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Analysis of Hydrolysis Reaction of N-Phosphoryl Phenylalanine by HPLC-ESI-MS/MS

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Abstract: Hydrolysis procedure of N-phosphoryl phenylalanine (DIPP-Phe) was studied by HPLC-ESI-MS/MS. The results showed that (HO)(*i*-PrO)P(O)Phe was the main intermediate and the hydrolysis of DIPP-Phe also occurred through a penta-coordinate transition state.

Keywords: N-Phosphoryl amino acids, hydrolysis reaction, HPLC-ESI-MS.

Phosphoamino acids are the smallest models of phosphoproteins. Insights into the mechanism of phosphorylation and dephosphorylation might be obtained partially by studying the properties of these simple phospho- amino acids. Previously, our group had investigated the hydrolysis reaction of N-diisopropyloxyphosphoryl amino acids (DIPP-AA) by ^{31}P-NMR[1,2]. In this paper, the hydrolysis reaction of N-phosphoamino acids was studied by HPLC-ESI-MS/MS. DIPP-Phe was as a sample and was studied in detail.

Analysis was performed using Bruker Esquire 3000 ion trap mass spectrometer with connection to a HP1100 HPLC series. Acetonitrile and methanol were of HPLC grade. Pure water was prepared through an instrument (Labconco Company, USA). DIPP-Phe was synthesized according to the literature[3] and its solution was prepared with 10% methanol in water as solvent at a concentration of 2×10^{-4}mol/L.

The chromatographic conditions were optimized. Concentration of each component was calculated by HPLC peak area normalization method. Because peptide can be self-assembled from DIPP-AA[4,5], there was about 6% N-diisopropyloxyphosphoryl phenylphenylalanine dipeptide (DIPP- Phe-Phe) in original DIPP-Phe solution. Testing solutions were incubated at 50℃ and then traced by HPLC-ESI-MS. For example, it was found that there were about 36.7% DIPP-Phe, 5.7% DIPP-Phe-Phe, 11.4% (HO)-(*i*-PrO)P(O)Phe, 44.4% phenylalanine(Phe) and 2% (CH_3O)(*i*-PrO)P(O) Phe after 5.5

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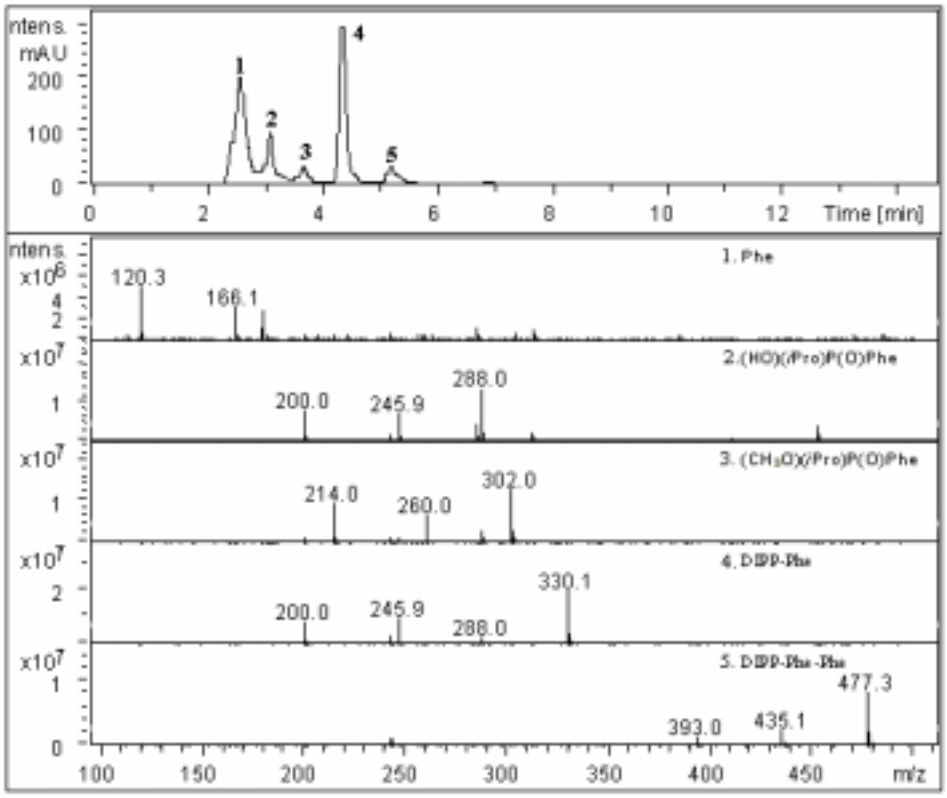
Figure 1 Analysis of DIPP-Phe solution by HPLC-ESI-MS after 5.5 h incubation



Conditions: Agilent ZORBAX Eclipse XDB-C8 column; 60% acetonitrile-water; detection wavelength: 254 nm; flow rate: 0.5 mL/min; nitrogen as nebulizer gas with a flow of 10 L/min (nebulizer pressure of 25 psi) at 350°C.

Table 1 MS/MS data of DIPP-Phe and its hydrolysis products

Entry	Compounds	t_R/min	$[M+H]^+$	Fragment ions (relative intensity)
1	Phe	2.9	166(36)	149(4), 120(100)
2	(HO)(i-PrO)P(O)Phe	3.2	288(25)	270(12), 246(100), 228(13), 200(46)
3	(CH_3O)(i-PrO)P(O)Phe	3.7	302(27)	260(100), 228(14), 200(100)
4	DIPP-Phe	4.4	330(11)	288(100), 246(99), 228(3), 200(34)
5	DIPP-Phe-Phe	5.2	477(5)	459(85), 435(89), 431(100), 417(50), 389(78), 347(52)

hours(**Figure 1**). (CH_3O)(*i*-PrO)P(O)Phe was ester-exchange product of DIPP-Phe[6]. The structures of hydrolysis products were identified by MS/MS and the data are given in **Table 1**.

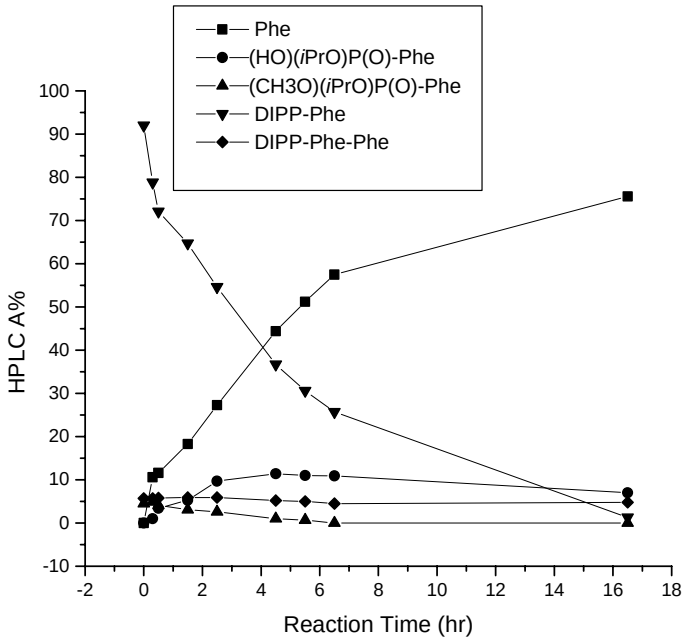
The kinetic data were obtained by the integral of the HPLC peaks. Test data were plotted in **Figure 2**, which tracked dynamic concentration changes of each compound in the solution.

According to the results in **Figure 2**, the curve lnC(DIPP-Phe)-t could be obtained

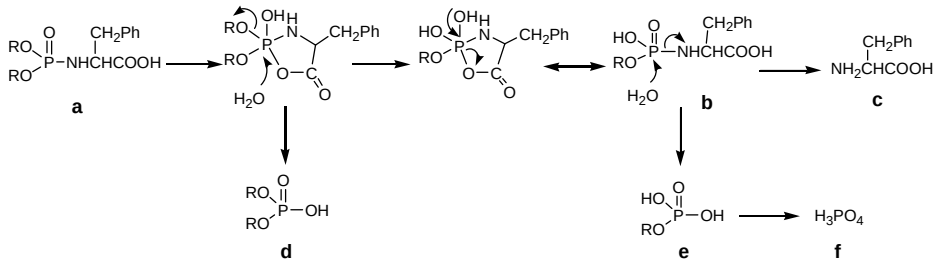
and it was found that the curve was a straight line. Therefore, the hydrolysis reactions of DIPP-Phe was kinetically first order. Kinetic constant *k* was obtained from the slope of the lnC-t line, which was 6.15×10^{-5} sec^{-1}. Interestingly, the concentration of DIPP- Phe-Phe was relatively stable under the same condition and that of (HO)(*i*-PrO)P(O)Phe was increased and then decreased. It was implied that (HO)(*i*-PrO)P(O)Phe was the main intermediate in the hydrolysis process. After comparing the hydrolysis rate of DIPP-Phe and DIPP-Phe -Phe, it was found that the hydrolysis rate of DIPP-Phe was much faster than DIPP-Phe-Phe. It is verified further that the hydrolysis mechanism of DIPP-Phe also occurred through a penta-coordinate transition state as reported in the literature[2]. DIPP-Phe-Phe could not form a penta- coordinate transition state so it was relatively stable under the same condition. The possible hydrolysis mechanism of DIPP-Phe is proposed in **Scheme 1**.

The literature[2] had identified hydrolysis products **b**, **d**, **e**, and **f** by ^{31}P-NMR while the hydrolysis products **b** and **c** were identified by HPLC-ESI-MS/MS in this letter. Different method identified different hydrolysis products. Hence, this method is complementary to study the hydrolysis reaction of DIPP-AA. Results from two methods were in accordance with each other. HPLC-ESI-MS is, therefore, recommended as an experimental tool to probe hydrolysis mechanism of DIPP-AA as ^{31}P-NMR.

Figure 2 Dynamic concentration changing data of each compound



Scheme 1 The hydrolysis mechanism of DIPP-Phe



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