A Novel Strategy for Oriented Protein Immobilization

Ching-Wei Tsai, Chih-I Liu, Ruoh-Chyu Ruaana

Abstract—A new strategy for oriented immobilization of proteins was proposed. The strategy contains two steps. The first step is to search for a docking site away from the active site on the protein surface. The second step is trying to find a ligand that is able to grasp the targeted site of the protein. To avoid ligand binding to the active site of protein, the targeted docking site is selected to own opposite charges to those near the active site. To enhance the ligand-protein binding, both hydrophobic and electrostatic interactions need to be included. The targeted docking site should therefore contain hydrophobic amino acids. The ligand is then selected through the help of molecular docking simulations. The enzyme α-amylase derived from *Aspergillus oryzae* (TAKA) was taken as an example for oriented immobilization. The active site of TAKA is surrounded by negatively charged amino acids. All the possible hydrophobic sites on the surface of TAKA were evaluated by the free energy estimation through benzene docking. A hydrophobic site on the opposite side of TAKA's active site was found to be positive in net charges. A possible ligand, 3,3',4,4' – Biphenyltetra- carboxylic acid (BPTA), was found to catch TAKA by the designated docking site. Then, the BPTA molecules were grafted onto silica gels and measured the affinity of TAKA adsorption and the specific activity of thereby immobilized enzymes. It was found that TAKA had a dissociation constant as low as 7.0×10^{-6} M toward the ligand BPTA on silica gel. The increase in ionic strength has little effect on the adsorption of TAKA, which indicated the existence of hydrophobic interaction between ligands and proteins. The specific activity of the immobilized TAKA was compared with the randomly adsorbed TAKA on primary amine containing silica gel. It was found that the orderly immobilized TAKA owns a specific activity twice as high as the one randomly adsorbed by ionic interaction.

Keywords—Protein Oriented immobilization, Molecular docking, ligand design, surface modification.

I. INTRODUCTION

PROTEIN immobilization technology has been widely applied to various biochemical assays and biotransformation processes[1, 2]. Most protein immobilization techniques resulted in protein attachment in various orientations, which causes considerable activity loss [1]. Oriented immobilization was then proposed to increase

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the efficiency of biotransformation and to enhance the sensitivity of bioassays. Two major techniques have been adopted for oriented immobilization. One strategy was to produce recombinant proteins containing histidine residues in either amino or carboxyl end of the protein. The recombinant proteins were then immobilized through metal chelating.[3, 4] The other strategy was to immobilize proteins through bio-affinity interaction. Frequently adopted interactions include antibody-antigen, biotin-avidin, DNA-DNA, aptamer-protein, affibody-protein interactions.[5-7] However, some bio-ligands themselves suffer from denaturization and misorientation.

A new strategy has been proposed for designing a synthetic affinity ligand for directed immobilization of proteins. The ligand is designed to grasp a specific spot on the protein surface far away from its active site. Both hydrophobic and electrostatic interactions are included in the protein-ligand contacts. Firstly, the amino acids and estimate the net charges near the active site of the targeted protein are examined. Secondly, molecular docking simulation of benzene molecule is performed to rank the possible hydrophobic sites on the protein surface. Then, a hydrophobic site away from the active site was selected and surrounded by amino acids of opposite charges to the active site. Taking this specific site as the target for ligand screening, molecular docking simulation is again performed to select the suitable ligands. In this study, the α-amylase derived from *Aspergillus oryzae* (TAKA) was selected as an example. The selected ligand was chemically attached to a silica gel support to test for adsorptive protein immobilization. The affinity of TAKA adsorption and the specific activity of the immobilized protein were measured to examine the ligand selection.

II. MATERIALS AND METHODS

A. Computational Methods

Molecular Docking. The AutoDock Program (version 4.0.1) was used for Ligand-Protein rigid docking calculations.[8] The conformation of TAKA was derived from the X ray diffraction results (pdb code: 2TAA). The initial coordinates of benzene and BPTA were obtained with AM1 method by using Gaussian 03 program for the energy minimization.[9] In the docking simulation, the AutoDock Tools (ADT) 4.0 was used to add the polar hydrogen and assign partial atomic charges with Kollman charges for protein and ligands. The atom type was also assigned by ATD

4.0. The docking calculations were performed by Genetic Algorithms. All the configuration figures were visualized by the VMD software.[10]

B. Experimental Methods

Ligand Grafting on Silica Gel. Two types of ligands were grafted in the carboxypropyl functionalized silica gel (CM-SiGel, 0.1 mmole/g silica gel, 6~12 μm). The amine containing silica gel was prepared by reacting diethylenetriamine (DETA) via N-(3-Dimethylaminopropyl) - N-ethylcarbodiimide hydrochloride (EDC) activation, denoted as DETA-SiGel. In brief, 0.5 g CM-SiGel were activated by 0.4 M EDC in 5 mL 0.1 M MES buffer (pH= 6.0) overnight. Then, the DETA-grafted silica gel were capped by 0.1 M ethanolamine after the free DETA removed by 0.1 M MES buffer and rinsed by DI-water. For the other ligand grafted SiGel, the ligand 3,3',4,4'-Biphenyltetracarboxylic dianhydride (BPDA) was also grafted in DETA-SiGel via EDC activation. In detail, the 0.5 g DETA-SiGel was reacted with 5 mL various concentration of BPDA (10, 20 & 40 mM) in DMAc overnight. The supernatant was collected to obtain the mounts of BPDA grafting by measuring the absorbance at 280 nm. This BPDA grafting silica gel is denoted as BPTA-SiGel.

Protein Adsorptive Isotherms and Activity Assay. The target protein, α-amylase from *Aspergillus oryzae* (TAKA), was used for orientated immobilization. The TAKA and three resins (DETA-SiGel, BPTA-SiGel-H (150.9 μmoles/g silica gel), BPTA-SiGel-L(96.7 μmoles/g silica gel)) were utilized to obtain protein adsorption isotherms. All the resins were first washed with di-ionized water and dying. The various initial concentration of 4mL protein solution was added to 0.1 g resins in the carried buffer, 10mM phosphate pH= 7.0 (Buffer A). The batch adsorption reactors were mixed in incubator with 50 rpm for 1hr at 25 °C. The supernatant was collect to obtain the mount of protein adsorption on resins by Bio-Rad Assay. For the adsorption affinity measurement, there were two kinds of Buffer for eluting the adsorbed protein, Buffer B: 10 mM tris pH= 9.0; Buffer C: 10mM Tris pH= 9.0 + 0.2 M NaCl, and the mount of desorption protein was also assay by Bio-Rad. On the other hand, the IFCC kit was used to assay the activity of adsorbed protein in resins [11].

III. RESULTS AND DISCUSSION

A. Active Site Analysis

The α-amylase from *Aspergillus oryzae* (TAKA) has a molecular weight around 52 KDa (478 amino acids) and an isoelectric point at pH 4.2. Its tertiary structure has been resolved by Swift et al. by X ray diffraction. As shown in Fig. 1, the protein contains two major domains. The active site is located at the carboxyl end of the parallel β-barrel in domain A [12]. If the shape of TAKA can be described as a fish, the domain B is the tail, the domain A comprises its head and body, and active side is located in the gill position. Figure 2 shows the amino acids surround the active site surface of TAKA. The hydrophobic amino acids are marked in gray; the positively charged ones are in blue; the negatively charged ones are in red, and the neutral hydrophilic amino acids are in green. It can be observed that there are only two hydrophobic amino acids, PHE 151 and LEU232, in the active site. However, the hydrophobic interaction may also be enhanced by the two tyrosines, TYR 75 and TYR 155, in vicinity. There are only two positively charged amino acids nearby, HIS 80 and LYS 209, but there are as many as five negatively charged amino acids, ASP 168, ASP 233, ASP 297, ASP 340 and GLU 156, located around the active site surface. Overall speaking, the active site may allow for moderate hydrophobic interaction and strong electrostatic interaction to its negative charges.

Surface hydrophobicity analysis by benzene docking

For searching a possible ligand docking site on protein surface that bound ligand through both hydrophobic and electrostatic interaction, the surface of a globular water soluble protein is occupied mostly by the hydrophilic amino acids. The possible hydrophobic exposures in TAKA were
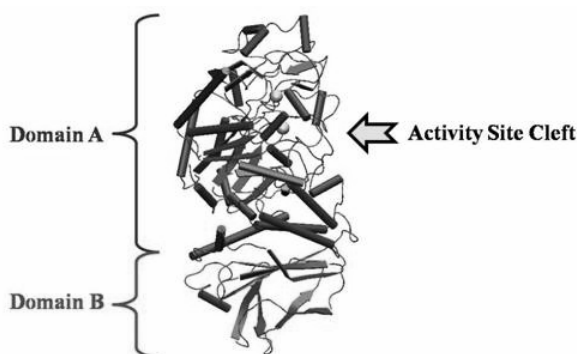

Fig. 1 Two major domains in the three dimensional secondary structure of α-amylase from *Aspergillus oryzae* (TAKA). Domain A constitutes with the first 380 residues from the N-terminus, and the rest make up the domain B. Two calcium ions are shown in ball. The activity site cleft is located at domain A and indicated by an arrow
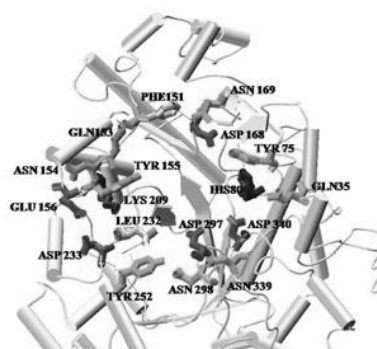

Fig. 2 The amino acids around the active site surface of the α-amylase from *Aspergillus oryzae*. The secondary structures of TAKA are shown as cartoon

determined through the help of molecular docking by the benzene molecule. The hydrophobic sites were ranked by the resulting free energy changes after interacting with benzene. Docking results revealed that top five hydrophobic sites of which the free energy was markedly decreased after benzene docking. The most hydrophobic site was very close to the active site. As shown in Figure 3, the benzene favorably docked in the location among PHE 151, TYR 155. The free energy was lowered by -2.71 kcal/mol. The site ranked No 3 was located on the top of domain A, near the mouth of the fish. The benzene probably interacted with TYR 125 residue nearby. The site ranked No 4 and 5 were located on the belly of the fish. One of them interacted benzene by its tyrosine and the other interacted benzene by its leucine residue. Mostly importantly, it could be found that the above four sites were mostly surrounded by negative charged amino acids. Interestingly, the 2nd most hydrophobic site was located in domain B, and contained positively charged amino acids, LYS 473 and LYS 375. Benzene interacted with LEU 469 and ILE 474 and the free energy was lowered by -2.5 kcal/mol. This site was far away from and owned opposite charges to the active site. Therefore, it was a suitable location for ligand binding.

Ligand design and ligand docking

After the ligand binding site was determined, a suitable ligand that binds the predetermined binding site strongly and specifically need to be designed. The desirable ligand was supposedly to contain nonpolar aliphatic or aromatic residues to interact with LEU 469 and ILE 474 and also negative charges to interact with LYS 375 and LYS 473 in the binding site. Through the help of molecular docking simulation, It could be considered that 3,3',4,4'- Biphenyltetra-carboxylic acid (BPTA) might be a suitable candidate. Then, the BPTA to TAKA molecular docking
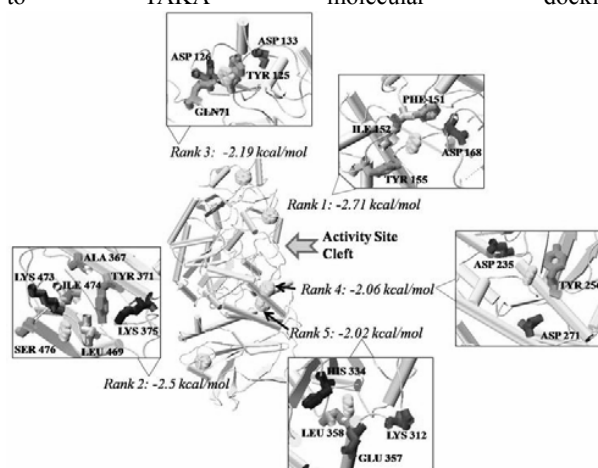

Fig. 3 The top 5 energy ranking of benzene docking to the α-amylase from *Aspergillus oryzae* (TAKA). The secondary structures of TAKA are shown as cartoon. The activity site cleft is marked by an arrow. The critical amino acids are shown as bond and the hydrophobic probes, benzene, are marked in spherical ball
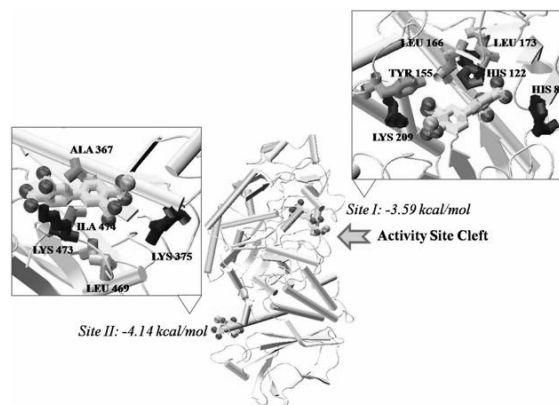

Fig. 4 The top 2 energy ranking of BPTA docking to the α-amylase from *Aspergillus oryzae* (TAKA). The secondary structures of TAKA are shown as cartoon. The activity site cleft is marked by an arrow. The critical amino acids are shown as bond and the hydrophobic probes, benzene, are marked in spherical ball. The bonding in BPTA is shown as bond, the oxygen atoms in BPTA are in heavy dark spheres, the methylated groups in BPTA are in light dark spheres

simulations were further performed. To simulate the immobilized ligand, one of the four carboxyl groups of BPTA was methylated. The simulation results show that the most favorable ligand binding site in TAKA was the predetermined site in domain B of the protein. As shown in Figure 4, the free energy was lowered by -4.14 kcal/mol. On the other hand, BPTA might also bind to the active site, but the free energy drop after binding was only -3.59 kcal/mol. Hence, this could be considered that BPTA was a suitable ligand to implant to substrates for the oriented adsorption of TAKA.

Ligand immobilization on silica gel

Amine containing silica gel was prepared by reacting DETA with thecarboxyl groups on CM-silica via EDC activation. Excess DETA was used for complete amidation of the carboxylate groups. Then, the BPDA were reacted with the primary amines on the resins in DMAc. The effect of BPDA concentration on ligand attachment was showed in Figure 5. It could be observed that the maximum ligand density obtained was 150.9 μ mole/g silica gel.

Adsorption affinity and specific activity measurements

Three adsorbents were used to adsorb TAKA. Two of them were BPTA ligand containing SilGels. The ligand densities were 150.9 and 96.7μ moles/g silica gel, respectively, namely BPTA-SiGel-H and BPTA-SiGel-L. The DETA derivatized CM-SiGel (DETA-SiGel) were also used as comparisons. Figure 6 shows the isotherms of TAKA adsorption on these three resins at pH 7.0. It can be observed that the positively charged DETA-SilGel has the highest amount of TAKA adsorption. It might be due to the low pI of TAKA. As expected, the higher ligand density gel, BPTA-SiGel-H, had a higher adsorption capacity than the low ligand density one,

BPTA-SiGel-H. The fitting parameters in Langmuir model showed that the two BPTA-SiGels owned the similar dissociation constants toward TAKA.

The specific activities of enzymes adsorbed on these gels were also compared. The specific activities of all the samples are also listed in Table I. The specific activity of the enzyme immobilized onto BPTA-SiGel-H was 10.5 U/mg and that onto the BPTA-SiGel-L was 14.3 U/mg. Both are lower than the specific activity of the free enzyme in solution, 30.0 U/mg, but were higher than that onto the DETA-SiGel.
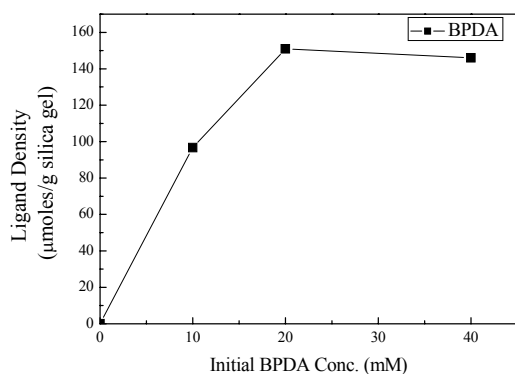
TABLE I
ADSORPTION CONSTANTS AND SPECIFIC ACTIVITIES OF TAKA ADSORBED ON VARIOUS RESINS

	q_{max} (mg/g-SiGel)	K_d ($\times 10^{-6}$ M)	Specific Activity(U/mg)
DETA-SiGel	20.97	10.07	6.6
BPTA-SiGel-L	7.9	7.18	14.3
BPTA-SiGel-H	10.61	7.01	10.5

Effect of pH's and ionic strength on protein adsorption

Three different buffer solutions were used to elute adsorbed TAKA from the resins. Buffer A was a pH 7.0 buffer of 10 mM sodium phosphate; Buffer B was a pH 9.0 buffer made of 10mM Tris; Buffer C is a buffer of higher ionic strength which was composed of 10 mM Tris buffer and 0.2M sodium chloride. It can be observed from Table 2 that the pH 7.0 buffer could not elute TAKA from any of the resins. The buffer of a higher pH value eluted small amount of protein from the BPTA-SiGel-H and DETA-SiGel resins. The buffer of high ionic strength elute large amount of TAKA from the positively charged DETA-SiGel but had no effects on the BPTA ligand containing resins. The results indicated that the hydrophobic interaction played an important part in the adsorption of TAKA to BPTA.

Fig. 5 The grafting density of BPDA on the SiGel as the function of initial reaction concentration

TABLE II
EFFECT OF PH'S AND IONIC STRENGTH ON PROTEIN ADSORPTION

Percentage desorption	BPTA-SiGel-L	BPTA-SiGel-H	DETA-SiGel
Buffer A (%)[*]	0	0	0
Buffer B (%)[*]	0	3.978	10.7
Buffer C (%)[*]	0	0	31.6

* Buffer A: 10mM phosphate pH= 7.0; Buffer B: 10mM Tris pH= 9.0; Buffer C: 10mM Tris pH= 9.0 + 0.2M NaCl
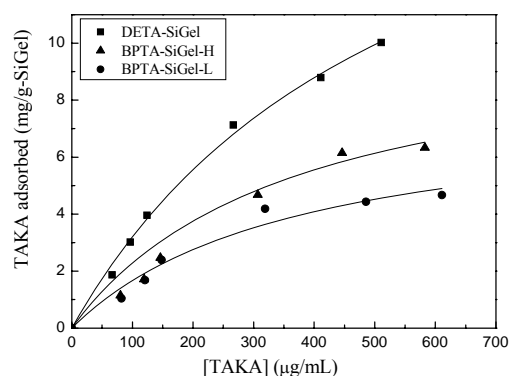

Fig. 6 Adsorption isotherm of TAKA on various adsorbents. The mounts of protein adsorption at each equilibrium concentration of TAKA for DETA-SiGel is denoted in square, BPTA-SiGel-H in triangle, BPTA-SiGel-L in circle. The line is fitting by Langmiur equation

IV. CONCLUSION

A new strategy for protein immobilization was proposed. The immobilization of α-amylase derived from *Aspergillus oryzae* (TAKA) is taken as an example for designing a ligand that grasp a spot on TAKA surface far from the active site. To enhance the ligand-protein interaction, a ligand is designed to interact with the protein by both hydrophobic and electrostatic interaction. To avoid docking to the active site, a ligand is designed to have the same charges as those near the active site. Molecular simulation is performed to predict the possible docking spot. The adsorption affinity and the specific activity of immobilized TAKA were measured to confirm the strategy. It was found that the TAKA adsorption to the ligand containing resin exhibited extraordinarily high affinity. The

TAKA immobilized by this methodology also has a much higher specific activity than the randomly oriented enzymes immobilized purely by electrostatic interaction. The experimental result indicates that the proposed method may be a possible alternative for oriented immobilization of proteins. But more examples are needed to support its applicability.

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