Chinese Chemical Letters Vol. 15, No. 3, pp 343-346, 2004
http://www.imm.ac.cn/journal/ccl.html

Direct Observation of Non-covalent Complexes for Phosphorylated Flavonoid-protein Interaction by ESI

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Absract: Diethyl flavon-7-yl phosphate was synthesized by modified Atheron-Todd reaction. The result of ESI shows that the phosphated flavonoids possess stronger binding affinities toward proteins such as myoglobin, insulin and lysozyme and are easier to form the non-covalent complexes with them.

Keywords: DEPH, 7-hydroxyflavone, phosphorylation, ESI-MS, myoglobin, hen egg white lysozyme (HEWL), bovine insulin, non-covalent complexes.

Cellular function is often triggered by weak non-covalent interactions between enzyme and substrate, protein and ligand[1]. It is known that esters of phosphoric acid have wide bio-activities and play a vital role in many biological processes. They appear to be synthesized and to undergo interconversion in living organisms with great ease[2-4]; in recent years, flavonoids have also attracted increasing interest due to their various beneficial pharmacological effects. The development of electrospray ionization and the discovery that highly charged ions of proteins are readily formed has lead to dramatic growth in the application of mass spectrometry to bio-molecules[5-7]. Eelctrospray ionization is sufficiently gentle to allow the ionization and detection of intact non-covalent complexes between proteins and small molecules and of multi-unit protein structures[8-9]. We selected one representative member of flavones, 7-hydroxyflavone to synthesize its phosphate ester through simplified Atheron-Todd reaction and then the affinities of the flavonoid and its phosphate ester towards proteins were compared through electrospray ionization mass spectroscopy.

Experimental

Diethyl flavon-7-yl phosphate (compound **3**): 0.5 g 7-hydroxyflavone (compound **2**) was added to a solution of 40 mL ethanol and 10 mL triethylamine. The mixture was stirred until 7-hydroxyflavone was dissolved. Then the solution of 0.5 mL diethylphosphite

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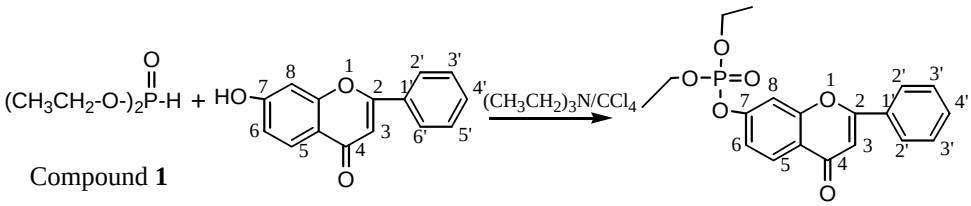
(DEPH) in 10 mL of CCl_4 was added to the above solution of 7-hydroxyflavone with stirring in ice-water bath. Then the reaction proceeded for 24 hours at room temperature. The resulting salt of triethyl amine was filtrated off. The filtrate was evaporated *in vacuo* below 50°C. Then 10 mL water was added. The water solution was extracted with ethyl acetate**.** The crude product was further purified by column chromatography ($CHCl_3$:EtOH=100:1). White plate compound **1** was obtained. m.p 60-61^o from light petroleum ether (b.p. 40-60℃). All the spectral data are reported for the first time. ^{1}H NMR (400MHz, CD_3Cl) δ 8.23 (d, 1H, J=8.7 Hz, H-5), 7.92 (dd, 2H, J=7.3, 1.6Hz, H-2′), 7.54 (m, 4H, H-3′, H-4′, H-8), 7.72 (dd, 1H, H-6, J=8.7, 1.4Hz), 6.82 (s, 1H, H-3), 4.28 (m, 4H, CH_2), 1.40 (m, 6H, CH_3). ESI-MS/MS, *m/z* 375 [M+H^+], 347[M-C_2H_4+H^+], 319[M-2C_2H_4+H^+]. Element analysis (found C, 60.8, H, 5.2, P 8.2%, calcd. for $C_{19}H_{19}O_6P$: C 61.0, H, 5.1, P, 8.3%)

Scheme 1



Compound **1** Compound **2** Compound **3**

Bovine insulin, myoglobin, hen egg white lysozyme (HEWL) were purchased from Sigma Company and were used without further purification.

Mass Spectrometric Conditions

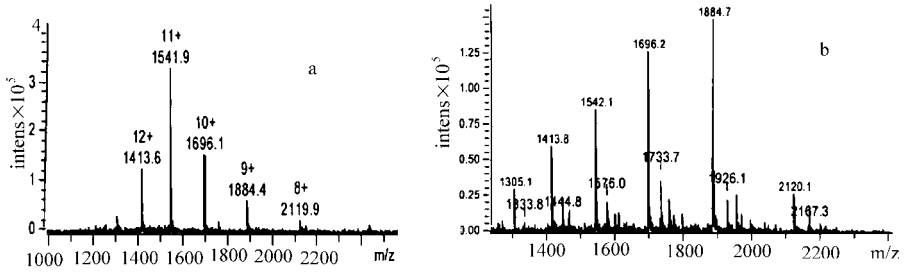
Solutions of the complexes were analyzed on Bruke-ESQUIRE 3000 fitted with an ion spray source working in the positive mode. Bruke ESQUIRE-LC ion trap spectrometer was equipped with a gas nebulizer prob, capable of analyzing ions up to *m/z* 6000.

Results and Discussion

Electrospray mass spectrum of myoglobin is shown in **Figure 1(a)**. Myoglobin solution (My Ⅰ) was prepared by mixing 100 μL myoglobin (0.29 mmol/L) with 900 μL NH_4OAc (3.47 mmol/L). Then the solution was infused through the ion-spray interface. The capillary exit was at 168.4 volt. The envelope of multiply protonated, multiply charged ions ranges from (M+8H) $^{8+}$ to the (M+12H) $^{12+}$ charge states of myoglobin. The average molecular mass measured from these peaks is 16951[10], corresponding to the calculated mass of apomyoglobin(16951.5 *u*; without heme) [11-12]. Electrospray mass spectrum obtained from the mixed solution of myoglobin and compound **3**(**CI**) is shown in **Figure 1(b).** The solution was prepared by mixing an equal volume of 0.27 mmol/L methanol solution of compound **1** and My Ⅰ (0.029 mmol/L). Then the solution was infused through the ion-spray interface. The capillary exit was set at 106.0 volt. Besides the expected multiply protonated molecule ions at *m/z* 1305 (myoglobin+13H)$^{13+}$, 1414 (myoglobin +12H)$^{12+}$, 1542 (myoglobin+11H)$^{11+}$, 1696 (myoglobin+10H)$^{10+}$,

1885(myoglobin+9H)$^{9+}$ and 2120 (myoglobin +8H)$^{8+}$, the mass spectrum reveals five new protonated ions *m/z* 1334, 1445, 1576, 1734, 1926 and 2167, corresponding to (myoglobin +CI)$^{13+}$, (myoglobin +CI)$^{12+}$, (myoglobin +CI)$^{11+}$, (myoglobin +CI)$^{10+}$ and (myoglobin +CI)$^{9+}$, (myoglobin +CI)$^{8+}$, respectively.

Figure 1 Electrospray ionization mass spectrum of myoglobin(**a**) and non-complexe of myoglobin with compound **3** (**b**)



Electrospray mass spectrum of bovine insulin is shown in **Figure 2(a).** 0.26mmol/L (0.1% HAc) bovine insulin solution (BIS) was infused through the ion-spray interface. The capillary exit was at 119.0 volt. The envelope of multiply protonated, multiply charged ions ranges from (M+3H)$^{3+}$ to the (M+6H) $^{6+}$ charge states of insulin. The spectrum shows a single distribution of peaks, the average molecular mass measured from these peaks is 5731 $\pm$ 1*u*. **Figure 2(b)** shows an electrospray mass spectrum obtained from the mixed solution of bovine insulin and compound **3**(**CI**). The mass spectrum reveals three new protonated ions *m/z* 1527, 1621 and 1715, corresponding to (insulin +CI)$^{4+}$, (insulin +CI_2)$^{4+}$, and (insulin +CI_3)$^{4+}$, respectively.

Figure 2 Electrospray ionization mass spectrum of bovine insulin (a) and non-complexes of bovine insulin with compound **3** (b)

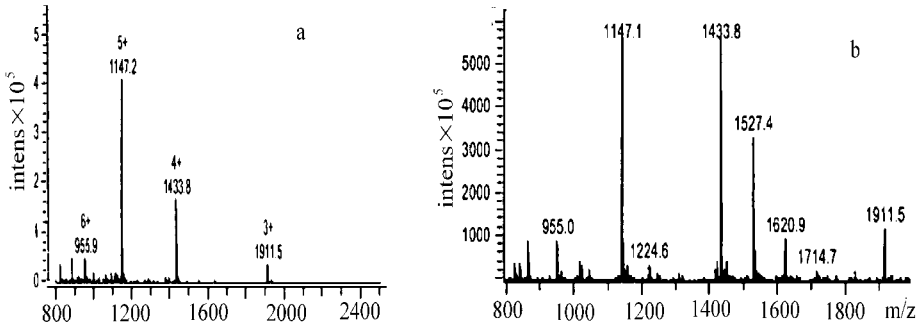
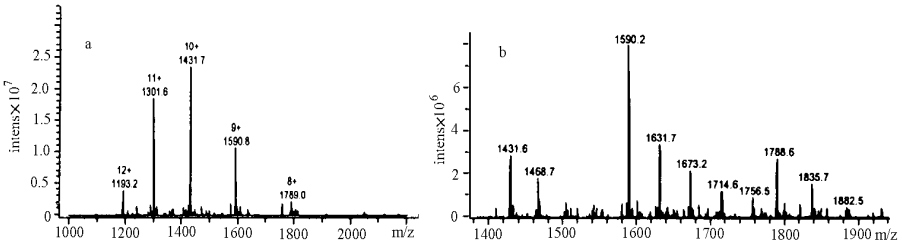


Figure 3 (a) shows the spectrum obtained from HEWL solution. The spectrum shows a single distribution of peaks, with protonation states ranging from 12+ to 8+, with 10+ the most intense. The average molecular mass measured from these peaks is 14307. These spectral characteristics are similar to those previously reported for the electrospray ionization of HEWL[13]. **Figure 3(b)** shows ESI of HEWL and compound 3 (**CI**). Besides

the expected multiply protonated molecule ions, a mass spectrum reveals significantly new protonated ions *m/z* 1469, 1632, 1573, 1715, 1757, 1835 and 1883, corresponding to $(HEWL +CI)^{10+}$, $(HEWL +CI)^{9+}$, $(HEWL +CI_2)^{9+}$, $(HEWL +CI_3)^{9+}$, $(HEWL +CI_4)^{9+}$, $(HEWL +CI)^{8+}$ and $(HEWL +CI_2)^{8+}$, respectively.

Figure 3 Ion-spray mass spectrum of HEWL(**a**) and non-complexes of HEWL with compound **3**(**b**)



There were no non-covalent complexes detected when the methods were applied to the mixed solutions of 7-hydroxyflvone with the different proteins mentioned above under the same condition.

Chrysin, as another represent member of flavonoids has also been phosphated. The phosphated chrysin also showed relatively strong affinity to the proteins. In a word, the phosphated flavonoids possess stronger affinities and are easier to form the non-covalent complexes with the proteins. These changes caused by the phosphated flavonoids might be important at the biological level.

Acknowledgment

The author would like to thank the NNSF of China (NO. 20175026) and Henan Academic Foundation of Science and Technology for their financial support.

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Received 3 March, 2003