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Comparative Studies of the Binding of Six Phthalate Plasticizers to Pepsin by Multispectroscopic Approach and Molecular Modeling

Yan-Qing Wang* and Hong-Mei Zhang

Jiangsu Provincial Key Laboratory of Coastal Wetland Bioresources and Environmental Protection Yancheng City, Jiangsu Province 224002, People's Republic of China

Institute of Applied Chemistry and Environmental Engineering, Yancheng Teachers University, Yancheng City, Jiangsu Province 224002, People's Republic of China

ABSTRACT: To explore the binding mechanism of phthalate plasticizers with digestive proteases, their effects on conformation and activity of pepsin by multispectroscopic approach and molecular modeling were investigated. Fluorescence spectra combined with UV−vis and circular dichroism (CD) spectra measurements indicated that the six phthalate plasticizers induced the changes of tertiary and secondary structure of pepsin. The solvent polarity of environment around both Trp and Tyr residues on pepsin were affected by phthalate plasticizers. By analyzing the fluorescence quenching and theoretical calculation data, it was concluded that a binding site exists for each phthalate plasticizer in pepsin with different binding ability. The hydrophobic, hydrogen bonding, and $\pi-\pi$ stacking interactions were involved in the interactions between pepsin and phthalate plasticizers. Moreover, the activity assay indicated that phthalate plasticizers were not powerfully inhibitors or activators for pepsin. These studies demonstrated that phthalate plasticizers could cause some negative effects on pepsin. The present studies may provide a way to analyze the biological safety of phthalate plasticizers on digestive proteases or other proteins.

KEYWORDS: *phthalate plasticizers, pepsin, spectroscopy, binding mechanism, conformation*

INTRODUCTION

As a class of industrial chemicals, phthalate plasticizers are widely used in consumer products such as building materials, food packaging, mouthing toys, children's articles, and medical devices that are made of polyvinyl chloride (PVC).[1,2] For example, diethylhexyl phthalate (DEHP) and diisonnonyl phthalate (DINP) constitute about 80% of phthalate production in plastic products, especially including food and beverage packaging and all PVC medical and surgical products.[3] Because phthalates are additives and not covalently bound to the plastic matrix of PVC, they are able to leach from PVC and enter into the environment.[4,5]

Therefore, concerns have been raised over the effects of phthalate exposure from PVC materials. For example, Fierens et al. have studied the effect of cooking at home on the levels of eight phthalates in foods. The results have shown that DEHP was the most abundant phthalate compound followed by diisobutyl phthalate (DiBP) and benzylbutyl phthalate (BBP).[6] Staden et al. have reviewed some effects of toxic phthalates used in plastic-packaged commercial herbal products.[7] Currently, there are limited studies that quantify the danger of phthalates to humans, but the binding interaction mechanism between phthalate and serum albumin have been studied in order to analyze the detailed physicochemical character of phthalate binding to serum albumin.[8,9]

Nowadays, human are exposed to thousands of different chemicals each day.[6] Pthalates are endocrine-disrupting chemicals, and they migrate from food contact materials into foods during processing or bioaccumulation and transfer through the food chain.[10,11] When phthalates enter the human stomach, the digestive proteases may be the indirect binding targets. However, little is known about the interaction of phthalates with digestive proteases and the effects of these phthalates on the activity of proteases. As one of digestive proteases, pepsin is released by the chief cells in the stomach to degrade food proteins into peptides by cleaving peptide bonds between hydrophobic and preferably aromatic amino acids.[12] Because pepsin plays an essential role in digestion deconstruction of food, it is often used as an important model of the digestive proteases to investigate the interactions of small molecule with proteins.[13−15] In this report, we selected six phthalate plasticizers as the investigated models to study the interactions of them with pepsin. The molecular structure of dimethyl phthalate (DMP), dibutyl phosphate (DBP), dinoctyl phthalate (DOP), DINP, dicyclohexyl phthalate (DCHP), and DEHP were shown in Figure 1. The activity and conformation of pepsin and the binding mechanism of above six phthalate plasticizers with pepsin were investigated by means of spectroscopic techniques and molecular modeling method in order to elucidate the effect of phthalates on the pepsin activity and their relationships to human health.

MATERIALS AND METHODS

Materials. Pepsin (from porcine stomach mucosa, Purified Enzymes, ≥3000 NFU/mg) and bovine hemoglobin (from bovine erythrocyte, SDS-PAGE purity, ≥95%) were purchased from Sangon Company (Shanghai, China) and used without further purification. The analysis standards of DMP, DBP, DOP, DINP, DCHP, and DEHP were obtained from Aladdin Industrial Corporation. The other chemical reagents such as citric acid, trichloroacetic acid, H_3PO_4, NaH_2PO_4, NaCl, etc. were all of analytical purity. The DMP (0.0125 M), DBP (0.0125 M), DOP (0.0125 M), DINP (0.0125 M), DCHP (0.0125 M),

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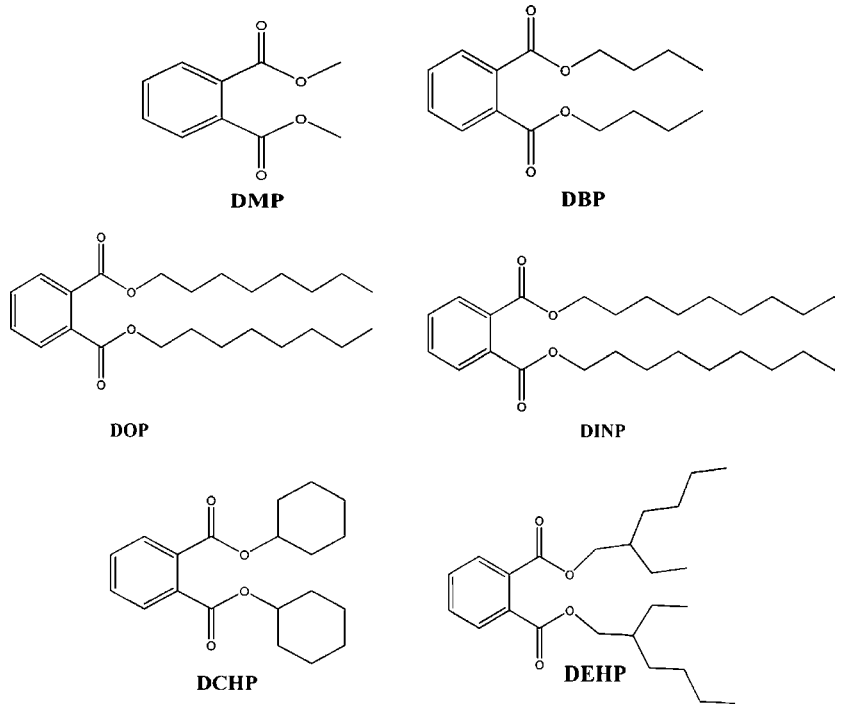

Figure 1. Molecular structure of DMP, DBP, DOP, DINP, DCHP, and DEHP.

and DEHP (0.0125 M) solutions were prepared in methanol. Pepsin solution (10 μM) was prepared in pH 2.00 citric acid buffer solution (0.025 M, 0.1 M NaCl). The 0.5 wt % solution of bovine hemoglobin was prepared in pH 2.00 citric acid buffer solution. The muriatic acid (reagent grade, 37%) was used to adjust pH of buffer solution. The 10% of trichloroacetic acid was prepared in water. During experiments, water was purified with a Millli-Q purification system.

Spectroscopic Measurements. *UV−Vis Absorption Spectroscopy.* The UV−vis spectra of pepsin (10.0 μM) in absence and presence of DMP, DBP, DOP, DINP, DCHP, or DEHP were recorded at 298 K on a SPECORD 50 (Jena, Germany) equipped with 1.0 cm quartz cells. The range of wavelength was from 250 to 400 nm.

Circular Dichroism Spectroscopy. The CD spectra of pepsin (10.0 μM) in absence and presence of DMP, DBP, DOP, DINP, DCHP, or DEHP were made at 298 K on a Chirascan spectrometer (Applied Photophyysics Ltd., Leatherhead, Surrey, U.K.) equipped with a temperature control quantum. The cell path length of 1 mm was used with the spectra range 190−250 nm. The scan speed was set at 20 nm/min with a bandwidth of 1 nm.

Fluorescence Spectroscopy. All fluorescence spectra of pepsin were recorded on LS−50B Spectrofluorimeter (Waltham, Massachusetts, U.S.A.) equipped with 1.0 cm quartz cells and a thermostat bath. Fluorescence emission spectra were recorded over a wavelength range of 300−500 nm at an excitation wavelength of 280 nm, the scan speed was set at 500 nm/min and the slit widths of emission and excitation were set at 5.0 nm and 5.0 nm. The wavelength interval $\Delta\lambda$ ($\lambda_{em} - \lambda_{ex}$) were set at 15 and 60 nm to obtain the synchronous fluorescence of pepsin in the same experimental conditions as the fluorescence emission spectra.

Molecular Modeling. The binding models between phthalate plasticizers and pepsin were generated by the molecular docking program Autodock 4.2.3.[16] The molecular structure of pepsin (PDB ID 5PEP) was obtained from RCSB Protein Data Bank, http://www.rcsb.org/pdb.[17,18] All water molecules were removed, and the polar hydrogen and the Gasteiger charges were added at the beginning of the docking study. The geometries of DMP, DBP, DOP, DINP, DCHP, and DEHP were optimized using Density Functional Theory (DFT) B3LYP/6-311^{+2}G(d, p) by Gaussian 03.[19] The lowest unoccupied molecular orbital's energy (E_{LUMO}), the highest occupied molecular orbital's energy (E_{HOMO}), the chemical potential (μ), the chemical harness (η), and the volume of molecule were obtained by analyzing the geometries data.[20] During molecular calculation study, the grid box size of pepsin−phthalate plasticizers systems were 100 Å × 100 Å × 100 Å, with grid spacing of 0.375 Å. The GA population size was set at 150, the maximum number of energy evaluation was set at 2 500 000, and others used were default parameters. Molegro Molecular Viewer software (Molegro-a CLC bio company, Aarhus, Denmark) was selected to analysis the docking conformation with the lowest binding free energy.[21]

Assay of Pepsin Activity. The Ason method was used to detect the pepsin activity.[22] Pepsin (1 mL; 30.0 μM) was mixed with different volumes of 0.008 M DMP, DBP, DOP, DINP, DCHP, or DEHP (0.00 mL, 0.05 mL, 0.10 mL, 0.15 mL, 0.20 mL) in a 10 mL centrifuge tube. Then, the different volumes of buffer solution (2.00 mL, 1.95 mL, 1.90 mL, 1.85 mL, 1.80 mL) were dropped into the pepsin solutions and made a constant volume of 3 mL. The pepsin−phthalate systems were set at 37 °C for 20 min. Then, 2 mL of bovine hemoglobin (0.5 wt %) solution was added in above solutions. After 20 min, 2 mL of 10% trichloroacetic acid was added to terminate the reaction. The supernatant was obtained by centrifuging at 12 000 rpm for 20 min twice and was measured via OD 275 nm. The each experiment was repeated three times in order to take the mean. The original activity of pepsin is 3000 NFU/mg, and the activity of pepsin in presence of DMP, DBP, DOP, DINP, DCHP, or DEHP was obtained by eq 1:

$$\text{Activity (NFU/mg)} = 3000\frac{\text{OD275(pepsin} - \text{phthalate)}}{\text{OD275(original pepsin)}} \quad (1)$$

RESULTS AND DISCUSSION

Molecular Properties of Phthalate Plasticizers. Theoretical calculation of the quantitative structure−activity relationship can be applied for the prediction of biological activity from chemical structure or properties.[20] For example, E_{LUMO} represents the ability to obtain an electron and E_{HOMO} represents the ability to donate an electron. The surface and contour of HOMO and LUMO molecular orbital plots of the optimized structures of various phthalates are shown in Figure 2. Table 1 listed the E_{LUMO}, E_{HOMO}, μ, η, and the volume of molecule for the series of selected phthalates. It can be seen that the charge density was mainly accumulated on the benzene ring,

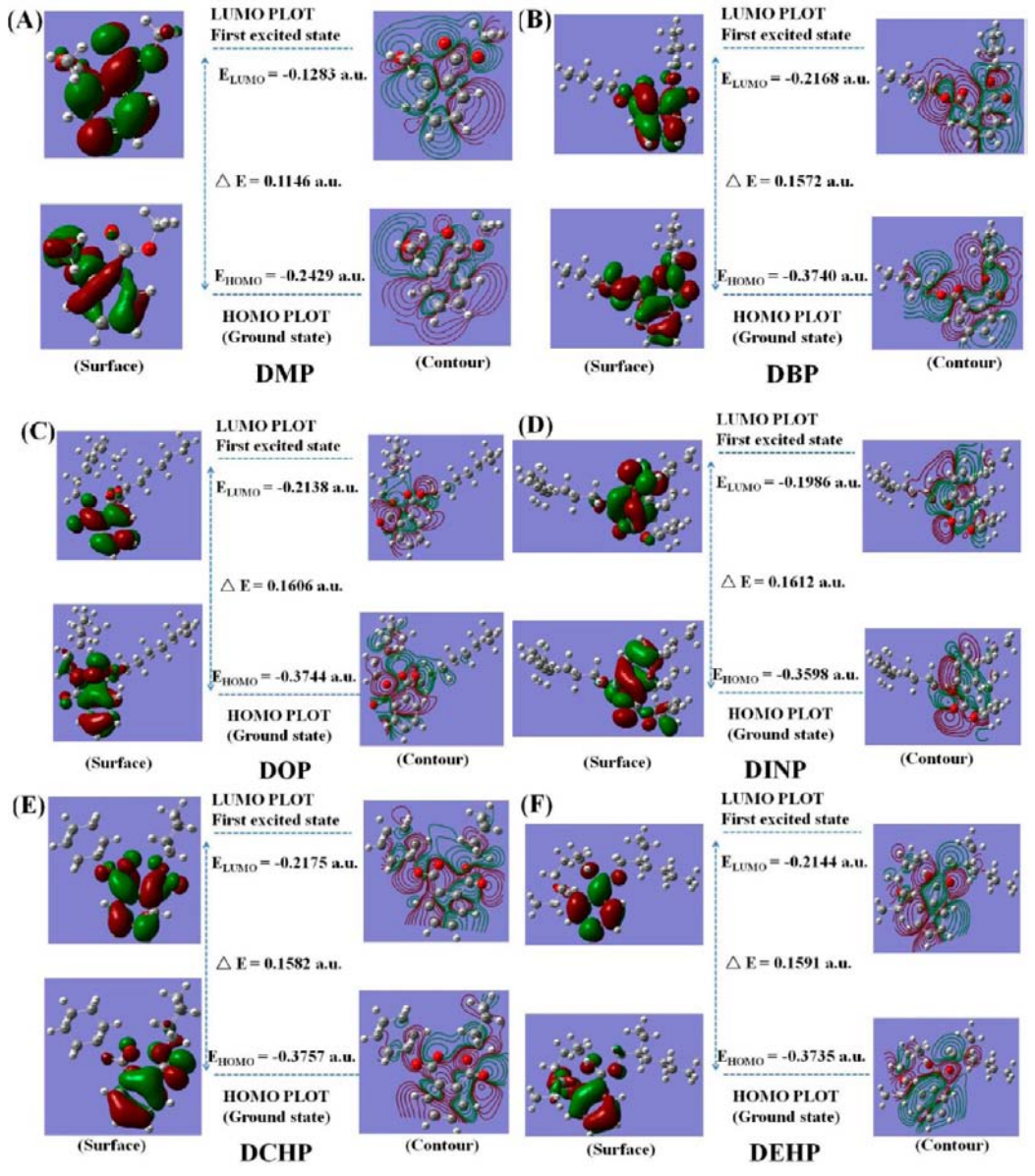

Figure 2. Surface and contour of the molecular orbital plots (HOMO and LUMO) of phthalates (A, DMP; B, DBP; C, DOP; D, DINP; E, DCHP; F, DEHP).

Table 1. Calculated Conceptual Density Functional Reactivity Descriptors in a.u. for the Series of Phthalates

molecule	E_{LUMO} (a.u.)	E_{HOMO} (a.u.)	chemical potential (μ)	chemical hardness (η)	vol. (cm^3/mol)
DMP	−0.1283	−0.2429	−0.1856	0.0573	118.237
DBP	−0.2168	−0.3740	−0.2954	0.0786	253.755
DOP	−0.2138	−0.3744	−0.2941	0.0803	348.613
DINP	−0.1986	−0.3598	−0.2792	0.0806	375.740
DCHP	−0.2175	−0.3756	−0.2965	0.0790	277.921
DEHP	−0.2144	−0.3735	−0.2939	0.0795	306.086

and —C═O in the HOMO and LUMO. However, the difference of the distribution of electric charge between HOMO and LUMO existed. The ester groups of phthalates partly influenced the distribution of electric charge. In cases such as DMP and DCHP, the electric charges distributed evenly over the molecule. However, with the increasing length of carbon chain of ester groups, little electric charges distributed in ester groups. In addition, the molecular volume of phthalates became bigger with the increasing length of carbon chain in ester groups. If the phthalates bind with the amino acid residues of pepsin by charge transfer, the benzene ring and —C═O are the main binding groups. The binding forces may be $\pi-\pi$ sticking or hydrogen bonding. The carbon chain of ester groups and the molecular volume of phthalates could affect the binding interactions of phthalates with pepsin for steric exclusion. In the light of the weak-interactions of phthalates with pepsin, the ester groups may be main contributor of hydrophobic binding force. The theoretical calculation data will be important for analyzing the following experimental results.

Effect of Phthalate Plasticizers on Pepsin Conformation. *UV–Vis Absorption Spectra.* Because pepsin has many aromatic amino acids including five tryptophan (Trp), sixteen tyrosine (Tyr), and fourteen phenylalanine (Phe), it gives an absorption peak at about 278 nm coming from the $\pi-\pi^*$ transition of aromatic amino acids.[23] The change of this absorption peak is often used as a conformational probe to

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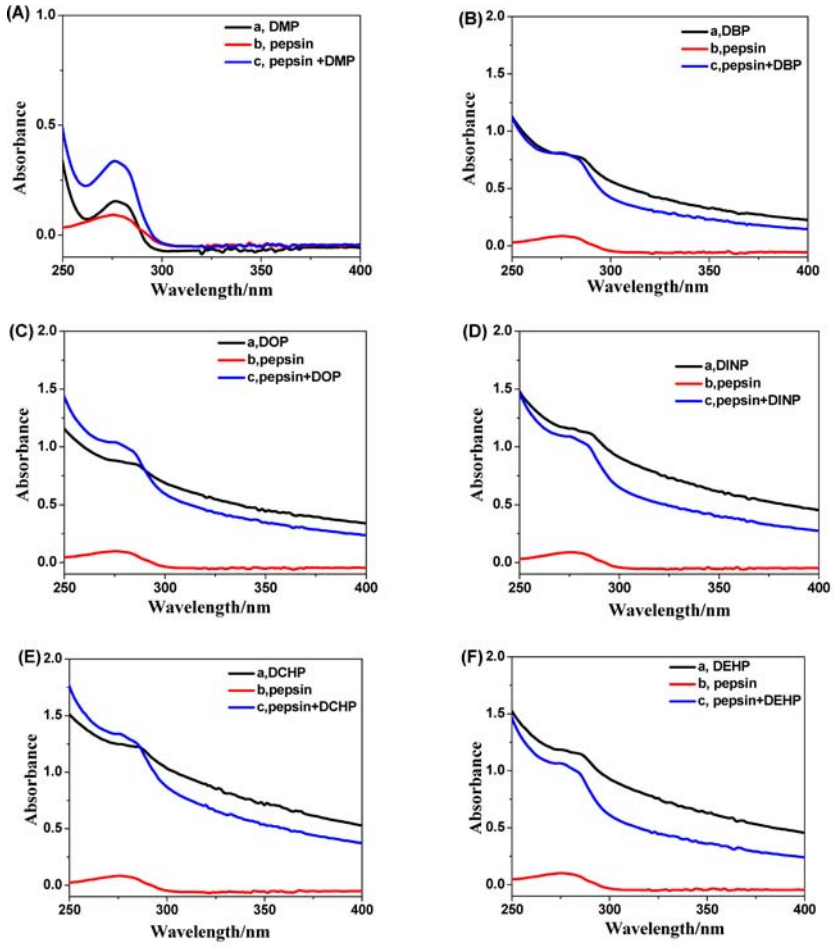

Figure 3. Absorption spectra of phthalate plasticizers, pepsin, and phthalate plasticizer−pepsin systems. (a, the absorption spectrum of phthalate plasticizers only; b, the absorption spectrum of pepsin; c, the absorption spectrum of phthalate plasticizer−pepsin system). *c* (pepsin) = 10.0 μM, *c* (phthalate plasticizers) = 250.0 μM (A, DMP; B, DBP; C, DOP; D, DINP; E, DCHP; F, DEHP).

explore the structure changes of protein. As shown in Figure 3, the intensity of absorption peak at 278 nm increased with addition of phthalate plasticizer and the absorption maximum took a slight blue shift toward lower wavelength region (from 278 to 274 nm). These results implied that the microenvironmental hydrophobicity of the amino acid residues were changed by the binding interactions between pepsin and DMP, DBP, DOP, DINP, DCHP, or DEHP. The conformation of pepsin was also changed.

CD Spectra. As one of well-known biophysical techniques, circular dichroism (CD) spectroscopy is often used to elucidate the secondary structures of proteins in solution.[24] Figure 4 showed the effects of six phthalate plasticizers on the far-UV CD spectra of pepsin. As can be seen from Figure 4, pepsin had a strong negative band at about 200 nm, which indicated that pepsin has significant amount of β-sheet conformation and adopts predominantly disordered structure.[24] With addition of phthalate plasticizers in pepsin solution, the shape of spectra did not change significantly, but the negative molar ellipticity showed decreases. The decreases in molar ellipticity at 200 nm suggested that phthalate plasticizers induced substantial secondary structure of pepsin. Furthermore, the phthalate plasticizers with different ester groups caused varying degrees of molar ellipticity decreases. From DMP to DINP, the degrees of molar ellipticity decreases increased gradually. DEHP induced the strongest decrease compared to other five phthalate plasticizers; the main reason may be that DEHP has two chain branches in ester groups. The different structure of ester groups in phthalate plasticizers not only induce change in molecular volume, but also give them different hydrophobic properties. These different molecular structures could change the ability of inducing substantial secondary structure of pepsin.

Fluorescence Spectra. If the binding interactions of small molecule with protein induced the changes around the aromatic amino acid residues in a protein, the fluorescence emission behavior of the protein are often affected.[25] Since pepsin consists of 5 Trp and 16 Tyr residues that are the major contributions of intrinsic fluorescence of pepsin, the fluorescence emission intensity and spectra properties are used to analysis the tertiary structure of pepsin. It can be obviously seen that the fluorescence intensity of pepsin at 344 nm decreased with an increase in phthalate plasticizers concentration (Figure 5). Moreover, the occurrence of an isoactinic point at about 425 nm indicated the existence of bound and free phthalate plasticizers in the equilibrium of the binding system.[26] In addition, the phthalate plasticizers with different ester groups caused varying degrees of fluorescence quenching. Of the six phthalate plasticizers, DMP induced the smallest degree of fluorescence quenching of pepsin compared to other five phthalate plasticizers because DMP had smallest chemical potential and molecular volume. The fluorescence quenching data also indicated that there were binding interactions between pepsin and phthalate plasticizers. The binding ability of phthalate plasticizers with pepsin were different each other because of their different molecular structure.

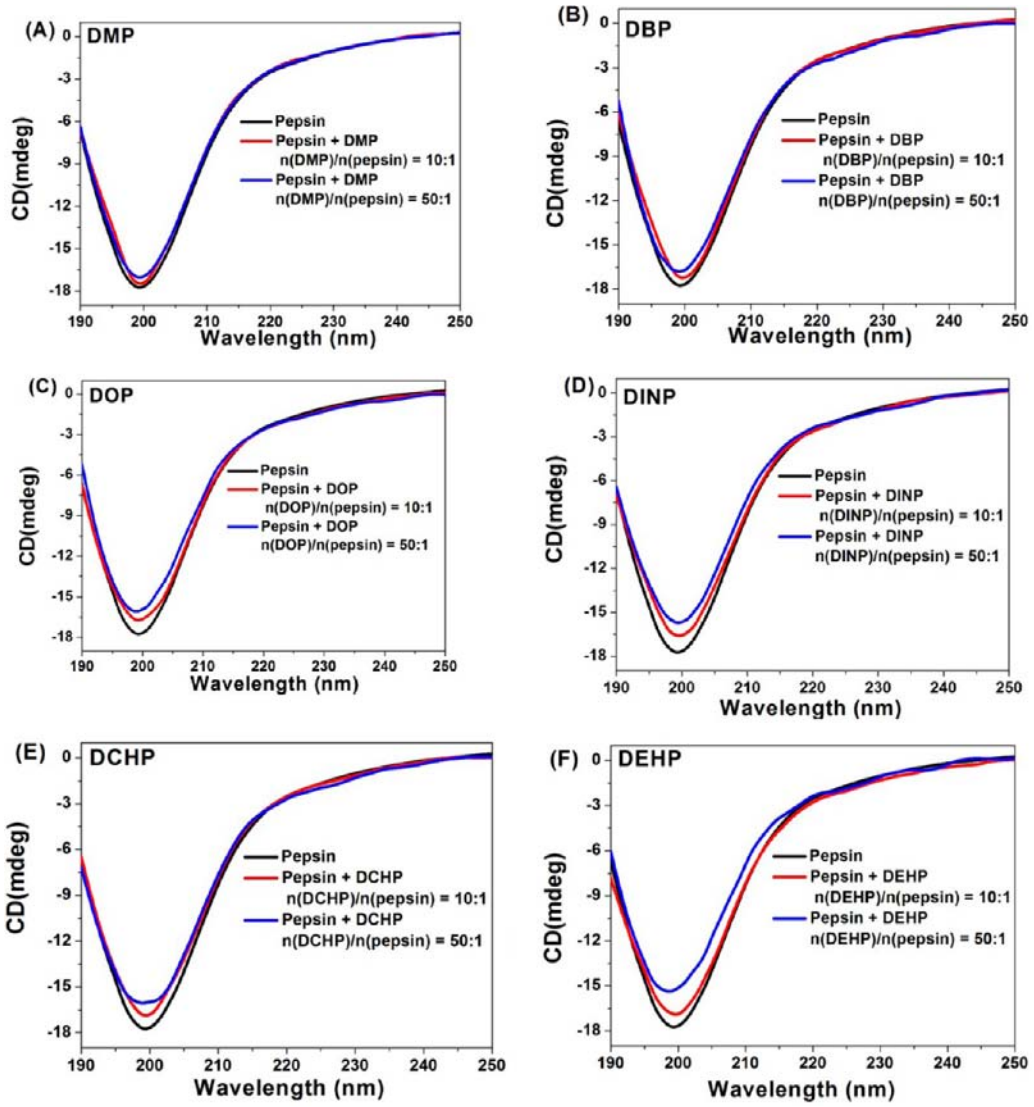

Figure 4. CD spectra of pepsin and pepsin−phthalate plasticizer systems. c (pepsin) = 10.0 μM, (A, DMP; B, DBP; C, DOP; D, DINP; E, DCHP; F, DEHP).

Because the fluorescence emission of pepsin with an excitation wavelength of 280 nm does the combination of Trp and Tyr fluorescence, the synchronous fluorescence measurements are often used to separate the Trp and Tyr fluorescence and to analyze thoroughly the effects of phthalate plasticizers on the molecular microenvironment of pepsin. Figure 6 illustrated the synchronous fluorescence spectra of pepsin treated with six phthalate plasticizers, respectively. Pepsin contains 5 Trp residues (Trp −39, 141, 181, 190, 300), which are located in the β-sheet regions of the protein. As shown in Figure 6(A-2, B-2, C-2, D-2, E-2, F-2), the synchronous fluorescence intensity decreased obviously in presence of phthalate plasticizers. In addition, the λ_{max} of Trp residues in pepsin did shift to longer wavelength (red shift); the observed red shift suggested the possibility of conformational changes of pepsin, which induced decrease in the hydrophobic environment around Trp residues. Except Trp residues, the λ_{max} of Tyr residues in pepsin also shifted to longer wavelength in presence of phthalate plasticizers. These results indicated that the binding interactions of phthalate plasticizers with pepsin induced the changes in the solvent polarity of environment around both Trp and Tyr residues.[27] However, DMP and DBP induced the fluorescence quenching of Tyr residues in pepsin, other four phthalate plasticizers induced the fluorescence increasing of Tyr residues in pepsin.

Binding Nature of Phthalate Plasticizers with Pepsin. *Binding Constants and Binding Sites.* By analyzing the UV−Vis, CD, and fluorescence data, a conclusion was draw that there were existences of binding interactions of phthalate plasticizers with pepsin. In order to analyze thoroughly the binding nature of them, binding constants and binding sites were obtained by calculating the fluorescence quenching data (Figure 5) using eq 2.[28]

$$\log \frac{F_0 - F}{F} = n \log K_A - n \log\left(\frac{1}{[Q_t] - (F_0 - F)[P_t]/F_0}\right) \quad (2)$$

where F_0 and F are the fluorescence intensities in the absence of phthalate plasticizer and the corrected fluorescence intensity according to ref 29 in the presence of phthalate plasticizer, respectively. $[Q_t]$ and $[P_t]$ are the total phthalate plasticizer concentration and the total pepsin concentration, respectively. The plot of $\log(F_0 - F)/F$ versus $\log(1/([Q_t] - (F_0 - F)[P_t]/F_0))$ were shown in Figure 7. The binding constants (K_A) and

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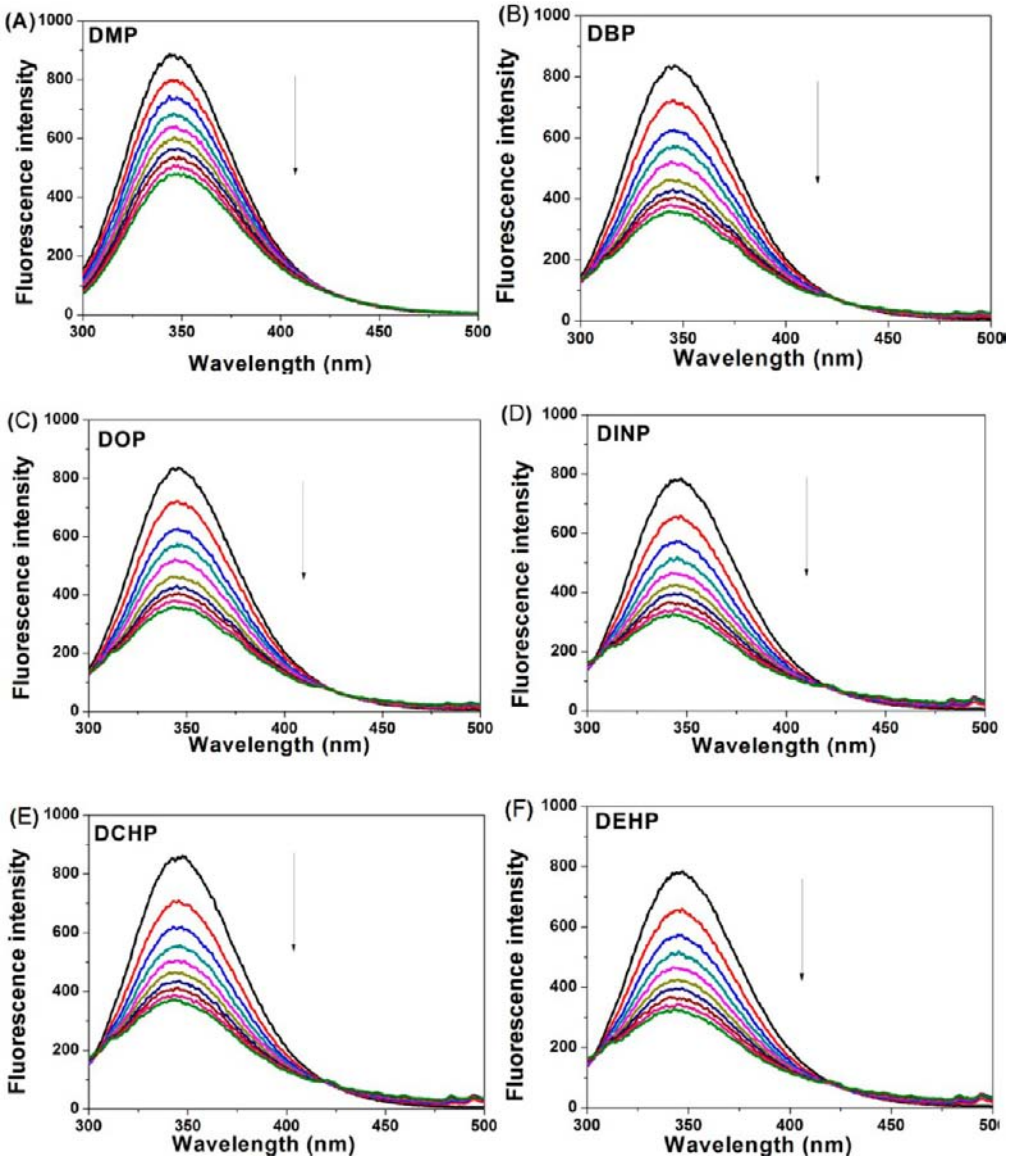

Figure 5. Effect of phthalate plasticizers on fluorescence spectra of pepsin (T = 298 K, λ_{ex} = 280 nm), c (pepsin) = 10.0 μM; c (phthalate plasticizers) (from top to bottom) = 0.0 μM, 50.0 μM, 100.0 μM, 150.0 μM, 200.0 μM, 250.0 μM, 300.0 μM, 350.0 μM, 400.0 μM, 450.0 μM, (A, DMP; B, DBP; C, DOP; D, DINP; E, DCHP; F, DEHP).

Table 2. Thermodynamic Parameters of the Interactions of Phthalate Plasticizer−Pepsin Systems

phthalate plasticizer	T (K)	K_A (L·mol^{-1})	n	R^a	$\Delta H°$ (kJ·mol^{-1})	$\Delta G°$ (kJ·mol^{-1})	$\Delta S°$ (J·mol^{-1}·K^{-1})
DMP	298	2.01×10^3	0.94	0.9983	−2.86	−18.84	53.63
	310	1.92×10^3	0.99	0.9967		−19.49	
DBP	298	3.05×10^3	0.96	0.9976	−9.88	−19.88	33.54
	310	2.62×10^3	0.99	0.9967		−20.28	
DOP	298	3.16×10^3	0.97	0.9975	−9.45	−19.97	35.31
	310	2.73×10^3	0.93	0.9980		−20.39	
DINP	298	3.36×10^3	0.89	0.9987	−11.10	−20.12	30.27
	310	2.83×10^3	0.88	0.9985		−20.48	
DCHP	298	2.25×10^3	0.84	0.9956	2.31	−19.12	71.95
	310	3.04×10^3	0.88	0.9957		−20.67	
DEHP	298	3.35×10^3	0.89	0.9987	−4.76	−20.12	51.54
	310	3.11×10^3	0.90	0.9988		−20.72	

[a]The correlation coefficient.

binding sites (n) can be obtained from the slope and intercept of the plots in Figure 7. The calculated results were showed in Table 2. It can be seen that the K_A values at 298 K increased in the order, K_A (DMP) < K_A (DCHP) < K_A (DBP) < K_A (DOP) < K_A

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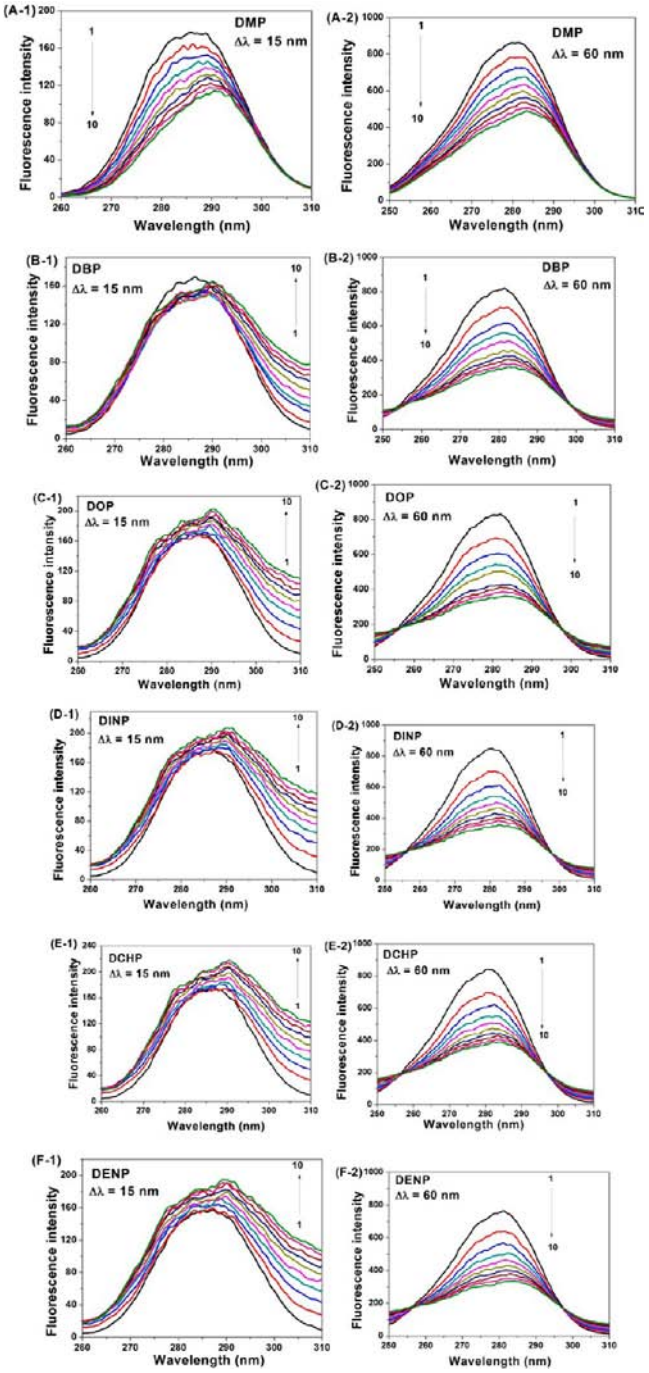

Figure 6. Effect of phthalate plasticizers on synchronous fluorescence spectra of pepsin at different $\Delta\lambda$ values (T = 298 K), c (pepsin) = 10.0 μM; c (phthalate plasticizers) (from 1 to 10) = 0.0 μM, 50.0 μM, 100.0 μM, 150.0 μM, 200.0 μM, 250.0 μM, 300.0 μM, 350.0 μM, 400.0 μM, 450.0 μM, (A, DMP; B, DBP; C, DOP; D, DINP; E, DCHP; F, DEHP).

(DINP) ≈ K_A (DEHP). In the light of structure-binding ability, the binding ability of phthalate plasticizers with pepsin increased with the increase of molecular volume of phthalate plasticizers. From DMP to DINP, the value of K_A obviously increased with the growth of carbon chain of ester groups in phthalate plasticizers. However, this difference did not affect the value of binding site (n). As also shown in Table 2, the values of n indicated the existence of one binding site in pepsin for DMP, DBP, DOP, DINP, DCHP, or DEHP. Compared with the binding interactions of phthalate plasticizers with serum albumin,

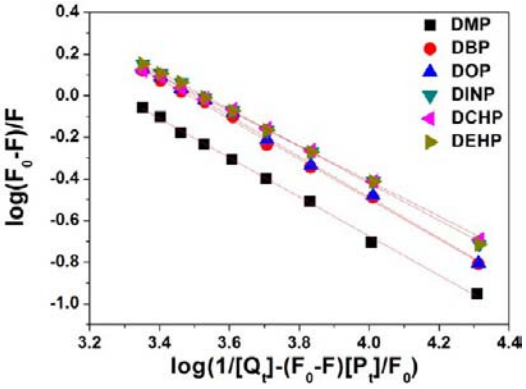

Figure 7. Plots of log $(F_0 - F)/F$ vs log $(1/([Q_t] - (F_0 - F)[P_t]/F_0))$ for phthalate plasticizer−pepsin systems. T = 298 K, pH = 7.40, and λ_{em} = 280 nm, c (pepsin) = 10.0 μM.

the values of K_A of pepsin with phthalate plasticizers were lower than those of serum albumin with phthalate plasticizers.[8,9] The difference of protein structures may result in the difference of ligand binding abilities.

The Binding Forces. The phthalate plasticizers consist of aromatic benzene ring, —C═O, and ester groups, which can bind with the amino acid residues of pepsin by different molecular forces. Such as the $\pi-\pi$ stacking between benzene ring of phthalate plasticizers and aromatic amino acid residues (Trp, Tyr, or Phe), the hydrogen interactions between the —C═O of phthalate plasticizers and pepsin or the hydrophobic interactions may be involved in the binding interactions of phthalate plasticizers with pepsin. In order to obtain the thermodynamic parameters, eqs 3−5 were used to calculate the enthalpy change (ΔH°), the entropy (ΔS°), and the free-energy change (ΔG°):[30,31]

$$\ln \frac{(K_A)_2}{(K_A)_1} = \frac{\Delta H^\circ}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \quad (3)$$

$$\Delta G^\circ = -RT \ln K_A \quad (4)$$

$$\Delta S^\circ = \frac{\Delta H^\circ - \Delta G^\circ}{T} \quad (5)$$

The calculated results are also listed in Table 2. From the table, according to the point of view of Ross and Subramanian, there were many interaction forces involved in the binding process.[32,33] First, the negative values of ΔG° indicated that the binding progress of phthalate plasticizers with pepsin were spontaneous. Second, the positive values of ΔS° were the main contribution of the source of ΔG°, which indicated that the hydrophobic interaction was one of main interaction forces. Third, the negative ΔH° values implied that there was hydrogen bonding in the interaction between pepsin and DMP (DBP, DOP, DINP, or DEHP). The positive ΔS° and ΔH° values for the binding interaction of DCHP with pepsin indicated that the dinoctyl enhanced the degree of hydrophobic interaction between DCHP and pepsin.

3.4. Theoretical Calculation of the Binding Interactions of Phthalate Plasticizers with Pepsin. The best docked results of phthalate plasticizer−pepsin systems were showed in Figure 8. Our results indicated that phthalate plasticizer can bind to pepsin with a similar binding domain. These results implied that phthalate groups played an important role in the binding interactions of phthalate plasticizers with pepsin. The values of the calculated binding energy were −63.861 kJ/mol, −76.311 kJ/mol,

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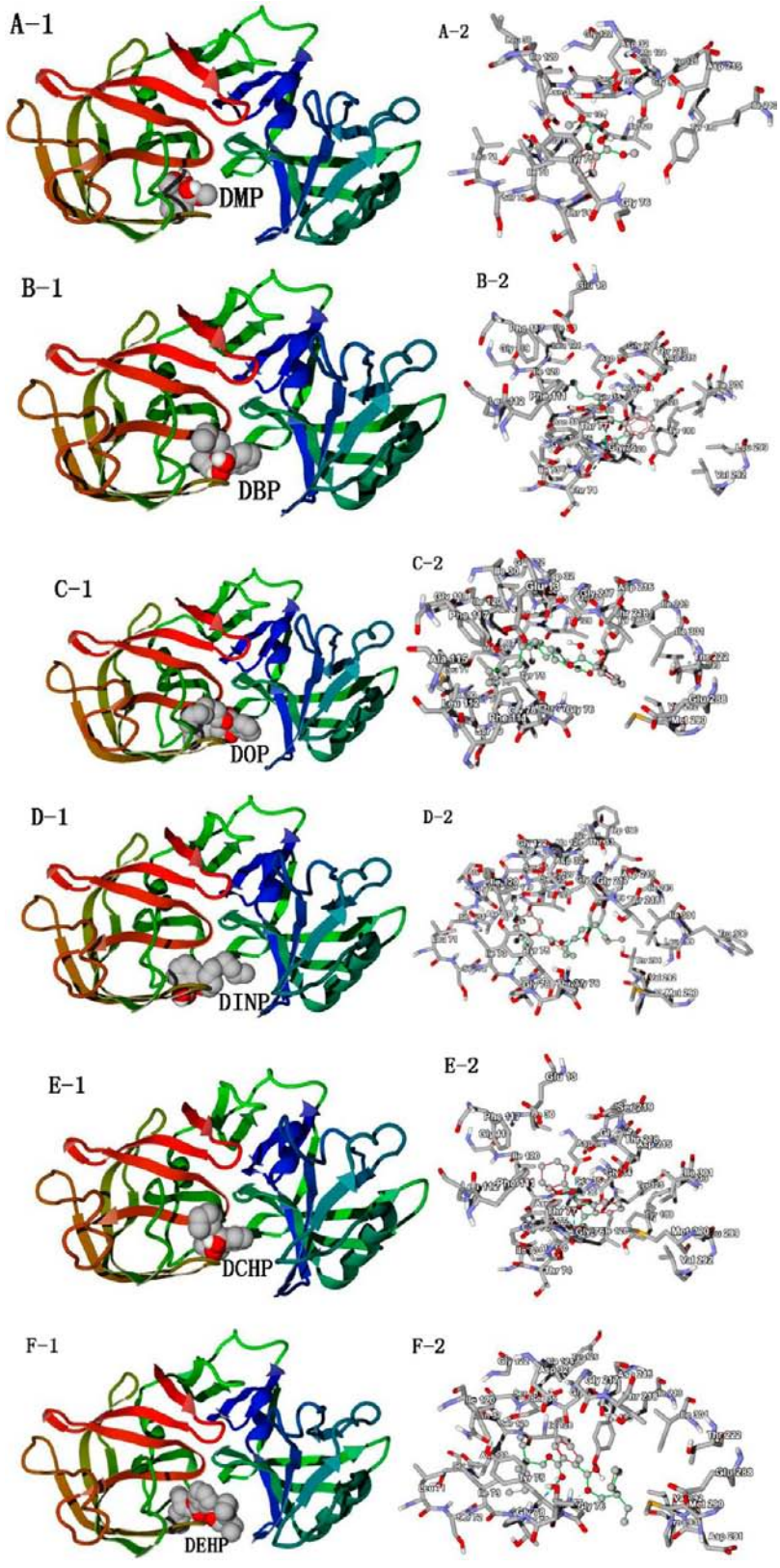

Figure 8. Overview structure and the best docked result of phthalate plasticizer−pepsin systems (A, DMP; B, DBP; C, DOP; D, DINPP; E, DCHP; F, DEHP).

−95.982 kJ/mol, −72.453 kJ/mol, −85.765 kJ/mol, and −68.280 kJ/mol for DMP, DBP, DOP, DINP, DCHP, and DEHP, respectively. The order of binding energy was DMP < DEHP < DINP < DBP < DCHP < DOP. This order was in accordance with the number of amino acid residues taking part in the binding interactions of phthalate plasticizers with pepsin. The number of amino acid residues taking part in the binding interactions of DMP with pepsin was 12; however, that of DOP with pepsin was 28. In

addition, compared with the experimental data, the similarity was that the binding energy of DMP with pepsin was smallest. The difference was that the binding energy of DOP with pepsin was biggest in theoretical calculation data. The above results implied that the binding interactions of phthalate plasticizers with pepsin were not only affected by the molecular structures of phthalate plasticizers but also affected by the actual states of phthalate plasticizers and pepsin in solution.

During the binding interactions of phthalate plasticizers with pepsin, Gly-34, Ile-73, 128, Ser-35, 36, 129, and Tyr-75, 189 all took part in their binding interactions. Especially Tyr-75 and Tyr-189, the binding energy of them with phthalate plasticizers were relatively stronger than those other amino acid residues. For Tyr-75, the binding energy were −5.589 kJ/mol (DMP), −25.141 kJ/mol (DBP), −28.069 kJ/mol (DOP), −12.989 kJ/mol (DINP), −24.283 kJ/mol (DCHP), and −23.358 kJ/mol (DEHP), respectively. For Tyr-189, the binding energies were −3.077 kJ/mol (DMP), −7.725 kJ/mol (DBP), −6.71 kJ/mol (DOP), −18.934 kJ/mol (DINP), −10.072 kJ/mol (DCHP), and −8.750 kJ/mol (DEHP), respectively. As a result, the $\pi-\pi$ stacking between benzene ring of phthalate plasticizers and Tyr residues were involved in the binding interactions. Further, there were hydrogen interactions between the phthalate plasticizers and pepsin: DMP, 11-O and Ile-128(2.677 Å), 12-O and Ser-35 (2.738 Å), Asn-37(3.173 Å), Ser-36 (2.839 Å) of pepsin; DBP, 7-O and Thr-74 (2.982 Å), 14-O and Gly-76(2.899 Å) of pepsin; DOP, 7-O and Asp-215(2.780 Å), 19-O and Tyr-189 (3.162 Å) of pepsin; DINP, 19-O and Thr-74 (3.105 Å), 8-O and Tyr-189 (3.015 Å) of pepsin; DCHP, 7-O and Gly-34 (2.946 Å), DCHP, 16-O and Gly-76 (2.997 Å) of pepsin; DEHP, 7-O and Thr-74 (3.067 Å), 18-O and Tyr-189 (3.208 Å) of pepsin. Meanwhile, the hydrophobic interactions between the phthalate plasticizers and hydrophobic amino acid residues were also involved in these binding. The calculated results were in accordance with the experimental results about the binding forces.

Effect of Phthalate Plasticizers on Pepsin Activity. From the experimental and calculated results, we found that there existed binding interactions of phthalate plasticizers with pepsin. Hence, the activity assays were carried out to study the effects of phthalate plasticizers on the activity of pepsin. We set the activity of pepsin in the absence of phthalate plasticizer as 1 and measured the enzyme activity changes induced by the presence of different concentration of phthalate plasticizers.

As showed in Figure 9, the pepsin activity did not change obviously in presence of phthalate plasticizers. For example, when the concentration of DOP reached 400 μM, that is to say, the values of ratio n(DOP)/n(pepsin) was 40, the enzyme activity increased about 16%. Similar to the cases of DBP, DCHP, and DEHP, the pepsin activity remained basically unchanged. Even the conformational changes of pepsin changed in presence of phthalate plasticizers, the pepsin has its intended function which degrades food proteins into peptides by cleaving peptide bonds between hydrophobic and preferably aromatic amino acids in the stomach. These results implied that phthalate plasticizers are not powerfully inhibitors or activators for pepsin.

In the present study, our in vitro analyses revealed that monitoring changes in pepsin fluorescence as a function of phthalate plasticizers binding provided qualitative and quantitative information about the binding of pepsin with the phthalate plasticizers. The molecular volume and the carbon chain of ester groups of phthalate plasticizers partly affected the binding ability of phthalate plasticizers with pepsin. The fluorescence and theoretical calculation data both indicated that there was the one main binding site in pepsin for phthalate plasticizers. Some microenvironmental and conformational changes in pepsin induced by phthalate plasticizers were monitored. In addition, the pepsin activity was not obviously affected. Although in vitro results could not completely represent the fully biochemical properties of phthalate plasticizers, these findings were helpful to understand the mechanism of some phthalate plasticizers affecting the conformation and activity of digestive proteases in biological processes in the sight of the food security.

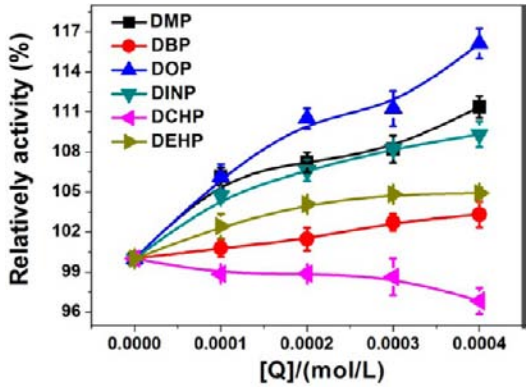

Figure 9. Pepsin activity in the absence and presence of phthalate plasticizers at different concentrations (pH = 2.0, T = 310 K), c (pepsin) = 10.0 μM.

AUTHOR INFORMATION

Corresponding Author

*Tel: +86 515 88233188. Fax: +86 515 88233188. E-mail: wyqing76@126.com.

Notes

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