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Direct Observation of Non-covalent Complexes Formed Through Phosphorylated Flavonoid Protein Interaction by Electrospray Ionization Mass Spectrometry

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7-Hydroxyflavone was phosphorylated by a modified Atherton–Todd reaction and the resulting structure determined by NMR, ESI-MS/MS and elemental analysis. The electrospray ionization results showed that phosphorylated flavonoids can form non-covalent complexes with many proteins such as lysozyme, cytochrome *c*, bovine insulin and myoglobin, while non-covalent complexes were not detected with a mixed solution of 7-hydroxyflavone and proteins. It is deduced that phosphorylated flavonoids possess relatively strong affinities for proteins and form non-covalent complexes with proteins more easily than the unphosphorylated compounds.

Keywords: 7-Hydroxyflavone; Phosphorylation; Myoglobin; DEPH; ESI-MS; Lysozyme; Cytochrome *c*; Bovine insulin; Non-covalent complexes

INTRODUCTION

Cellular functions are often triggered by weak non-covalent enzyme–substrate, protein–ligand, protein–protein or antibody–antigen interactions [1]. It is known that esters of phosphoric acid have wide-ranging bioactivities and play a vital role in many biological processes. They appear to be synthesized and to undergo interconversion with great ease in living organisms [2–5]; in recent years, flavonoids have attracted increasing interest due to their various beneficial pharmacological effects. In this paper we selected 7-hydroxyflavone, a representative flavone, to synthesize its phosphate ester through a simplified Atherton–Todd reaction. The binding affinities of the flavonoid and its phosphate ester with proteins were compared using electrospray ionization mass spectrometry (ESI-MS). The development of electrospray ionization and the discovery that highly charged ions of proteins are readily formed has led to a dramatic increase in the application of mass spectrometry to biomolecules [6–8]. Electrospray ionization is sufficiently gentle to allow the ionization and detection of intact non-covalent complexes between proteins and small molecules and of multiunit protein structures [9,10]. The key to success in the study of non-covalent complexes depends on careful understanding and manipulation of ESI source parameters and sample solution conditions. To study these non-covalent complexes, solutions of the flavonoid and its phosphate ester with different proteins, such as myoglobin, hen egg white lysozyme (HEWL), cytochrome *c* and bovine insulin, were injected individually into an ion-trap mass spectrometer. The results indicate that phosphorylated flavonoids possess relatively strong affinities for proteins and form non-covalent complexes with proteins more easily than the unphosphorylated compounds.

EXPERIMENTAL

Compound 1: Diethyl Flavon-7-yl Phosphate (FP). $C_{19}H_{19}O_6P$

7-Hydroxyflavone (0.5 g) was added to a solution of 40 ml ethanol and 10 ml triethylamine. The mixture

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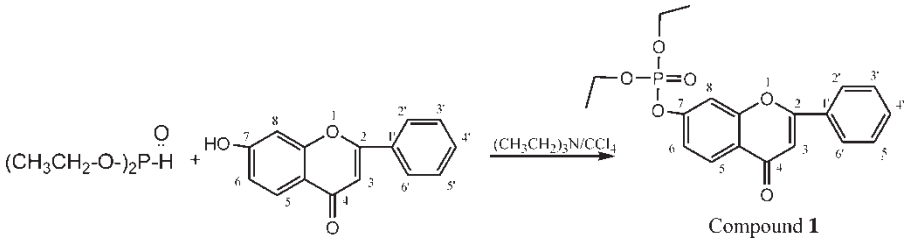

SCHEME 1 Synthesis of compound **1**. Bovine insulin, myoglobin, cytochrome *c* and HEWL were purchased from Sigma Chemicals and were used without further purification.

was stirred until the 7-hydroxyflavone had dissolved and then a solution of 0.5 ml diethyl phosphite (DEPH) and 10 ml CCl_4 was added dropwise with vigorous stirring in an ice–water bath. The reaction proceeded for 24 hours at room temperature. The resulting triethylamine salt was precipitated. The filtrate was evaporated *in vacuo* below 50°C and 10 ml water added. The solution was extracted with ethyl acetate. The product was further purified by column chromatography. A white plate compound (**1**) was obtained: mp 60–61°C from light petroleum (bp 40–60°C). The spectral data are reported here for the first time. ^{1}H NMR (400 MHz, CD_3Cl) 8.23 (d, 1 H, $J = 8.4$ Hz, H-5), 7.92 (dd, 2H, $J = 7.2, 1.6$ Hz, H-2′, H-6′), 7.54 (m, 4H, H-3′, H-4′, H-8), 7.72 (dd, 1H, H-6, $J = 8.3, 1.4$ Hz), 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, 347. Elemental analysis (found: C, 60.8; H, 5.2; P 8.2. $C_{19}H_{19}O_6P$ requires C, 61.0; H, 5.1, P, 8.3).

Mass Spectrometry Conditions

Solutions of the complexes were analysed using a Bruker-Esquire 3000 mass spectrometer fitted with an ion spray source working in the positive mode. The Bruker-Esquire-LC ion-trap spectrometer was equipped with a gas nebulizer probe, capable of analysing ions up to m/z 6000. Nitrogen was used as the drying gas at a flow rate of 4 μl min^{-1}. The nebulizer pressure was 17 psi and the dry gas flow rate was 9.00 l min^{-1}. The capillary was typically held at 4 kV and the source temperature was maintained at 300°C. Six spectra were averaged and the rolling average was 7. ICC was set at 30 000. The samples dissolved were continuously infused into the ESI chamber at a flow rate of 4 μl min^{-1} using a Cole-Parmer 744900 syringe pump (Cole-Parmer Instrument Co.).

RESULTS AND DISCUSSION

Mass spectrometry (MS) has been an indispensable tool for biomedical research involving protein and peptide structural analysis mainly due to the development of various gentle ionization methods. Nevertheless, the detection of non-covalent complexes by MS methods has been a challenging task. To successfully perform direct MS analysis of a preformed complex, the MS ionization technique used must satisfy certain criteria. The control of solution pH, organic solvent and stabilizing buffer additives is crucial in the detection of non-covalent complexes; moreover, the internal energy transfer to the macromolecule during the ionization process must be minimal to prevent dissociation of the complex. In this experiment, the solvation conditions for myoglobin, HEWL, cytochrome *c* and bovine insulin were adjusted appropriately; the orifice voltage is the most important parameter relating to detection of the complex, and special attention was paid to its effects. As expected, the stronger the binding energy of the non-covalent complex, the lower the effect of increasing the orifice potential on the stability of the complex produced. Increasing the orifice potential increases the internal energy imparted to the complex during the desolvation process and could induce dissociation of the complex. The stability of the phosphate–protein complex is influenced strongly by the orifice voltage used in the ion-spray analysis. As solutions of the phosphate ester with the different proteins were injected individually into an ion-trap mass spectrometer, different orifice voltages were tried and eventually suitable voltage ranges for successful detection of the different complexes were obtained. The results show that the myoglobin–FP complex started to dissociate at 110 V, insulin–FP at 105 V, HEWL-FP at 125 V and cytochrome *c*–FP at 100 V. These dissociation voltages show the different binding affinities of FP to different proteins and therefore show the different stabilities of the FP–protein complexes. In the following experiment, suitable voltages were applied individually to different FP–protein complexes. The corresponding electrospray mass spectra are shown in Figs. 2, 4, 6 and 8.

Because myoglobin is readily available commercially in large quantities, it was used as a "tuning" compound to optimize the experimental conditions, to maximize the sensitivity for ESI and to observe the non-covalent protein complexes [11]. Katta and Chait reported that positive-ion ESI-MS of the heme-globin

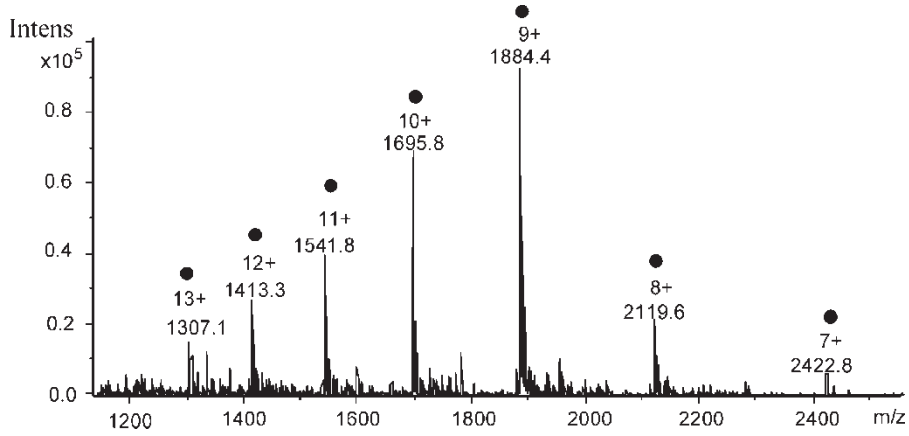

FIGURE 1 Electrospray ionization mass spectrum of myoglobin. Myoglobin solution was prepared by mixing 100 μl myoglobin (0. 29 mM) with 900 μl NH_4OAc (3.47 mM). The pH was adjusted to about 3.39 by adding HOAc. The orifice voltage was set at 106.0 V. ● identifies the multiply charged ion peaks of myoglobin.

complex in myoglobin could be demonstrated when pH $>$ 3.39, while positive-ion ESI-MS of apomyoglobin (without heme) could be demonstrated when pH $<$ 3.35 [12]. In our experiment, the pH of the myoglobin solution was adjusted in advance to about 3.35 for FP–apomyoglobin complex detection. Figure 1 shows the spectrum obtained from equine myoglobin solution. The spectrum shows a single distribution of peaks, with protonation states ranging from 13+ to 7+, with 9+ the most intense. The average molecular mass measured from these peaks is $16951 \pm 1\,u$ and corresponds to the calculated mass of apomyoglobin (16951.5 u; without heme). These spectral characteristics are similar to those previously reported for the electrospray ionization of myoglobin [13,14]. Figure 2 shows an electrospray mass spectrum obtained from the mixed solution of myoglobin and FP. Besides the expected multiply protonated molecule ions at m/z 1305 $(\text{myoglobin} + 13\text{H})^{13+}$, 1414 $(\text{myoglobin} + 12\text{H})^{12+}$, 1542 $(\text{myoglobin} + 11\text{H})^{11+}$, 1696 $(\text{myoglobin} + 10\text{H})^{10+}$, 1885 $(\text{myoglobin} + 9\text{H})^{9+}$ and 2120 $(\text{myoglobin} + 8\text{H})^{8+}$, the mass spectrum revealed six new protonated ions at m/z 1334, 1445, 1576, 1734, 1926 and 2167, corresponding to $(\text{myoglobin} + \text{FP})^{13+}$, $(\text{myoglobin} + \text{FP})^{12+}$, $(\text{myoglobin} + \text{FP})^{11+}$, $(\text{myoglobin} + \text{FP})^{10+}$, $(\text{myoglobin} + \text{FP})^{9+}$ and $(\text{myoglobin} + \text{FP})^{8+}$, respectively. The complexes $(\text{myoglobin} + \text{FP})^{10+}$ and $(\text{myoglobin} + \text{FP})^{9+}$ are more abundant than the other complexes. Even if the amount of FP was increased to a molar ratio of 9:1, no myoglobin–nFP ($n > 2$) complexes were observed. This may indicate that FP could interact selectively with specific sites on the myglobin.

Our experiment showed that insulin was easy to dissolve in dilute acetic acid. Thus 0.5% acetic acid was used to prepare this protein solution. Figure 3 shows the spectrum obtained from bovine insulin solution. The spectrum shows a single distribution of peaks, with protonation states ranging from 6 + to

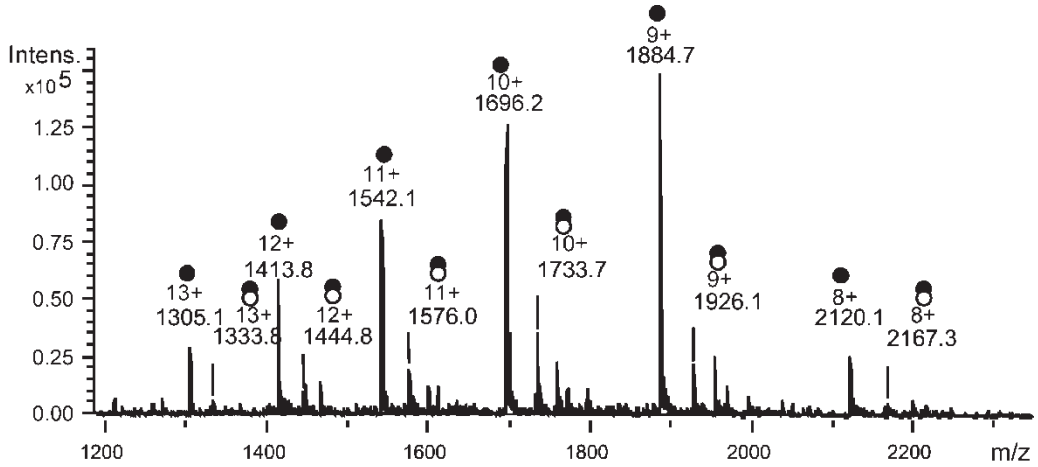

FIGURE 2 Ion-spray mass spectrum of myoglobin with FP. The solution was prepared by mixing an equal volume of 0.27 mM methanol solution of FP and myoglobin (0.029 mM). The pH was adjusted to about 3.39. Then the solution was infused through the ion-spray interface. The orifice voltage was set at 106.0 V. ● identifies the multiply charged ion peaks of myoglobin; ●○ identifies the multiply charged ion peaks of the myoglobin–FP complex.

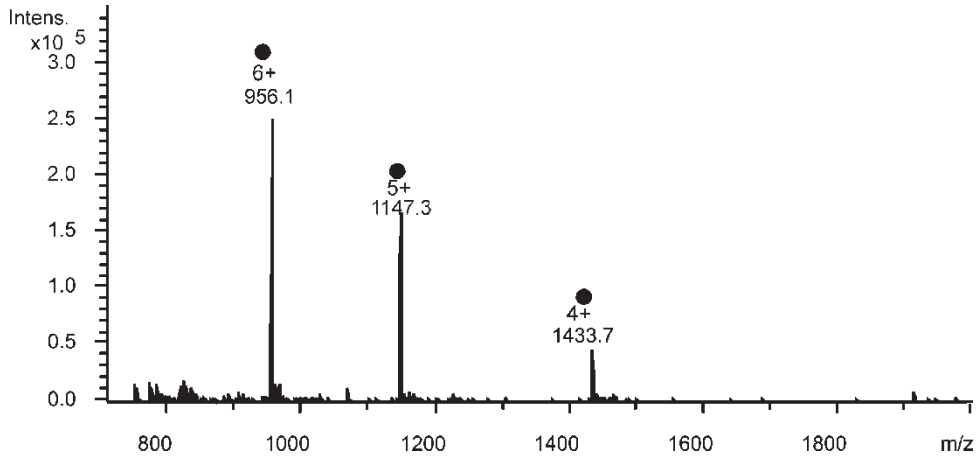

FIGURE 3 Electrospray ionization mass spectrum of insulin. Insulin solution was prepared by mixing equal volumes of methanol solution and an 0.5 mM (0.5% HAc) solution of insulin. Then the solution was infused through the ion-spray interface. The orifice voltage was 99.8 V. ● identifies the multiply charged ion peaks of insulin.

4 + . The average molecular mass measured from these peaks is $5731 \pm 1\,u$. Figure 4 shows an electrospray mass spectrum obtained from the mixed solution of bovine insulin and FP. The amount of FP was increased to a molar ratio of 10:1. Besides the expected multiply protonated molecule ions at m/z 955 (insulin + 6H)$^{6+}$, 1147 (insulin + 5H)$^{5+}$, 1434 (insulin + 4H)$^{4+}$ and 1912 (insulin + 3H)$^{3+}$, the mass spectrum reveals three new protonated ions at m/z 1527, 1621 and 1715, corresponding to (insulin + FP)$^{4+}$, (insulin + 2FP)$^{4+}$ and (insulin + 3FP)$^{4+}$, respectively. This result shows that the m/z region between the 4+ and 3+ charge states is the region of interest for detecting non-covalent complexes described in this work. Interestingly enough, no non-covalent complexes were observed in the m/z region between 5+ and 4+. In contrast to the FP–myoglobin complex observed in Fig. 2, 1:1–1:3 non-covalent insulin–FP complexes were observed, as shown in Fig. 4.

Decreasing the solution pH is reported to have little effect on the observed charge distribution of HEWL [15]. A neutral solution of equine HEWL was therefore used in our experiments. Figure 5 shows the spectrum obtained from the neutral solution of HEWL. The envelope of multiply protonated, multiply charged ions ranges from (M + 7H)$^{7+}$ to the (M + 12H)$^{12+}$ charge states of HEWL, with (M + 9H)$^{9+}$ being the most intense. The average molecular mass measured from these peaks is 14 307. These spectral characteristics are similar to those reported previously for the electrospray ionization of HEWL [16]. Figure 6 shows an electrospray mass spectrum obtained from a mixed solution of HEWL and FP. Besides the expected multiply protonated molecule ions at m/z 1432 (HEWL + 10H)$^{10+}$, 1590 (HEWL + 9H)$^{9+}$ and 1789 (HEWL + 8H)$^{8+}$, the mass spectrum reveals significantly new protonated ions at m/z 1469, 1632, 1573, 1715, 1757, 1835 and 1883, corresponding to (HEWL + FP)$^{10+}$, (HEWL + FP)$^{9+}$, (HEWL + 2FP)$^{9+}$, (HEWL + 3FP)$^{9+}$, (HEWL + 4FP)$^{9+}$, (HEWL + FP)$^{8+}$ and (HEWL + 2FP)$^{8+}$, respectively. Our results show that the FP–lysozyme complexes can survive relatively high orifice

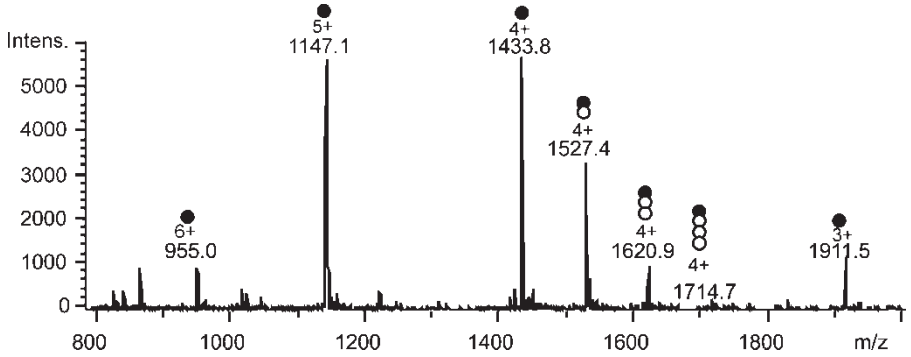

FIGURE 4 An ion-spray mass spectrum of insulin with FP. The solution was prepared by mixing equal volumes of 0.27 mM methanol solution of FP and 0.026 mM (0.5% HAc) solution of insulin. Then the solution was infused through the ion-spray interface. The capillary exit was set at 99.80 V. ● identifies the multiply charged ion peaks of insulin; ●○ identifies the multiply charged ion peaks of insulin–FP complex; ●○○ identifies the multiply charged ion peaks of insulin–2FP complex; ●○○○ identifies the multiply ion peaks of insulin–3FP complex.

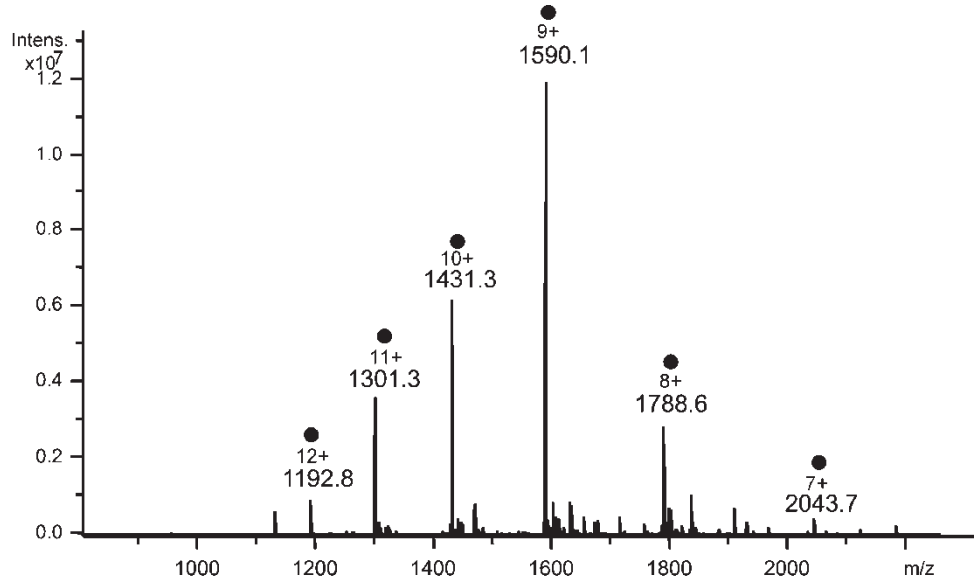

FIGURE 5 Ion-spray mass spectrum of HEWL recorded on an ESI Bruke-Esquire 3000. The solution was prepared by mixing equal volumes of methanol solution and HEWL (0.027 mM). The envelope of multiply protonated, multiply charged ions ranges from the $(M + 7H)^{7+}$ to the $(M + 12H)^{12+}$ charge states of HEWL. The orifice voltage was set at 118.7 V. ● dentifies the multiply charged ion peaks of HEWL.

voltages. An orifice voltage up to 118.7 V could be used to demonstrate the FP–lysozyme complexes (Fig. 6). Even though such a high voltage was used, non-covalent FP–lysozyme complexes appeared within the wide m/z region between the 10+ and 7+ charge states. Moreover, non-covalent FP-lysozyme complexes with ratios up to 4:1 were observed in the region between the 9+ and 8+ charge states. This observation suggests that lysozyme has more bonding sites to interact with FP and shows a relatively stronger bonding affinity to FP.

The solution of cytochrome *c* was prepared using biological buffer. The electrospray mass spectrum taken from infusion of the solution of cytochrome *c* is given in Fig. 7, yielding an average molecular weight of 12361.3 Da. The result is consistent with a previous report [17]; the spectrum taken from an infusion of the solution of the FP–cytochrome *c* complex is given in Fig. 8. A series of ions at m/z 1274, 1416 and 1593 was detected, corresponding to (cytochrome *c* + FP)$^{10+}$, (cytochrome *c* + FP)$^{9+}$ and (cytochrome *c* + FP)$^{8+}$, respectively.

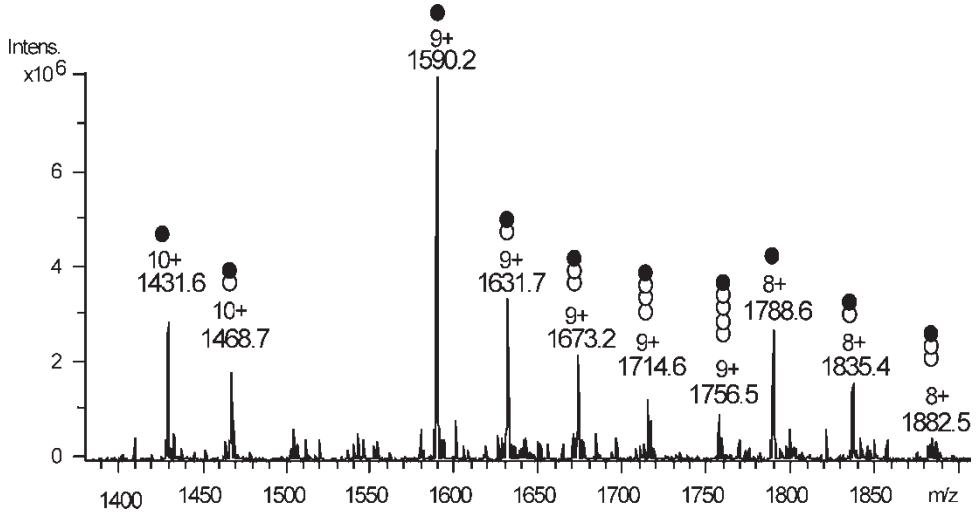

FIGURE 6 Ion-spray mass spectrum of HEWL with FP. The solution was prepared by mixing equal volumes of 0.27 mM methanol solution of FP and HEWL (0.027 mM). Then the solution was infused through the ion-spray interface. The orifice voltage was set at 118.7 V. ● identifies the multiply charged ion peaks of HEWL; ●○ identifies the multiply charged ion peaks of HEWL–FP complex; ●○○ identifies the multiply charged ion peaks of HEWL–2FP complex; ●○○○ identifies the multiply charged ion peaks of HEWL–3FP complex; ●○○○○ identifies the multiply charged ion peaks of HEWL–4FP complex.

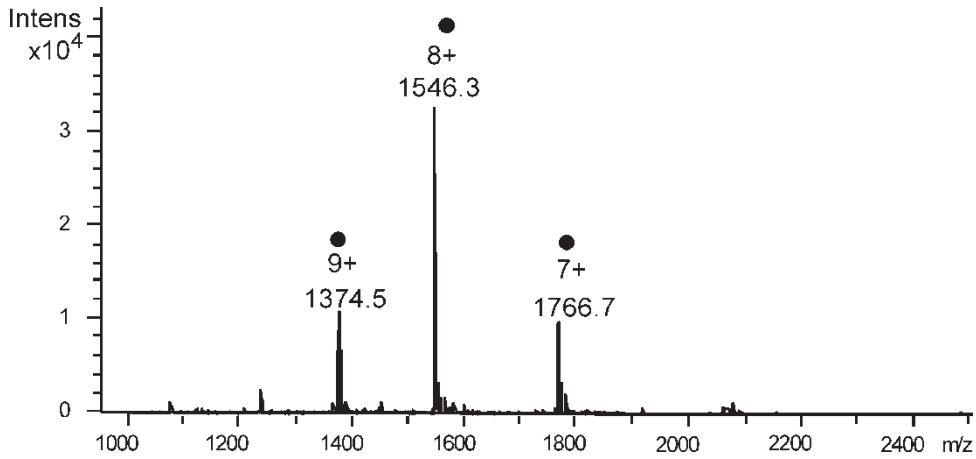

FIGURE 7 Electrospray ionization mass spectrum of cytochrome *c*. Cytochrome *c* solution was prepared by mixing equal volumes of methanol and 0.032 mM cytochrome *c* (2 mM NH_4OAc). Then the solution was infused through the ion-spray interface. The orifice voltage was 106.0 V. ● identifies the multiply charged peaks of cytochrome *c*.

The experiment described above shows that FP could interact with all the proteins. However, different bonding affinities for FP were shown with the different proteins; ratios of up to 1:4 were observed in the case of the lysozyme–FP non-covalent complex whereas a 1:1 ratio was observed with the myoglobin–FP and cytochrome *c*-FP complexes. These differences show the specificity of the different proteins. For comparison, 0.19 mM 7-hydroxyflavone was also mixed with the protein solutions mentioned above, that is myoglobin (0.029 mM), insulin (0.026 mM, 0.5% HAc), HEWL (0.026 mM) and cytochrome *c* (0.032 mM), and infused to ESI individually. Even though different parameters were tried, no corresponding non-covalent protein–flavonoid complexes were detected. The experiments described herein suggest that the phosphorylated flavonoid could form non-covalent complexes with myoglobin and bovine insulin, HEWL and cytochrome *c* more easily than the unphosphorylated compound.

In order to obtain further information relating to the interactions between FP and the proteins, the following experiment was carried out in two steps: first, the experiment was designed to investigate whether FP and 7-hydroxyflavonoid could selectively recognize some amino acids and interact with them; then to see if the reactive amino acid residues might be involved in explaining the FP–protein interaction. To ensure that the results were indeed typical and not the result of electrospray conditions leading to the clustering of all species, the amino acids were analysed under identical conditions. The results show that no 7-hydroxyflavone-amino acid complexes were detected, but FP clearly selected all of the basic amino acids (histidine, arginine and lysine) and formed non-covalent complexes with them (Figs. 9–11).

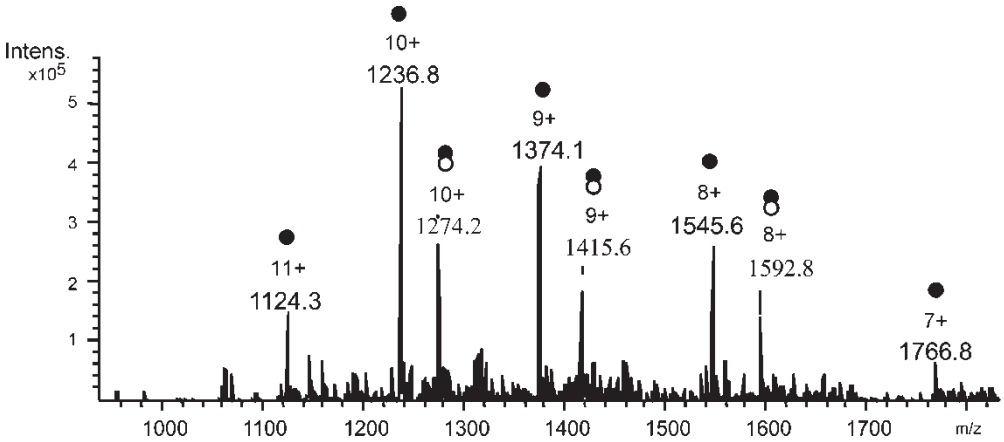

FIGURE 8 An ion-spray mass spectrum of cytochrome *c* with FP. The solution was prepared by mixing equal volumes of 0.27 mM methanol solution of FP and 0.032 mM cytochrome *c* (2 mM NH_4OAc). Then the solution was infused through the ion-spray interface. The orifice voltage was 97.7 V. ● identifies the multiply charged ion peaks of cytochrome *c*; ●○ identifies the multiply charged ion peaks of cytochrome *c*–FP complex.

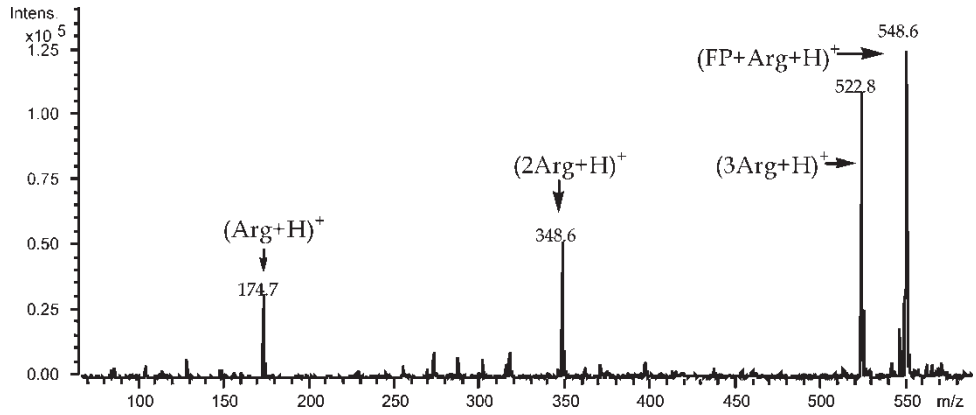

FIGURE 9 Electrospray mass spectrum of Arg–FP.

It is concluded that protonated arginine, histidine and lysine form strong hydrogen bond complexes with electron-donating groups, such as the oxygen of the P═O of the phosphate group in FP. Basic amino acids are known to play an important role in the charged regions and interactions of many biologically relevant systems. For most peptides and proteins with molecular weights ranging from a few hundred to ~40 000 Da, the maximum number of observed charges for peptides and smaller proteins correlates well with the number of basic amino acid residues, except for disulfide-containing molecules [18]. The protonated arginine, histidine and lysine residues of the proteins used in our experiment may act as binding sites and contribute to the survival of these complexes in the gas phase.

To determine whether the stability of the gas-phase complexes was due to the interaction of protonated basic groups of the basic amino acid residues with electron-donating groups from FP, FP was mixed with a peptide (CANPEDARLHGGPQY) and their interaction was examined. The pH was adjusted to 3.0 using HOAc prior to analysis to allow the two basic amino residues in the peptide to "relax" into a more extended conformation and then to be more readily protonated. Increasing the ratio of FP to peptide to 10:1 allowed the detection of protonated 1:2 peptide–FP complexes (Fig. 12). The ions at m/z 749.1 and 1144.9 were assigned to complexes $(2FP + H)^+$ and $(3FP + Na)^+$ respectively. The peptide gave a relatively weak MH^+ ion at m/z 1628.4 and an abundant doubly protonated molecule $(M + 2H)^{2+}$ at m/z 814.7. The relatively intense peak at m/z 1001.7 was attributed to the 1:1 complex $(peptide + FP + 2H)^{2+}$. The doubly charged ion at m/z 1188.3 was attributed to the doubly protonated 1:2 complex $(peptide + 2FP + 2H)^{2+}$. Even though FP was added in excess, no peptide–3FP complexes were detected. Thus, the protonated basic amino acid residues of the peptide may form binding sites and contributed to the survival of these FP–protein complexes in the gas phase. However, the results of our experiment show not only that different proteins have different binding affinities for FP but also that none of the proteins mentioned above interact with many FP molecules, as might be expected from the above result. This contradiction may be explained by the complexity of the protein structure. Non-covalent FP-protein

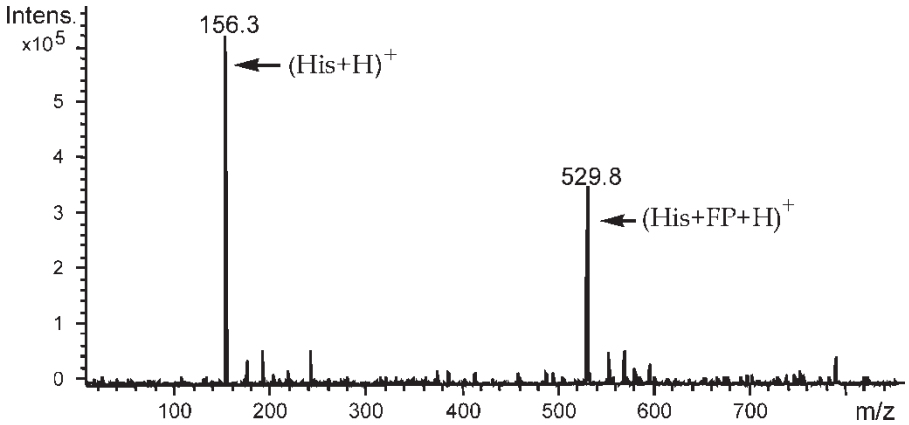

FIGURE 10 Electrospray mass spectrum of His–FP.

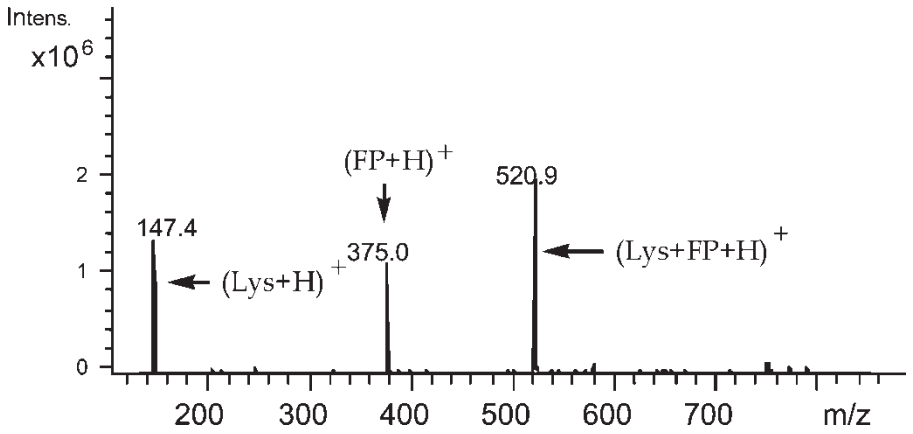

FIGURE 11 Electrospray mass spectrum of Lys–FP.

interactions can be influenced not only by the primary structure but also by the secondary, tertiary and quaternary structures of the proteins. Conformation is therefore another important factor affecting the formation of non-covalent complexes and contributes to the specificity of such interactions. Moreover, because of the high flexibility of proteins, dynamic changes in conformation may enable them to adopt a steric complementarity involving creation of additional van der Waals bonds. However, because of the aqueous medium used and the speed of attainment of equilibrium between free and bound molecules [19], an evaluation of van der Waals bonds is particularly difficult.

In the gas phase the external medium (vacuum) is widely considered to be hydrophobic. This reinforces the strength of the hydrogen bonds compared to hydrophobic and van der Waals interactions [20]. Therefore, caution is required in any structural analogy between gas-phase and solution complexes: this is part of a general debate on the mass spectrometry of non-covalent complexes [21]. To elucidate this problem further, the binding reaction between lysozyme and FP in an aqueous phase was studied in detail using fluorescence spectrometry (Fig. 13) as shown below. When FP concentration was increased, the emission peak of lysozyme decreased in each case and at the same time the maximum emission wavelength increased by about 10 nm (Fig. 13). This result indicates that an interaction between FP and lysozyme did occur in the liquid phase. This experiment demonstrated that as the temperature is increased, the slope of the quenching curves of lysozyme in the presence of different amounts of FP increases. It can be concluded that the binding power of FP with lysozyme is a static quenching process. Although the data do not imply that the protein conformation in solution is preserved in the gas phase, the results suggest that non-covalent associations of proteins and cofactors in solution can be preserved into the gas phase and can be observed by mass spectrometry.

In conclusion, FP can form non-covalent complexes with the various proteins mentioned in this paper and indeed showed stronger binding affinities with proteins than with flavonoids. The protonated basic amino acid residues of the peptide may form binding sites and contributed to the survival of these FP–protein complexes in the gas phase. This suggests that phosphate esters of flavonoids can enhance the interaction with proteins. Thus, ESI-MS provides useful information about phosphate ester-protein interactions. Moreover, the rapidity of data acquisition and the relative simplicity of interpretation make ESI-MS a powerful tool for screening numerous small molecule–protein complexes.

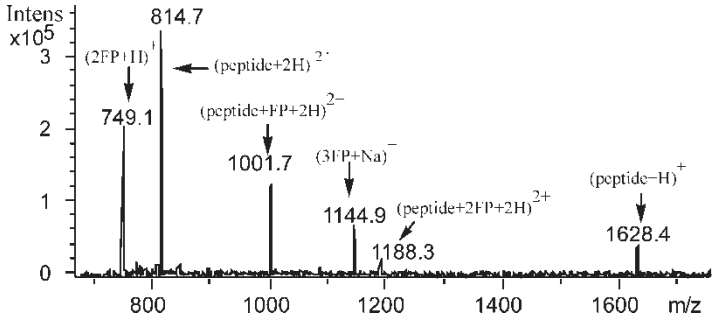

FIGURE 12 Electrospray mass spectrum of peptide–FP.

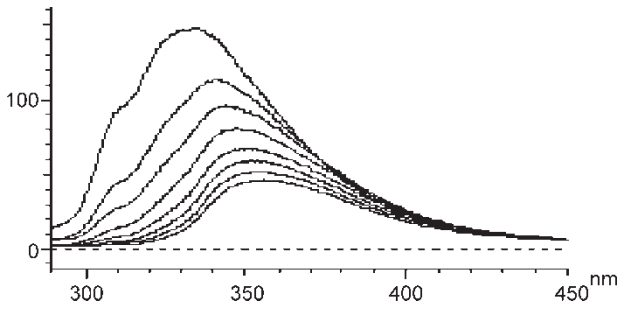

FIGURE 13 Fluorescence emission spectrum of FP–lysozyme for different amounts of FP.

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