 0% (B)–100% (B) in 45 min was performed for purification. The main fraction was collected and desalted by ultrafiltration with Ultrafree3 (MW cutoff 5,000; Millipore Ltd., Tokyo, Japan) using 5 mM ammonium acetate (pH 4.5). The final concentration of papain stock solution was 50 pmol/μL determined by amino acid analysis.

A 2.5 μL of cystatin stock solution was mixed with 5 μL of papain stock solution. The molar ratio of cystatin to papain was expected to be equal. The solution in the molar ratio of *ca.* 1 : 3 and 3 : 1 were also prepared by varying the volume of cystatin or papain stock solution. The cystatin–papain solution was kept standing for more than 30 min at room temperature and diluted with 0.1 M acetic acid and water, resulting in the concentration of 50 mM of acetic acid (pH 3.0), prior to ESIMS analysis.

The actual concentration of each protein in the sample solution for ESIMS was calculated using RP-HPLC by comparing with the standard solution whose concentration had been determined by amino acid analysis. In the case of the equivalent mixture of cystatin and papain, the concentration of each protein was determined as 3.6 and 4.7 pmol/μL, respectively. For the 3 : 1 mixture, the actual concentration of cystatin and papain was 14.2 and 5.1 pmol/μL, respectively. In the case of 1 : 3 mixture, the concentration was determined as 4.2 (cystatin) and 14.2 pmol/μL (papain).

2.2 ESI-FTICR MS analysis

The sample solution was introduced into the spectrometer at a flow rate of 1 μL/min using a syringe pump. Spectra were obtained using a Bruker (Billerica, MA) Bio-Apex II Fourier transform ion cyclotron resonance mass spectrometer, equipped with a 7 T magnet and an external electrospray ion source (Analytica of Branford, Branford, CT), by accumulating 32 scans for each sample. External calibration was carried out using ubiquitin. The voltages on the end-plate and the non-heated capillary entrance were set to −2450 V and −2960 V, respectively. For obtaining ESI mass spectrum of cystatin in the absence of papain, capillary-skimmer potential was set to 77 V and trapping time in an rf-only hexapole ion guide was 0.5 s. For ESI mass spectra of cystatin–papain complex, capillary-skimmer potential was set to 77 V and trapping time in the hexapole ion guide was 3 s. For spectra of dissociated compounds, capillary-skimmer potential was set to 220 V (for the mixture of cystatin : papain = 1 : 1 or 3 : 1) or 240 V (for the mixture of cystatin : papain = 1 : 3) and trapping time in the hexapole ion guide was 4 s.

3. Results and Discussion

An ESI-FTICR mass spectrum of Sigma cystatin (Lot #78H40891), shown in Fig. 1, indicated the presence of cystatin with N-terminally truncated forms. Intense multiply charged molecules (M^{13+}–M^{9+}) were observed for the full-length cystatin (1–116). Some phosphorylated species were also recognized in the spectrum. For the full-length cystatin, 11+ charged species showed the most intense signals among the observed multiply protonated molecules. Multiply charged molecules of the N-terminally truncated forms, such as 10–116, 9–116, 8–116, 7–116, and 6–116, were found in relatively low intensity. There are two arginine residues at the 4 th and 6th positions from the N-terminus. Thus, the number of basic amino acid residues present in the truncated forms is smaller than the intact one. The most intense signals were seen with the charge state of 9+ on each truncated form except for 6–116. Since the 6th residue from the N-terminus is arginine, the truncated form of 6–116 contains one more arginine residue than the other truncated forms and the most intense species for 6–116 was 10+. Phosphorylation on some population of the N-terminally truncated forms was recognized, as the full-length cystatin. Partial phosphorylation of each truncated form made the spectrum more complicated. Although there might be some effect on the ionization efficiency of cystatin isoforms with small differences in the number of basic amino acid residues, the relative peak intensity should roughly represent the abundance of each component. Thus, the content of the truncated forms is estimated to be less than 10% compared with that of the intact one.

An ESI mass analysis was carried out for the diluted sample mixture of cystatin and papain pre-incubated for more than 30 min at room temperature. Figure 2(a) shows an ESI-FTICR mass spectrum of the equivalent mixture of cystatin and papain at low capillary-skimmer potential. Multiply protonated cystatin–papain complexes were observed together with those of free cystatin and papain. Although it was expected that equivalent amount of cystatin and papain should exist in the solution, the actual ratio of cystatin to papain was 1 : 1.3 (see Experimental). The K_d value for the full-length cystatin–papain complex is 6.0×10^{-14} M at pH 7.4.[15] If the interaction between cystatin and papain is strong enough to survive the experimental condition, about 80% population of papain is expected to be bound to cystatin while almost all the cystatin should be in a complex form. Thus, 20% population of papain was expected to be unbound and gave distinct signals of M^{10+}, M^{11+}, and M^{12+}, as in Fig. 2(a). Relative intensity of multiply charged molecules of N-terminally truncated cystatin increased in the presence of papain (Fig. 2(a)) compared with that in the absence of papain (Fig. 1).

To increase the desolvation efficiency and to decrease the adduct formation, high capillary-skimmer potential is preferable. However, higher capillary-skimmer voltage promotes the dissociation of the complex. Thus, the capillary-skimmer potential was first kept at 77 V to minimize the dissociation of the enzyme–inhibitor complex for the analysis of the native-like state. Multiply charged cystatin–papain non-covalent complexes (M^{13+}, M^{14+}, and M^{15+}) were observed in low intensity accompanied with several adduct peaks in Fig. 2(a). Low capillary-skimmer potential tends to lead to poor ionization efficiency of a complex with large molecular mass, despite of higher concentration than that of free cystatin or papain. In the case of cystatin–papain mixture, the intensity of

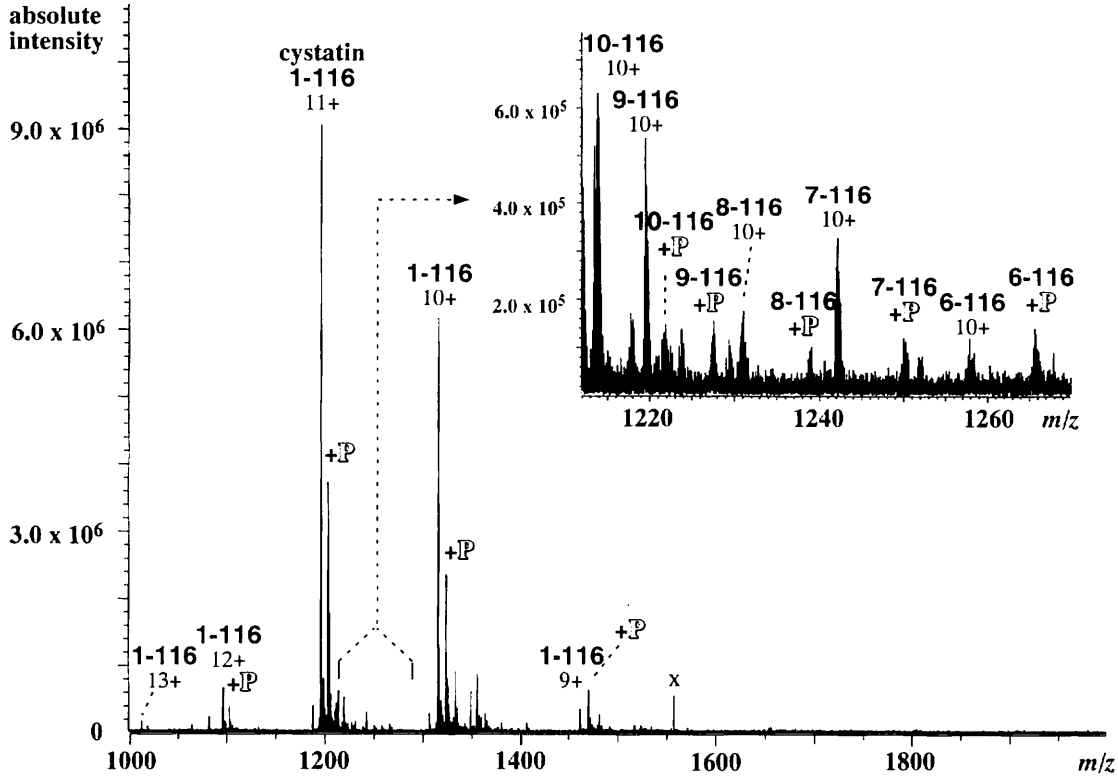

Fig. 1. ESI-FTICR mass spectrum of cystatin (Sigma, Lot#78H40891). Multiply protonated molecules of the full-length cystatin are indicated as "1-116" accompanied by the number of charges. Multiply charged molecules of the N-terminally truncated forms are indicated as "10-116", "9-116", "8-116", "7-116", or "6-116" accompanied by the number of charges. Multiply charged phosphorylated cystatin are indicated with a mark of "+P". A noise peak is marked with a letter ×.

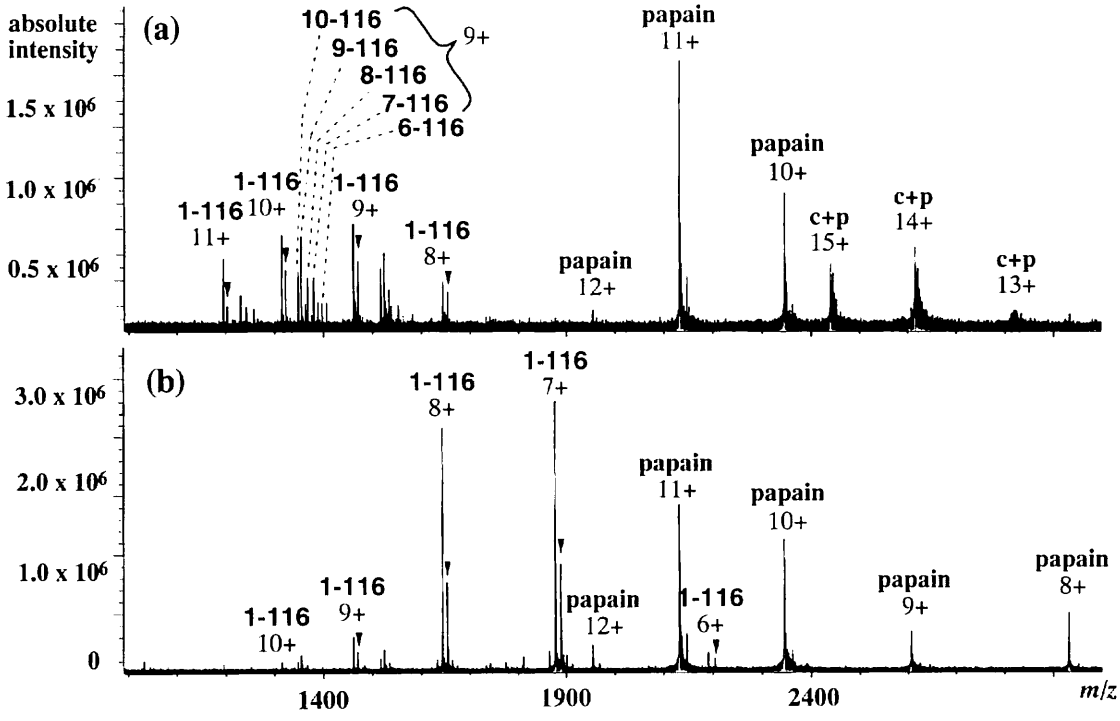

Fig. 2. ESI-FTICR mass spectra of 1 : 1.3 mixture of cystatin and papain obtained at low (77 V) (a) and high (220 V) (b) capillary-skimmer potential. Multiply charged cystatin-papain complexes are indicated as "c−p" accompanied by the number of retaining charges. Multiply charged papain is noted as "papain" with the number of charges. Multiply protonated molecules of cystatin in different length are indicated with the positions of the N- and C-terminal residues. An arrowhead indicates the multiply protonated molecules of phosphorylated species of the intact cystatin.

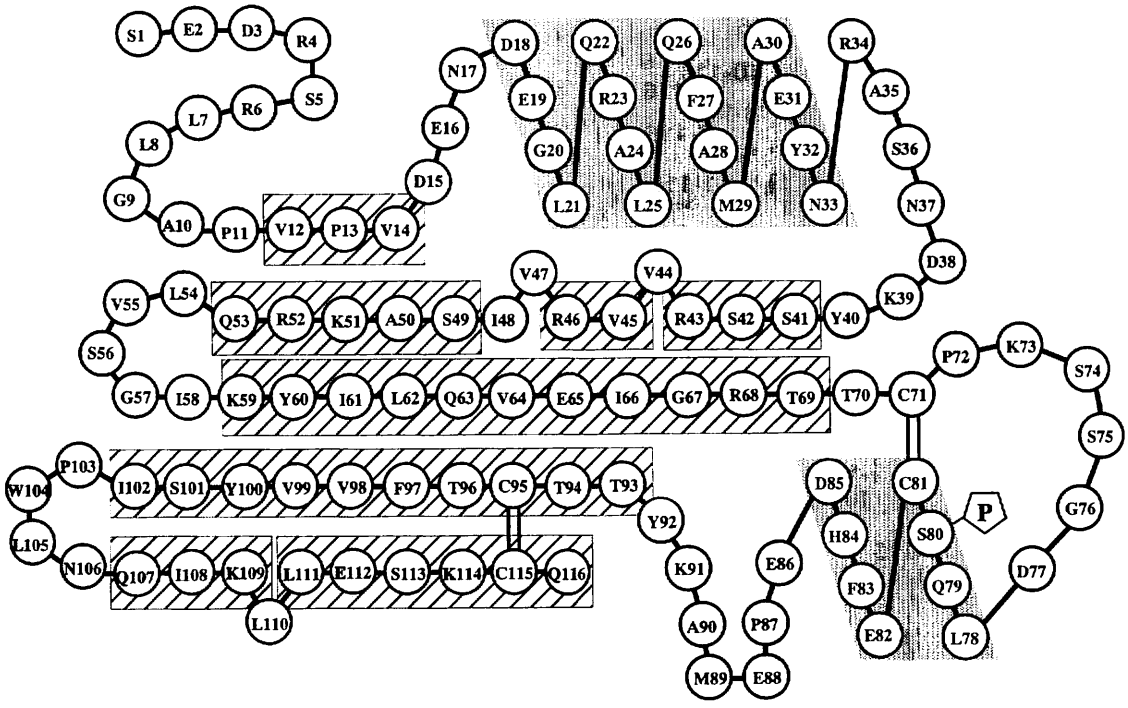

Fig. 3. Amino acid sequence and polypeptide fold arrangement of egg cystatin. Gray background indicates the position of α-helices. Rectangular boxes with slashed lines indicate the position of β-strands. Disulfide bonds are shown with double lines. Some population of cystatin is phosphorylated at Ser80, which is indicated by a pentagon.

the multiply protonated molecules not only of the complex but also of the component protein decreased if the same trapping time was applied. To increase the ion intensity, trapping time in the hexapole region was set to 3 s, longer than that for cystatin alone. Longer trapping time in a hexapole might have caused charge stripping of multiply charged cystatin molecules.

When the capillary-skimmer potential increased to 220 V, no multiply charged cystatin-papain complex was observed in the spectrum, as shown in Fig. 2(b). Only the multiply protonated molecules of free cystatin and papain were observed. Since the higher capillary-skimmer potential and longer trapping time in the hexapole is apt to cause charge stripping of the multiply protonated molecules, cystatin and papain in Fig. 2 (b) retained fewer charges than those in Figs. 1 and 2(a) did. Masking by complex formation might also have contributed to fewer charges of the component protein. Relative intensity of the truncated cystatin decreased in Fig. 2(b) while that of the full-length cystatin dramatically increased.

Binding mechanism of cystatin with papain might have been reflected on the relative abundance of the unbound cystatin isoforms in Figs. 2(a) and 2(b). The mechanism of the cystatin-papain interaction has been proposed on the basis of X-ray crystallography data of the complex of papain-stefin B, another thiol protease inhibitor.[18] Stefin B is a member of the stefin family, whose characteristics are MW~11 kDa and no disulfide bonds, while cystatin has MW of ~13 kDa and two disulfide bonds.[11] Figure 3 shows the amino acid sequence and the secondary structure of cystatin. Though several different structural characteristics were found between the crystal structure of cystatin and that of stefin B complexed with papain, considerably large parts are identical in their tertiary structure except for an α-helix 78-85 that is located on the opposite side of the interface with papain.[18, 19] Conserved amino acid residues of stefin B occur in equivalent positions to those in cystatin.[18] Thus, binding mechanism to papain is considered to be almost identical. Three regions in cystatin sequence, N-terminal four residues (Leu7, Leu8, Gly9, and Ala10) and the first and second hairpin loops (53-57 and 102-107), are thought to be important for the cystatin-papain interaction from the assay results of the mutants.[14, 17, 20] The N-terminal region is involved in the formation of hydrogen bonds with the main chain of papain. On the other hand, two hairpin loops, QLVSG (53-57) and IPWLNQ (102-107), are participating mainly in hydrophobic interactions.

In Fig. 2(a), multiply protonated molecules of the unbound cystatin were observed in lower intensity than those in Fig. 1. Relative intensity of the N-terminally truncated forms turned higher while that of the full-length cystatin dramatically decreased. This suggests that the addition of papain affected on the relative abundance of the unbound cystatin in different length. The K_d values for the truncated forms at pH 7.4 are reported to be 5.1×10^{-13} M (7-116), 1.2×10^{-10} M (8-116), 1.2×10^{-8} M (9-116), and 1.5×10^{-7} M (10-116), respectively, while that for the intact cystatin is 6.0×10^{-14} M.[15] This indicates that the binding affinity of these truncated forms for papain is much lower than that of the intact one, especially when more than seven residues are lost from the N-terminus. The K_d value in the order of 10^{-8} M does not represent very weak binding. However, electrostatic interactions *via* hydrogen bonds might be necessary for maintaining the complex during the ESI process. Therefore, the

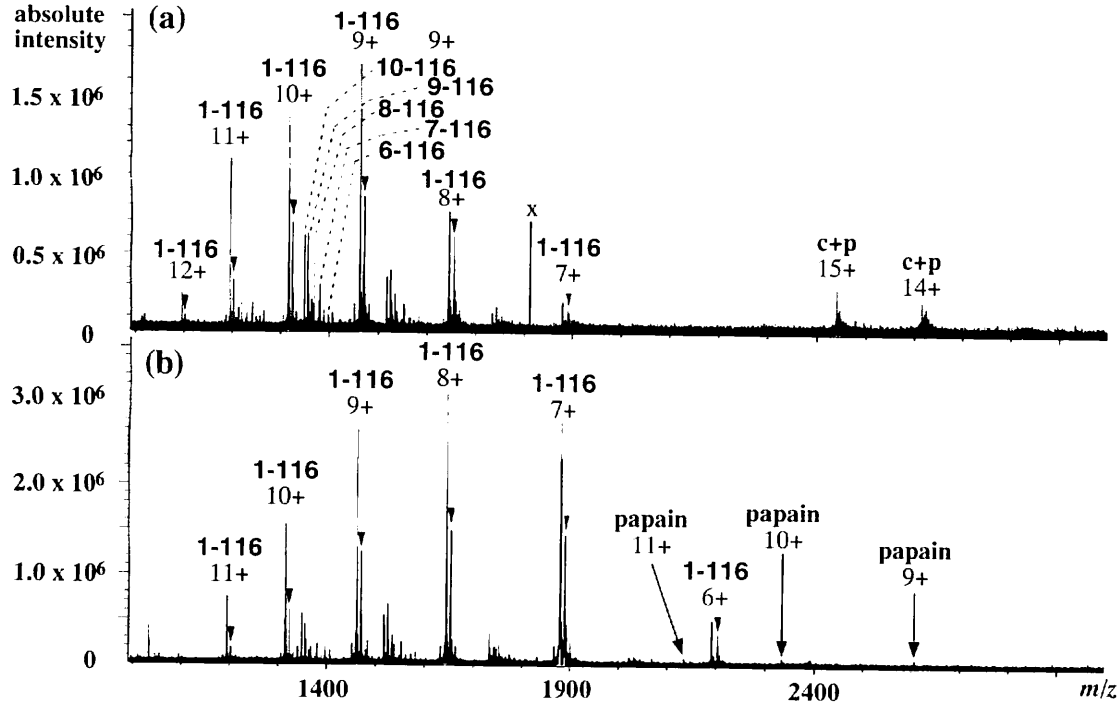

Fig. 4. ESI-FTICR mass spectra of 2.8 : 1 mixture of cystatin and papain obtained at low (77 V) (a) and high (220 V) (b) capillary-skimmer potential. Annotations are identical to those in Fig. 2. A noise peak is marked with a letter ×.

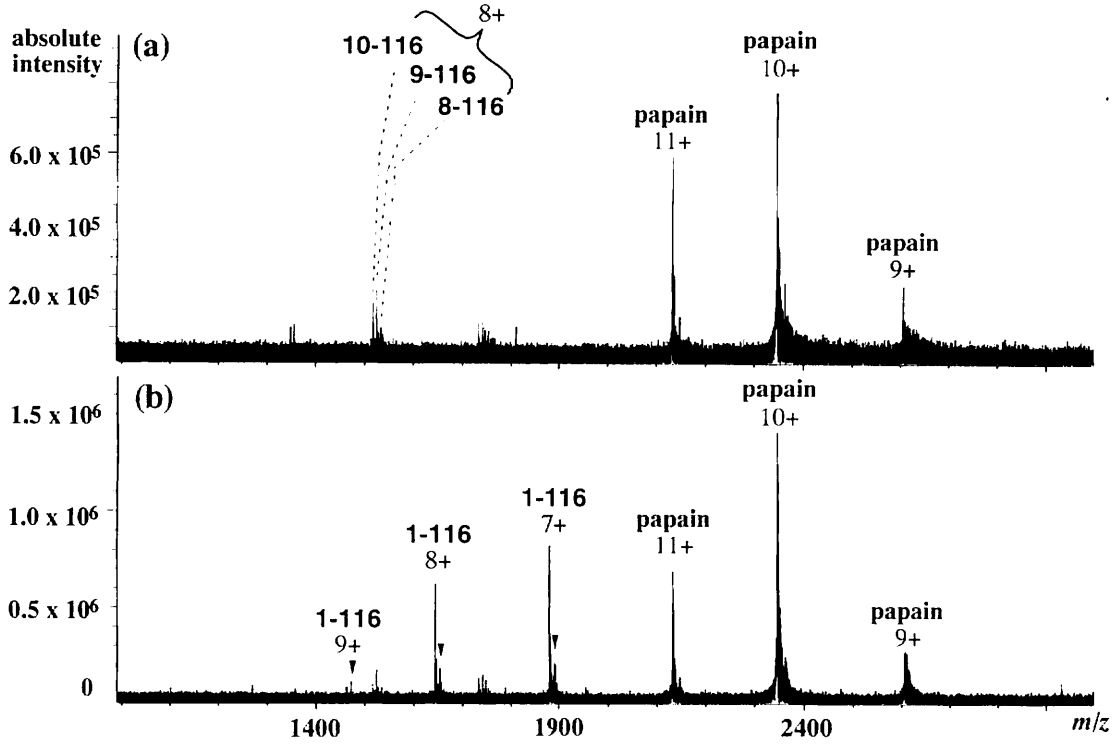

Fig. 5. ESI-FTICR mass spectra of 1 : 3.2 mixture of cystatin and papain obtained at low (77 V) (a) and high (240 V) (b) capillary-skimmer potential. Annotations are identical to those in Fig. 2.

N-terminally truncated forms, which have only two hydrophobic hairpin loops among three important regions for binding, should have been easily dissociated during the spraying process and showed relatively high intensity. On the other hand, the intact cystatin was possible to remain in a complex form, and its relative intensity extremely decreased in Fig. 2(a). The present results suggest that ESI analyses can be used for the investigation of the non-covalent bindings of enzyme–inhibitor complex, even in the mixture of inhibitors with different K_d values. It might be possible to rank the interaction strength by ESIMS analyses.

In order to verify the possibility mentioned above, the ratio of inhibitor to enzyme (I : E) was changed to 2.8 : 1 or 1 : 3.2, and the sample solution was subjected to ESIMS analysis. Figure 4(a) shows an ESI mass

spectrum of the 2.8 : 1 mixture of cystatin and papain. In Fig. 4(a), no protonated molecules of papain were observed. Relative peak intensity of the cystatin molecules with different length has been changed compared with Figs. 1 and 2(a). The ratio of peak intensity of M^{9+} of the full-length cystatin to that of the N-terminally truncated forms was *ca.* 3 : 1 in Fig. 4(a), while that of M^{10-} in Fig. 1 was *ca.* 10 : 1. Relative intensity of the truncated forms turned higher in the ESI mass spectra in the presence of papain, as observed in Fig. 2(a). Considering from that the molar ratio of cystatin to papain was 2.8 : 1, only *ca.* 35% population of cystatin was supposed to be bound with papain and more than 60% of the intact cystatin was free in the solution. As a result, the decrease in relative peak intensity of the intact cystatin in Fig. 4(a) was not so remarkable as observed in Fig. 2(a). Figure 4(b) shows an ESI mass spectrum of 2.8 : 1 mixture of cystatin and papain obtained at 220 V of capillary-skimmer potential. It seems that cystatin was preferentially ionized, and excess amount of cystatin affected on ionization efficiency of papain, resulting in virtual absence of the protonated molecules of papain in Fig. 4(b).

Figure 5(a) shows an ESI mass spectrum of the 1 : 3.2 mixture of cystatin and papain at low capillary-skimmer potential. Multiply charged papain molecules were mainly observed while the full-length cystatin was not detected at all. Multiply charged molecules of the truncated forms of cystatin, 10–116, 9–116, and 8–116, were still observed in the spectrum. Observation of multiply protonated molecules only of the unbound truncated forms verifies that their binding with papain is not so strong as to maintain the complex during ESI. Though cystatin–papain complex should have existed in the solution, no multiply charged cystatin–papain complex was observed in the spectrum. Excess amount of papain might have reduced the ionization efficiency not only of the complex but also of the constituent protein, resulting in poor S/N ratio of the spectrum, as shown in Fig. 5(a). Figure 5(b) shows an ESI mass spectrum of 1 : 3.2 mixture of cystatin and papain obtained at 240 V of capillary-skimmer potential, 20 V higher than that for Figs. 2(b) and 4(b). Poor ionization efficiency of papain, the major component, should have required higher capillary-skimmer potential. In Fig. 5(b), multiply charged molecules of cystatin and papain were clearly observed. Peaks of the truncated forms of cystatin were observed in low intensity, as observed in Fig. 2(b).

The decrease in binding affinity of the truncated forms should have been caused by the loss of electrostatic interaction *via* hydrogen bonding between the main chains of cystatin and papain. It is not possible to conclude just from the present data that compounds bound mainly with hydrophobic interactions, such as the N-terminally truncated cystatin and papain, are difficult to detect in ESI mass spectra. It is worth examining a mutant of the hydrophobic loop regions (53–57 and/or 102–107). The mutant is expected to have less hydrophobic affinity for papain, resulting in distorted binding. Kinetic modeling of temporary inhibition of such mutants was studied and K_d values of the complex with mutants were estimated as 360–0.92 $\times 10^{-9}$ M.[20] On the basis of the K_d values, these mutants might show identical behavior in ESI mass spectra to the N-terminally truncated forms. According to the temporary inhibition model by cystatin mutants, however, the active site, Cys25, of papain is not fully deactivated and it cleaves the N-terminal region of cystatin, resulting in the N-terminally truncated mutants.[20] Consequently, it might be impossible to discuss the difference in the stability of the complex during the ESI process, between the complex bound only with electrostatic interactions and that only with hydrophobic interactions in the case of cystatin–papain binding.

These results suggest that binding affinity of inhibitor for a target enzyme can be investigated using ESIMS. Though it might be impossible to estimate the K_d value of the enzyme–inhibitor complex only from ESIMS analyses, it seems that there is a good correlation between solution phase K_d and proportions of complex and dissociated protein generated by hexapole CID, as Rostom *et al.* reported.[7] The greatest advantage of ESIMS analyses for such a kind of study is that investigation can be carried out without isolating each component. In addition, some applications for adduct removal for clear observation of the multiply charged non-covalent complex have been developed for FTICR MS.[10, 21, 22] The present study demonstrates that ESIMS is an effective tool for the primary check for binding affinity of non-covalent complex.

Acknowledgments

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References

1) J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong, and C. M. Whitehouse, *Science*, 246, 64–71 (1989).
2) R. D. Smith, J. A. Loo, C. G. Edmonds, C. J. Barinaga, and H. R. Udseth, *Anal. Chem.*, 62, 882–899 (1990).
3) J. A. Loo, *Mass Spectrom. Rev.*, 16, 1–23 (1997).
4) B. Ganem, Y.-T. Li, and J. D. Henion, *J. Am. Chem. Soc.*, 113, 6294–6296 (1991).
5) B. Ganem, Y.-T. Li, and J. D. Henion, *J. Am. Chem. Soc.*, 113, 7818–7819 (1991).
6) N. Potier, P. Barth, D. Tritsch, J.-F. Biellmann, and A. Van Dorselaer, *Eur. J. Biochem.*, 243, 274–282 (1997).
7) A. A. Rostom, J. H. R. Tame, J. E. Ladbury, and C. V. Robinson, *J. Mol. Biol.*, 296, 269–279 (2000).
8) A. K. Ganguly, B. N. Pramanik, A. Tsarbopoulos, T. R. Covey, E. Huang, and S. A. Fuhrman, *J. Am. Chem. Soc.*, 114, 6559–6560 (1992).
9) B. N. Pramanik, P. L. Bartner, U. A. Mirza, Y.-H. Liu, and A. K. Ganguly, *J. Mass Spectrom.*, 33, 911–920 (1998).
10) J. E. Bruce, V. F. Smith, C. Liu, L. L. Randall, and R. D. Smith, *Protein Sci.*, 7, 1180–1185 (1998).
11) A. J. Barrett, N. D. Rawlings, M. F. Davies, W. Machleidt, G. Salvesen, and V. Turk, "Proteinase Inhibitors," ed. by A. J. Barrett and G. Salvesen, Elsevier, Amsterdam (1986), pp. 515–569.
12) A. J. Barrett, *Trends Biochem. Sci.*, 12, 193–196 (1987).

13) M. J. H. Nicklin and A. J. Barrett, *Biochem. J.*, 223, 245–253 (1984).
14) W. Machleidt, U. Thiele, B. Laber, I. Assfalg-Machleidt, A. Esterl, G. Wiegand, J. Kos, V. Turk, and W. Bode, *FEBS Lett.*, 243, 234–238 (1989).
15) P. Lindahl, M. Nycander, K. Ylinenjärvi, E. Pol, and I. Björk, *Biochem. J.*, 286, 165–171 (1992).
16) E. A. Auerswald, D. K. Nägler, A. J. Schulze, R. A. Engh, G. Genenger, W. Machleidt, and H. Fritz, *Eur. J. Biochem.*, 224, 407–415 (1994).
17) I. Björk, E. Pol, E. Raub-Segall, M. Abrahamson, A. D. Rowan, and J. S. Mort, *Biochem. J.*, 299, 219–225 (1994).
18) M. T. Stubbs, B. Laber, W. Bode, R. Huber, R. Jerala, B. Lenarcic, and V. Turk, *EMBO J.*, 9, 1939–1947 (1990).
19) W. Bode, R. Engh, D. Musil, U. Thiele, R. Huber, A. Karshikov, J. Brzin, J. Kos, and V. Turk, *EMBO J.*, 7, 2593–2599 (1988).
20) W. Machleidt, D. K. Nägler, I. Assfalg-Machleidt, M. T. Stubbs, H. Fritz, and E. A. Auerswald, *FEBS Lett.*, 361, 185–190 (1995).
21) D. P. Little, J. P. Speir, M. W. Senko, P. B. O'Connor, and F. W. McLafferty, *Anal. Chem.*, 66, 2809–2815 (1994).
22) L. Paša-Tolić, J. E. Bruce, Q. P. Lei, G. A. Anderson, and R. D. Smith, *Anal. Chem.*, 70, 405–408 (1998).

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