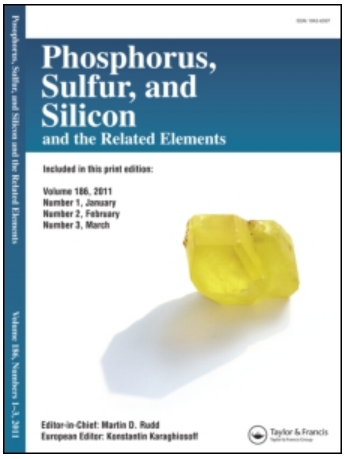

Phosphorus, Sulfur, and Silicon and the Related Elements

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713618290

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To cite this Article Chen, Xiaolan , Shi, Xiaona , Qu, Lingbo , Yuan, Jinwei and Zhao, Yufen(2008) 'ESI Investigation of Non-Covalent Complexes between Phosphorylated Daidzein Derivatives and Insulin', Phosphorus, Sulfur, and Silicon and the Related Elements, 183: 2, 527 — 537
To link to this Article: DOI: 10.1080/10426500701761763
URL: http://dx.doi.org/10.1080/10426500701761763

Phosphorus, Sulfur, and Silicon, 183:527–537, 2008

ISSN: 1042-6507 print / 1563-5325 online
DOI: 10.1080/10426500701761763

ESI Investigation of Non-Covalent Complexes between Phosphorylated Daidzein Derivatives and Insulin

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Since the end of the last century, ESI-MS has begun to be used to reveal the existence of non-covalent complexes, providing important stoichiometric information. Many researchers have reported the use of ESI-MS to determine dissociation constants of such complexes. Gas phase dissociation constants measured in this way have been found to correlate well with those measured by solution based techniques.[1,2] *In this article, ESI results show that all the phosphorylated daidzein derivatives can form non-covalent complexes with the protein insulin, while non-covalent complexes were not detected in solutions of unphosphorylated daidzein and insulin. The relative affinity of each non-covalent complex was obtained according to its different decomposition orifice voltage. Compound* ***e*** *has the highest disappearing orifice voltage and therefore the strongest binding affinity. The relative stability of the non-covalent complexes was closely associated with the length of the hydrophobic chain.*

Keywords Daidzein; ESI-MS; insulin; non-covalent complex; phosphorylation

INTRODUCTION

Cellular functions are often triggered by weak non-covalent enzyme-substrate, protein-ligand, protein-protein or antibody-antigen.[3] In

The authors thank the National Natural Science Foundation of China (No. 20472076 and 20175026) and Henan Academic Foundation of Science and Technology for their financial support.

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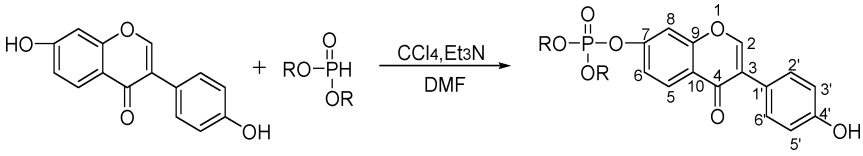
recent years, isoflavonoids have attracted increasing interest due to their various beneficial pharmacological effects. Daidzein (7, 4′-dihydroxyisoflavone) is one of the most abundant isoflavonoids, found in legumes, especially soybeans. It has been implicated in the prevention of cardiovascular disease,[4] lessening the symptoms of the menopause[5] and protection against osteoporosis.[6] It was also shown to inhibit proliferation of different cancer derived cell lines in culture and reduced the multiplicity, but not incidence, of mammary tumors in rats.[7–8] Our previous researches indicated that the phosphorylated flavonoid derivatives possess relatively strong affinities, form non-covalent complexes with the proteins more easily than the non-phosphorylated compounds.[9–10] Preliminary biological activity screening tests also indicated that the phosphorylated flavone derivatives indeed had stronger activity against Hela tumor cells than the corresponding flavones in vitro.[11] Five solutions of the phosphorylated daidzein derivatives were mixed with the solution of insulin, which were then injected individually into an ion trap mass spectrometer. The results show that the phosphorylated daidzein derivatives could form non-covalent complexes with insulin. Through modulating the orifice voltage the covalent complexes could be decomposed, different phosphorylated daidzein derivative has different decomposition orifice voltage due to its different affinity. Therefore, the stability of different non-covalent complex can be obtained by measuring their decomposition orifice voltage.

EXPERIMENTAL



a R= CH_3 **b** R= CH_2CH_3 **c** R= $CH_2CH_2CH_3$

d R= $CH(CH_3)_2$ **e** R= $CH_2CH_2CH_2CH_3$

SCHEME 1

These five phosphorlated daidzein derivatives were synthesized by our laboratory (Scheme 1), and their structures were determined by X-ray, IR, NMR, and ESI-MS.

Mass spectrometric conditions: Solutions of the complexes were analyzed on a Bruker-Esquire 3000 mass spectrometer fitted with an ion spray source working in the positive ion mode. Nitrogen was used as drying gas at a flow rate of 4 μ L/min. The nebulizer pressure was 110.3 kPa and the dry gas flow rate was 6.5 L/min. The capillary was typically held at 4 kV. Nine spectra were averaged, and the rolling average was 20. The ICC was set at 40,000.

RESULTS AND DISCUSSION

The development of electrospray ionization and the discovery that highly charged ions of proteins are readily formed have led to dramatic growth in the application of mass spectrometry to biomolecules. 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.[12–13] ESI-MS has served as a powerful tool in providing evidence in support of the existence of non-covalently associated macromolecular complexes in the gas phase. For many years, ESI-MS has been used to reveal the existence of non-covalent complexes, providing important stoichiometric information. Some examples of non-covalent complexes studied by ESI-MS are protein-ligand,[14,15,16] protein-metal,[17,18] protein-protein,[19,20] and protein-sugar[21] complexes. An excellent review concerning the use of mass spectrometry for analyzing non-covalent interactions has been published by Loo.[22] Many researchers have reported the use of ESI-MS to determine dissociation constants of such complexes. Gas phase dissociation constants measured in this way have been found to correlate well with those measured by solution based techniques.[1,2]

Detection of non-covalent complexes by ESI requires that the complex tolerate the interface conditions (i.e., heat, collisional activation due to electrical voltage) during the desolvation/desorption processes. In the work described in this article, the solution of different phosphorylated daidzein derivative and insulin was first injected in suitable orifice voltages and capillary temperature, and then the orifice voltage was increased gradually until the peak of the phosphorylated daidzein-insulin complex disappeared. Relative affinity of every non-covalent complex was obtained according to its different decomposition orifice voltage. The stability of different no-covalent complex was therefore compared.

The mass spectra of phosphorylated daidzein derivative-insulin noncovalent complexes are shown in Figure 2 and the decomposition orifice voltages of the corresponding complexes in Table I.

Figure 1 shows an ESI mass spectrum obtained from a neutral solution of insulin. The charge-state distribution comprises four charged

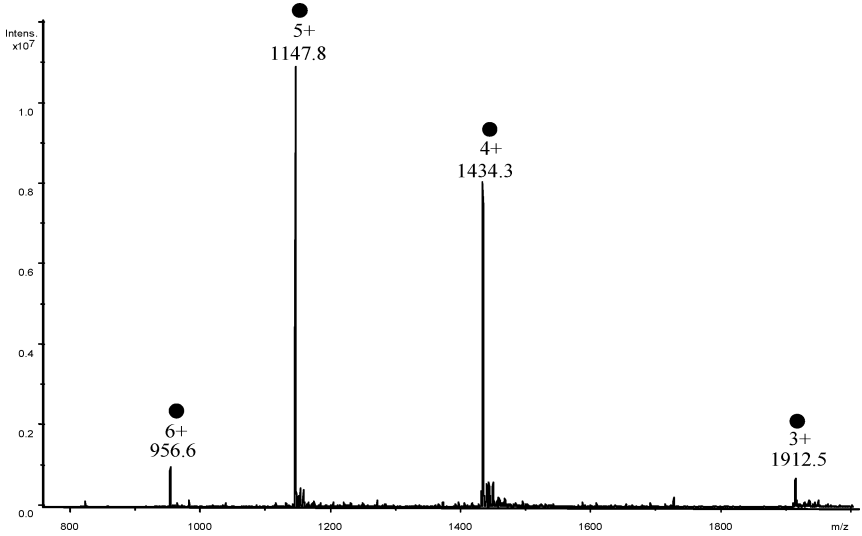


FIGURE 1 Electrospray ionzation mass spectrum of insulin. The solution was prepared by mixing equal volumes of methanol and 0.04 mmol · L^{-1} insulin. The orifice voltage was set at 113.0 V. The source temperature was maintained at 250°C. ● means multiply charged ion peaks of insulin.

states ranging from 6+ to 3+, with 5+ the most intense. The average molecular mass measured from these peaks is 5734 Da.

Figure 2a shows an ESI mass spectrum obtained from a mixed solution of insulin and compound **a**. Besides the expected mulitiply protonated molecule ions, a mass spectrum revealed several groups of new protonated ions, corresponding to several kinds of highly charged multiple adducts, respectively, for example, ions at *m/z*: 1220 and 1296, corresponding to (insulin +a +5H)$^{5+}$, (insulin +2a +5H)$^{5+}$.

Figure 2b shows an ESI mass spectrum obtained from a mixed solution of insulin and compound **b**. The mass spectrum also revealed several groups of new protonated ions, corresponding to several kinds of highly charged multiple adducts, respectively, for example, there is a set of ions at *m/z* 1226, 1304, 1382, 1460, 1532, corresponding to (insulin +b +5H)$^{5+}$, (insulin +2b +5H)$^{5+}$, (insulin +3b +5H)$^{5+}$, (insulin +4b +5H)$^{5+}$, (insulin +b +4H)$^{4+}$.

Figure 2c shows an ESI mass spectrum obtained from a mixed solution of insulin and compound **c**. The mass spectrum also revealed several groups of new protonated ions, corresponding to several kinds of highly charged multiple adducts, respectively, for example, there is a set of ions at *m/z* 1231, 1315, 1398, 1482, 1566, 1539, corresponding to

$(\text{insulin} + \text{c} + 5\text{H})^{5+}$, $(\text{insulin} + 2\text{c} + 5\text{H})^{5+}$, $(\text{insulin} + 3\text{c} + 5\text{H})^{5+}$, $(\text{insulin} + 4\text{c} + 5\text{H})^{5+}$, $(\text{insulin} + 5\text{c} + 5\text{H})^{5+}$, $(\text{insulin} + \text{c} + 4\text{H})^{4+}$.

Figure 2d shows an ESI mass spectrum obtained from a mixed solution of insulin and compound **d**. The mass spectrum also revealed several groups of new protonated ions, corresponding to several kinds of highly charged multiple adducts, respectively, for example, there is

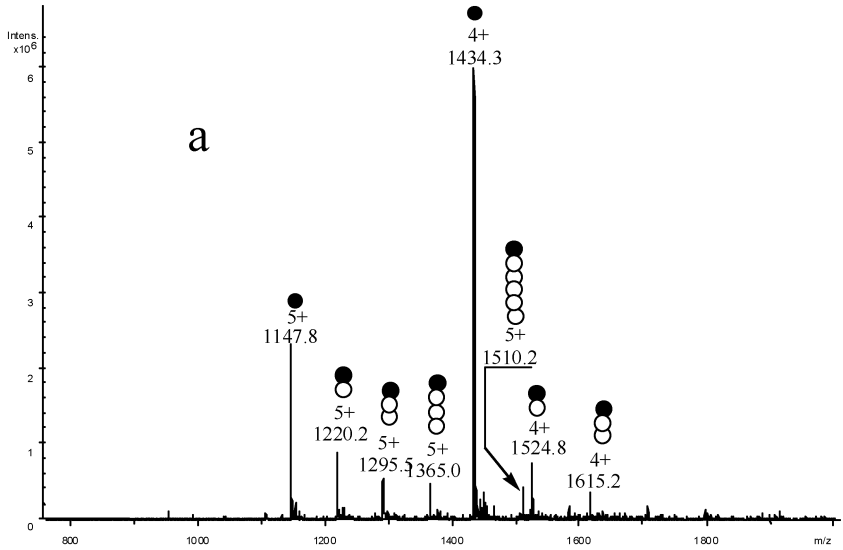


FIGURE 2 (a) ESI mass spectrum of insulin with pa. The solution was prepared by mixing equal volumes of 0.04 m mol · L^{-1} inlulin and 0.4 m mol · L^{-1} methanol solution of compound a. (b) ESI mass spectrum of insulin with compound b. The solution was prepared by mixing equal volumes of 0.04 m mol · L^{-1} insulin and 0.4 m mol · L^{-1} methanol solution of compound b. (c) ESI mass spectrum of insulin with compound c. The solution was prepared by mixing equal volumes of 0.04 m mol · L^{-1} insulin and 0.4 m mol · L^{-1} methanol solution of compound c. (d) ESI mass spectrum of insulin with compound d. The solution was prepared by mixing equal volumes of 0.04 m mol · L^{-1} insulin and 0.4 m mol · L^{-1} methanol solution of compound d. (e) ESI mass spectrum of insulin with compound e. The solution was prepared by mixing equal volumes of 0.04 m mol · L^{-1} insulin and 0.4 m mol · L^{-1}M methanol solution of compound e. The orifice voltage was set at 113.0 V. The source temperature was maintained at 250°C. ● means multiply charged ion peaks of insulin. ●○ means multiply charged ion peaks of insulin-phosphorylated isoflavonoid (PF) complex; ●○○ means multiply charged ion peaks of insulin-2PF complex; ●○○○ means multiply charged ion peaks of insulin-3PF complex; ●○○○○ means multiply charged ion peaks of insulin-4PF complex, ●○○○○○ means multiply charged ion peaks of insulin-5PF complex; and ●○○○○○○ means multiply charged ion peaks of insulin-6PF complex. *(Continued)*

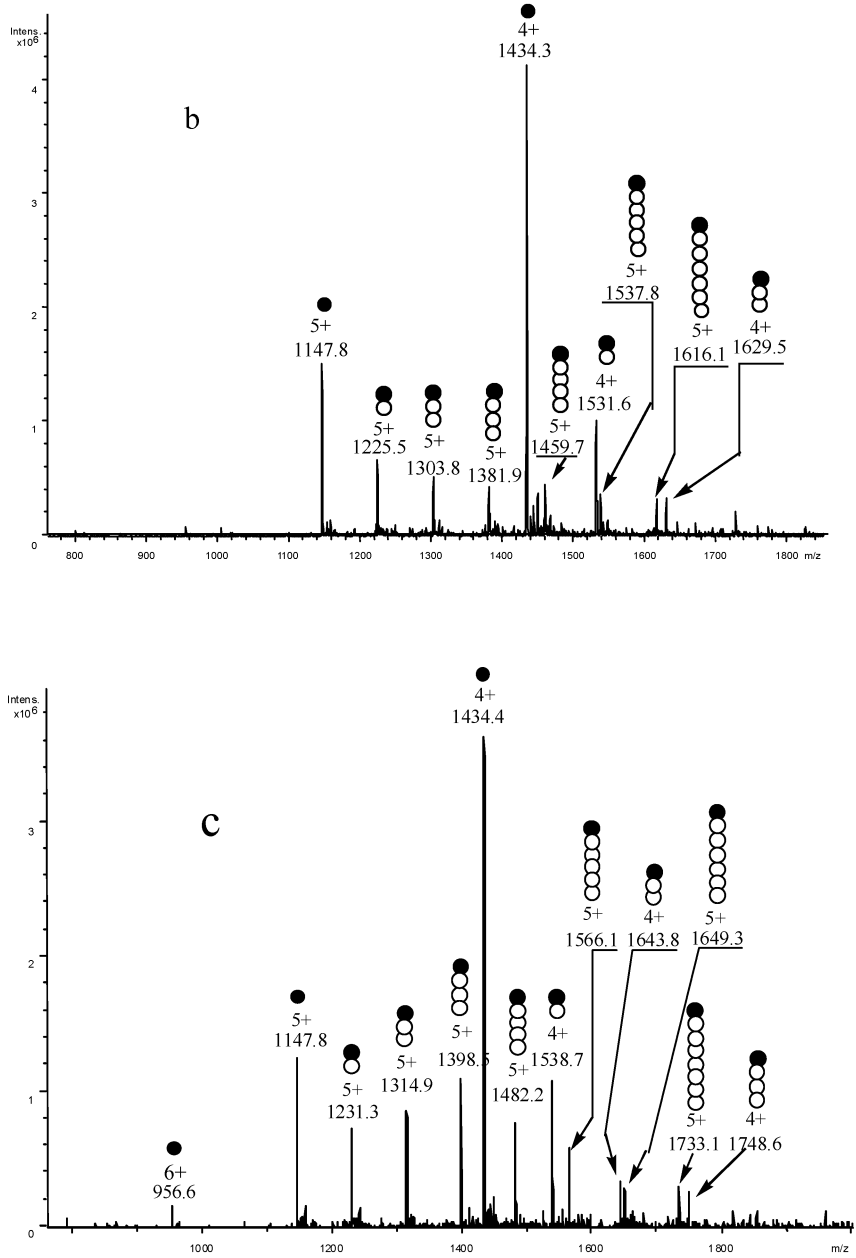


FIGURE 2 (Continued).

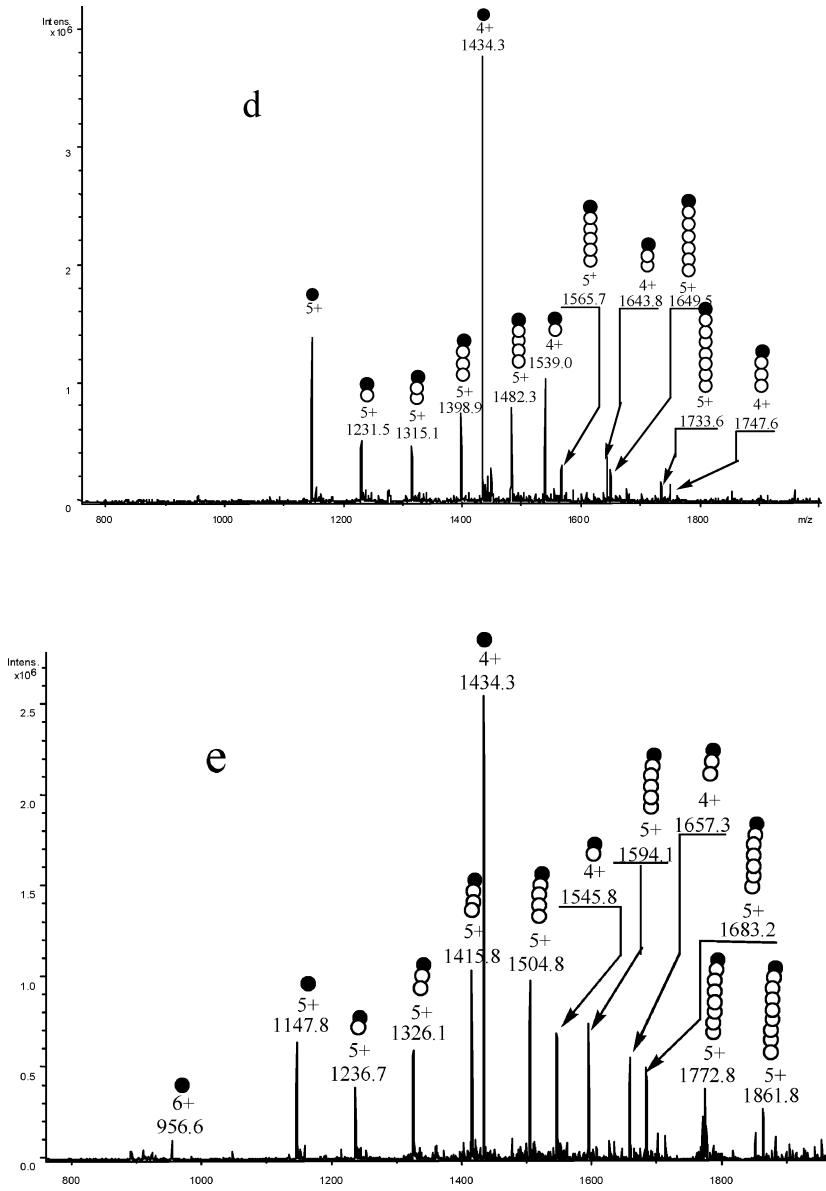


FIGURE 2 (Continued).

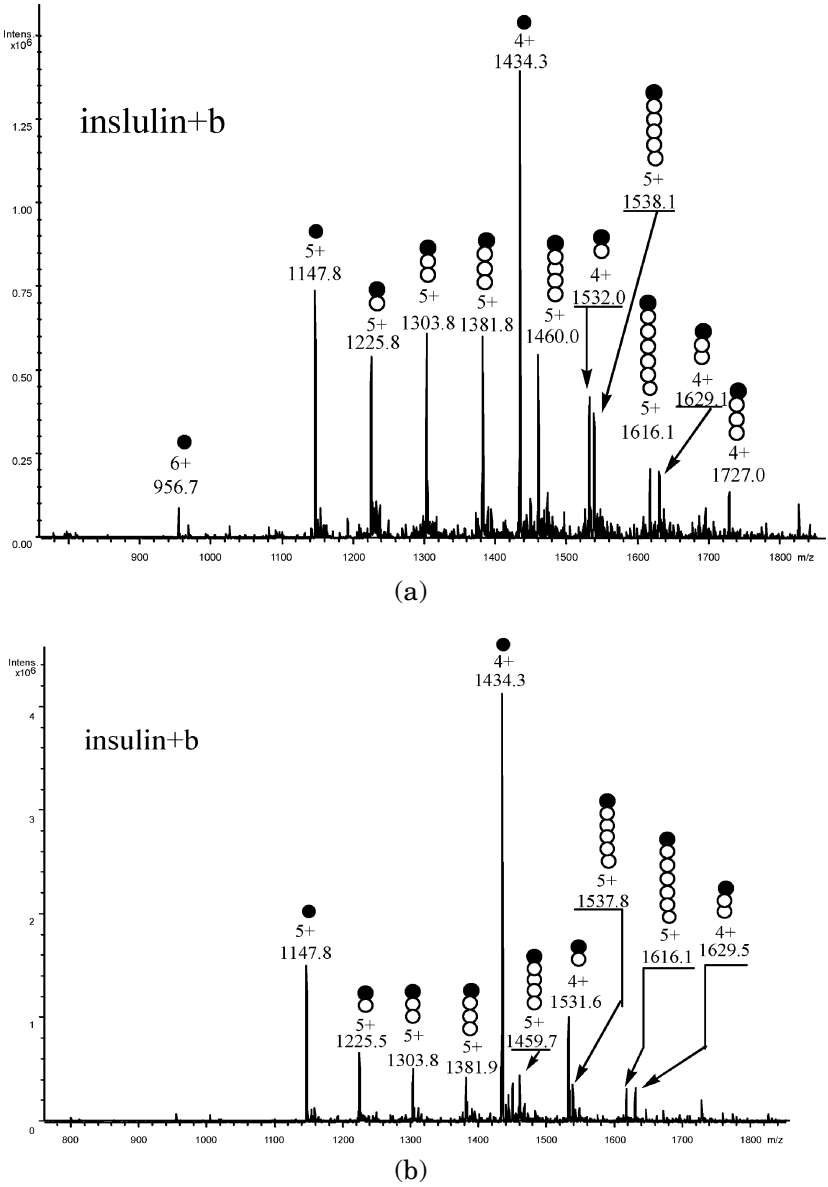


FIGURE 3 (a) ESI mass spectrum of insulin with $_{P}$b. The orifice voltage was set at 105.0 V; (b) ESI mass spectrum of insulin with $_{P}$b. The orifice voltage was set at 113.0 V; and (c) ESI mass spectrum of insulin with $_{P}$b. The orifice voltage was set at 120.4 V.

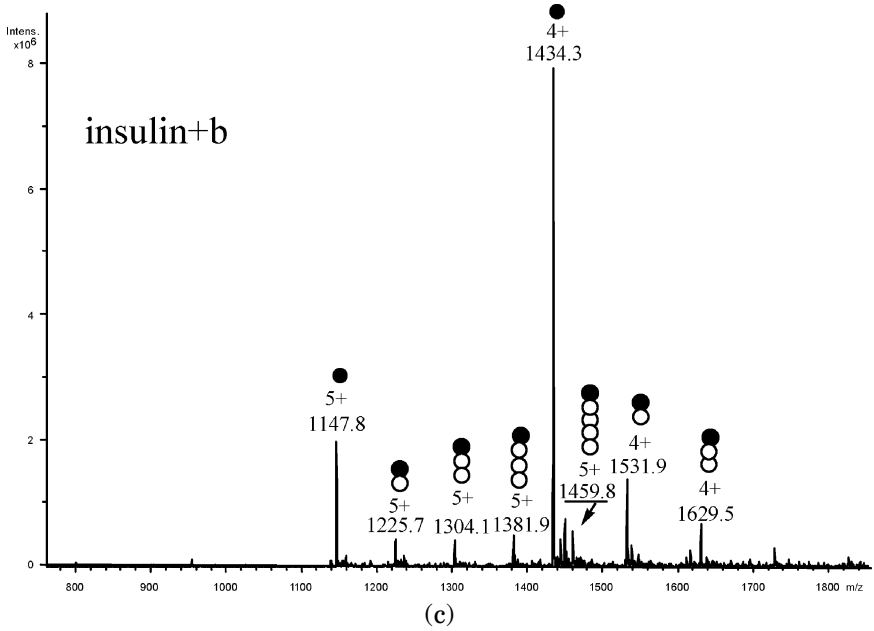


FIGURE 3 (Continued).

a set of ions at m/z 1232, 1315, 1399, 1539, corresponding to (insulin +d +5H)$^{5+}$, (insulin +2d +5H)$^{5+}$, (insulin +3d +5H)$^{5+}$, (insulin +d +4H)$^{4+}$.

Figure 2e shows an ESI mass spectrum obtained from a mixed solution of insulin and compound **e**. The mass spectrum also revealed several groups of new protonated ions, corresponding to several kinds of highly charged multiple adducts, respectively, for example, there is a set of ions at m/z 1237, 1326 and 1539, corresponding to (insulin +e +5H)$^{5+}$, (insulin +2e +5H)$^{5+}$, (insulin +e +4H)$^{4+}$.

The experiment described above shows that phosphorylated daidzein derivative could interact with the protein insulin in the gas phase. Even though different parameters were tried, no corresponding non-covalent protein-daidzein complexes were detected. The experiment described herein suggests that the phosphorylated daidzein derivatives could form non-covalent complexes with insulin more easily than the unphosphorylated daidzein. The phosphate group of the phosphorylated daidzein played important role in the processes of phosphorylated daidzein-protein interactions.[10,23]

From the Figure 3, it is clear that the intensity of the non-covalent complexes was reducing when the orifice voltage was increased. The

TABLE I The Orifice Voltage of the Non-Covalent Complexes Disappearing

Compound	**a**	**b**	**c**	**d**	**e**
Orifice voltage/V	133.8	146.0	151.8	157.4	162.9

interaction between the phosphorylated daidzein derivatives and insulin was destroyed when the orifice voltage was increased. If the non-covalent complexes were disappeared at higher orifice voltage, the binding affinity is stronger. We found that different phosphorylated daidzein derivative could tolerance different high orifice voltage. The relative affinity of each non-covalent complex was obtained according to its different decomposition orifice voltage. From the Table I, it is clear that compound **e** has the highest disappearing orifice voltage and therefore the strongest binding affinity. The relative stability of the non-covalent complexes was closely associated with the length of the hydrophobic chain.

CONCLUSION

The phosphorylated daidzein derivatives could form non-covalent complexes with the protein insulin. Different phosphorylated daidzein derivative shows different binding affinity with the same protein. The compound **e** shows stronger binding affinity to insulin than others. The relative stability of the non-covalent complexes was closely associated with the length of the hydrophobic chain.

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