Monitoring the Hydrolysis of *p*-Nitrophenyl Acetate Catalyzed by Seryl-histidine with Electrospray Ionization Mass Spectrometry

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The hydrolysis of *p*-nitrophenyl acetate (*p*-NPA) catalyzed by seryl-histidine or histidine has been monitored by electrospray ionization mass spectrometry in the presence of the internal calibration, 8-anilino-1-naphthalenesulfonic acid ammonium salt (ANS). The half-life of *p*-NPA in the presence of 10 mmol·L^{-1} seryl-histidine or histidine at 25 ℃ was 370 min and 70 min respectively. With the occurrence of acetyl seryl-histidine and acetyl histidine in the reaction, and the fact that *p*-NPA was stable in the presence of 10 mmol·L^{-1} serine, an imidazolysis mechanism has been proposed, which is in accordance with the reported work.

Keywords reaction monitoring, hydrolysis, *p*-nitrophenyl acetate, ESI-MS

Introduction

In the past decade, it was found that two amino acid residues, Ser and His, work as the active sites in the serine protease.[1-3] In our previous work, a dipeptide seryl-histidine (Ser-His) was found to have the protein and nucleosides cleavage activity.[4-7] *p*-Nitrophenyl acetate (*p*-NPA) was also used as the substrate to identify the cleavage activity of Ser-His on ester. The reaction process was monitored by detecting the change of UV absorbance by UV-visible spectrometry and a first order reaction was presented.[7] To further clarify the cleavage mechanism of *p*-NPA, a method to monitor the occurrence of reaction products was essential. With the characteristic of studying the impure compounds and mixtures, mass spectrometry is a suitable means for this purpose. Since its introduction by Yamashita and Fenn in 1984,[8] electrospray ionization mass spectrometry (ESI-MS) has become a powerful tool in biological research recently. More specifically, ESI-MS has been used in the sequencing of protein[9] and polynucleotide,[10] the elucidation of protein folding pathways,[11] detecting non-covalent complex,[12] quantitative study of enzyme-catalyzed reactions[13] and so on. In this paper, ESI-MS was used to monitor the hydrolysis of *p*-NPA catalyzed by Ser-His and its analogues. A possible mechanism for this reaction was proposed.

Materials and methods

Chemicals

p-nitrophenyl acetate (*p*-NPA), *p*-nitrophenol (*p*-NPO) and 8-anilinonaphthalene-1-sulfonic acid ammonium salt (ANS) are purchased from Fluka Co.. Seryl-histidine (Ser-His) is from ICN Biomedicals Inc.. Serine (Ser) and histidine (His) are purchased from Sigma Chemical Co..

Mass spectrometry

Mass spectra were acquired on a Bruker ESQUIRE ~ LC™ ion trap spectrometer (Bruker, Bremen, German) equipped with a gas nebulizer probe capable of ana-

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lyzing ions up to m/z 6000. The samples dissolved in methanol were ionized by electrospray ionization and continuously infused into the ESI chamber at a flow rate of 4 μL min^{-1} by a Cole-Parmer 74900 syringe pump (Cole-Parmer Instrument Company, Illinois, USA). Ions were gated into the ion trap for each scan at injection time of 20 ms. Three spectra on each sample were acquired and the average value was used to analyze the reaction. All of the experiments were acquired in negative mode to simplify the spectra, since the protonated compounds and sodium adducts will exist in the positive mode, which will complicate the spectra and quantification.

Kinetic measurements

The kinetics of *p*-NPA hydrolysis rate were determined by measuring the *p*-NPO concentration with ESI-MS in the presence of an internal standard 8-anilinonaphthalene-1-sulfonic acid ammonium salt (ANS). A calibration curve with the intensity ratio between *p*-NPO and ANS as y coordinates and *p*-NPO concentration as x coordinates was generated by mixing 10 μL of *p*-NPO at different final concentration (0—2 mmol·L^{-1}) with 10 μL of ANS (0.08 mmol·L^{-1}) and then diluted with 400 μL of methanol for analysis by ESI-MS in the above mass conditions. The monitoring of hydrolysis reaction was acquired by the following procedure. *p*-NPA was mixed with the water solutions of Ser-His or its analogues to the final concentration of 2 mmol·L^{-1}(*p*-NPA) and 10 mmol·L^{-1} (Ser-His or its analogues). 10 μL of reaction mixture was loaded and added into 10 μL of ANS (0.08 mmol·L^{-1}) at different time. The solution was diluted with 400 μL of methanol and analyzed by ESI-MS immediately. The intensity ratio between *p*-NPO and ANS was transferred into *p*-NPO concentration with the help of calibration curve.

Results and discussion

The calibration curve of the *p*-NPO concentration against intensity ratio between *p*-NPO and ANS was showed in Fig. 1 with a good linearity, providing the possibility of monitoring the *p*-NPA hydrolysis by mass spectrometry.

In our previous work, *p*-NPA hydrolysis in the presence of seryl-histidine was monitored by UV-visible spectrometry, which proved to be a first order reaction. In this work, with the addition of internal calibration ANS, this

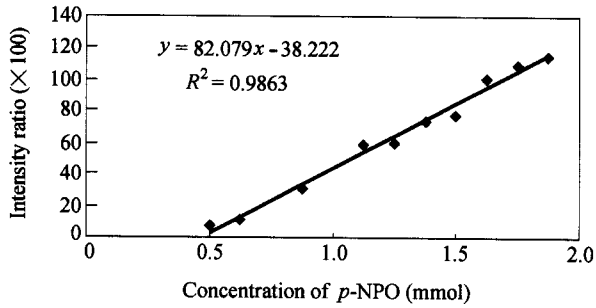


Fig. 1 Calibration curve of *p*-NPO concentration against intensity ratio between *p*-NPO and ANS in ESI-MS.

reaction was carried out at room temperature (25 ℃) and monitored by mass spectrometry (Fig. 2). Since negative mode was used in all the experiments, *p*-NPA could not be detected. With the calibration curve showed in Fig. 1, the intensity ratios between *p*-NPO and ANS in mass spectra were changed into the *p*-NPO concentration, which increased with the reaction time (Fig. 2). The half-life of *p*-NPA was about 370 min. To our surprise, while the optical density at 400 nm during UV-visible monitoring of the *p*-NPA hydrolysis increased quickly from 0 to 0.7 within just 12 min, in this work, the *p*-NPO could be detected by mass spectrometry after 20 min. Buffer effect could be used to interpret the difference. In our previous work, Britton-Robinson buffer (BR buffer, 40 mmol·L^{-1} phosphate, 40 mmol·L^{-1} acetate, 40 mmol·L^{-1} borate, adjusted with NaOH to pH 6.0) was used in all the reactions. While in this experiment, BR buffer has been excluded and replaced by water because salts, especially phosphate and borate strongly decrease the signal intensity in ESI-MS and are harmful to the life of mass spectrometer. Since we have found that the phosphate could catalyze the cleavage of protein by seryl-histidine,[4] we proposed that the reaction rate difference of *p*-NPA hydrolysis might come from the effect of

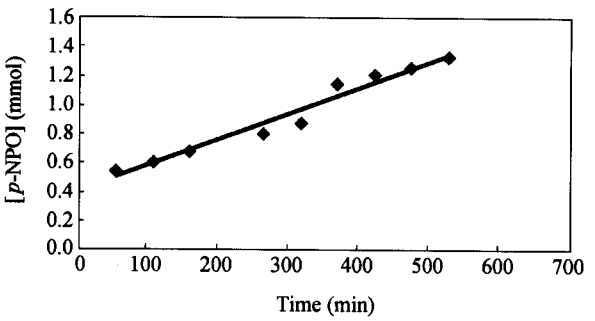


Fig. 2 *p*-NPA hydrolysis catalyzed by Ser-His.

BR buffer.

To study the stability of *p*-NRA, seryl-histidine was replaced with the same volume of water. It was found that *p*-NPA was very stable in this case and there was not any *p*-NPO detected in mass spectrometry even after 24 h at 25 ℃. This result supported the catalyzer role of seryl-histidine in the hydrolysis of *p*-NPA. However, It was really surprising to find that *p*-NPA was stable in the presence of 10 mmol·L^{-1} serine (even after 24 h at 25 ℃), although serine residue has been proved to be essential for the cleavage of protein and DNA by seryl-histidine.[5,6] It could be concluded that the cleavage mechanism of *p*-NPA was different from protein and DNA cleavage by seryl-histidine. Histidine residue should be the working domain. To test the proposal, 10 mmol·L^{-1} histidine was used in the reaction. It was clear that histidine really catalyzed the hydrolysis at much faster rate than seryl-histidine did (Fig. 3). The half-life of *p*-NPA was only 70 min, 6 folds faster than in the presence of seryl-histidine. The reasons for this reaction rate difference still remain unclear and much work should be carried out on this project.

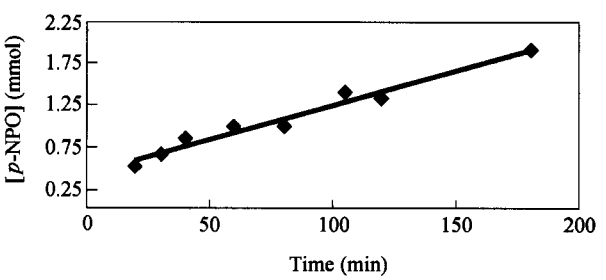


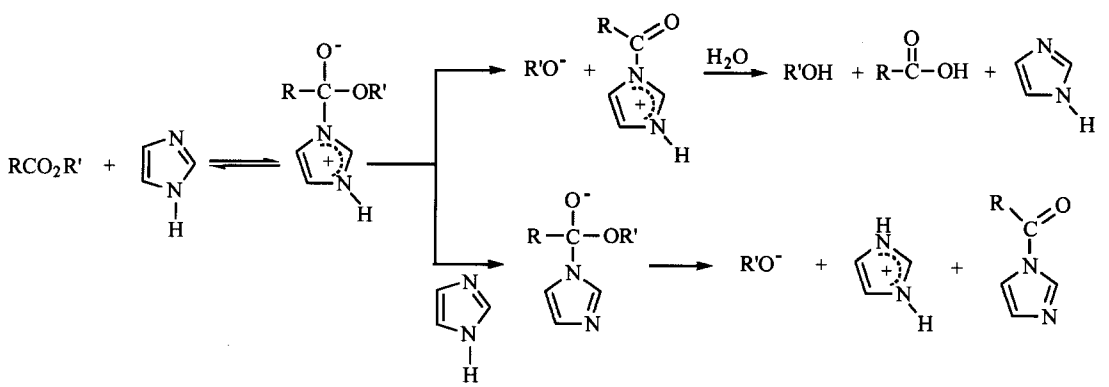
Fig. 3 *p*-NPA hydrolysis catalyzed by His.

In the past several years, the hydrolysis of *p*-NPA catalyzed by imidazole has been reported.[14-17] Two kinds of mechanisms of ester imidazolysis have been proposed,[14] in which imidazole works as a nucleophilic reagent (Scheme 1). Under the assistance of water or another imidazole molecule, *p*-NPA is hydrolyzed into *p*-NPO with the formation of acetic acid or acetyl-imidazole.

From scheme 1, it can be seen that an imidazole group was included in the intermediate, which could easily interpret the catalyzing ability of histidine and seryl-histidine on *p*-NPA. Indeed, the acetyl histidine *m/z* 195 or acetyl seryl-histidine *m/z* 283 presented respectively in the hydrolysis reaction mixture showed the mechanism (Fig. 4, Fig. 5), which was in accordance with the imidazole group assistant route in Scheme 1. The characteristic fragment of $[M-42]^-$ in the ESI-MS/MS spectra, which corresponds to losing of ketene from the acetyl group revealed the acetylation reaction (Scheme 2). Since serine did not catalyze the hydrolysis of *p*-NPA, the acetylation reaction should occur on the imidazole group and not amino group, which accords with the other group's work.

In conclusion, the hydrolysis of *p*-NPA catalyzed by seryl-histidine and histidine in aqueous media at 25 ℃ was monitored by ESI-MS with the half-life of 370 min and 70 min respectively. With the support of tandem mass spectrometry of reaction product, an imidazolysis mechanism has been proposed. From this work, it can be concluded that mass spectrometry can be used in the reaction monitoring, which will be helpful in some biological studies.

Scheme 1 Two mechanisms of ester imidazolysis of *p*-NPA (from Ref. 14)



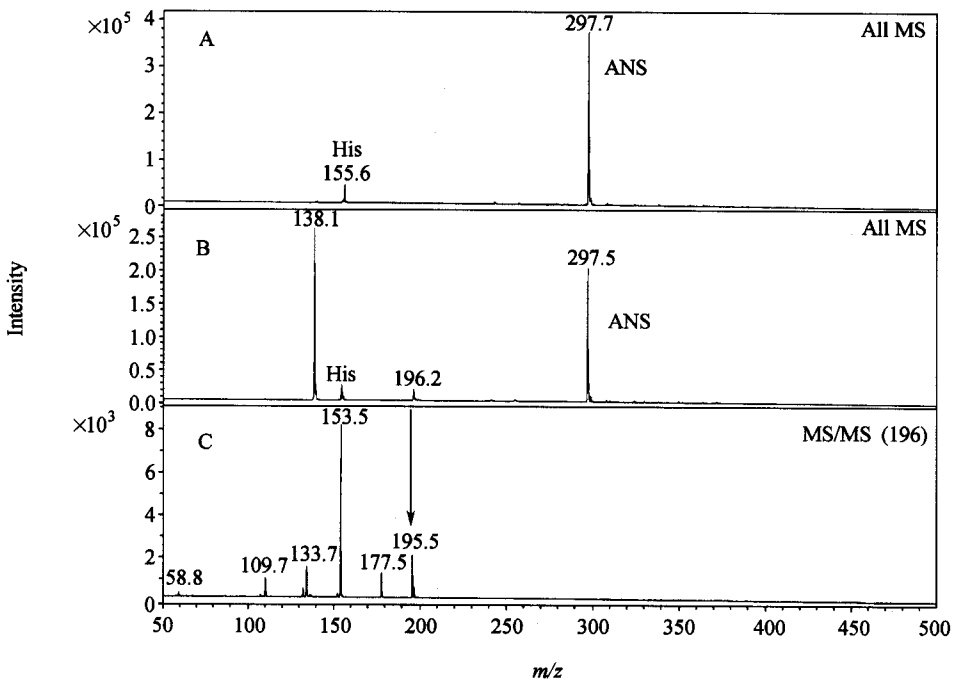


Fig. 4 Histidine catalyzing hydrolysis of *p*-NPA. A. hydrolysis reaction of *p*-NPA at 0 min; B. Hydrolysis reaction of *p*-NPA at 180 min; C. ESI-MS/MS of the peak in accordance with acetyl histidine.

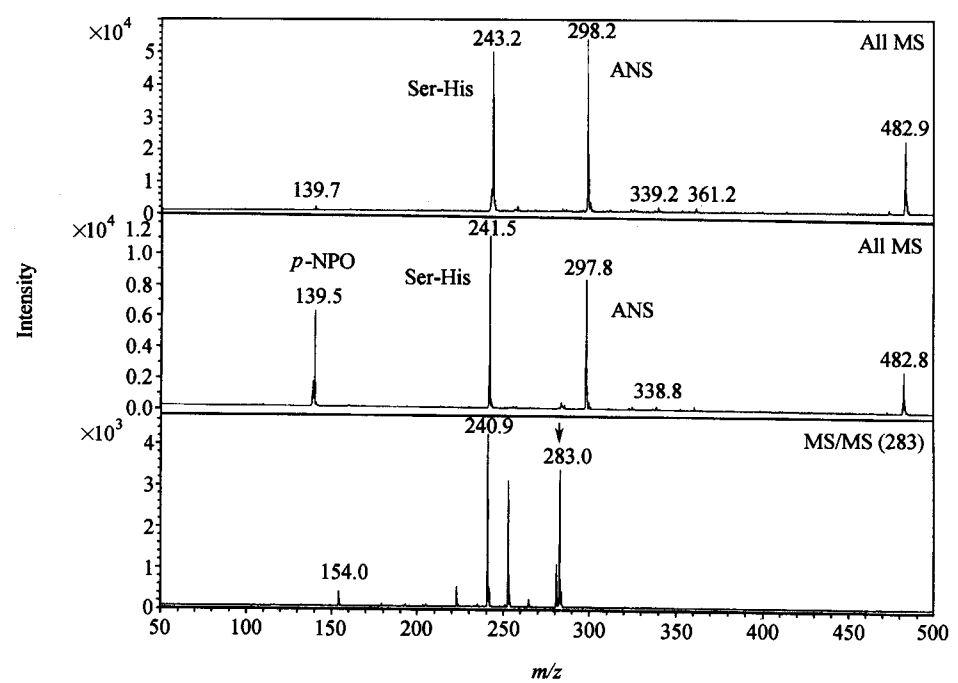
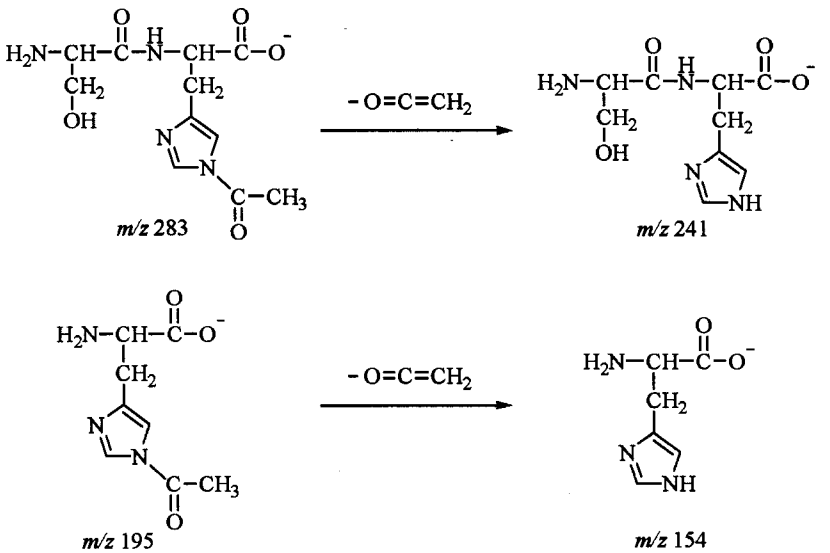


Fig. 5 Seryl-histidine catalyzing hydrolysis of *p*-NPA. (A) hydrolysis reaction of *p*-NPA at 0 min; (B) hydrolysis reaction of *p*-NPA at 600 min; (C) ESI-MS/MS of the peak in accordance with acetyl seryl-histidine.

Scheme 2 Losing of ketene from acetylated seryl-histidine and histidine



References

1 Schrag, J. D.; Li, Y.; Wu, S.; Cygler, M. *Nature* **1991**, *351*, 761.

2 Frey, P. A.; Whitt, S. A.; Tobin, J. B. *Science* **1994**, *264*, 1927.

3 Brady, L.; Brzozowski, A. M.; Derewenda, Z. S.; Dodson, E.; Dodson, G.; Tolley, S.; Turkenburg, J. P.; Christiansen, L.; Jensen, B. H.; Norskov, L.; Thim, L.; Menge, U. *Nature* **1990**, *343*, 767.

4 Chen, J.; Wang, R.; Liu, H.; Cheng, C.-M.; Zhao, Y.-F. *Lett. Pept. Sci.* **2000**, *7*, 325.

5 Wan, R.; Wang, N.; Zhao, G.; Zhao, Y.-F. *Chem. J. Chin. Univ.* **2000**, *21*, 1864 (in Chinese).

6 Wan, R.; Wang, N.; Zhao, Y.-F. *Chem. J. Chin. Univ.* **2001**, *22*, 598 (in Chinese).

7 Li, Y.; Zhao, Y.-F.; Hatfield, S.; Wan, R.; Zhu, Q.; Li, X.-H.; McMills, M.; Ma, Y.; Li, J.; Brown, K. L.; He, C.; Liu, F.; Chen, X.-Z. *Bioorg. Med. Chem.* **2000**, *8*, 2675.

8 Yamashita, M.; Fenn, J. B. *J. Phys. Chem.* **1984**, *88*, 4671.

9 Bauer, M. D.; Sun, Y. P.; Wang, F. *J. Protein Chem.* **1999**, *18*, 337.

10 Wu, H.-Q.; Aboleneen, H. *Anal. Biochem.* **2001**, *290*, 347.

11 Miranker, A.; Robinson, C. V.; Radford, S. E.; Dobso, C. M. *FASEB J.* **1996**, *10*, 93.

12 Krebs, J. F.; Siuzdak, G.; Dyson, H. J.; Stewart, J. D.; Benkovic, S. J. *Biochemistry* **1995**, *34*, 720.

13 Takayama, S.; Martin, R.; Wu, J. Y.; Laslo, K.; Siuzdak, G.; Wong, C. H. *J. Am. Chem. Soc.* **1997**, *119*, 8146.

14 Schmeer, G.; Six, C.; Steinkirchner, J. *J. Solution Chem.* **1999**, *28*, 211.

15 Bowden, K.; Brownhill, A. *J. Chem. Soc., Perkin Trans. 2* **1997**, *2*, 219.

16 Broo, K. S.; Nilsson, H.; Nilsson, J.; Baltzer, L. *J. Am. Chem. Soc.* **1998**, *120*, 10278.

17 Lee, W. S.; Ueno, A. *Chem. Lett.* **2000**, *3*, 258.

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