BIOCELL
2020 44(2): 247-255

Tech Science Press

Combined molecular docking, homology modeling and DFT method for the modification of bovine serum albumin (BSA) to improve fluorescence spectroscopy for phthalate acid esters chelated with BSA

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Key words: Protein modification, Amino acid residues, Fluorescence enhancement, Environmental detection

Abstract: While phthalate acid esters (PAEs) cannot fluoresce alone, they can be detected by fluorescence spectroscopy after chelation with bovine serum albumin (BSA). In this study, the types of amino acid residues at the active site of PAEs chelated with BSA were determined using molecular docking technology. A modification scheme of BSA with higher detection sensitivity fluorescence spectroscopy for PAEs was proposed based on the docking results and constructed for a novel BSA structure with a higher detection sensitivity of fluorescence spectroscopy using a homologous modeling method. Density functional theory (DFT) was employed to explore the influence before and after BSA modification on PAEs' detection through fluorescence spectroscopy. The results showed that the docking scores between BSAs and dimethyl phthalate (DMP), dibutyl phthalate (DBP) and di-n-octyl phthalate (DNOP) were increased up to 26.45%, 16.82% and 16.30%, respectively, indicating that the active site modification of BSA could enhance the binding affinity between BSA and PAEs. The fluorescence intensity of PAEs chelated with modified BSAs were calculated. The fluorescence intensity of fluorescence spectroscopy for DMP, DBP and DNOP chelated with BSAs after modification was increased up to 2.8-, 104.51- and 62.43-fold, respectively, which achieved the purpose of theoretically modifying BSA to improve the detection sensitivity of fluorescence spectroscopy for PAEs.

Introduction

Phthalate Acid Esters (PAEs) are usually synthesised by the esterification of phthalic anhydride with various alcohol compounds (González-sálamo *et al.*, 2018a) and they have high lipid solubility and low water solubility (González-Sálamo *et al.*, 2018b). PAEs are widely used as plasticisers in food packaging, toys, medical supplies and personal care products as they have a characteristic offering significant improvement of the strength and plasticity of plastic products (Staples *et al.*, 1997). The estimated worldwide annual production of plastic products is 150 million tons, while about 8 million tons of PAEs are consumed each year (Net *et al.*, 2015). Because PAEs are not covalently bonded to polymers (Bope *et al.*, 2019) and easily released into the environment, studies have shown that PAEs have endocrine disrupting, teratogenic, carcinogenic and mutagenic properties (Wang *et al.*, 2015; Guo and Kannan, 2011) and their extensive use further increases the risk to human health. Therefore, finding efficient ways of detecting PAEs in the environment to avoid their potential harm to biological health has become an environmental problem that need to be solved.

Presently, the main methods for detecting PAEs are Gas Chromatography (GC), High-performance Liquid Chromatography (HPLC), Gas Chromatography–Mass Spectrometry (GC-MS) and Liquid Chromatography–Mass Spectrometry (LC-MS). Liang *et al.* (2009) used the GC method to detect PAEs content in water and soil in Wuhan. However, the GC method is only suitable for PAE samples that can be volatilised below 300°C, and GC detectors are vulnerable to contamination with poor sensitivity (Beens and Brinkman, 2014). Li *et al.* (2017) utilised HPLC and LC-MS to detect PAEs degradation intermediates. The detection process in this method is complex and requires the high pretreatment of samples. Shao *et al.* (2013) employed GC-MS to detect PAEs in water and sediments. The sample preparation and operation steps of the traditional detection method are complicated and require a lot of manpower, financial resources and time, and since there are more than

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Received: 15 October 2019; Accepted: 3 February 2020

one type of PAE in the environment, the PAE spectral detection results obtained by traditional detection methods are susceptible to interference and can easily mistake environmentally friendly and harmful contaminants. Therefore, there is a need to find ways to quickly and further improve the detection sensitivity of PAEs.

Compared with traditional detection methods, fluorescence spectrometry has the advantages of high sensitivity, simplicity and high selectivity (Zhang *et al.*, 2011). However, since PAEs cannot emit fluorescence alone and cannot be directly detected by fluorescence methods, the protein's endogenous fluorescence spectrum can be studied by the interaction of PAEs with proteins, thereby indirectly realising the detection of PAEs (Xie *et al.*, 2011). Phthalates combine with serum albumin in plasma after entering the human body and gradually play a toxic role in the receptor sites and the transport of plasma (Varshney *et al.*, 2010; Ryan *et al.*, 2011; Wagner *et al.*, 2010). Therefore, in this study, Bovine Serum Albumin (BSA), which has low molecular weight, high solubility, high stability, low cost, high affinity with the ligand and high homology with Human Serum Albumin (HSA), was selected as the target protein to improve the detection sensitivity of PAEs for analysis (Duan *et al.*, 2013; Bourassa *et al.*, 2010) and thus study the mechanisms and laws of the two and provide guidance for theoretical transformation. The molecular docking method has been widely used in the research field of interactions between small molecules and proteins (Karjiban *et al.*, 2014). As a recognised method for the predictable synthesis of receptor proteins, the homologous modeling method can rapidly and effectively construct the structure of novel proteins (Zhou *et al.*, 2013; Klepeis *et al.*, 2009; Liu *et al.*, 2006) and density functional theory has been widely used in the theoretical calculation of spectral recognition (Kohn and Sham, 1965; Egger *et al.*, 2015). The DMP, DBP and DNOP were limited in the list of priority control pollutants by the Environmental Protection Agency (EPA) in 1977 (Amir *et al.*, 2005). In China, DMP, DBP and DNOP were also identified as priority control pollutants for control in 1990 (Yang *et al.*, 2013; Jin *et al.*, 2013). In this paper, three representative PAEs (DMP, DBP and DNOP) were selected to dock with BSA.

Firstly, since phthalate acid esters (PAEs) cannot fluoresce alone, they can be detected by fluorescence spectroscopy after chelation with bovine serum albumin (BSA). Therefore, in order to improve fluorescence spectroscopy for phthalate acid esters chelated with BSA, three representative PAEs (DMP, DBP and DNOP) were employed to dock with BSA through molecular docking technology by Discovery studio software. The docking scores between PAEs (DMP, DBP and DNOP) and BAS were generated, and the levels of the docking scores were used to evaluate the binding affinity between them. The interaction module in the software were used to identify the types of the key amino acids between PAEs and BSAs.

Secondly, a modification scheme of BSA with higher detection sensitivity fluorescence spectroscopy for PAEs was proposed based on the docking results. The hydrophilic amino acid residues near the low affinity active sites were replaced with hydrophobic amino acid residues which could improve the affinity between the molecule and the protein, whereas the hydrophobic amino acid residues were replaced with hydrophilic amino acid residues.

In addition, the novel BSA structures based on the modification schemes were constructed with a higher detection sensitivity of fluorescence spectroscopy using a homologous modeling method, and the density functional theory (DFT) was employed to evaluate the influence before and after BSA modification on PAEs' detection through fluorescence spectroscopy.

As shown in Fig. 1, the design of the novel BSA with higher detection sensitivity fluorescence spectroscopy for PAEs were summarized.

Materials and Methods

Data sources

In this paper, the receptor protein used for molecular docking with dimethyl phthalate (DMP), dibutyl phthalate (DBP) and di-n-octyl phthalate (DNOP) was a commonly used bovine serum (bovine serum albumin, BSA: 3V03) (Shi *et al.*, 2016). The BSA (PDB ID: 3V03) and the sequence of its amino acid residues were obtained using the NCBI database query and stored in PDB format.

Molecular docking method used to ascertain the key amino acid residues of PAEs binding to BSA

Discovery Studio 4.0 software was used to explore the docking conformation of PAEs with BSA and novel modified BSAs. Firstly, the protein model in the form of PDB was removed from the water molecules and hydrogenated and charged. Secondly, the receptor binding cavity of the protein was defined, and the PAEs were optimised using Gaussian 09 software to obtain the optimal configuration. In the case of unknown receptors, the lowest energy conformation of the molecule was regarded as the dominant stable conformation. The molecular program called Minimize was adopted to optimise the energy of each molecule following the Tripos force and MMFF94 charge loading (Halgren, 1996). Using the Powell energy gradient method, the maximum number of optimisations was set to 10,000, and the energy convergence limit was 0.001 kJ/mol; the rest were default values. The optimised PAEs were loaded into Discovery Studio 4.0 software. To compare the change in binding affinity between BSA and target PAEs before and after modification, the LibDock module of Discovery Studio 4.0 software was used to dock the target DMP, DBP and DNOP with BSA and their novel modified BSAs, respectively. The docking scores was obtained by considering the polarity, hydrophobicity, entropy and solvation between ligand and receptor. Docking scores were used to evaluate the docking results of molecules. The higher the score was, the stronger the interaction between molecules and the more stable the binding (Wang *et al.*, 2017).

Homologous modeling for BSA modification with high detection sensitivity of fluorescence spectroscopy for PAEs

Homology modeling is a computational method for establishing a three-dimensional structural model based on the amino acid sequence of a protein (Yan *et al.*, 2016). In

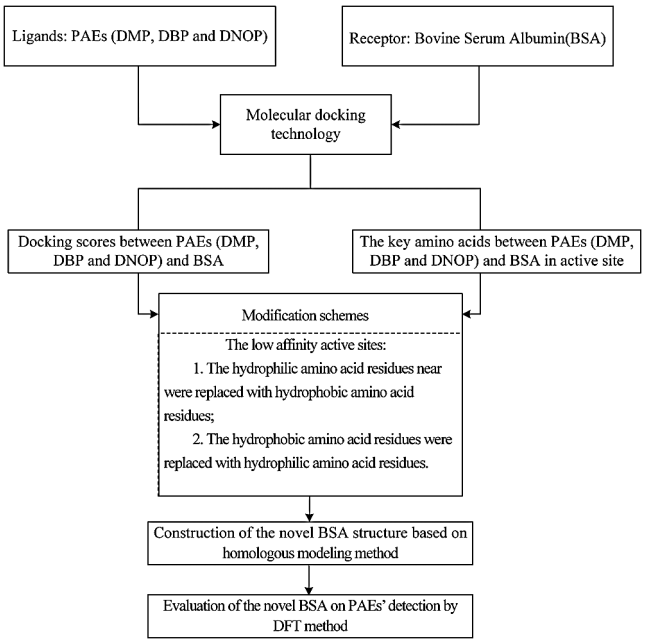

FIGURE 1. The design of the novel BSA with higher detection sensitivity fluorescence spectroscopy for PAEs.

this paper, the sequences of amino acid residues of BSA (PDB ID: 3V03) were obtained using the NCBI database query (https://www.ncbi.nlm.nih.gov/protein/3V03_A). The key amino acid residues at the active sites of BSA (PDB ID: 3V03) were replaced by a rational design and other molecular biological means to obtain a modified sequence of BSA amino acid residues.

SWISS-MODEL module, as a method to protein homology modelling, is based on the principle is based on the principle of selecting proteins with homology and known structure with unknown structure as the template of predicting structure, comparing the multiple alignment of target sequence with all homologous sequences, and identifying conservative segments. Then, the three-dimensional structure corresponding to these conservative segments is copied from the template structure, and the final three-dimensional structure is optimized by energy minimization technology. Using the SWISS-MODEL module in the automatic protein modeling server provided by Glaxo Smith Kline centre in Geneva, Switzerland (http://www.swissmodel.expasy.org), the novel modified BSA amino acid sequences and template BSA molecules were submitted to the above swiss-model server respectively.

Finally, the novel modified BSA structure was obtained using a homologous modeling method. To further verify the structural rationality of the model, the Ramachandran conformation map (http://services.mbi.ucla.edu/SAVES/) in the online evaluation server PROCHECK was used to evaluate the structural rationality of the novel modified BSA model. The condition signifying qualified model quality is if the basic group's proportions in these three regions (core region + allowable region + maximum allowable region) are greater than 95% (Arnold *et al.*, 2006).

DFT for calculation of PAEs' fluorescence emission spectra

Firstly, the time-dependent density functional theory (TD DFT) method was used to optimise the structure of excited state (Qiu and Li, 2018). Secondly, the structures of target PAEs were optimised and the fluorescence spectrum intensity of PAEs were calculated at the basis group level of B3LYP/6-31g * using Gaussian 09 software. (Gu *et al.*, 2018; Jiang *et al.*, 2017; Qiu and Li, 2018; Yang, 2016). The fluorescence emission spectra of the optimised target DMP, DBP and DNOP molecules combined with BSA and novel modified BSAs were calculated to further explore the influence of novel BSAs before and after modification of the fluorescence spectrum detection of target PAEs.

Results

Identification of key amino acids at the binding sites between PAEs and BSA

In this paper, the NCBI database was used to query the structure information of BSA (PDB ID: 3V03) and to identify the binding sites of the target PAEs to BSA (PDB ID: 3V03). The LibDock module of Discovery Studio 4.0 software was used to dock the optimised target DMP, DBP and DNOP with BSA, respectively. As shown in Fig. 2, the amino acid residues surrounding the binding sites of the target DMP, DBP and DNOP molecules to the BSA included Arg198, Arg217, Arg194, Ser214, Asp450, Arg256, Trp213, Leu197, Leu454 and Val342. Studies have shown that when a compound binds to a receptor, there are non-bond interactions such as hydrophobic interactions, hydrogen bonding, electrostatic interactions and van der Waals forces (Johnson *et al.*, 2010; Wang, 2013). Amino acid residues (could be identified as key amino acid residues) acted in main roles at a certain distance from the binding site (Yang *et al.*, 2010). Therefore, the above amino acid residues can be preliminarily identified as key amino acid residues for direct interactions between BSA and DMP, DBP and DNOP.

The degradation mechanism of aromatic pyrene based on the structure of the target enzyme JPN2-NDO shows that hydrophobic interactions play an important role in the binding of aromatic pyrene and the target enzyme JPN2-NDO (Jin, 2017). When the hydrophilic amino acid Thr308 of template 1O7N was replaced by the hydrophobic amino acid Val232 of target enzyme JPN2-NDO, the hydrophobic

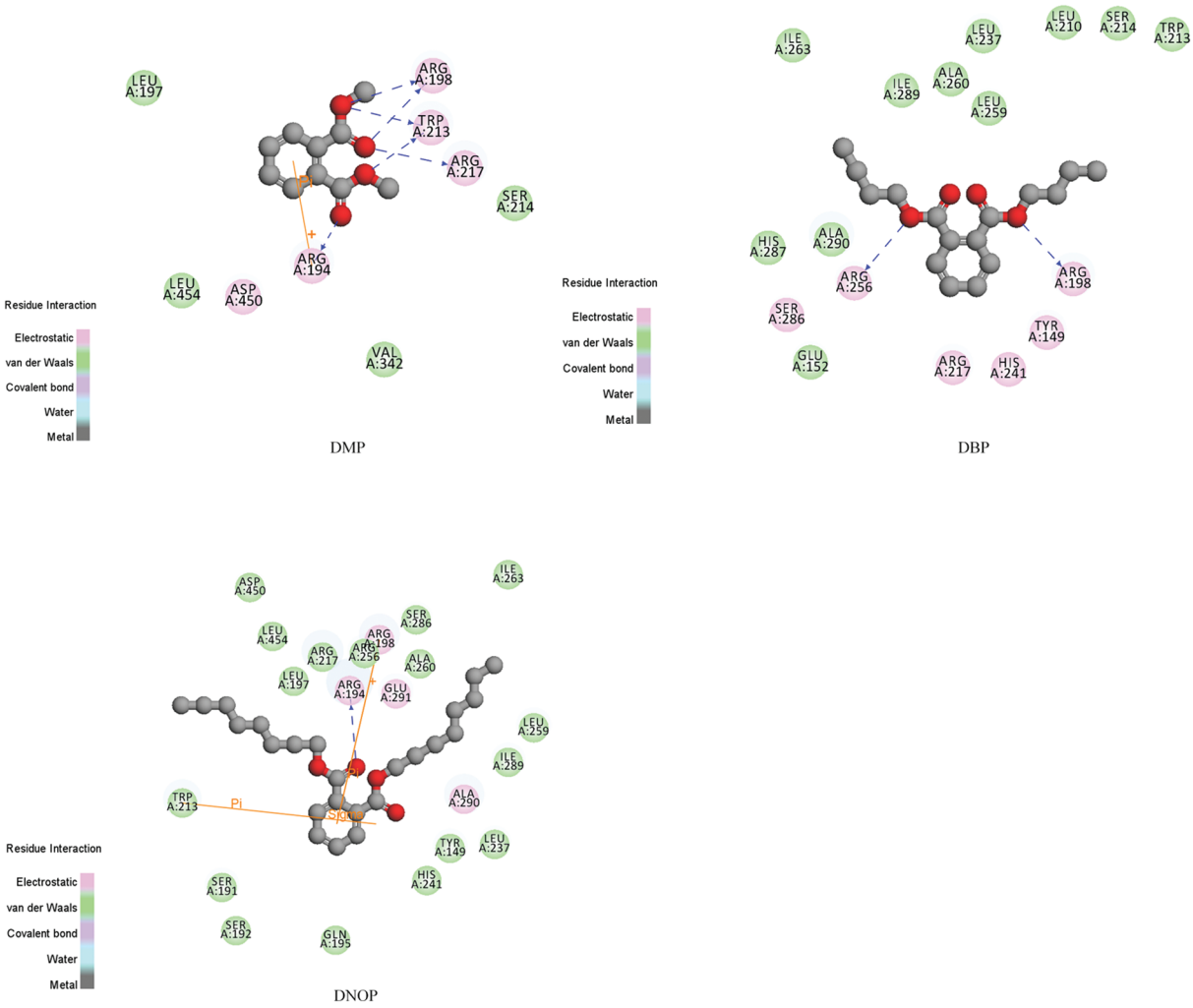

FIGURE 2. Binding conformation at the docking active sites of the target PAEs with BSA.

interactions at the active site were enhanced, which is more favourable to the binding of pyrene. Therefore, this paper intended to replace hydrophilic amino acid residues at the active site of BSA with hydrophobic amino acid residues to improve the affinity between the target PAEs and BSA, and thereby improve the efficient detection of target PAEs by BSA.

Design of targeted modification scheme of BSA with high detection sensitivity of fluorescence spectroscopy for PAEs

Reasonable protein design can identify key amino acid residues related to protein properties based on the protein structure, function and catalytic mechanism (Samant *et al.*, 2014). The mutation sites in its sequence were designed to change the specific amino acid residues in the protein through biological means such as substitution or deletion (Johnson *et al.*, 2010) to modify the characteristics of the protein molecules. The cellulase Asn179, Asp194 and Glu137 were mutated into Lys through site-specific modification and their thermal stability was significantly improved (Hakamada *et al.*, 2001). The effects of amino acids in Cel6A of thermobifida fusca cellulase on its catalytic activity showed that the mutant modified with Arg237, Glu263, Lys259 and His159 amino acid residues could improve the hydrolysis activity of the enzyme (Zhang *et al.*, 2010). As shown in Tab. 1, according to the hydrophilic and hydrophobic characteristics of 20 natural amino acid residues (Jiang, 2016), the hydrophilic amino acids Arg198 and Arg256 at the active site of BSA (PDB ID: 3V03) were replaced with hydrophobic amino acids Ile, Phe, Val, Leu, etc. A targeted modification scheme of single amino acid residues of eight novel modified BSAs was designed.

Homology modeling and rationality verification of novel BSA with high detection sensitivity of PAEs

The amino acid sequence of BSA (PDB ID: 3V03) was obtained from the NCBI database (Fig. 3). The homology of the modified novel BSAs and template proteins was more than 90%, indicating that the selected template protein was reasonable (Benkert *et al.*, 2011).

The Ramachandran diagram (Figs. 4 and 5) showed that 95.3% of the amino acid residues of the constructed BSA-1 structure were in the optimal region; 4.7% were in the allowable region; 94.0% of the constructed BSA-2 structure were in the optimal region; and 5.8% were in the allowable region. The above analysis indicates that the three-dimensional structure of BSA-1 and BSA-2 constructed by homology modeling was reasonable and reliable. The Ramachandran conformational maps of the eight modified BSAs were also analysed; the results verified that the novel modified BSA structure meets the rationality of the model

TABLE 1

Pro-/hydrophobic characteristics of 20 amino acid residues

Amino acid residues	Abbreviation	Type	Hydrophobic value	Amino acid residues	Abbreviation	Type	Hydrophobic value
Alanine	Ala	hydrophobic	0.62	Methionine	Met	hydrophobic	0.64
Cysteine	Cys	hydrophilic	0.29	Asparagine	Asn	hydrophilic	−0.85
Aspartic acid	Asp	hydrophilic	−1.05	Proline	Pro	hydrophobic	0.12
Glutamate	Glu	hydrophilic	−0.87	Glutamine	Gln	hydrophilic	−0.78
Phenylalanine	Phe	hydrophobic	1.19	Arginine	Arg	hydrophilic	−1.73
Glycine	Gly	neutral	0.48	Serine	Ser	hydrophilic	−0.18
Histidine	His	hydrophilic	−0.40	Threonine	Thr	hydrophilic	−0.05
Isoleucine	Ile	hydrophobic	1.38	Valine	Val	hydrophobic	1.08
Lysine	Lys	hydrophilic	−1.35	Tryptophan	Trp	hydrophobic	0.81
Leucine	Leu	hydrophobic	1.06	Tyrosine	Tyr	hydrophobic	0.26

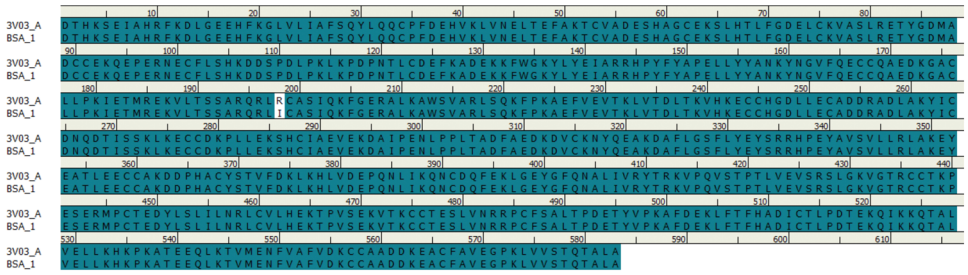

FIGURE 3. Amino acid sequence of BSA (PDB ID: 3V03) and novel BSA after modification (BSA-1).

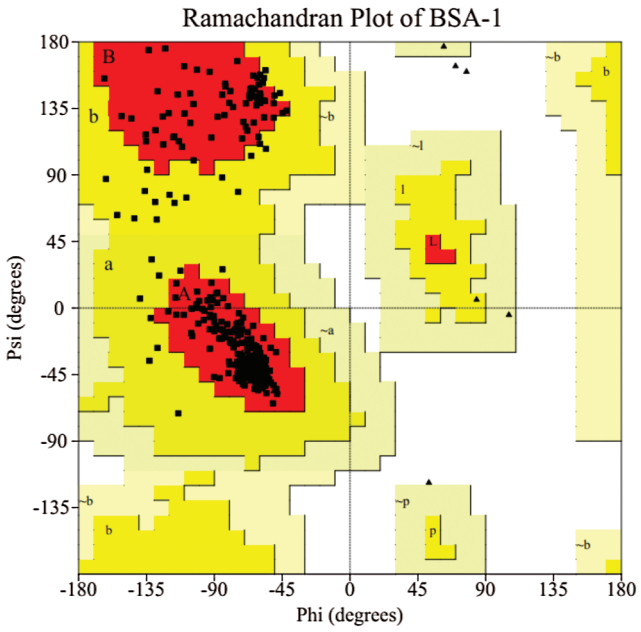

FIGURE 4. Ramachandran conformational maps of BSAs after modification BSA-1.

with an amino acid percentage greater than 95% in the optimal region + allowable region + maximum allowable region (Morris *et al.*, 1992).

Evaluation of binding affinity between BSAs after modification and PAEs

The target molecules (DMP, DBP and DNOP) were molecularly docked with the eight modified modified BSAs using the LibDock module in Discovery Studio 4.0 software. The scoring functions of the BSA (PDB ID: 3V03) docked with the DMP, DBP and DNOP molecules were 61.10, 91.02 and 109.49, respectively. As shown in Tab. 2, comparisons of the docking scores between the BSAs after modification with the target DMP, DBP and DNOP molecules showed that the hydrophilic amino acid residues on the active site of BSAs (PDB ID: 3V03) were replaced by hydrophobic amino acid residues. The docking scores of the BSA with target PAEs before and after modification were showed a difference. After the active sites

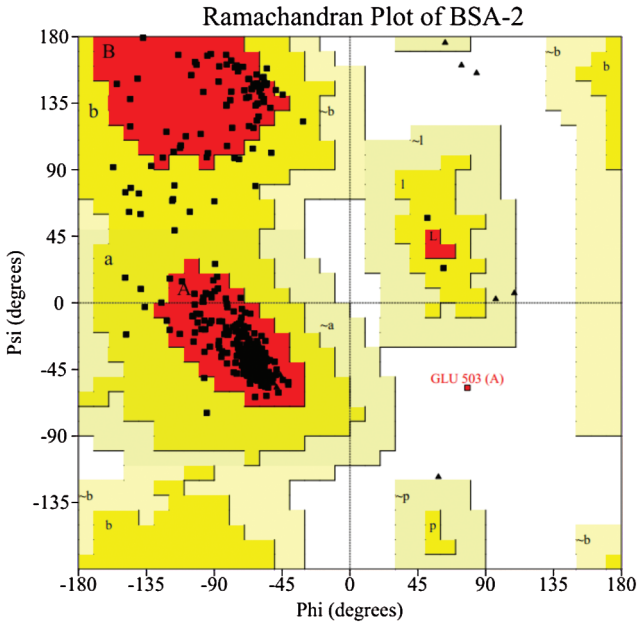

FIGURE 5. Ramachandran conformational maps of BSAs after modification BSA-2.

TABLE 2

Docking scores of BSAs with target PAEs (DMP, DBP and DNOP) before and after modification

Type	Original amino acid residues	New amino acid residues	DMP		DBP		DNOP	
			Docking scores	Change rate (%)	Docking scores	Change rate (%)	Docking scores	Change rate (%)
BSA			61.10		91.02		109.49	
BSA-1	Arg198	Ile	68.34	11.85	98.75	8.49	115.87	5.83
BSA-2	Arg198	Phe	74.92	22.61	99.00	8.77	93.58	−14.53
BSA-3	Arg198	Val	63.16	3.37	102.74	12.88	127.34	16.30
BSA-4	Arg198	Leu	77.27	26.45	99.19	8.97	107.89	−1.46
BSA-5	Arg256	Ile	74.33	21.65	104.67	15.00	108.82	−0.61
BSA-6	Arg256	Phe	74.00	21.12	106.33	16.82	100.41	−8.29
BSA-7	Arg256	Val	75.80	24.05	105.35	15.75	94.00	−14.15
BSA-8	Arg256	Leu	75.84	24.13	104.65	14.98	95.73	−12.57

Arg198 and Arg256 were replaced by hydrophobic amino acid residues such as Ile, Phe, Val and Leu, the scoring functions of all the modified BSAs docked with the target molecules (DMP and DBP) were improved at ranges between 3.37–26.45% and 8.49–16.82%, respectively, and only two modified BSAs docked with DNOP increased by 5.83% and 16.30%, respectively. Most docking scores are improved, indicating its corresponding BSA (PDB ID: 3V03). Site-directed modification could enhance the binding affinity between BSA after modification with target PAEs.

In summary, eight modification schemes to replace hydrophilic amino acids with hydrophobic amino acids at the active site of BSA could simultaneously increase the binding of BSA to target DMP, DBP and DNOP molecules to varying degrees, and promote the detection of BSA and target PAEs. The modification of the BSA (PDB ID: 3V03) with Arg198 at the active site instead of the hydrophobic amino acid Val was significant.

Changes in the fluorescence intensity of PAEs with BSA before and after modification

To further explore the influence of the novel modified BSAs on the fluorescence spectrum detection of target PAEs, Gaussian 09 software was used to calculate the fluorescence spectrum intensity of the target DMP, DBP and DNOP molecules combined with the novel modified BSAs under the TD-DFT method B3LYP/6-31G (d) base groups (Qu *et al.*, 2016). The calculated results are listed in Tab. 3.

After binding the target DMP, DBP and DNOP molecules with BSA (PDB ID: 3V03), the fluorescence intensity of the target PAEs was found to be weak (40.44, 32.35 and 28.31, respectively; Tab. 3). Compared to the fluorescence intensity of PAEs after binding with the original BSA, the fluorescence intensity of all DMP, DBP and DNOP molecules after binding to the novel BSAs increased. The fluorescence spectral intensities of DMP, DBP and DNOP molecules were increased by 2.10–2.80,

TABLE 3

Fluorescence intensity changes after binding of target PAEs to BSA before and after modification

Type	DMP		DBP		DNOP	
	Fluorescence intensity	Change rates (time)	Fluorescence intensity	Change rates (time)	Fluorescence intensity	Change rates (time)
BSA	40.44		32.35		28.31	
BSA-1	84.93	2.10	44.49	1.38	177.95	6.29
BSA-2	84.93	2.10	424.65	13.13	554.07	19.57
BSA-3	90.62	2.24	88.97	2.75	732.01	25.86
BSA-4	101.21	2.50	400.38	12.38	1767.34	62.43
BSA-5	105.15	2.60	101.11	3.13	469.13	16.57
BSA-6	112.34	2.78	1759.26	54.38	1532.78	54.14
BSA-7	113.24	2.80	962.53	29.75	509.58	18.00
BSA-8	89.21	2.21	3381.02	104.51	1633.88	57.71

1.38–104.51 and 6.29–62.43 times, among which the fluorescence intensities were significantly enhanced after binding with novel modified BSA-7, BSA-8 and BSA-4. In conclusion, the selected modification schemes of BSA (PDB ID: 3V03) could effectively improve the fluorescence spectrum intensity of DMP, DBP and DNOP molecules combined with BSA so that the target PAEs could be detected more easily. A comprehensive analysis of the changes in the fluorescence intensity of the target DMP, DBP and DNOP showed that the modification of the novel BSA-7, BSA-8 and BSA-4 at the designed active sites of BSA (Arg25 instead of Val, Arg256 instead of Leu and Arg198 instead of Leu) had the most significant improvement in the fluorescence spectrum intensity of the target PAEs. Compared to the DMP and DNOP, the effects of the novel BSAs has a more significant effect on the fluorescence intensity of DBP molecules.

Discussion

In this paper, the theoretical modifications of BSA with high detection sensitivity of PAEs was carried out, and the influence of novel modified BSA on the fluorescence intensity of PAEs was analysed. The results showed that the binding affinity of eight novel modified BSAs with high detection sensitivity to target DMP, DBP and DNOP was improved to varying degrees. The modification of novel BSA with Arg198 replaced with Val at the active site was very significant. Three modified BSAs with high detection sensitivity (BSA-7, BSA-8 and BSA-4) showed the most significant improvement in the fluorescence spectrum intensity of the target DMP, DBP and DNOP. The novel BSAs had a more obvious effect on the fluorescence intensity values of DBP. Theoretical verification showed that the modified BSAs designed using this method satisfies the purpose generating high detection sensitivity of PAEs and could provide a theoretical basis for the design of modified BSAs with high detection sensitivity of PAEs.

Acknowledgement: The authors would like to thank Wordvice (https://wordvice.cn/) for their English language editing.

Funding Statement: The authors received no specific funding for this study.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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