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In vitro assessment of phthalate acid esters-trypsin complex formation

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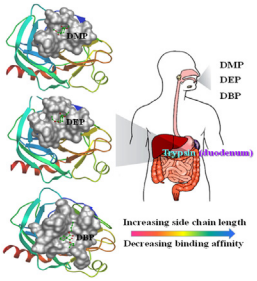
HIGHLIGHTS

- The interactions between PAEs and the trypsin are studied *in vitro*.
- Multi-spectroscopic techniques and molecular docking are used.
- The affinity order of DMP > DEP > DBP is opposite to the side-chain length order.
- The effect of PAEs on the trypsin construction is studied.

GRAPHICAL ABSTRACT



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ABSTRACT

In this work, interactions of three phthalate acid esters (PAEs), including dimethyl phthalate (DMP), diethyl phthalate (DEP) and dibutyl phthalate (DBP), with trypsin have been studied *in vitro*, under simulated physiological conditions using multi-spectroscopic techniques and molecular modeling. The results show that these PAEs can bind to the trypsin, forming trypsin-PAEs complexes, mainly via hydrophobic interactions, with the affinity order of DMP > DEP > DBP. Binding to the PAEs is found to result in molecular deformation of trypsin. The modeling results suggest that only DBP can bind with the amino acid residues of the catalytic triad and S1 binding pocket of trypsin, leading to potential competitive enzyme inhibition.

1. Introduction

Phthalate acid esters (PAEs), namely phthalates, are widely used as plasticizer in industries including toy making, skincare cosmetics, food packaging and medical equipments (Shi et al., 2012). PAEs can be easily released from plastics into environment during the period of use, since PAEs are not polymerized onto the carbon chain of the polyvinyl chloride (PVC) polymers (Cheng et al., 2013). Currently, PAEs have been found in the air (up to μg m^{-3} level), the soil and sediment (up to μg g^{-1} dw level), the water (up to μg L^{-1} level), the food (up to mg kg^{-1} level) as well as tissues and body fluids of animals and humans (up to μg L^{-1} level) (Li et al., 2016; Martine et al., 2013; Wang et al., 2013; Xu et al., 2014;

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Zhang et al., 2015), which has raised potential toxicological concerns worldwide (Sun et al., 2013). The PAEs can permeate into the human body through respiration, skin contact and ingestion, and may pose toxicological threat to the health (Wang et al., 2013). PAEs are class of chemicals in which have well known effects in disrupting endocrine system; PAEs can also result in liver and kidney failure, and defects in human reproductive system including mutagenic and carcinogenic defects (Yokota et al., 2008). In consideration of the toxicity of PAEs, the U.S. Environmental Protection Agency has deemed PAEs to be one of the priority toxic pollutants. China also has blacklisted dimethyl phthalate (DMP), dibutyl phthalate (DBP) and dimethyl octyl phthalic (DOP) as environmental priority pollutants.

Trypsin (Fig. 1A), is a protease secreted by the pancreas, plays a vital role in digestion of food proteins in duodenum (Ghosh, 2008; Gombos et al., 2008). This enzyme which cleaves peptide bonds at the carboxylic groups of arginine and lysine has a wide application in wound-healing, cleaning and other biotechnologies (Ghosh, 2008; Zhang et al., 2010). Trypsin contains 223 amino acid residues, forming two domains with almost an equal size, between which the activated sites of trypsin (catalytic triad, His-57, Asp-102, and Ser-195) are located (Hu et al., 2013; He et al., 2014). With frequent exposures, PAEs can easily enter the duodenum, and induce interactions with the digestive proteases leading to toxic effects.

Previous works have reported the potential interaction of some PAEs with trypsin (Wang et al., 2015), however, these interactions with trypsin have not been elucidated. The aim of this study is to establish an *in vitro* modeling of three members of PAEs, including DMP, DEP and DBP, with the trypsin under simulated physiological conditions. Fig. 1A shows molecular structures of these PAEs with the increasing order of the side-chain length. The binding constants, binding forces and numbers of binding sites have been determined for the interactions of PAEs with the trypsin via the fluorescence spectroscopy and molecular modeling studies. The effect of PAEs on trypsin conformation has also been investigated

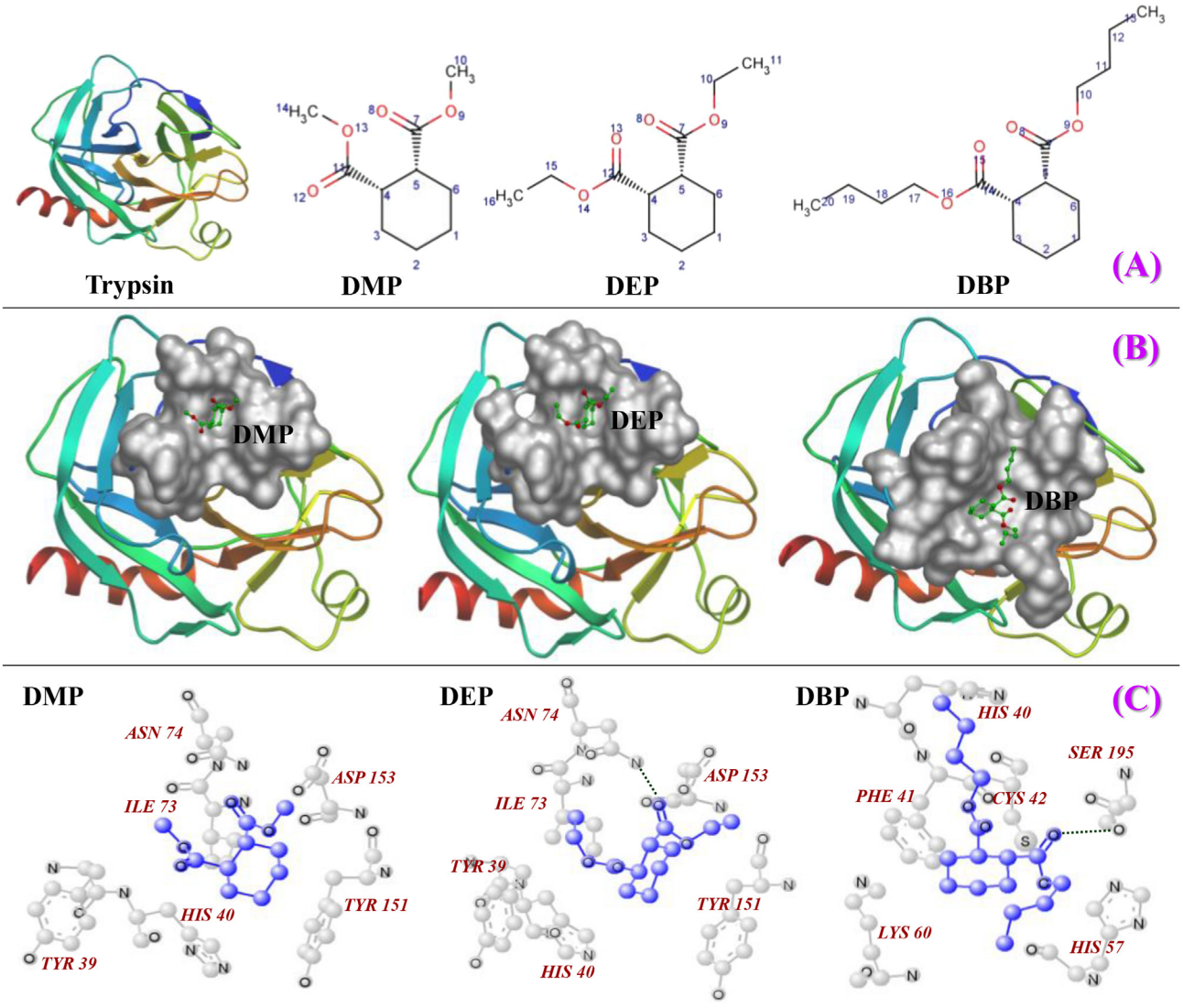

Fig. 1. (A) Molecular structures of trypsin and PAEs including with atom numbers; (B) The binding mode of different PAEs with the trypsin. Interacting catalytic domain of trypsin are displayed in the surface mode. PAEs are represented using balls and stick. The atoms of PAEs are color-coded as follows: O, red; C, green; (C) The molecular modeling of interaction between PAEs and trypsin. The atoms of PAEs are marked with blue and the atoms of amino acid residues of trypsin are labeled with gray. The hydrogen bonds between PAEs and trypsin are indicated by green dashed line. The electrostatic forces are illustrated with pink dashed line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

by the means of the UV–Vis absorption and circular dichroism (CD) spectrometry. The *in vitro* results may contribute to further understanding of potential toxicity of different PAEs to humans and will definitely provide some useful information for further toxicological works in related fields.

2. Materials and methods

2.1. Reagents

Trypsin (from bovine pancreas, AMRESCO) was dissolved in ultrapure water to form a 1.0×10^{-4} M solution, preserved at 0–4 °C, and diluted as required. Stock solutions of DMP, DEP and DBP (1.5×10^{-4} M, Sinopharm Chemical Reagent Co., Ltd) were prepared and diluted as required. Phosphate saline buffer (PBS) (0.2 M, a combination of $NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_4 \cdot 12H_2O$, pH 7.6) was used to control the pH. $NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_4 \cdot 12H_2O$ were of analytical reagent grade. All other chemicals were of analytical grade and were purchased from Tianjin Damao Chemical Reagent Factory. Ultrapure water was used in the experiments.

2.2. Fluorescence measurements

Fluorescence measurements were performed using a PerkinElmer spectrofluorometer, model LS4 (USA), equipped by a thermostat bath with a 1.0 cm quartz cell. The scan speed was 1200 nm min^{-1}. The photomultiplier tube voltage was 700 V. The excitation and emission slit widths were 10 nm. In a typical operation, PBS (1.0 mL, pH 7.6) and trypsin (1.0 mL, 1.0×10^{-4} M) were added to a 10 mL colorimetric tube. Different doses of 1.5×10^{-4} M solutions of PAEs (DMP, DEP and DBP) were then added into the colorimetric tube. The fluorescence spectra were measured after stabilization for 20 min. The excitation wavelength was set at 278 nm and the excitation wavelength was set from 280 to 450 nm.

In order to determine the quenching mechanism, the fluorescence quenching data were analyzed using the Stern-Volmer equation (eq (1)), examining the possibility for the interaction of PAEs with trypsin to form a trypsin-PAEs complex (Cui et al., 2004; Hu et al., 2004).

$$\frac{F_0}{F} = 1 + K_{sv}[Q] = 1 + k_q\tau_0[Q], \quad (1)$$

where F_0 and F are the fluorescence intensity in the absence and presence of the quencher, separately, [Q] is the concentration of the quencher, τ_0 (usually 10^{-8} s) is the fluorescence lifetime in the absence of the quencher, K_{sv} is the Stern-Volmer quenching constant, and k_q is the quenching rate constant of biomacromolecule.

For static quenching, the binding constant (K_a) and the number of binding sites (n) can be obtained from the equation (eq (2)) (Hu et al., 2004).

$$\lg\frac{(F_0 - F)}{F} = \lg K_a + n\lg[Q]. \quad (2)$$

The fluorescence lifetime spectra were observed using an FLS920 spectrophotometer (Edinburgh Instruments, UK). The excitation wavelength was 278 nm and the emission wavelength was 349 nm.

2.3. UV–Vis absorption spectra measurements

A U-2900 spectrophotometer (Hitachi, Japan) equipped with 10 mm quartz cells was used to record the UV–vis absorption spectra (ranging from 190 to 320 nm) of trypsin in the presence and absence of PAEs at 290 K.

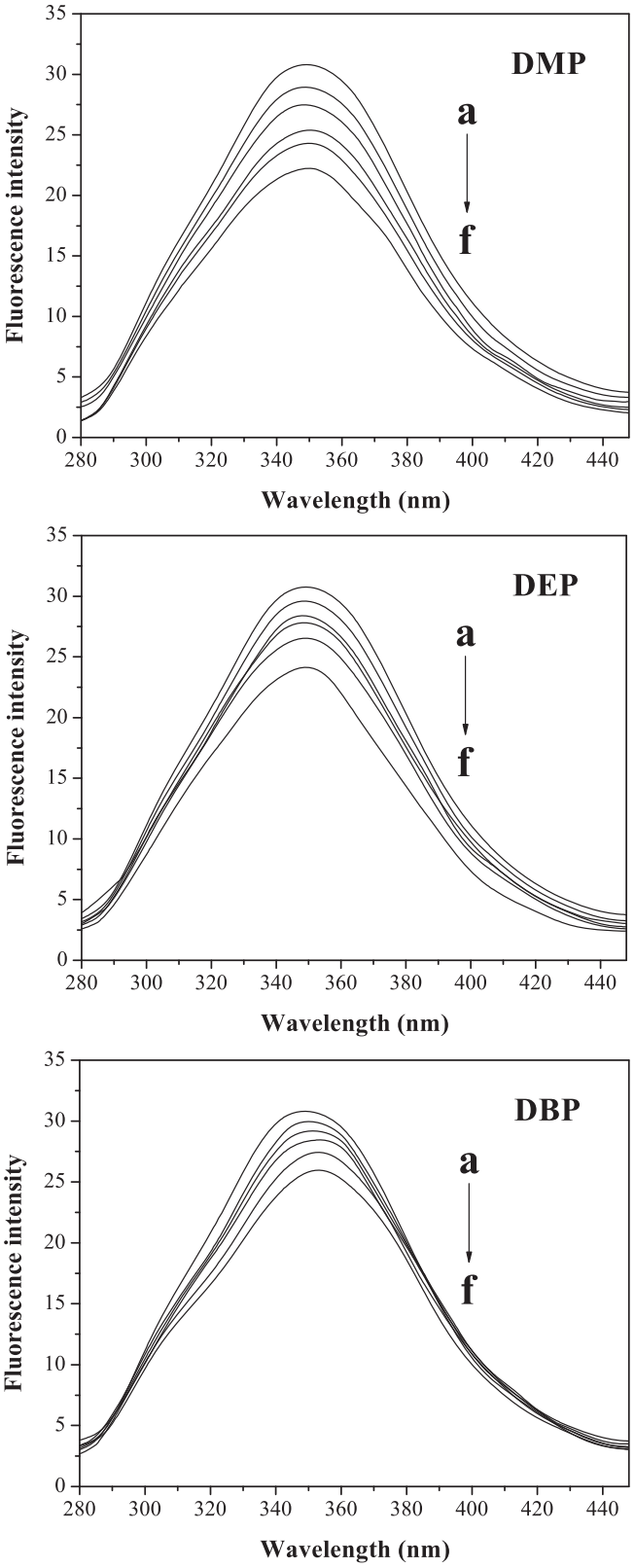

Fig. 2. Trypsin fluorescence spectra under different PAEs concentrations. Conditions: trypsin: 1.0×10^{-5} M; PAEs/(10^{-5} M): a, 0; b, 0.75; c, 1.5; d, 2.25; e, 3; f, 4.5; pH 7.6; T = 290 K.

2.4. Circular dichroism (CD) measurements

The CD spectra were measured by a JASCO J-810 CD spectrometer using a quartz cell with the path length of 1 cm. The scan wavelength range was set from 190 to 260 nm with 0.2 nm intervals at the temperature of 290 K. Two scans were equally performed to obtain the CD spectra. The CD spectra were investigated based on the CONTINLL program offered by CDPro software (Provencher and Glockner, 1981).

2.5. Molecular docking

The docking calculations were carried out using Docking Server (Bikadi and Hazai, 2009). The MMFF94 force field (Halgren, 1996) was used for energy minimization of the ligand molecules (PAEs). Gasteiger partial charges were added to the ligand atoms. Nonpolar hydrogen atoms were merged, and rotatable bonds were defined.

The trypsin protein model was processed using molecular docking methodology. With the aid of AutoDock tools (Morris et al., 1998), essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added into the docking calculations. Affinity (grid) maps (100 × 100 × 100 Å grid points) with the 0.375 Å spacing were applied in the Autogrid program (Morris et al., 1998). AutoDock parameter set- and distance-dependent dielectric functions were applied in the calculation of the van der Waals and electrostatic terms, respectively.

The docking simulations were conducted using the Lamarckian genetic algorithm and the Solis and Wets local search modes. The initial positions, orientations, and torsions of the ligand molecules were randomly set. Each docking experiment was derived from 10 different runs that were set to terminate after a maximum of 250 000 energy evaluations. The population size was 150. During each search, a translational step of 0.2 Å and 5 quaternion and torsion steps were used.

3. Results and discussion

3.1. Fluorescence measurements

Fluorescence spectroscopy has previously been used to unveil binding of various chemicals with proteins (Chi et al., 2016). There are mainly two fluorophore residues-, Trp (tryptophan) and Tyr (tyrosine) that contribute to the inherent fluorescence of the trypsin (Dockal et al., 2000; Wang et al., 2014). The fluorescence quality of trypsin with different PAEs (DMP, DEP and DBP) concentrations are shown in Fig. 2. With the increasing concentration of PAEs, the maximum emission wavelength of trypsin remains around 349 nm, however the fluorescence intensity gradually decline, suggesting that the PAEs has a fluorescence quenching effect on the trypsin.

In order to investigate whether the PAEs interact with the trypsin to form a complex, the quenching mechanism was further studied. The fluorescence quenching data were analyzed according to the Stern-Volmer equation (eq (1)). The Stern-Volmer plots are shown in Fig. 3A, and the calculated values of K_{sv} and k_q are listed in Table 1. According to the results, K_{sv} values decreased following the order of DMP > DEP > DBP. The values of k_q are much larger than the maximum scattering collision quenching constant (2.0×10^{-10} L mol s^{-1}), which suggested that the fluorescence quenching of the trypsin was initiated by the complex formation rather than a dynamic collision (Xu et al., 2008).

Furthermore, rapid quenching (dynamic or static) can be differentiated by the lifetime measurements (Zhang et al., 2009). Fig. 4 shows the time-resolved fluorescence decay profile of trypsin and trypsin-PAEs systems. These data correspond well to the sum of single exponential decay with a χ^2 value close to 1.00. Interestingly, the lifetime values of trypsin with and without PAEs remained nearly unchanged. Therefore, it can be concluded that the quenching is static and participates in the formation of trypsin-PAEs complexes (Chai et al., 2013).

Eq (2) is used to calculate the binding constants (K_a) and the number of binding sites (n). Y-intercept of the fitted logarithmic curve (Fig. 3B) is lg K_a, and the slope is equal to n. The K_a values decrease with the order of DMP > DEP > DBP, opposite to the order of the side-chain length increases (Table 1). The number of binding sites, n, is approximately equal to 1, indicating one single binding site on the trypsin for the bound PAE.

Together these data show interaction between all three PAEs with trypsin via a single binding site. Therefore, based on these findings, it is evident that the formation of trypsin-PAEs complexes follow the affinity order of DMP > DEP > DBP, opposite to the order of side-chain lengths.

3.2. Modeling of the trypsin-PAEs complexes

To identify the specific binding sites of the PAEs on trypsin,

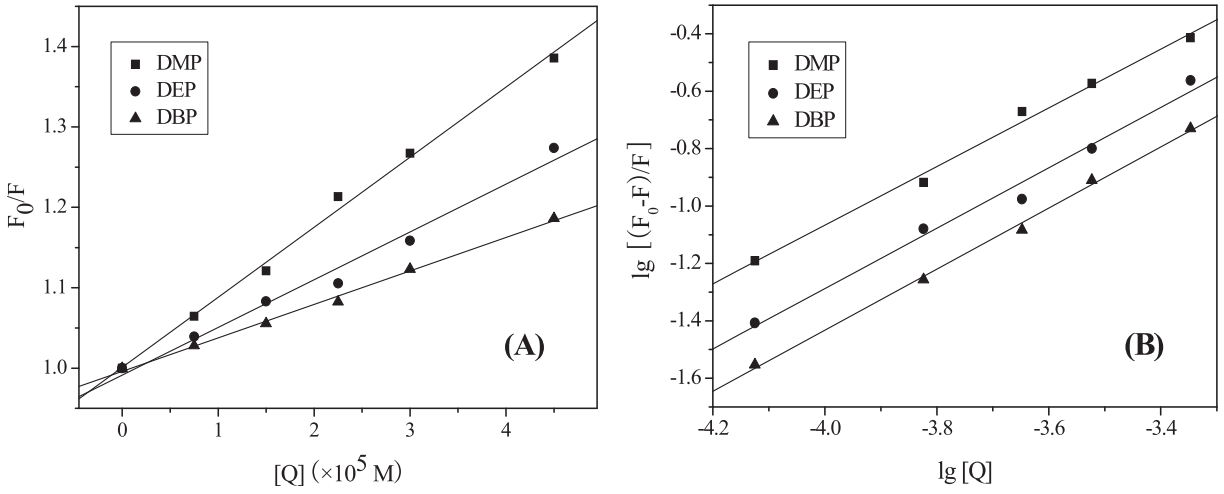

Fig. 3. (A) Stern-Volmer plots for the quenching of trypsin by the PAEs under different conditions; (B) Plot of log [(F_0 − F)/F] vs. log [PAEs] for the binding of PAEs to the trypsin. Conditions: trypsin: 1.0×10^{-5} M; pH 7.6; T = 290 K.

Table 1
The Stern-Volmer quenching constants, binding constants and the number of binding sites for the interaction of PAEs with the trypsin.

	DMP	DEP	DBP
K_{sv} ($\times 10^4$ L mol^{-1})	0.8715	0.5936	0.4166
k_q ($\times 10^{11}$ L mol^{-1} s^{-1})	8.715	5.936	4.166
R[a]	0.99767	0.99127	0.99805
S.D.[b]	0.0108	0.0143	0.00472
K_a ($\times 10^4$ L mol^{-1})	1.052	0.832	0.667
n	1.02228	1.05198	1.06421
R[a]	0.99662	0.99272	0.99851
S.D.[b]	0.02896	0.04389	0.02

[a] R is the correlation coefficient.
[b] S.D. is the standard deviation.

molecular docking method was applied. The crystal structure of trypsin is derived from the protein database (Protein coding 2ZQ1). The best energy ranking results are shown in Fig. 1B and C. The catalytic triad of trypsin consists of His-57, Asp-102 and Ser-195, with residues 189–195, 214–220 and 225–228 form primary substrate-binding pocket (S1 binding pocket) (Chi et al., 2011). From Fig. 1B, all PAEs can bind with trypsin, however, only DBP can bind with residues of the catalytic triad and S1 binding pocket (Fig. 1C, HIS-57 and SER-195), resulting in a competitive inhibition for trypsin activity.

Furthermore, from Table 2, results show that DEP and DBP interaction with trypsin is mainly via hydrophobic interactions notably, DMP with HIS-40, ILE-73, TYR-151; DEP with TYR-39, HIS-40, ILE-73, and TYR-151; DBP with HIS-40, PHE-41, CYS-42, and HIS-57. Besides hydrophobic forces other forces also exist in the interaction between PAEs with trypsin. The DMP combined with ASN-74 interact mainly through electrostatic forces, with other forces (DMP with TYR-39, HIS-40, TYR-151, ASP-153) were also observed, but at weaker interactions. The DEP interacted with the ASN-74 via hydrogen bonds and bonded with the ASP-153 by the electrostatic force, while other forces (DEP with HIS-40, ASN-74, TYR-151 and ASP-153) were also present. The DBP interacted with the trypsin through hydrogen bonds (DBP with SER-195) and some other forces (DBP with PHE-41, HIS-57, LYS-60).

From this a conclusion can be made that all three PAEs can bind to trypsin primarily through hydrophobic forces. However, only DBP interacted with the amino acid residues of the catalytic triad and S1 binding pocket of the trypsin, showing a high inhibition of the enzyme activity.

3.3. Effect of PAEs on the conformation of trypsin

Structure determines function. In this part, the role of PAEs in the conformation of PAEs was examined by the UV–vis absorption and CD spectroscopy.

3.3.1. UV–Vis Absorption Spectroscopy

As a simple but effective technique, UV–visible absorption spectroscopy has been adopted to explore the structural changes of a protein and investigate protein-ligand complex formation (Chi

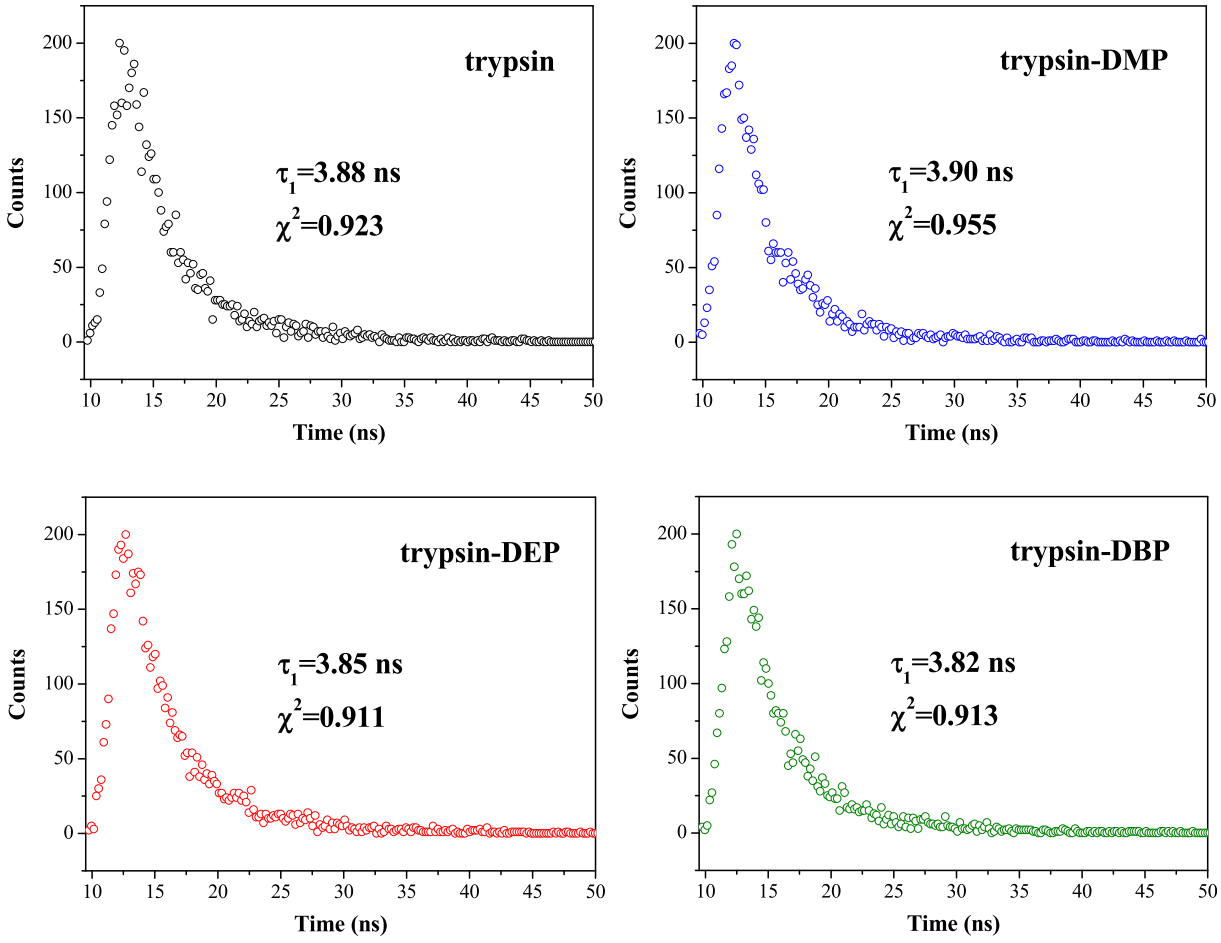

Fig. 4. Time-resolved fluorescence decay profile of trypsin and trypsin-PAEs system. **Conditions**: trypsin: 5×10^{-6} M; PAEs: 1.5×10^{-5} M; pH 7.6; T = 290 K.

Table 2
The interaction of PAEs with the trypsin.

	force	ligand atom (DMP)	protein atom (trypsin)	Distance (Å)	interaction energies (kcal/mol)
DMP	electrostatic	O8	ASN74 (ND2)	3.58	−0.5248
	hydrophobic	C3	HIS40 (CB, CD2, CG)	3.10	−0.7633
		C2	HIS40 (CD2)	3.36	
		C1	ILE73 (CD1)	3.31	−0.5366
		C2	ILE73 (CD1)	3.59	
		C3	ILE73 (CD1)	3.44	
		C1	TYR151 (CB)	3.86	−1.0812
		C5	TYR151 (CB)	3.38	
		C2	TYR151 (CD2)	3.61	
	other	O12	TYR39 (CB)	3.73	−0.4075
		O13	HIS40 (CB)	3.83	
		O9	TYR151 (CB)	3.71	
		C5	ASP153 (OD1)	3.28	−0.4238
		C6	ASP153 (OD1)	3.81	
DEP	hydrogen bonds	O8	ASN74 (ND2)	3.35	−0.6702
	electrostatic	O8	ASP153 (OD1)	3.78	−0.4984
	hydrophobic	C15	TYR39 (CB)	3.76	−0.5552
		C3	HIS40 (CB, CD2, CG)	3.24	−1.0562
		C2	HIS40 (CD2)	3.51	
		C1	ILE73 (CD1)	3.29	−0.7528
		C2	ILE73 (CD1)	3.77	
		C3	ILE73 (CD1)	3.77	
		C16	ILE73 (CD1)	3.88	
		C1	TYR151 (CB)	3.88	−1.4208
		C6	TYR151 (CB)	3.40	
		C11	TYR151 (CB)	3.49	
		C2	TYR151 (CD2)	3.42	
	other	O14	HIS40 (CB)	3.55	
		C16	ASN74 (CG, OD1)	3.22	
		O9	TYR151 (CB)	3.74	
		C5	ASP153 (OD1)	3.80	
		C6	ASP153 (OD1)	3.18	
		C7	ASP153 (OD1)	3.88	
DBP	hydrogen bonds	O8	SER195 (OG)	3.34	−0.4593
	hydrophobic	C19	HIS40 (CD2)	3.84	−0.3937
		C3	PHE41 (CB, CD2)	3.58	−1.4397
		C1	PHE41 (CD2, CE2)	3.25	
		C2	PHE41 (CD2)	3.41	
		C1	CYS42 (SG)	3.51	−0.1658
		C5	CYS42 (SG)	3.72	
		C6	CYS42 (SG)	3.47	
		C6	HIS57 (CD2)	3.84	−0.8991
	other	O16	PHE41 (CB)	3.62	
		O9	HIS57 (CD2)	3.43	
		C2	LYS60 (CE, NZ)	3.61	−0.4571

et al., 2016). The absorption spectrum of the trypsin have two absorption bands, namely the framework absorption at nearly 200 nm and the aromatic amino acids absorption at around 274 nm (Wu et al., 2007). The UV–Vis absorption spectra of the trypsin were observed in distinctive concentrations of all three members of PAEs. With the increasing PAEs concentration, there was an apparent decline in the intensity of the framework absorption peak of trypsin to about 195 nm following the order of DMP > DEP > DBP (Fig. 5). A redshift of the framework absorption peak was also observed, suggesting that the loosening and unfolding of the protein skeleton can be natural outcome during the interactions between PAEs and trypsin (Wu et al., 2007; Chai et al., 2013).

3.3.2. Circular dichroism Spectroscopy

To further investigate whether the binding of PAEs have any impact on the structure of trypsin, CD spectroscopy were utilized (Wang and Zhang, 2014). The secondary structure containing

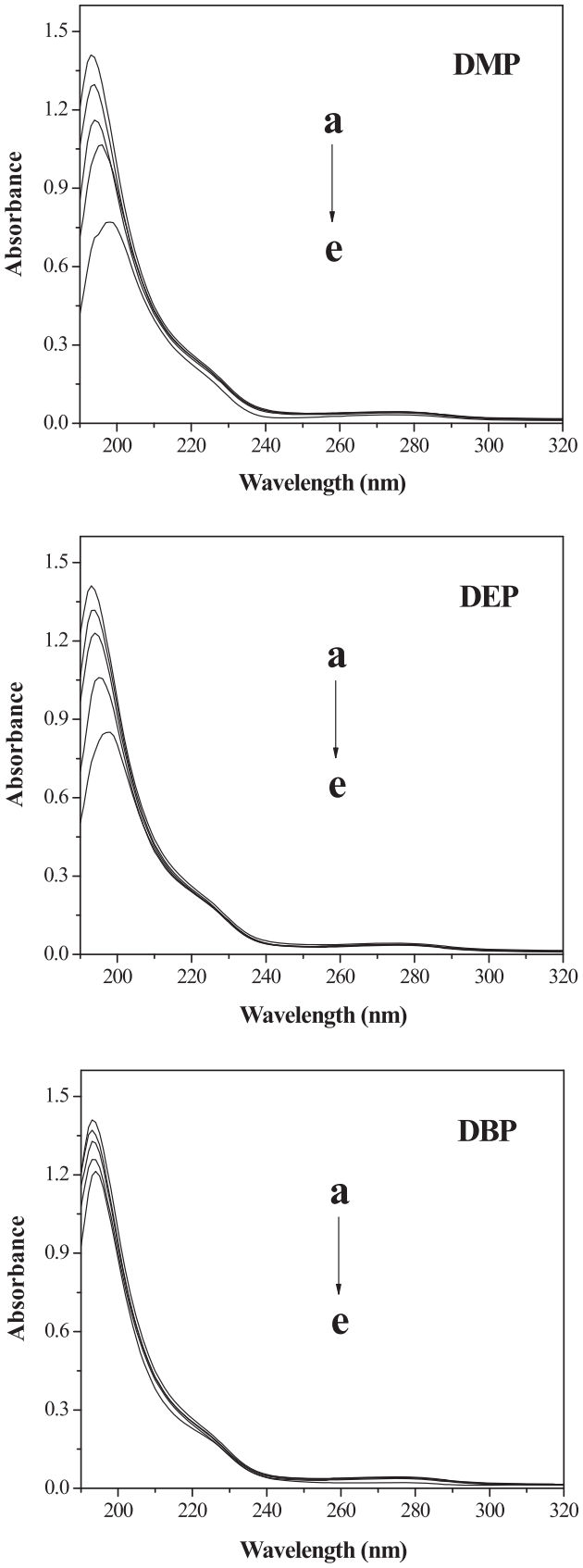

Fig. 5. UV-Vis spectra of trypsin under different PAEs concentrations (vs the same concentration of PAEs solution). **Conditions**: trypsin: 1.0×10^{-5} M; PAEs/(10^{-5} M): a, 0; b, 0.75; c, 1.5; d, 3; e, 4.5; pH 7.6; T = 290 K.

trypsin in the absence of PAEs was 4.7% α-helix, 44.3% β-pleated sheet, 11.0% β-turn and 40% random coil (trypsin: 5×10^{-6} M; pH = 7.6; T = 290 K), in accordance with the fact that trypsin is one of the all-β proteins (Liu et al., 2008). With the addition of PAEs at a trypsin/PAEs ratio of 1:9, the content of secondary structure of the trypsin remained fairly the same, with no significant structural change after binding of PAEs to trypsin.

4. Conclusion

The present study has provided new insight into the interactions between PAEs and trypsin in light of spectroscopy and computational modeling systems. The study revealed that PAEs (DMP, DEP, DBP) interacted with trypsin to form trypsin-PAEs complexes via a single binding site, mainly dependent on the hydrophobic forces, following the affinity order of DMP > DEP > DBP. The bound PAEs resulted in the conformational change of the protein with the order of DMP > DEP > DBP. The molecular docking results revealed that, unlike DMP and DEP, DBP combined with the residues of catalytic triad and S1 binding pocket of the trypsin could possibly cause competitive enzyme inhibition. This work may offer scientific evidence to assess the potential risk of PAEs pollutants exist in the environment in conjunction with healthcare purposes.

Acknowledgements

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