Journal of Chromatography A, 1217 (2010) 7539–7546

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Design, optimization and evaluation of specific affinity adsorbent for oligopeptides

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ARTICLE INFO

Article history:
Received 30 June 2010
Received in revised form
24 September 2010
Accepted 4 October 2010

Keywords:
Affinity adsorbent
Specific adsorption
Middle molecular peptides
Hydrophobic inclusion
Cooperation

ABSTRACT

A major challenge in the development of affinity adsorbents is the design of specific adsorbents for target molecules. In this paper, a two-step strategy was used to design a specific adsorbent for oligopeptides. Based on the structural characteristics of target peptide DFLAE (DE5), the affinity ligand CDenHis bearing hydrophobic inclusion and electrostatic interaction sites was prepared by grafting histidine onto β-cyclodextrin (CD) using ethylenediamine; ligands with single hydrophobic inclusion or electrostatic interaction sites (CDen and HisOMe) were used as reference ligands. Results indicated that the binding affinity (K_a) of CDenHis with DE5 was $6.23 \times 10^4\ M^{-1}$, 23- and 61-fold higher than that of CDen and HisOMe, respectively. Computer simulations were used to further optimize the steric configuration of CDenHis. It was found that the optimized ligand CDdnHis exhibited a much improved binding affinity for DE5 ($K_a = 1.02 \times 10^5\ M^{-1}$). Moreover, the corresponding adsorbent A-CDdnHis not only showed much better adsorption ability compared with A-CDenHis, but also excellent adsorption specificity for DE5-containing peptides. Kinetic analysis and adsorption mechanism studies suggested that the configuration matching of CDdnHis with DE5 and the cooperation of multiple interactions led to the fast and selective adsorption of DE5-containing peptides to A-CDdnHis.

1. Introduction

Since affinity adsorbents were first used for the purification of staphylococcal nuclease in 1968 [1], they are predicted to be useful in the specific adsorption and elimination of target molecules from solution. Conventionally, affinity adsorbents are prepared by the immobilization of affinity ligands on a matrix. Two main types of affinity ligands are used: biomolecular ligand and synthetic ligands. Biomolecular ligands can have good affinity and selectivity for the target proteins and peptides, but are associated with low structural stability and high costs. Synthetic ligands have been investigated extensively in recent years; some synthetic ligands can form stable binding with target molecules, and show high durability and low cost. However, the specificity of synthetic ligands needs to be further improved. To our knowledge, weak interactions, such as hydrophobic interactions, electrostatic interaction, hydrogen bonding, and van der Waals interactions, are thought to be responsible for the adsorption [2]. An effective approach to regulate these weak interaction sites on synthetic ligand is of particular interest for the preparation and improvement of specific affinity adsorbents.

Middle molecular weight toxins ranging from 500 to 5000 Da are the main toxic substances that accumulates in uremic patients, among which middle molecular peptides have been assumed to be one type of major uremic toxin [3]. The accumulation of these middle molecular peptides in the human body is thought to be toxic and contribute to many pathological changes in patients [4,5], and these toxins are expected to be eliminated by affinity adsorption in hemoperfusion. As reported by Kaplan et al., DFLAE (DE5) is a fragment of many middle molecular peptides that accumulate in the serum of uremic patients and has been shown to be related to uremia [6]. Therefore, specific affinity adsorbents for DE5 and DE5-containing peptides would be helpful for the elimination of middle molecular peptides in the treatment of uremia.

Cyclodextrins (CD) are a class of cyclic oligosaccharides consisting of several cyclic α-(1-4)-linked D-glucooligosaccharides units. They are water-soluble and have hydrophobic cavities into which a hydrophobic guest molecule of an appropriate size and shape can be included [7], such as adamantine [8,9], aromatic amino acid [10,11] and so on [12,13]. Recently, cyclodextrins have been widely used in many areas, including the food and pharmaceutical industries and analytical chemistry owing to their inclusion properties and excellent biocompatibility [14]. Moreover, cyclodextrins also have specific features as core molecules in adsorption and separation processes because their hydrophobic cavity can provide an important interaction site [15]. Thus, cyclodextrin derivatives bearing hydrophobic inclusion and other multiple interaction sites could be

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doi:10.1016/j.chroma.2010.10.007

promising specific ligand for peptides containing aromatic amino acid.

In this study, two steps were considered in the design of a specific affinity adsorbent for DE5-containing peptide. First, based on the structural characteristics of DE5, we presented a biocompatible affinity ligand (CDenHis) designed as cyclodextrin derivatives; this ligand has both hydrophobic inclusion and electrostatic interaction sites. Isothermal titration calorimetry (ITC) experiments and adsorption experiments were performed to examine the effects of these interaction sites. Second, with the help of computer simulation, further optimization to CDenHis was performed by molecular docking analysis. The optimized ligand CDdnHis and its corresponding adsorbent A-CDdnHis were designed and synthesized, and their binding affinity, adsorption kinetic, adsorption ability and specificity were examined. Furthermore, the detailed adsorption mechanism of A-CDdnHis to DE5 was also assessed.

2. Materials and methods

2.1. Materials and measurements

β-CD was purchased from Tianjin Chemical Co. and recrystallized from water twice and total dried in vacuo for 24 h at 100 °C using P_2O_5 trap. p-Toluenesulfochloride was recrystallized from petroleum ether. Ethylenediamine was dried with Mg_2SO_4 and distilled before use. Agar powder was BR grade and purchased from Reagents Company of National Pharmaceutial Group Company (Shanghai, PRC). Peptides and Boc-Histidine were purchased from GL Biochem (Shanghai) Ltd. The other reagents and solvents were analytical grade and commercial available without further purification.

^{1}H NMR spectra were recorded on a Varian UNITY plus 400 MHz NMR spectrometer.

2.2. Preparation of affinity ligands and affinity adsorbents

2.2.1. Tosylation of β-cyclodextrin (tosyl-CD)

The preparation of Tosyl-CD was according to the method in Ref. [16]. Typically, CD (40 g) were dissolved in aqueous sodium hydroxide solution (0.4 M, 600 mL), p-toluenesulfochloride (30 g) was added and reacted under vigorous agitation at 0 °C for 2 h. After filtering off the unreacted p-toluenesulfochloride, the solution was adjusted to neutralize using hydrochloric acid, and tosyl-CD was obtained as a precipitate. The pure tosyl-CD was obtained from recrystallization of crude Tosyl-CD from hot water. ^{1}H NMR (400 MHz, DMSO-d_6): δ7.76–7.74 (d, 2H), δ7.44–7.42 (d, 2H), δ5.83–5.63 (m, 14H), δ4.83(s, 4H), δ4.77 (s, 3H), δ4.51–4.31 (br, 6H), δ3.66–3.43(m, 28H), δ2.43 (s, 3H).

2.2.2. Amination of β-cyclodextrin (CDen and CDdn)

Tosyl-CD (5 g) was dissolved in ethylenediamine (60 mL), the reaction was carried out at 80 °C for 4 h. Then, approximately half of the unreacted ethylenediamine was removed by distilling. The condensed solution was added dropwise to acetone (500 mL), and crude CDen was obtained as a precipitate. The precipitate was dissolved in 10 mL water; the pure CDen was precipitated from acetone twice. ^{1}H NMR (400 MHz, D_2O): δ5.12 (s, 7H), δ4.03–3.89 (m, 28H), δ3.72–3.61 (m, 14H), δ2.94(s, 2H), δ2.81 (s, 2H).

CDdn was prepared by Tosyl-CD and diethylenetriamine with the same procedure. ^{1}H NMR (400 MHz, D_2O): δ5.12 (s, 7H), δ4.03–3.89 (m, 28H), δ3.72–3.61 (m, 14H), δ2.78–2.51 (br, 8H).

2.2.3. Histidine modified amination of β-cyclodextrin (CDenHis and CDdnHis)

Boc-Histidine (0.325 g) was dissolved in 15 mL water, the solution was cooled to 0 °C. EDC (0.488 g) and NHS (0.293 g) were added, and the reaction was maintained with stirring for 1 h. Then, the solution was warmed to 25 °C, CDen (3 g) was added, the reaction was carried out at 25 °C for a period of 24 h. The crude CDen-His-Boc was obtained by precipitation of the solution from acetone. CDen-His-Boc was then purified by column (20 × 600 mm) CM-sephadex C-25 (NH_4^+ form), the appropriate fraction were concentrated to give CDen-His-Boc.

For the deprotecting step, CDen-His-Boc was dissolved in neat trifluoroacetic acid, the solution was stirred at room temperature for 1 h and the solvent was evaporated. The product was purified by CM-sephadex C-25, the appropriate fraction were concentrated to give CDen-His. ^{1}H NMR (400 MHz, D_2O): δ8.17 (s, 1H), δ7.20 (s, 1H), δ5.07 (m, 7H), δ4.03–3.89 (m, 28H), δ3.72–3.61 (m, 14H), δ3.41–3.23 (m, 2H), δ3.21–3.04 (br, 4H).

CDdnHis was prepared from CDdn and Boc-Histidine according to the above procedure. ^{1}H NMR (400 MHz, D_2O): δ8.17 (s, 1H), δ7.20 (s, 1H), δ5.07 (m, 7H), δ4.03–3.89 (m, 28H), δ3.72–3.61 (m, 14H), δ3.20–2.61(br, 10H).

2.2.4. Histidine methyl ester

Histidine (6.23 g) was suspended in 75 mL CH_3OH, the mixture was cooled to −10 °C. $SOCl_2$ (5.64 mL) was added dropwise to the mixture (approximately 40 min). After that, the mixture was warmed to 40 °C and maintained for 4 h, and then heated up to 80 °C and maintained for 12 h. The suspending liquid changed to a clear solution. Histidine methyl ester was obtained after the evaporation of solvent. ^{1}H NMR(400 MHz, D_2O): δ8.74 (s, 1H), δ7.48 (m, 1H), δ4.54 (t, 1H), δ3.86 (s, 3H), δ3.51(m, 2H).

2.2.5. Preparation of agar gel beads

Two grams of tween-80 (as a dispersion agent) was dissolved in 250 mL solution of toluene and carbon tetrachloride (3:1, V/V) at 75 °C. 4 g agar powder and 70 mL distilled water were mixed and heated to reflux, and then the sticky liquid was poured into the dispersed phase under stirring at 75 °C for 1 h. After cooling to room temperature, the resultant agar beads were sieved (20–40 meshes) and washed with distilled water.

2.2.6. Preparation of affinity adsorbents

Before modification by affinity ligands, 20 g of agar beads was incubated in a mixture of 13.3 mL epichlorohydrin and 23.5 mL NaOH solution (2 M), and the reaction was performed at 40 °C for 2 h. After washing with distilled water, 2 g beads were suspended in 10 mL aqueous solution, which contained 0.5 g affinity ligand. The mixture was adjusted to pH 12 and shaken at 60 °C for 4 h. The final adsorbents were washed with distilled water adequately. The fry adsorbents were characterized by element analysis (Table 1).

Table 1
Element analysis of the dry adsorbents.

	A-HisOMe	A-CDen	A-CDenHis	A-CDdnHis
N%	3.00%	1.1%	0.90%	0.93%
C%	39.93%	40.99%	40.39%	40.89%
H%	6.81%	6.04%	7.28%	6.23%

The molar amount of ligand modified can be calculated by the equation:

$$Q = \frac{mN\%}{nM}$$

where Q stands for the molar amount of ligand modified, m stands for the mass of the dry adsorbent. $N\%$ stands for the content of the nitrogen. n stands for the number of nitrogen in a ligand. M is the atomic weight of nitrogen.

2.3. ITC experiments

ITC experiments were performed on a VP-ITC (Microcal) calorimeter in H_2O at 298.15 K. In general, a solution of peptide (1.5 mM) was injected in portions (9 μL × 30 times) to a solution of ligand (0.15 mM). The heat flow was recorded, plotted against time, and converted into enthalpies (ΔH) by integration of the appropriate reaction heat peaks. Dilution heat was determined by a blank experiment, all the data were calculated by Origin ITC Data Analysis Software using one set of sites model.

2.4. Adsorption experiments

0.1 g of wet adsorbents was added into peptide solution (3.5 mL, 0.2 mol/mL) in a 10 mL glass bottle, and the bottle was oscillated at 25 °C. The absorbance of the peptides solution was determined with a Unico UV-4802 spectrometer at 220 nm. A series of peptide solutions with different concentrations were utilized to make a calibration curve and the concentration of each sample was determined by comparison with the calibration curve. Because the amount of ligand modified on the agar gel was different, adsorption ability was used to characterize the adsorption of each adsorbent. The adsorption ability was calculated by the equation:

$$Aa = \frac{(c_1 - c_2)V}{Q}$$

where Aa stands for the adsorption ability, c_1 and c_2 stand for the concentration before and after adsorption, respectively, V stands for the volume of peptide solution used in adsorption, Q stands for the molar amount of ligand modified on the agar beads.

2.5. Molecular docking

The Sillicon Graphics SGI INDIGO 2 workstation was used for the stimulation. Molecular building, geometry optimization, conformational search were done using the molecular modeling package Sybyl version 6.6. The lowest energy conformations of peptides and ligands were obtained by simulated annealing, respectively. Each molecule was subjected to simulated annealing for 100 cycles. During the simulated annealing, each molecule was heated to 1000 K for 1000 fs and then cooled to 0 K for 1000 fs. The conformations with the lowest energy for peptides and ligands were energetically minimized by Powell's method using Tripos force field with Gasteiger–Hückel charges added until a terminating gradient of 0.005 kcal mol^{-1} $Å^{-1}$ was reached. The DOCK module of the Sybyl package was used to perform the docking between ligand and peptide. In calculation, the molecular mechanics method and molecular dynamics method were used [17]. DE5 docking to the ligands with different conformations was performed. In all cases lowest energy conformation was applied as the initial molecular configuration. The results of computer simulation were expressed using binding energy (E_{bind}, kJ/mol), which was determined as following:

$$E_{bind} = E_{complex} - E_{peptide} - E_{ligand}$$

where $E_{complex}$ is the energy of complex formed by docking of DE5 and ligand, $E_{peptide}$ and E_{ligand} represent the energy of DE5 and ligand, respectively. The $E_{complex}$, $E_{peptide}$ and E_{ligand} were calculated by the energy minimization method in the Sybyl package.

2.6. SPR kinetic analysis

Kinetic binding measurements were performed on a BIAcore 3000 SPR measurement apparatus on CM5 chips. The ligands were immobilized on the chips by a conventional amine coupling. Briefly, the CM5 chip was initially treated with EDC/NHS, and affinity ligand CDenHis and CDdnHis were immobilized at a constant flow rate of 5 mL/min at 25 °C. A total of 223 RU CDenHis and 257 RU CDdnHis were immobilized (RU: pg/mm^2, immobilized protein concentration [18]).

All SPR kinetic experiments were performed at 298 K with diluted DE5 solutions running over the ligand-immobilized chip surfaces at a constant flow rate of 10 μL/min. Deionized water was used as eluant. In each run, different concentration of DE5 were injected over the ligand-modified sensor surface, the injection time was 60 s, followed by a 300 s dissociation phase. The chip was regenerated with a solution of NaOH (50 mM) using a flow rate of 10 μL/min for 2 min, and then 0.02% sodium dodecyl sulfate for 1 min. The binding and dissociation data were analyzed in BIA evaluation Software version 4.0 with a nonlinear regression analysis of the measured sensorgrams (1:1 Langmuir binding model).

2.7. NMR experiments

^{1}H NMR study of CDdnHis with DE5 was carried out on a Varian UNITY plus 400 MHz NMR spectrometer at room temperature. The interaction sample was prepared by dissolving DE5 and CDdnHis (1:1, mol/mol) in D_2O.

3. Results and discussion

3.1. Preliminary design of affinity ligands

The structure–function relationship of affinity ligand has received much attention and been well characterized. However, owing to the difficulties associated with realizing complete spatial matching of ligands with target molecules and the competition from solvent, actual specific adsorption by affinity adsorbent remains a challenge. Lowe and co-workers outlined that the basic stage for the design of affinity ligands was the selection of target binding sites on target molecules for the modeling of ligands [19]. Previous work by Schmuck and which demonstrated the efficiency of multiple interactions on the design of ligands [20]. Moreover, considering the molecular recognition principle of "lock–key" [21], the realization of specific adsorption should focus on two points: first, the ligands could provide multiple weak interaction sites to target molecules; second, the steric configuration of ligands must match the appropriate geometric organization of reciprocal functional groups on the target molecule.

In this study, the frequent sequence DE5 was used as the target peptide and affinity adsorbents were designed to selectively eliminate DE5 and DE5-containing peptides. DE5 has a special structure with hydrophobic phenylalanine residues in the middle and two acidic amino acid residues (Asp and Glu) at each terminus (Fig. 1(A)). Based on its structural features, ligands bearing both hydrophobic and electrostatic interaction sites are expected to display multiple binding interactions with DE5. Cyclodextrin, which has a hydrophobic cavity, has a remarkable capacity to form inclusion complexes with hydrophobic moieties through host–guest interactions. Here, it is proposed to form hydrophobic inclusion with the Phe residue of DE5. Electrostatic attractions are strong weak interactions and can be easily achieved in aqueous solution. We attempted to introduce electrostatic interaction sites by the modification of a histidine on CD. Thus, an affinity ligand (CDenHis) bearing cyclodextrin and basic histidine was designed and synthesized; CD and histidine were connected by an ethylenediamine molecule, which provided an interval between the functional groups to form a proper steric configuration. Ligands with only CDen and histidine were used as a reference; the carboxyl groups of the histidine ligand were esterified to supply only basic imidazolyl groups, as in CDenHis (Fig. 1(B)).

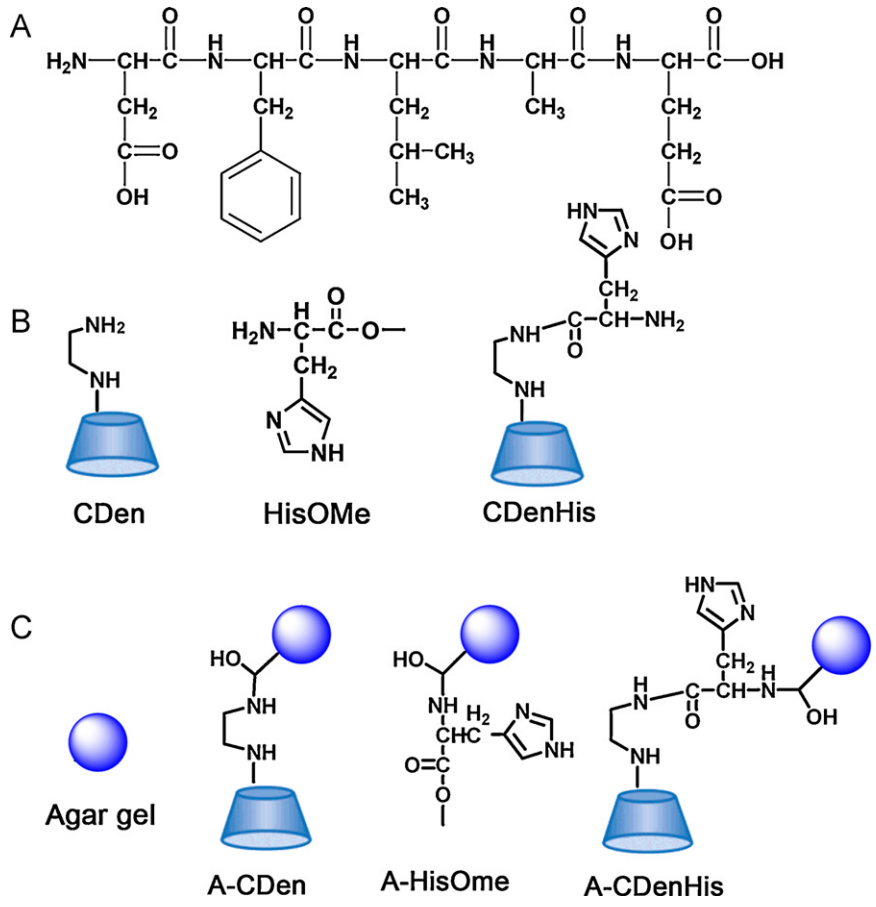

Fig. 1. Chemical structures of DE5 (A), affinity ligands (B) and corresponding affinity adsorgbents (C).

3.2. Binding affinities between ligands and DE5

The binding affinities of DE5 and ligands were assessed by ITC analysis. Fig. 2 shows the heat flow of each injection when DE5 was injected into cells containing CDen, HisOMe or CDenHis solutions. All data were analyzed using ITC analysis software with one set of sites model after subtracting the reference. The resulting affinity constants (K_a) are shown in Fig. 2. The K_a of DE5-CDenHis was $6.23 \times 10^4\ M^{-1}$, about 23- and 60-fold higher than that of DE5-CDen and DE5-HisOMe ($2.66 \times 10^3\ M^{-1}$, $1.02 \times 10^3\ M^{-1}$), respectively. It can be seen that there is a great improvement in the binding affinity of DE5-CDenHis with multiple interaction sites. As mentioned above, two important possible interaction sites were designed for CDenHis; compared with the single interaction site for each of CDen and HisOMe, the multiple interactions would be expected to lead to an improvement in the binding affinity. The much higher K_a of DE5-CDenHis provides preliminary evidence to support the efficiency of the multiple interaction sites.

3.3. Structural optimization by molecular docking

Computer simulation is a simple and rapid technique to mimic the complexation of molecules and it has also been used to design affinity adsorbents, as reported by Labrou and Liu [22,23]. Computer simulation can provide binding information at the molecular level, which can direct the improvement in the adsorbents. In this study, a molecular docking method was used to further optimize the steric structure of the ligands.

ITC experiments have demonstrated the efficiency of the two interaction sites on CDenHis, and the distance between these two interaction sites has a key role in the cooperation of multiple interactions. Here, molecular docking results provided the rational interpretation of the interactions. The lowest energy conformation of DE5 and ligand were obtained by simulated annealing respectively. The DOCK module of the Sybyl package was used to perform the docking between ligand and peptide. The most favorable binding configuration of DE5 with CDenHis is shown in Fig. 3(A). For CDenHis, computer simulation showed that its binding with DE5 was favorable with a binding energy −64.51 kJ/mol. However, it can also be seen that the distance between the CD and His on CDenHis was slightly shorter than the distance between Phe and Glu of DE5 (Fig. 3(A)), which means the distance between the two functional groups when separated by an ethylenediamine should not be optimal. Therefore, another ligand CDdnHis, in which CD and His were connected by a diethylenetriamine, was designed by computer. The lowest binding energy of CDdnHis-DE5 was −79.67 kJ/mol, which is a more negative energy than that of CDenHis-DE5, indicating that CDdnHis is a more preferable structure for binding to DE5. As shown in Fig. 3(B), CDdnHis could form near-perfect matching with DE5 both for the two major weak interactions. With the help of computer simulation, CDdnHis was shown to be a more closely matched ligand by structural optimization.

3.4. Adsorption properties of adsorbents

To verify their adsorption ability to DE5, the affinity ligands CDen, HisOMe, CDenHis and CDdnHis were grafted onto agar gel beads (20–40 mesh) by epichlorohydrin; the resulting adsorbents were named A-CDen, A-HisOMe, A-CDenHis and A-CDdnHis, respectively. We evaluated their adsorption abilities to DE5 by UV–vis spectroscopy at 25 °C by static adsorption experiments.

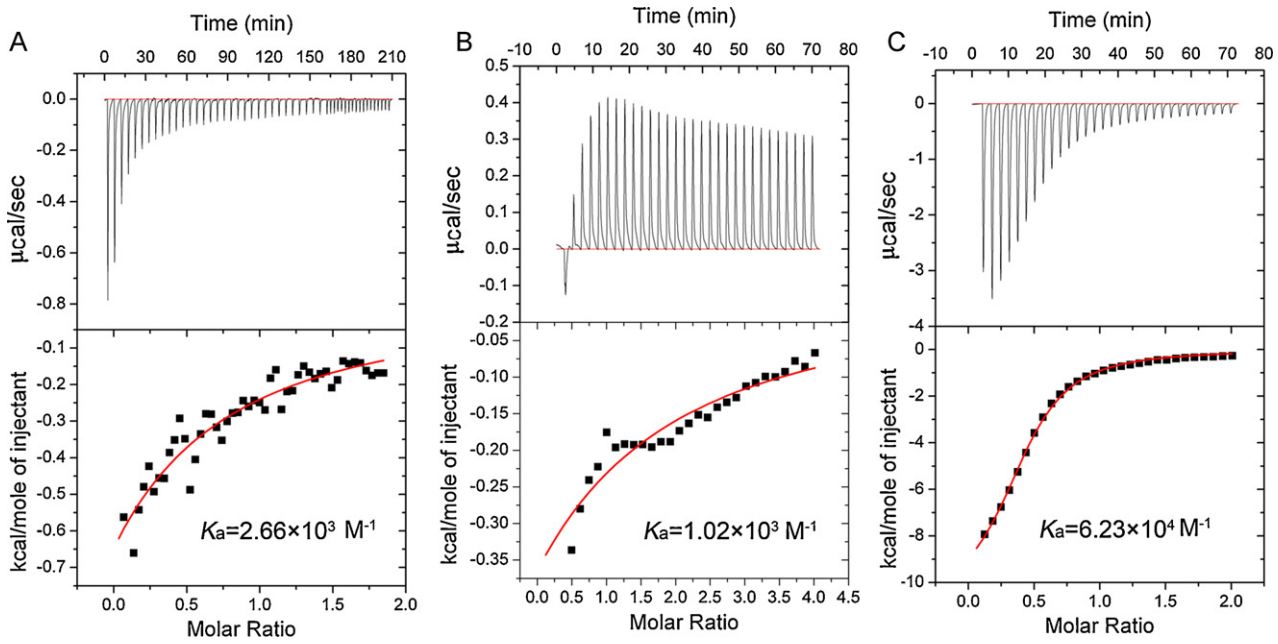

Fig. 2. ITC titration of DE5 into cells filled with solutions of CDen (A), HisOMe (B) and CDenHis (C). The upper panels show the raw data for sequential injections of DE5 into different ligand solutions. The bottom panels show the apparent reaction heat obtained from the integration of calorimetric traces. The binding affinity was obtained by the fitting of one set of site models after subtracting the reference values.

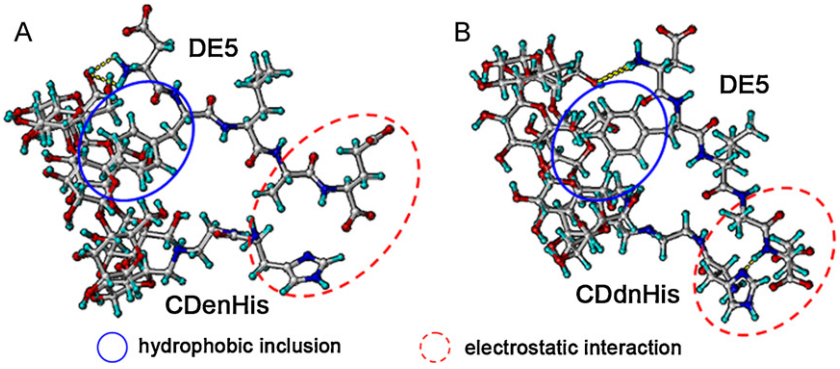

Fig. 3. The most favorable binding of DE5 docked to CDenHis (A) and CDdnHis (B) using molecular docking.

As shown in Fig. 4, for defined aqueous solutions with excess DE5, all static adsorption experiments reached an equilibrium state within 30 min. As there was no obvious adsorption on pure agar gels, the adsorption of DE5 onto affinity adsorbents should be attributed to the efficiency of the ligands grafted on the agar gel. We represented their adsorption ability to DE5 as the molar ration of DE5 adsorbed versus the affinity ligands grafted on agar gel. The modification by CDen and HisOMe improved the adsorption ability of the adsorbents. However, compared with A-CDenHis, the improvements in adsorption were limited. A-CDenHis exhibited the best adsorption ability to DE5, more than 2.5-fold that of A-CDen and 16-fold that of A-HisOMe. The adsorption results were in agreement with the ITC results. The adsorption ability also indicated that the multiple interactions on A-CDenHis led to improved adsorption ability. After optimization, ITC experiments revealed that the affinity constant of CDdnHis-DE5 was $1.02 \times 10^5\ M^{-1}$, which was higher than that of CDenHis-DE5. Moreover, it was found that there was a considerable increase in the adsorption ability of A-CDdnHis compared with A-CDenHis and other adsorbents (Fig. 4), the ratio of DE5 to ligand was up to 0.74, which means 74% of CDdnHis grafted onto agar gel bonded a target peptide in the adsorption. Together, these results indicated the viability of computer optimization and A-CDdnHis was shown to have the best spatial matching with DE5.

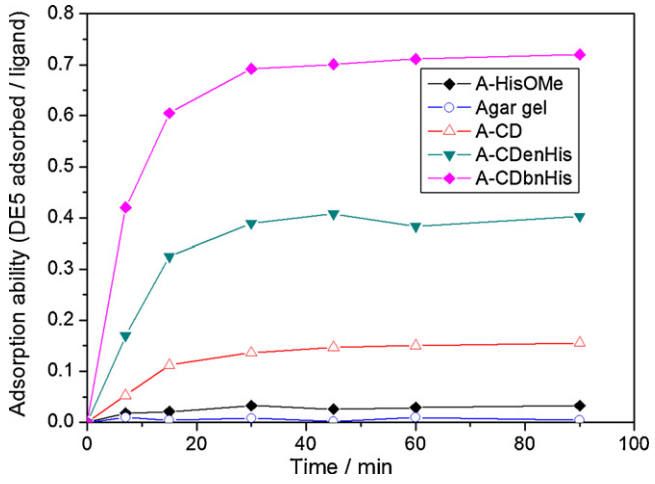

Fig. 4. Adsorption of DE5 on A-CDen, A-HisOMe, A-CDenHis, and A-CDdnHis.

SPR biosensor techniques provide fast, automatic, real-time examination of kinetic binding, and can provide both association and dissociation data for the formation of molecular complexes

Table 2
SPR kinetic parameters for the binding of DE5 to CDenHis and CDdnHis at 298 K.

Complex	Kinetic parameters			
	k_a (1/Ms)	k_d (1/s)	K_a (1/M)	K_d (M)
DE5/CDenHis	76	2.5×10^{-3}	3.0×10^{4}	3.3×10^{-5}
DE5/CDdnHis	670	5.8×10^{-3}	1.2×10^{5}	8.6×10^{-6}

k_a and k_d stand for the kinetic association and dissociation constants, respectively whereas K_a and K_d stand for the thermodynamic association and dissociation constants, respectively.

[24,25]. Because affinity adsorption, by definition, occurs mostly at the interface of adsorbents, SPR sensor surfaces immobilized with CDenHis and CDdnHis appeared to be ideal adsorption models to evaluate surface adsorption. In this study, CDenHis and CDdnHis were immobilized on the sensor surface and a solution of DE5 flowed across the surface to investigate its binding properties to adsorbents. A 1:1 Langmuir kinetic model was used to fit the binding curves. Table 2 summarized the kinetic parameters for the binding of DE5 to the sensor modified with CDenHis and CDdnHis. By comparing the kinetic association constants (k_a), it can be seen that the k_a of DE5-CDdnHis is about nine times that of DE5-CDenHis, which means the binding of DE5-CDdnHis is much faster than that of DE5-CDenHis. Moreover, the thermodynamic association constant (K_a) of DE5-CDdnHis from SPR experiments is comparable to that in ITC experiments, but for DE5-CDenHis, the K_a from SPR experiments is half that from ITC experiments. These variations may be arising from the principle of these two methods, the examination by ITC focuses on the thermodynamics of the binding, while SPR detects the dynamic process. It can be seen from the obtained K_a and kinetic parameters that the binding of DE5 with CDdnHis is fast and stable. In SPR experiments, peptide solution flows through the chip surface modified with ligand, the flow of the peptide solution (10 μL/min in SPR experiment) should have little influence on the binding of DE5 to CDdnHis. Whereas, in contrast, the binding of DE5 to CDenHis is a slow complexation, possibly because DE5 and CDenHis need more time to match their steric conformations to form a more stable complex, so the flow of the solution may shorten the contact time of DE5 with CDenHis and decreased the stability of the complex in some extent. But in the ITC experiments, long enough contact time can provide sufficient binding between DE5 and ligands, which lead to the differences of their affinity constants compared with SPR results. Taking these results into consideration, A-CDdnHis exhibits an excellent fast and stable adsorption to DE5, and these properties make A-CDdnH a suitable affinity adsorbent for DE5.

Furthermore, proper affinity adsorbents should be specific to target molecules. To examine the adsorption selectivity of A-CDdnHis to DE5 and DE5-containing peptides, another six peptides were used. Their amino acid sequences are summarized in Table 3; DE8, DG12 and SR14 are middle molecular peptides containing the DE5 sequence also found in uremic serum; SH5 is a pentapeptide with double acidic residues but without the hydrophobic residues; VW8 is a basic peptide with double hydrophobic Try residues; and GF6 contains both acid amino acid residues and a

Table 3
The amino acid sequence of peptides.

Peptide	Amino acid sequence
DE5	**Asp-Phe-Leu-Ala-Glu**
DG8	**Asp-Phe-Leu-Ala-Glu**-Gly-Gly-Gly
DG12	Asp-Ser-Gly-Glu-Gly-**Asp-Phe-Leu-Ala-Glu**-Gly-Gly
SR14	Ser-Gly-Glu-Gly-**Asp-Phe-Leu-Ala-Glu**-Gly-Gly-Gly-Val-Arg
SH5	Ser-Glu-Ala-Asp-His
GF6	Gly-Arg-Trp-Met-Asp-Phe
VW8	Val-Val-Arg-Gly-Cys-Thr-Trp-Trp

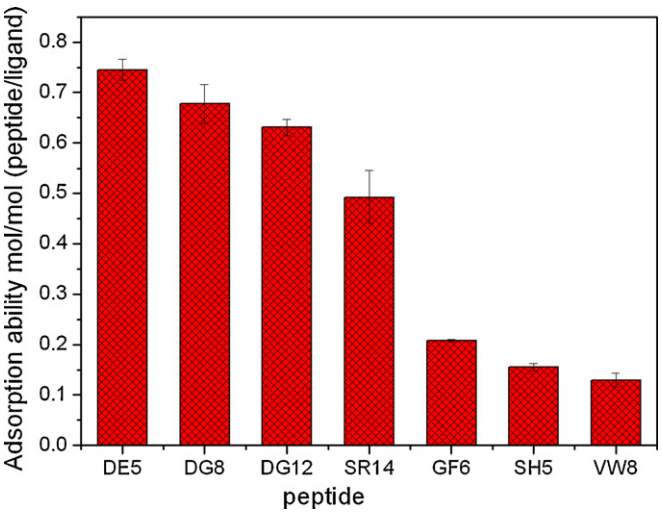

Fig. 5. Adsorption selectivity of A-CDdnHis to DE5-containing peptides.

hydrophobic Phe residue. We used static adsorption experiments to test the adsorption ability of A-CDdnHis to these peptides. As shown in Fig. 5, A-CDdnHis showed greater specificity for DE5 and DE5-containing peptides compared with GF6, SH5 and VW8. As for SH5 and VW8, although they contain the potential electrostatic interaction or hydrophobic inclusion sites, it is difficult to form stable binding with A-CDdnHis by a single weak interaction, and for GF6, although it contains electrostatic interaction and hydrophobic inclusion sites, the spatial mismatching of GF6 with A-CDdnHis likely led to the poor adsorption ability. In summary, the optimized A-CDdnHis also showed good adsorption specificity to DE5 and DE5-containing peptides.

Furthermore, the adsorption selectivity of A-CDdnHis to DE5 from mixed peptide solution was also examined. As displayed in Fig. 6, it can be seen that whether in single peptide solution or in mixed solution, A-CDdnHis exhibits much better adsorption capacity to DE5 compared with VW8 and SH5. This result also provides further evidence to the adsorption selectivity of A-CDdnHis to DE5.

3.5. Adsorption mechanism and binding sites

To further characterize the binding sites, ^{1}H NMR analysis was used to characterize the chemical shifts of DE5 and CDdnHis. Fig. 7(A) displays the expansions of ^{1}H NMR spectra of DE5, CDdnHis, and CDdnHis-DE5. Upon complexation, 0.09 ppm upfield shifts were observed for the protons of the phenyl group of DE5. These chemical shifts are likely due to the shielding effects induced by the hydrophobic cavity of CD, which indicate that the phenyl

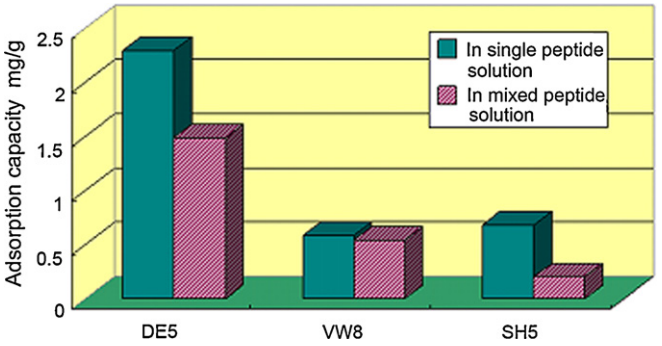

Fig. 6. Adsorption capacity of A-CDdnHis to peptides in their single solution and in the mixed peptides solution.

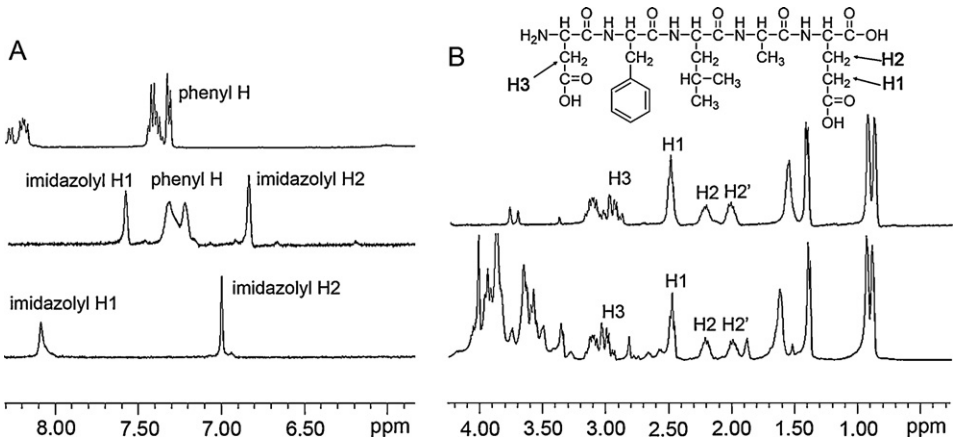

Fig. 7. ^{1}H NMR spectra of DE5 (A, top), CDdnHis (A, bottom), CDdnHis-DE5 (A, middle, and the chemical shifts in the upfield, DE5 (B, top) and CDdnHis-DE5 (B, bottom).

ring was included in the hydrophobic cavity. For the proton of imidazolyl group on CDdnHis, an obvious chemical shift was also observed for imidazolyl H1 and H2 (Fig. 7(A)), indicating the existence of an electrostatic interaction between DE5 and the imidazolyl group of CDdnHis. In the upfield of the ^{1}H NMR spectra of CDdnHis-DE5 (Fig. 7(B)), there were clear chemical shifts for H1, H2′ and H3 (0.028, 0.03 and 0.05 ppm, respectively). All of these protons are located next to the carboxyl groups and their chemical shifts are likely due to the interaction of carboxyl groups with CDdnHis. The proton shifts of DE5 and CDdnHis give us a clear image of their binding sites, the phenyl ring and the carboxyl groups of the amino acid residues on DE5 that are involved in the binding. These results provide clear evidence for the hydrophobic inclusion and electrostatic interaction sites.

Another peptide DGLAE was chosen as a reference to examine the mechanism of DE5 adsorbed to A-CDdnHis; the only difference between DGLAE and DE5 is a hydrophobic benzyl on the peptide DGLAE. Fig. 8 shows the adsorption ability of different adsorbents to DE5 and DGLAE; there was a clear decrease in the adsorption ability of adsorbents containing CD (A-CDen, A-CDenHis and A-CDdnHis). The hydrophobic inclusion of the benzene ring into the CD cavity is thought to be one of the major interactions between DE5 and adsorbents. The lack of a hydrophobic amino acid in the peptide may decrease the interaction sites, which further decreases the binding affinity of DGLAE with ligands and thereby the adsorption ability. Moreover, although the adsorption ability of A-CDdnHis to DE5 is twice that of A-CDenHis, the adsorption ability of A-CDdnHis and A-CDenHis to DGLAE are similar and are both less than that to DE5. The configuration matching of DE5 with CDdnHis and the cooperation of hydrophobic inclusion and electrostatic interaction led to the good adsorption ability of A-CDdnHis to DE5. As for DGLAE, without hydrophobic inclusion interaction the electrostatic interaction between DGLAE and ligands was the main driving force for the binding, so the distance between CD and His on ligand did not affect the binding; therefore, A-CDenHis and A-CDdnHis showed similar adsorption ability to DGLAE. These adsorption phenomena indicated that the cooperation of hydrophobic inclusion and electrostatic interactions is the main driving force for the fast and tight binding of DE5 to A-CDdnHis.

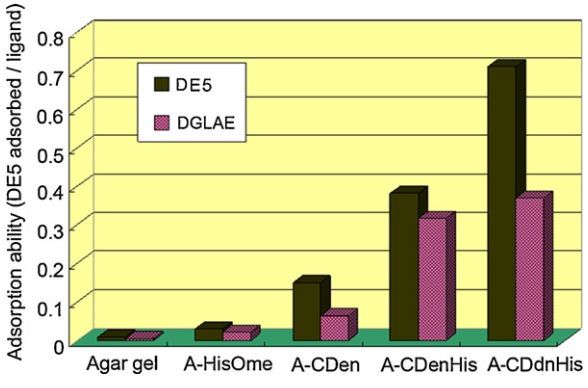

Fig. 8. Adsorption ability of different adsorbents to DE5 and DGLAE.

4. Conclusions

To identify a specific affinity adsorbent for the elimination of DE5 and DE5-containing peptides, a two-step strategy to regulate weak interaction sites was successfully developed as a promising approach to direct the design of specific affinity adsorbents. A ligand was primarily designed with double major weak interaction sites and then computer simulation was used to further optimize the steric structure of ligands. The results indicate that the obtained affinity adsorbent A-CDdnHis has excellent adsorption ability and adsorption specificity to DE5 and DE5-containing peptides due to the cooperation of hydrophobic inclusion and electrostatic interactions. This adsorbent is likely to have application in the elimination of middle molecular peptides. Our work not only identified a specific affinity adsorbent for DE5-containing middle molecular peptides but also developed an efficient approach for the design of specific affinity adsorbents.

Acknowledgements

We are grateful to Dr. Zhaofeng Luo at University of Science and Technology of China for the ITC testing and Prof. Xin Wen at College of Chemistry, Nankai University for beneficial discussions. This work was supported by the National Natural Science Foundation of China (Nos. 20634030 and 50573034).

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