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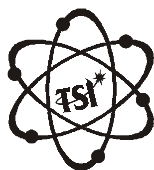

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Probing The Hydrolytic Function Of Lysozyme On Phosphate Ester Bound By Ion-Spray Mass Spectrometry

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ABSTRACT

The paper showed the first observation relating to hen egg white lysozyme (HEWL) as a special hydrolytic enzyme to hydrolyze the phosphate ester bond of the phosphated chrysin (PC). The experiments were carried on by injecting the solution of PC and HEWL, the solution of PC and cytochrome c and the solution of PC and bovine insulin respectively in an ion trap mass spectrometer and then examined by ESI-MS. The results show that HEWL could hydrolyze the phosphated chrysin and then formed non-covalent complex with the hydrolyzed product, while bovine insulin and cytochrome c couldn't exert any hydrolytic action and only form the non-covalent complexes with the phosphated chrysin. © 2007 Trade Science Inc. - INDIA

KEYWORDS

Hen egg white lysozyme (HEWL);
Phophated chrysin;
ESI-MS;
Non-covalent complexes;
Hydrolytic function.

INTRODUCTION

Phosphates have wide bio-activities and play a vital role in many biological process. They appear to be synthesized and to undergo interconversion in living organisms with great ease[1-4]. Here one of flavones, chrysin was converted into its phosphate through simplified Atheron-Todd reaction. The solution of the phosphated chrysin (PC) was mixed with different solution of proteins such as hen egg white lysozyme (HEWL), cytochrome c and bovine insulin. These solutions were injected in an ion trap mass spectrometer respectively and then examined by ESI-MS. The results show that HEWL could hydrolyze (PC) and then formed non-covalent complex with the hydrolyzed product, while bovine insulin and cytochrome c couldn't exert any hydrolytic action and only form the non-covalent complexes with PC under the same condition. It has long been known that lysozyme functions as an antibacterial agent by catalyzing the hydrolysis of β(1-4) glycosidic bonds of bacterial cell walls. Its active site con-

tains six subsites A-F and it interacts with a number of structurally similar saccharides[5-6]. But we couldn't see any report relating to the hydrolytic function of HEWL to special phosphate ester bond. So we here present our first observation about HEWL as a special hydrolytic enzyme to hydrolyze the phosphate ester bond of the phosphated chrysin.

RESULTS AND DISCUSSION

Firstly 2.08 mM PC solution was prepared by adding an equal volume of water into the 5.17 mM methanol solution of PC. The diluted solution was examined separately by ESI-MS using different mass spectrometric conditions. First the mass spectrometric conditions mentioned above were set, and then we changed different compound stability (CS) values (100%, 90%, 80%, 70%, 65%, 60%, 55% and 50%) to observe the mass spectrum of PC. Three of them are shown in figure 1. The result showed that when the CS was set bellow 70%, no fragment ions could be produced and detected by ESI-MS.

The reaction of HEWL with PC can be examined by ion-spray MS, as illustrated in figure 3. The CS was set at 60% to make sure that PC couldn't be split under mass spectrometric conditions. The result showed that HEWL could hydrolysis phosphate ester bond of PC. The hydrolytic product of PC (HPC), with molecular weight 499, was then produced by loss of molecular ethane after mixing. Besides the expected multiply protonated molecule ions at m/z 1590$(HEWL+9H)^{9+}$ and 1789 $(HEWL+8H)^{8+}$, a mass spectrum after mixing reveals seven new protonated ions m/z 1645, 1701, 1851, 1913, 1976, 2038 and 2101, corresponding to $(HEWL+HPC)^{9+}$, $(HEWL+HPC_2)^{9+}$, $(HEWL+HPC)^{8+}$, $(HEWL+HPC_2)^{8+}$, $(HEWL+HPC_3)^{8+}$, $(HEWL+HPC_4)^{8+}$ and $(HEWL+HPC_5)^{8+}$, respectively. $(HEWL+HPC)^{8+}$ and $(HEWL+HPC_2)^{8+}$ complexes are significantly more abundant than other complexes. No complex is observed between HEWL and PC.

Other proteins such as cytochrome c and bovine insulin were also selected for comparison. The mass spectrum of cyctochrome c, shown in figure 4 exhibits four peaks, each one corresponding to a different protonation state of cytochrome c. These protonation states ranges from +9 to +7 being most intense. A mixture of cyctochrome c and PC was infused through the ion-spray interface. Additional ions m/z 1162, 1678, 1743, 1841 and 1916, corresponding to $(cytochrome\ c+PC)^{8+}$, $(cytochrome\ c+PC_2)^{8+}$, $(cytochrome\ c+PC_3)^{8+}$, $(cytochrome\ c+PC)^{7+}$, $(cytochrome\ c+PC_2)^{7+}$, respectively, are obviously observed in figure 5. No complex is showed between cytochrome c and hydrolytic product of PC.

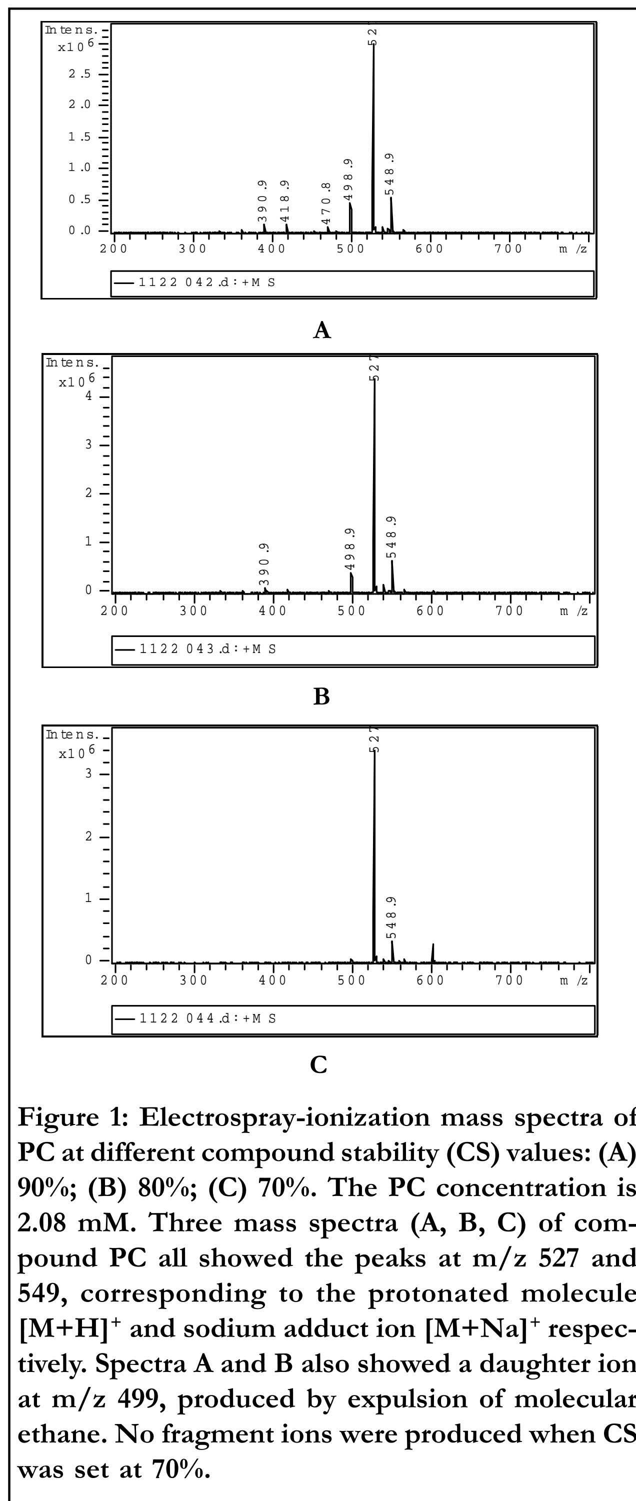


Figure 1: Electrospray-ionization mass spectra of PC at different compound stability (CS) values: (A) 90%; (B) 80%; (C) 70%. The PC concentration is 2.08 mM. Three mass spectra (A, B, C) of compound PC all showed the peaks at m/z 527 and 549, corresponding to the protonated molecule $[M+H]^+$ and sodium adduct ion $[M+Na]^+$ respectively. Spectra A and B also showed a daughter ion at m/z 499, produced by expulsion of molecular ethane. No fragment ions were produced when CS was set at 70%.

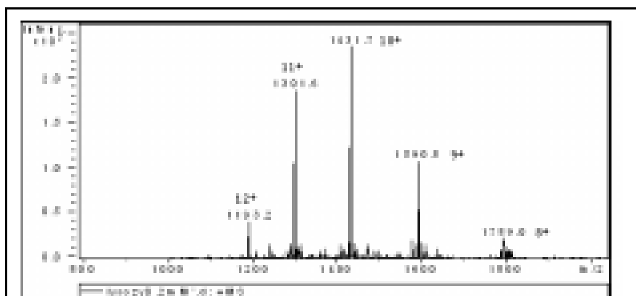


Figure 2: Ion-spray mass spectrum of HEWL recorded on ESI-esquire 3000. 0.01mM HEWL was infused through the ion-spray interface. The envelope of multiply protonated, multiply charged ions ranges from $(M+7H)^{7+}$ to the $(M+11H)^{11+}$ charge states of HEWL. The mass-to-charge region between the 8+ and 7+ charge states is the region of interest for detecting noncovalent enzyme-product complexes described in this work.

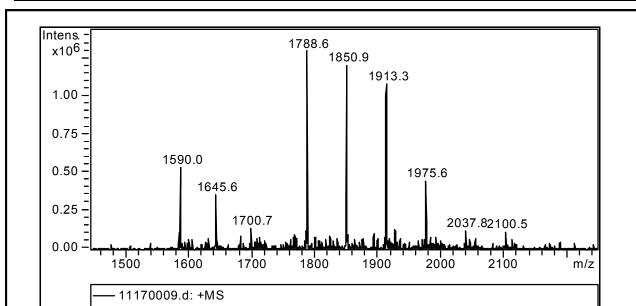


Figure 3: An ion-spray MS study for the hydrolysis of PC by HEWL. HEWL-PC solution was prepared by mixing an equal volume of 5.17mM methanol solution of PC and 0.02mM water solution of HEWL. Then the solution was infused through the ion-spray interface. The compound stability (CS) was set at 60%. Ion-spray mass spectrum of HEWL (0.02mM) with the hydrolyzed product was obtained.

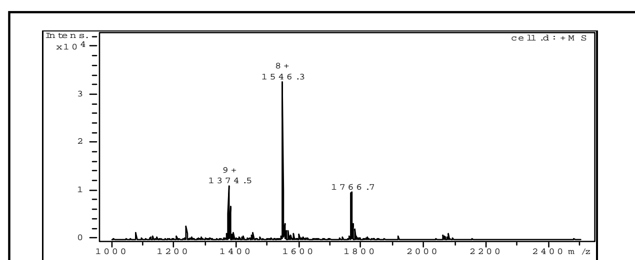


Figure 4: Electrospray ionization mass spectrum of bovine cytochrome c. Cytochrome c solution was prepared by mixing an equal volume of methanol and 0.032mM water solution of cytochrome c. Then the solution was infused through the ion-spray interface. The compound stability (CS) was set at 60%.

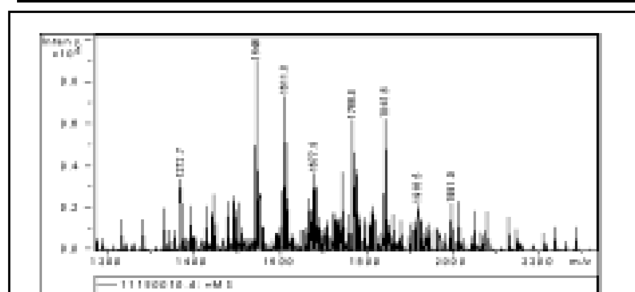
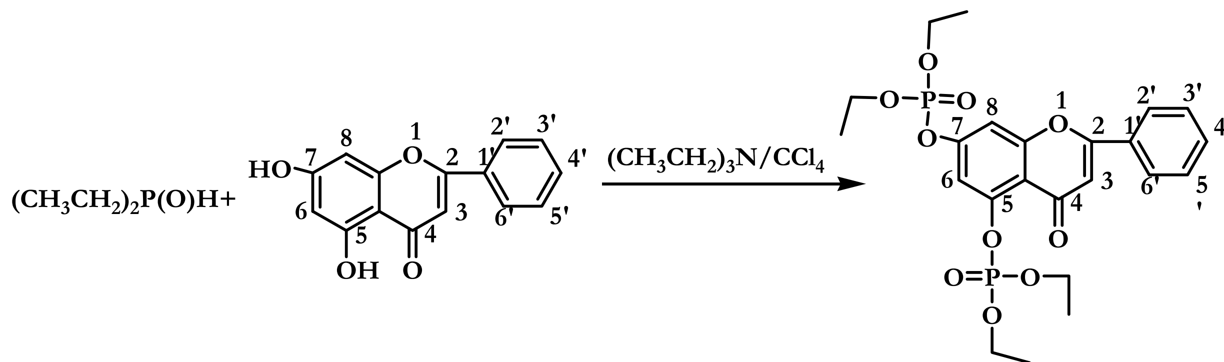


Figure 5: An ion-spray MS for non-complexes of cytochrome c with PC. The solution was prepared by mixing an equal volume of 5.17mM methanol solution of PC and 0.032mM water solution of Cytochrome c. Then the solution was infused through the ion-spray interface. The compound stability (CS) was set at 60%. Ion-spray mass spectrum of cytochrome c with PC was obtained.

Furthermore when scanning was performed from m/z 50 to 1000, there were no any hydrolytic ions, for example, m/z 499 detected. Similar results were also obtained for bovine insulin.

EXPERIMENTAL

Preparation of PC (SCHEME 1): $C_{23}H_{28}O_{10}P_2$. 0.5g chrysin was added to a solution of 40ml dioxane 10ml triethlamine. The mixture was stirred until chrysin was dissolved and a solution of diethylphosphite (DEPH) and CCl_4 (0.6ml DEPH +10mlCCl_4) was then added room temperature. The resulting salt of triethyl amine was added dropwise vigorous stirring in ice-water bath. The reaction proceeded for 24 hours. The filtrate was evaporated in vacuo below 50°C. Then 10ml water was added. The solution was first extracted by petroleum ether (b.p.60~90°C) and then by ethylacetate. The needle crystalline residues were separated out. m.p 83-84°C. All the spectral data is reported for the first time. $C_{23}H_{28}O_{10}P_2$ All the spectral data is reported for the first time. ^1H NMR (400MHz, CD_3Cl) δ 7.87 (dd, H-2' and H-6', J=2.0, 7.6Hz, Total 2H), 7.54-7.52 (m, H-3', H-4' and H-5', 3H), 7.41(s, H-6, 1H), 4.44-4.26 (m, $-CH_2$, 8H), 1.43-1.38 (m, $-CH_3$, 12H). ESI-MS/MS, m/z 527 $[M+H]^+$, 499, 471, 453, 419, 391,



SCHEME 1

363, 335. The structure of the target molecule was also proved by x-ray data.

Bovine insulin, cytochrome C and hen egg white lysozyme (HEWL) were purchased from Sigma Company and were used without further purification. 5.17 mM methanol solution of PC, 0.02mM water solution of HEWL, 0.026 mM (0.5% HAc) solution of insulin, 0.032 mM water solution of cytochrome C were prepared respectively.

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. Nitrogen was used as drying gas at a flow rate of 8 L/min. The neublizer pressure was 15 psi. The capillary was typically held at 4kV and the source temperature was maintained at 300°C. About 5 spectra were averaged. The samples dissolved were continuously infused into the ESI chamber at a flow rate 4 µL/min using a Cole-Parmer 744900 syringe pump (Cole-Parmer Instrument Co.).

CONCLUSION

The experiments described herein suggest that HEWL could hydrolyze the phosphated chrysin and then formed non-covalent complex with the hydrolyzed product, but bovine insulin and cytochrome c can't hydrolyzed the phosphate ester bond of the phosphated chrysin and only form the non-covalent complexes with it. Here we report for the first time the successful detection of HEWL as a special hydrolytic enzyme to hydrolyze the phosphate ester bond of the phosphated chrysin. The ion-spray and electrospray mass spectrometery (MS) technologies, using an extremely mild means for the ionization of molecules[7-10], can provide information on non-covalent complexes as their protonated, multiply charged species. Although the data do not imply the solution conformation is preserved into gas phase, the result suggest that non-covalent associations of proteins and cofactors in solution can be preserved into the gas phase and observed by mass spectrometry.

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