Exploring Peptide Space for Enzyme Modulation

by

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ABSTRACT

Enzymes which regulate the metabolic reactions for sustaining all living things, are the engines of life. The discovery of molecules that are able to control enzyme activity is of great interest for therapeutics and the biocatalysis industry. Peptides are promising enzyme modulators due to their large chemical diversity and the existence of well-established methods for library synthesis.

Microarrays represent a powerful tool for screening thousands of molecules, on a small chip, for candidates that interact with enzymes and modulate their functions. In this work, a method is presented for screening high-density arrays to discover peptides that bind and modulate enzyme activity. A viscous polyvinyl alcohol (PVA) solution was applied to array surfaces to limit the diffusion of product molecules released from enzymatic reactions, allowing the simultaneous measurement of enzyme activity and binding at each peptide feature. For proof of concept, it was possible to identify peptides that bound to horseradish peroxidase (HRP), alkaline phosphatase (APase) and β-galactosidase (β-Gal) and substantially alter their activities by comparing the peptide-enzyme binding levels and bound enzyme activity on microarrays. Several peptides, selected from microarrays, were able to inhibit β-Gal in solution, which demonstrates that behaviors selected from surfaces often transfer to solution. A mechanistic study of inhibition revealed that some of the selected peptides inhibited enzyme activity by binding to enzymes and inducing aggregation.

PVA-coated peptide slides can be rapidly analyzed, given an appropriate enzyme assay, and they may also be assayed under various conditions (such as temperature, pH and solvent). I have developed a general method to discover

molecules that modulate enzyme activity at desired conditions. As demonstrations, some peptides were able to promote the thermal stability of bound enzyme, which were selected by performing the microarray-based enzyme assay at high temperature. For broad applications, selected peptide ligands were used to immobilize enzymes on solid surfaces. Compared to conventional methods, enzymes immobilized on peptide-modified surfaces exhibited higher specific activities and stabilities. Peptide-modified surfaces may prove useful for immobilizing enzymes on surfaces with optimized orientation, location and performance, which are of great interest to the biocatalysis industry.

DEDICATION

I would like to dedicate this work to my family for their steady support and encouragement. I want to express my sincere love and acknowledgement to my parents Zhiguan Fu and Fengqin Jin who have raised me up to what I am today. I am grateful to my amazing wife Ting, whose love has made my life complete, and who has devoted her time, patience and efforts to supporting me during the entire Ph.D study.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Neal Woodbury, for his steady guidance and support through my entire Ph.D study. Dr. Woodbury always encouraged me to think independently and respected my ideas. When I presented new ideas to him, he seldom made “YES” or “NO” judgments. Instead, he used his knowledge to help me define the possible problems and applications that might be associated with these ideas. Neal often talked with me as a good friend or family elder, sharing his invaluable experience with me.

I also want to thank Dr. Stephen Johnston for his insightful suggestions and mentorship as my co-advisor. Dr. Johnston encouraged me to explore unconventional approaches to learn science and taught me how to form questions from my observations. I still remember his statement: “For the same set of data, some people might publish it in *Science*; others might just treat it as rubbish. It depends on how you present the question.”

I also need to thank Drs. Phillip Stafford, Chris Diehnelt and Zhan-Gong Zhao for helpful discussions on microarray analysis and fabrication. Drs .Su Lin and Douglas Daniel gave me a great deal of help implementing laser detection and imaging technologies. I also thank Drs. Giovanna Ghirlanda and Hao Yan for the guidance that they have provided as my committee members.

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I want to thank all of my laboratory colleagues with whom I have had many interesting discussions and good friendships: Matt Greving, Pallav Kumar, Zhi Guo, Jie Pan, Wei Wang, Anil Prasad, Katherine Cai and Jeremy Reinhold. I also owe many thanks to the people in the Center for Innovations in Medicine: John Lainson and Mitch Magee for peptide array production; Seongmin Hahn, Donnie Shepard and Loren Howell for peptide synthesis and purification; Rebecca Halperin and Bart Legutki for help in microarray assays and analysis.

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CHAPTER 1: GENERAL INTRODUCTION

ABSTRACT

In this chapter, the basic concepts of enzymes and enzyme modulation are introduced. The general methods for discovering molecules that modulate enzyme function are briefly summarized. This chapter discusses the potential applications of peptide-based molecules in ligand development and drug discovery. Finally, microarray-based profiling of protein interactions and enzyme activity is described, which is closely related to the subsequent chapters.

Enzymes

The metabolism of living systems involves complex synthetic pathways with numerous multi-step reactions that possess extraordinary yields and specificities[1-2]. In these complex systems, enzymes play key roles as catalysts and are essential to biological functions. No enzymes, no life. In general, enzymes are proteins or protein-based molecules that speed up chemical reactions in living organisms.[3-4] Enzymes do not initiate reactions with unfavorable changes in free energy and that would not naturally occur. They accelerate reactions toward equilibrium with rate enhancements of 10^6 – 10^{17}, compared to uncatalyzed reactions.[5-6] In Figure 1, an enzyme catalyzes a reaction by first binding to the substrate molecules, then lowering the activation energy in the microenvironment created at the active site, converting substrate to product molecules, releasing the product molecules from the active site, and being ready to accept the next substrate.[7]

Most enzymes are globular proteins and consist of one or several poly peptide chains ranging from just 60 amino acid residues[8] to over 2,500 residues in length.[9] For a given enzyme, the polypeptide chains are folded into a specific

three-dimensional structure, under physiological conditions, forming an active site which generally consists of 3-4 key amino acids.[10-11] In Figure 2a, the protein folding of ketosteroid isomerase is shown as an example. In its active site, an enzyme catalyzes a reaction by favoring binding to the substrate transition state, which is a high-energy, unstable arrangement of atoms formed during a reaction, and essential for product generation (Figure 2b).[12] As shown in Figure 3, an enzyme stabilizes the transition state of the substrate and lowers the energy barrier (activation energy), making the reaction much more likely to occur. Recently, catalytic nucleic acids (RNA or DNA-based) have been discovered which catalyze some chemical reactions, like nucleic acid cleavage or synthesis.[13]

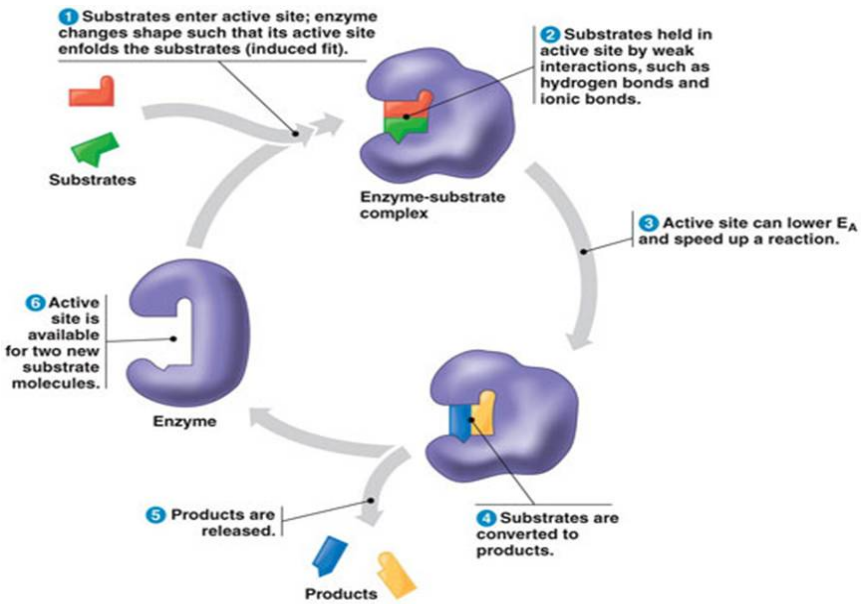

Figure 1. Description of the basic processes of enzyme catalysis.[14]

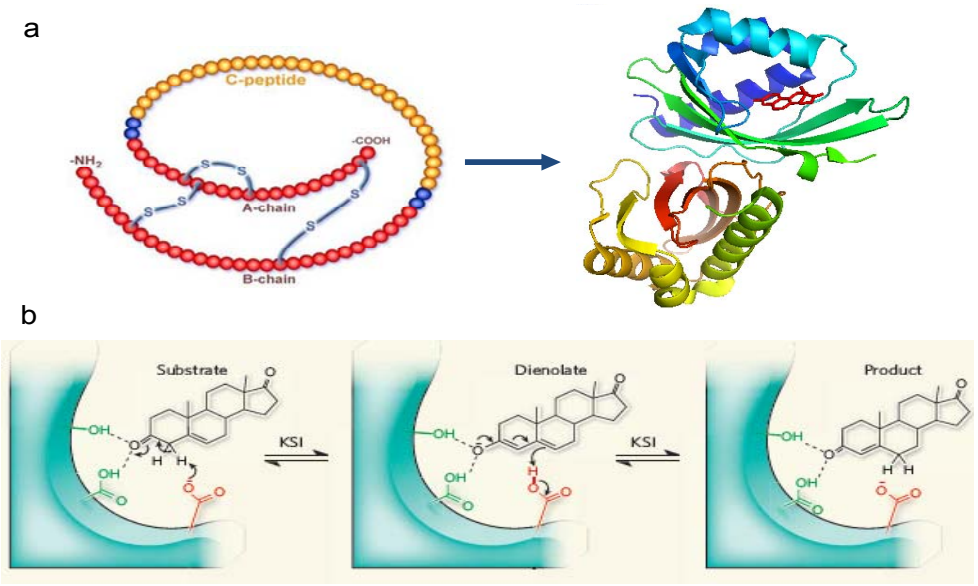

Figure 2. Example of (a) an enzyme folding from a linear poly peptide chain to a 3-D structure and (b) stabilization of the substrate transition state and conversion of substrate to product. The example enzyme is ketosteroid isomerase.[12]

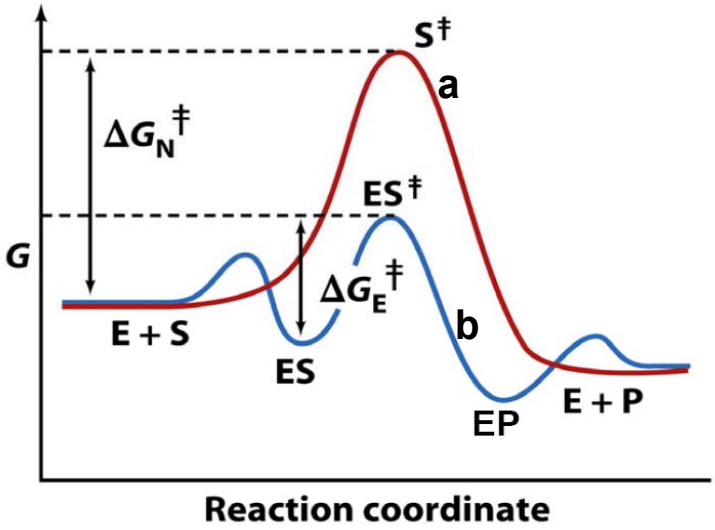

Figure 3. Gibbs free energy change for (a) an uncatalyzed reaction and (b) a catalyzed reaction. $\Delta G_N^{\ddagger}$ and $\Delta G_E^{\ddagger}$ indicate the activation energy required to induce the transition for the uncatalyzed (*red*) and catalyzed (*blue*) reactions, respectively.[3,15]

Enzymes are crucial in maintaining health. Many diseases are related to the malfunction of certain key enzymes, for instance, beta-secretase has recently been implicated in the early development of Alzheimer's disease.[16] Many drugs target specific enzymes, either inhibiting or activating them.[17-18] Enzymes, themselves, can also be used as therapeutic drugs. For example, Adagen® is a bovine adenosine deaminase that is used to treat a type of severe combined immunodeficiency disease.[19]

The strong catalytic efficiency and high specificity of enzymes make them ideal candidates for industrial applications. The use of enzymes dates back several thousand years, when ancient Egyptians were making beer from flour, although they were likely unaware of the concept of enzymes.[20] In 1897, Eduard Buchner discovered the ability of yeast extracts to ferment sugar as the start of using purified enzymes in industry.[21] Today, enzymes are widely used in detergents and paper manufacturing as well as food, textile, fuel and alcohol production.[22] More and more organic synthesis processes are starting to use enzymes as catalysts to obtain higher yields and regioselectivity.[23-24] The use of enzymes in synthesis may reduce the use of organic solvents with similar product yields and selectivity as traditional organic synthesis, and will be very important for green chemistry.[25-26]

Most enzymes used today originally come from Nature or have evolved from naturally-existing enzymes. High-throughput screening of living organisms for interesting catalytic targets is still one of the major sources of novel enzymes.[27-28] Directed evolution, which mimics Darwinian evolution on an accelerated time-scale, has been widely used to discover new enzyme variants by combining random mutagenesis and recombination with screening or

selection for desired protein target functions.[29-30] Site-directed mutagenesis is used as a probe to study enzyme structure and function.[31-33] Szostak's group recently reported the selection and evolution of an enzyme from a partially-randomized, non-catalytic scaffold using mRNA display.[34]

Enzymes catalyze reactions by stabilizing the substrate transition state. Catalytic antibodies that bind to transition-state analogues have been developed as new enzymes with desired functions (Figure 4).[35-36] Recent developments in rational protein design make it possible to design novel enzymes completely through computational approach. Baker's group reported the design of an artificial enzyme catalyzing the Diels-Alder reaction, which was predicted from the computational analysis of protein binding to the transition state of the reaction (Figure 5).[37]

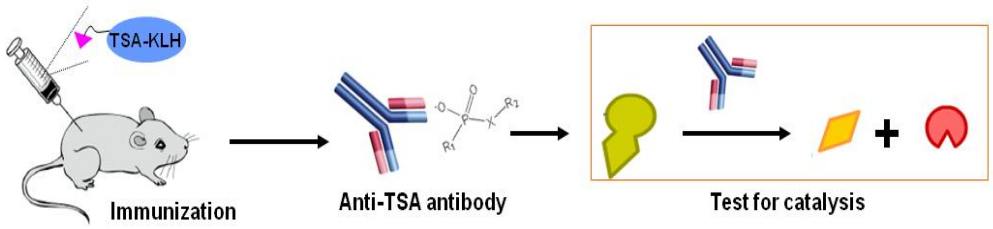

Figure 4. The generation of a catalytic antibody that binds to a Transition-State Analogue (TSA) to promote specific catalytic activities.

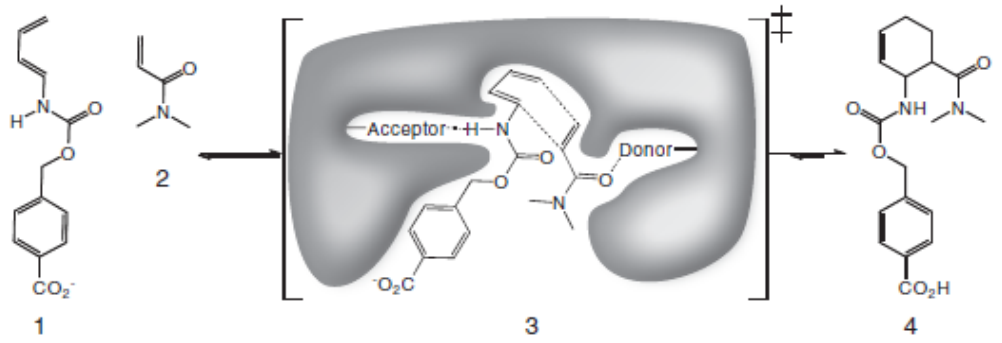

Figure 5. Computational design of an enzyme that stabilizes the transition state and catalyzes the stereoselective Diels-Alder reaction.

Enzyme Modulation

Enzyme modulation plays an important role in biological metabolism[38], and regulates metabolic pathways through inhibition or activation of key enzymes. Most specific enzyme inhibition happens through binding to small-molecule inhibitors, which either stop substrates from entering enzyme active sites, or hinder enzymes from catalyzing their respective reactions. In some special cases (e.g. cellular enzyme inhibitors), proteins can also act as enzyme inhibitors, like trypsin inhibitor[39] and ribonuclease inhibitor.[40] Inhibitor binding can be reversible or irreversible. Reversible inhibitors bind to specific sites on enzymes through non-covalent interactions, such as hydrogen bonding, hydrophobic interactions and charge interactions. Non-covalent interactions between enzymes and reversible inhibitors dissociate when competitor molecules are added to the system or when the system undergoes dialysis, and enzyme activity is thereby recovered.

Generally, there are four types of reversible enzyme inhibitions which are classified according to their kinetics: competitive inhibition, non-competitive inhibition, uncompetitive inhibition and mixed inhibition,[3] as shown in Figure 6. A competitive inhibitor and the substrate molecule cannot bind to an enzyme at the

same time (Figure 7a); usually because the inhibitor possesses the similar structure as the substrate and binds to the enzyme active site (good inhibitors bind to enzymes stronger than substrates). This results in an increased apparent K_m with unchanged V_{max} (Figure 6b). In Figure 7b, a noncompetitive inhibitor generally binds to an enzyme at sites other than the active site, and inhibits the enzyme activity. It does not affect the ability of enzymes to bind with substrates, but bound substrates cannot be converted to products. Kinetically, this decreases V_{max} with little effect on K_m (Figure 6c). Uncompetitive inhibitors bind to the enzyme-substrate complexes and hinder the conversion of substrate to product (Figure 7c). Both apparent V_{max} and K_m will decrease because of the removal of activated enzyme-substrate complexes (Figure 6d). Mixed inhibition possesses more complicated kinetics, in which inhibitor binding affects both substrate binding and the function of the active site. Kinetically, both the apparent V_{max} and K_m are altered by inhibitor binding but, unlike the above three types of inhibitions, it does not possess a constant K_m/V_{max} value.

Irreversible enzyme inhibitors usually covalently modify target enzymes and inhibit their activity. Inhibition cannot be removed by increasing substrate concentration or through dialysis. In Figure 8a, most irreversible inhibitors contain electrophilic reactive groups which react with nucleophilic amino acid side chains, such as hydroxyl, amine and thiol groups, inside or near enzyme active sites.[43] Example reactive groups include nitrogen mustards, aldehydes, haloalkanes, alkenes, phenyl sulfonates or fluorophosphonates.[43] Some irreversible inhibitors are used as probes to study the mechanisms of enzyme catalysis.[44] Irreversible inhibitors exhibit time-dependent inhibition and their potency cannot be characterized by IC_{50}. Instead, irreversible inhibitors are evaluated using second

order rate constants for inactivation (K_{obs} /[I]),[45-46] where K_{obs} is the pseudo-first order rate constant of inactivation (Figure 8b), and [I] is the inhibitor concentration.[47] Irreversible enzyme inhibitors are widely used as antibiotics, such as penicillin.

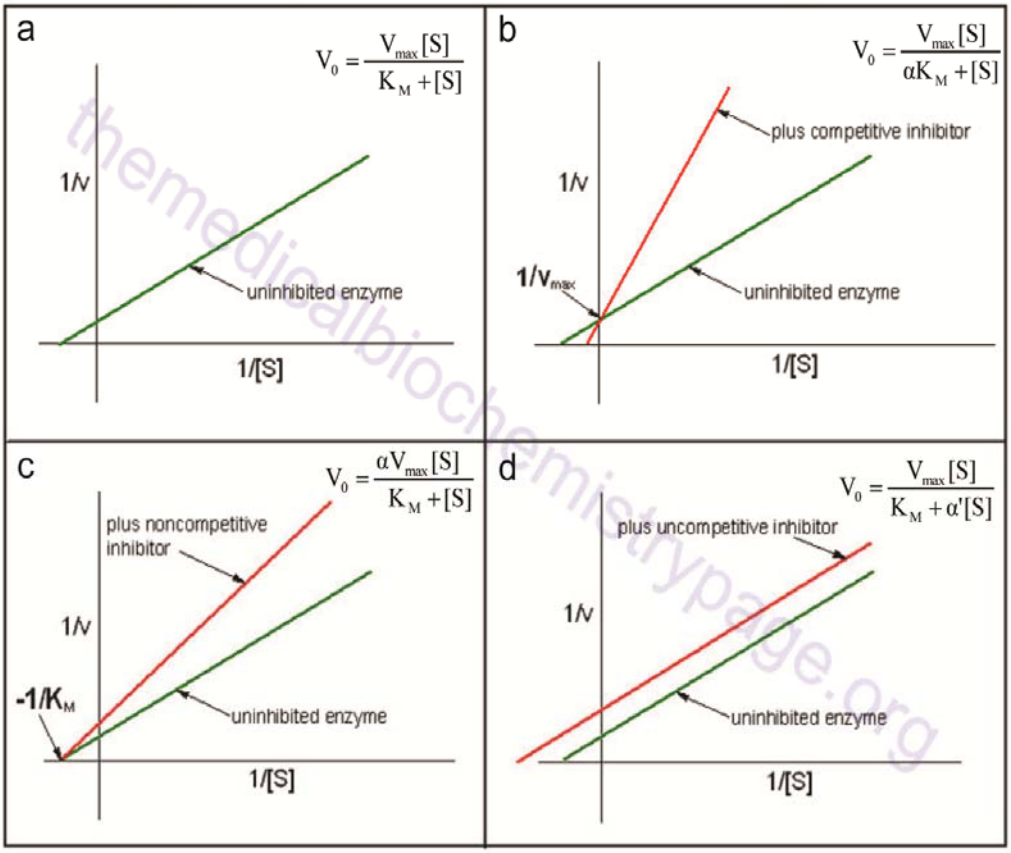

Figure 6. Enzyme inhibition kinetics of (a) uninhibited enzyme, (b) competitive inhibition, (c) noncompetitive inhibition and (d) uncompetitive inhibition (Figure copied from ref [41]).

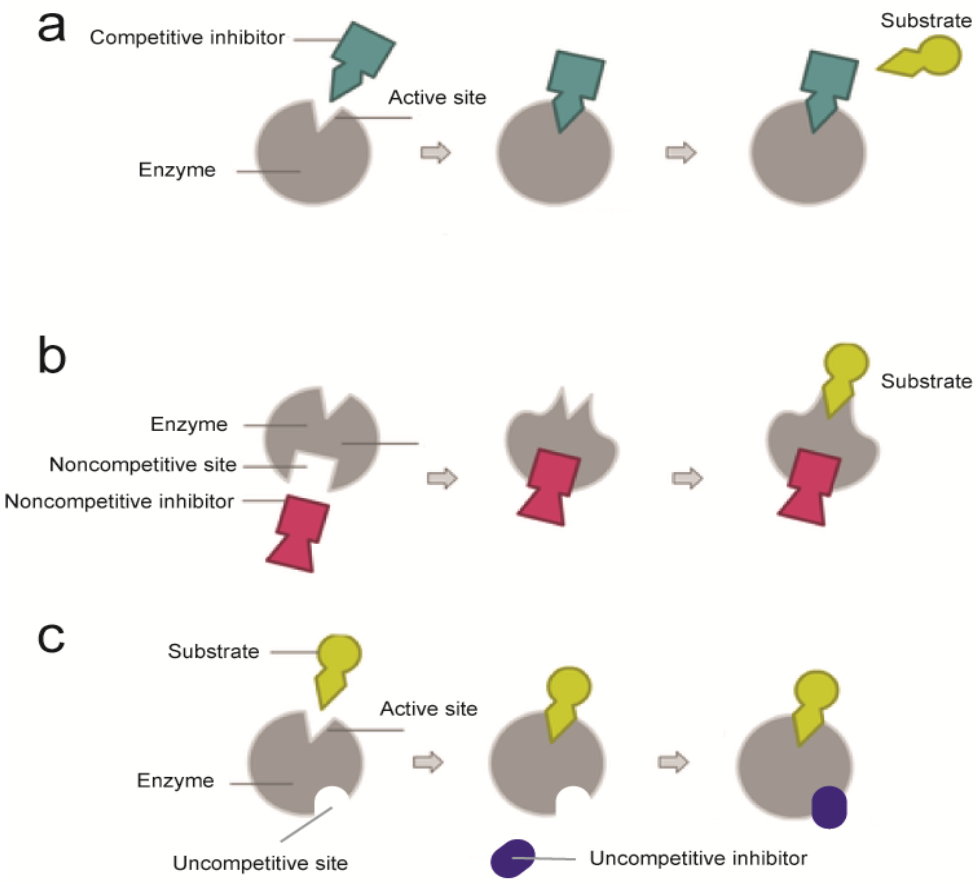

Figure 7. Mechanisms of (a) competitive inhibition, (b) noncompetitive inhibition and (c) uncompetitive inhibition (originally copied from ref [42]).

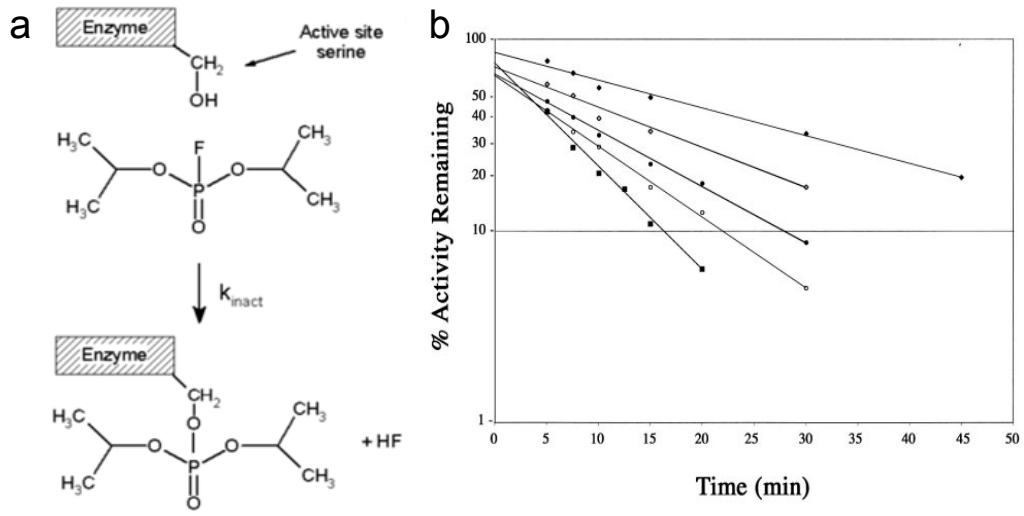

Figure 8. (a) Irreversible inhibition of enzymes through covalent modification of amino acids residues inside or near the active site. (b) Time-dependent inhibition of an irreversible inhibitor. The slope of the inactivation curve is the pseudo-first order rate constant, K_{obs}.[46]

Allosteric regulation controls enzyme or protein activity through the binding of an effector molecule at a protein's allosteric site, which is located at a site other than the active site (Figure 9).[3] Generally, allosteric regulators are structurally different from substrate molecules, and can trigger conformational changes of whole proteins. Allosteric inhibitors can behave as competitive or noncompetitive inhibitors, kinetically.[48] Molecules that target the allosteric sites of enzymes are promising drug candidates; possessing the advantages of high specificity, lower toxicity and cooperativity with other ligands.[49-50]

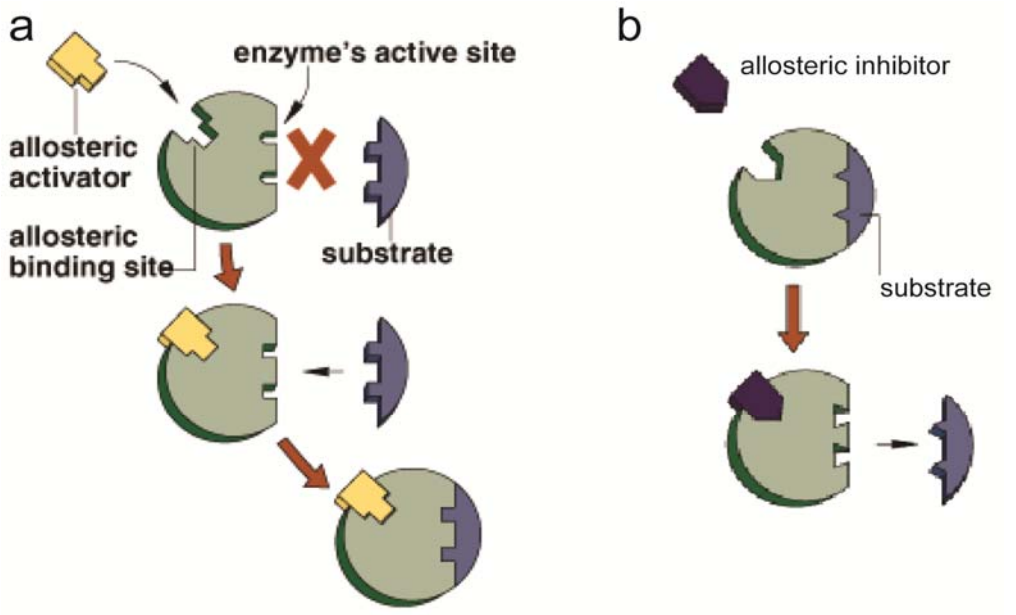

Figure 9. Allosteric regulation of (a) enzyme activation and (b) enzyme inhibition.

Discovery of Enzyme Modulators

Discovery of molecules that are able to control enzyme activity through noncovalent/covalent interactions is central to therapeutics[51]. Many drugs are enzyme inhibitors, or molecules capable of inhibiting or activating protein-protein interactions[52], so their discovery and development is an active area of research in biochemistry and pharmacology.

In early drug development, the only way to discover enzyme modulators is by trial and error. Combinatorial chemistry which is evolved from early organic chemistry research focused on strategies for generating molecular diversity becomes a powerful tool for developing and optimizing small-molecule modulators.[53] Combinatorial chemistry generally involves the rapid synthesis of a large number of different, but structurally-related molecules that have been designed to have potential effects on target proteins. Diversity-oriented synthesis

has recently been developed to generate large libraries of small molecules with structural and functional diversity.[54]

High-throughput screening (HTS) technologies have become more and more important in the rapid identification of active lead compounds from a large molecule library.[55] HTS is defined as the process in which large batches of compounds are tested for binding activity or biological activity against target molecules. The test compounds can act as enzyme inhibitors or activators, ligands for binding, agonists or antagonists for receptor-mediated intracellular processes, etc. Several detection approaches are applied to the screening process, like absorbance, fluorescence, polarization[56], dynamic light scattering[57], surface plasma resonance[58] and mass spectrometry.[59-60] Today, most HTS processes are highly automated and robotic with screening levels as high as 100,000 per day[61]. Many lead compounds selected from HTS are found to inhibit enzymes through aggregation-based mechanisms with poor specificity.[62-64] Developing new screening strategies to differentiate drug leads from aggregation-inducing compounds is a challenge for HTS technology.

Recently, rational design of small molecules has been applied to discovering enzyme modulators utilizing the knowledge in three-dimensional protein structure and advances in computer simulation of protein-small molecule interactions (e.g. molecular docking and molecular mechanics).[65-66] Novel strategies, like click chemistry, are used to design and select multi-valent enzyme inhibitors (Figure 10).[67]

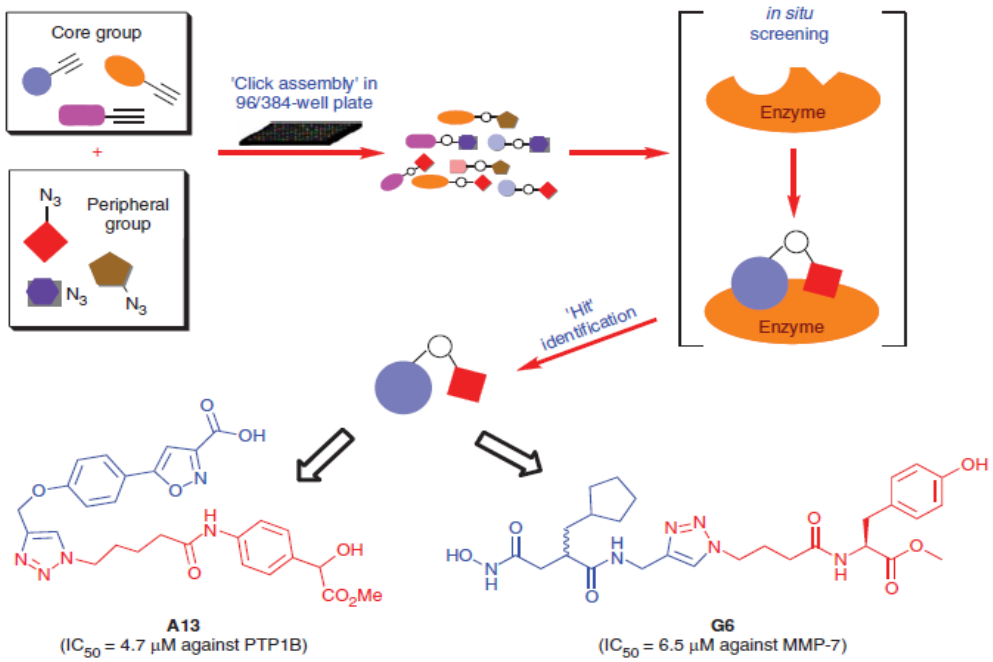

Figure 10. Inhibitor discovery by click chemistry.[67]

Peptides for Ligand Discovery

Peptides represent a very promising class of potential protein-modulating molecules due to their large chemical diversity[68] and the existence of well-established methods for library synthesis[69]. Peptides and their derivatives are found to inhibit many important enzymes[70], like dehydrogenases[71], protein kinases[72] and proteases.[73] Cell-permeable peptides are becoming more and more useful in blocking cellular signaling pathways and drug delivery.[74-75] There is a growing realization that, by using peptides as building blocks, it is possible to create synthetic structures with affinities and specificities comparable to natural antibodies[76-77]. It has also been shown that a peptide or small-molecule ligand that binds to a unique region of an enzyme can be used for orientation-specific enzyme immobilization on a solid support[78-80] in order to optimize its activity and stability.

More and more drug discovery studies have shown that the chemical diversity of a molecular library is crucial for discovering potential lead compounds that affect target enzymes.[53-54,81] Peptides possess huge sequence diversity as the size of peptide chemical space is defined by: $Y = X^n$, where Y is the total number of molecules in the space, X is the size of the set of unique amino acid substituents, and n is the number of residues in a peptide. For example, the entire space of a natural peptide library containing 10-mer peptides is 20^{10}, or ~ 10^{13}. Considering that there are hundreds of unnatural amino acids that have been developed so far[82-83], potential library sizes are actually even bigger. The magnitude of possible peptide sequence combinations is the basis of protein functional diversity.

Several strategies have been developed to create large libraries of peptides. In Figure 11, phage display[84-85] and mRNA display[86] are two widely used *in vitro* selection methods for peptide ligand discovery, which harness molecular biology and peptide synthesis to generate peptide libraries with sizes from 10^6 to more than 10^{13}. Purely chemical strategies for generating large peptide libraries are developed based on the one-bead one-compound (OBOC) approach or "split and mix".[87] In Figure 12, for OBOC approach, solid-phase supports (bead) are first separated into vials with a single amino acid coupled in each vial. After the first coupling, the beads from different vials are mixed together in a random fashion, then separated and a second amino acid is coupled. A library size with more than 10^6 unique compounds will be generated after several rounds of "split and mix".

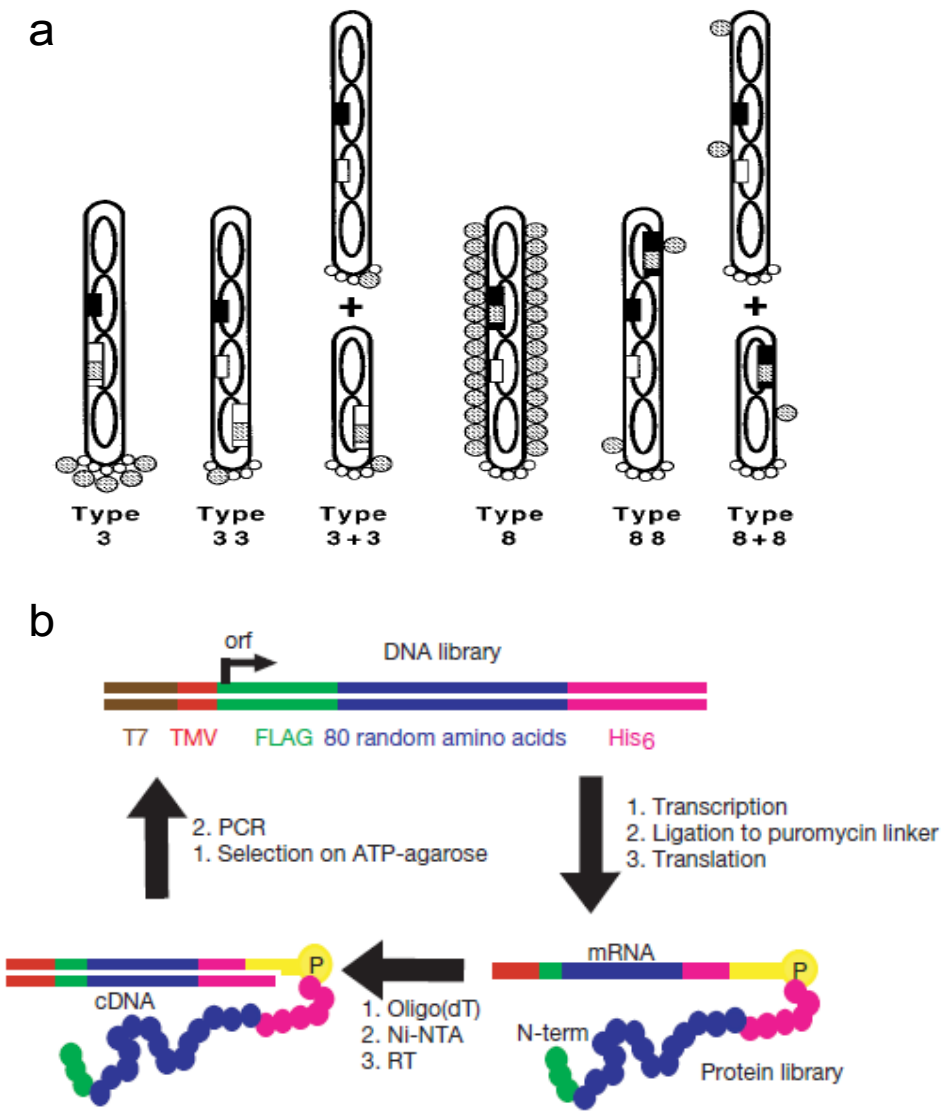

Figure 11. Two popular in vitro selection methods of peptide ligand discovery. (a) Types of phage display[85] and (b) mRNA display.[86]

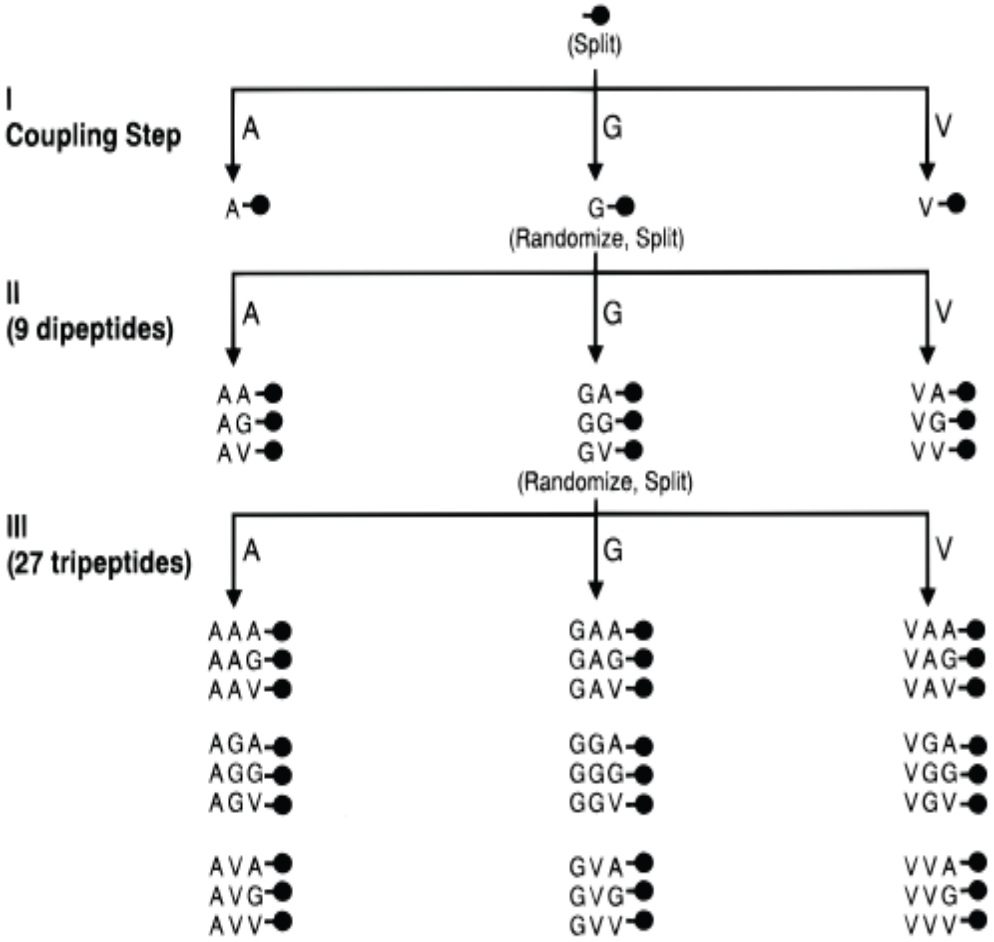

Figure 12. Steps in peptide library synthesis based on OBOC strategy.[87]

Discovery of Ligands on Microarray

Microarrays have been in use as high-throughput screening platforms to study molecule-molecule interactions with addressable patterns for each compound on an array surface since the 1990s (Figure 13).[69] A typical microarray can contain a library of molecules from a few hundred up to more than one million on a chip area of several square centimeters, with picomoles of compound at each spot.[88-91] Compared to selection library methods (e.g. mRNA and phage display), microarrays give out the information of each individuals in a given molecular library.

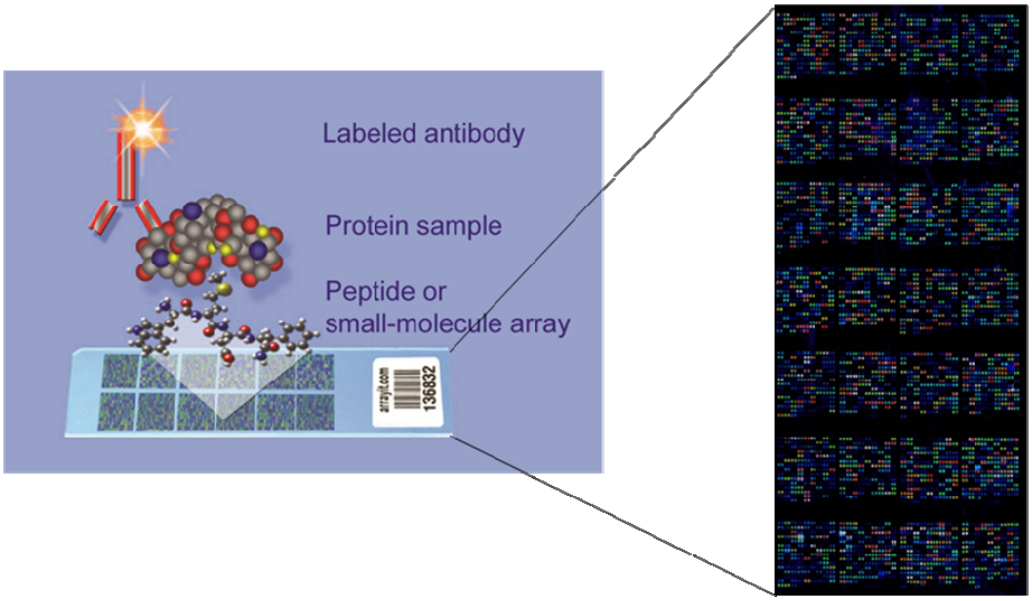

Figure 13. A high-density microarray.

High-density microarrays represent a powerful approach to screening for molecules that alter enzymatic functions[92-94]. Microarrays have been used for this purpose in the past, by constructing arrays of small molecules [95-97], peptides[98-99] and nucleic acids.[100] A typical small-molecule array contains more than 10,000 compounds synthesized on functionalized glass slides (25 mm × 75 mm) with

features ranging from 50 – 300 μm in diameter.[101] The most commonly-used approaches for immobilizing small molecules are shown in Figure 14, including epoxide-, isocyanate- and fluoroalkysilane-coated surfaces, and PNA (peptide nucleic acid) encoding small-molecule arrays.[100,102] Small-molecule arrays have been used to discover inhibitors of enzymes[103], like protein kinase C[103], cysteine proteases[102], metalloproteases[104] and histone deacetylases.[95, 105]

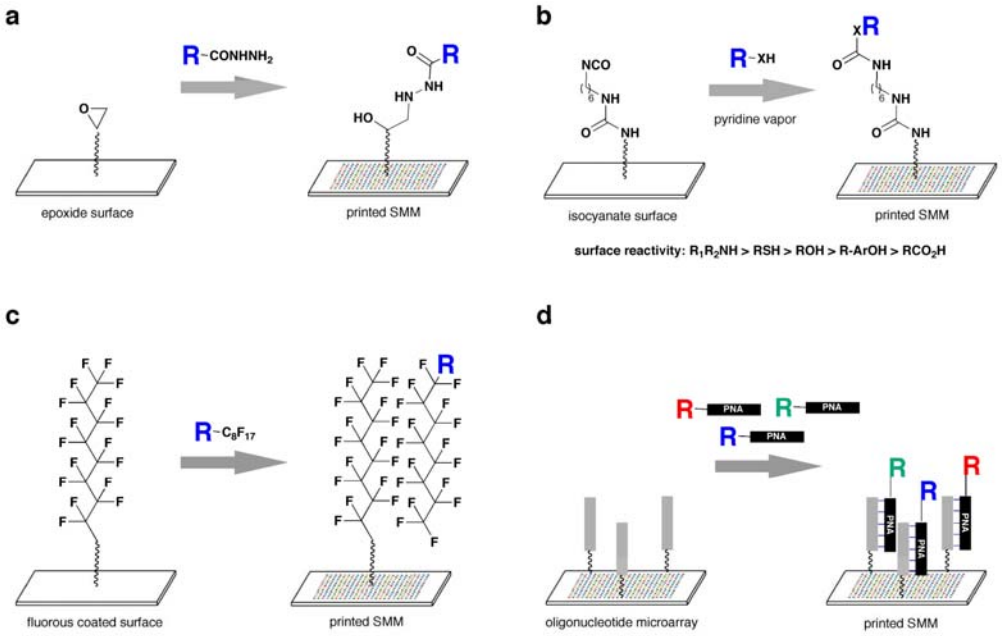

Figure 14. Representative approaches used to immobilize small molecules including (a) epoxide-coated surfaces, (b) isocyanate-coated surfaces, (c) fluoroalkylsilane-coated slides and (d) PNA (peptide nucleic acid) encoding small-molecule arrays.[101]

Peptide microarrays were first introduced by Stephen Fodor who synthesized of an array of 1024 peptides for binding to a monoclonal antibody.[69] There are three generally-used approaches for the preparation of peptide microarrays, printing of pre-synthesized peptides[106], SPOT synthesis[107] and light-

directed, spatially-addressable synthesis.[98] In Figure 15, several strategies for the immobilization of pre-synthesized peptides on glass slides are presented.[108] Commercial devices for printing molecules on activated surfaces are available, for instance, the Telechem Nanoprint System. SPOT- synthesis, which follows standard Fmoc chemistry, is another easy technique that permits the parallel synthesis of large numbers of addressable peptides on a cellulose membrane support, as shown in Figure 16.[109-110] Light-directed *in situ* sythesis generally utilizes photolabile protecting groups, photo-generated acid[111] and masks to selectively deprotect features on a microarray, and can generate a high-density microarray containing more than 100,000 peptides (Figure 17).[98, 108]

In an effort to understand the enzymatic pathways and functions, substrate profiling of protein kinase, phosphatase and protease has been performed on peptide microarrays by printing or synthesizing peptide-based molecules on surfaces (Figure 18).[99,112-115] More importantly, substrate specificity determination greatly aids the design of novel enzyme substrates and inhibitors.[113] It is possible to identify new enzyme inhibitors and evaluate their IC_{50} values using peptide- or peptoid-arrays.[90,116] In Figure 19, activity-based protein profiling (ABPP), which utilizes active-site-directed probes to profile the functional states of enzymes in proteomes, has also been performed on microarrays with improvements in sensitivity, resolution and enzyme identification as compared to the traditional liquid chromatography-mass spectrometry (LC-MS) method.[114] Recently, a reactome array was developed to sense the metabolic phenotypes and networks for cell populations and communities.[117] In Figure 20, the reactome array contains 1,676 dye-linked substrate molecules which are

designed to represent central metabolic pathways and detect enzymatic activity in the pathways.

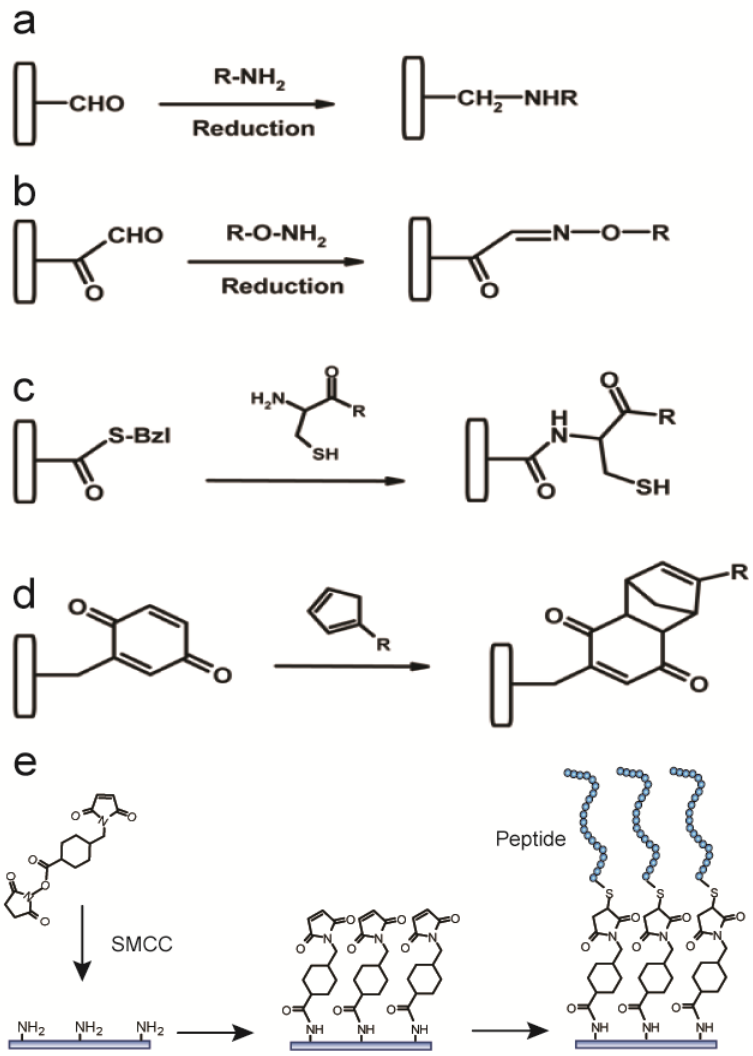

Figure 15. Methods for immobilizing pre-synthesized peptides on surfaces using (a) an amino group to aldehyde linker, (b) an aminooxyacetyl group to glyoxylyl linker, (c) a cysteine residue to thioester linker , (d) a cyclopentadiene residue to benzoquinone linker and (e) a cysteine residue to maleimide linker.[108]

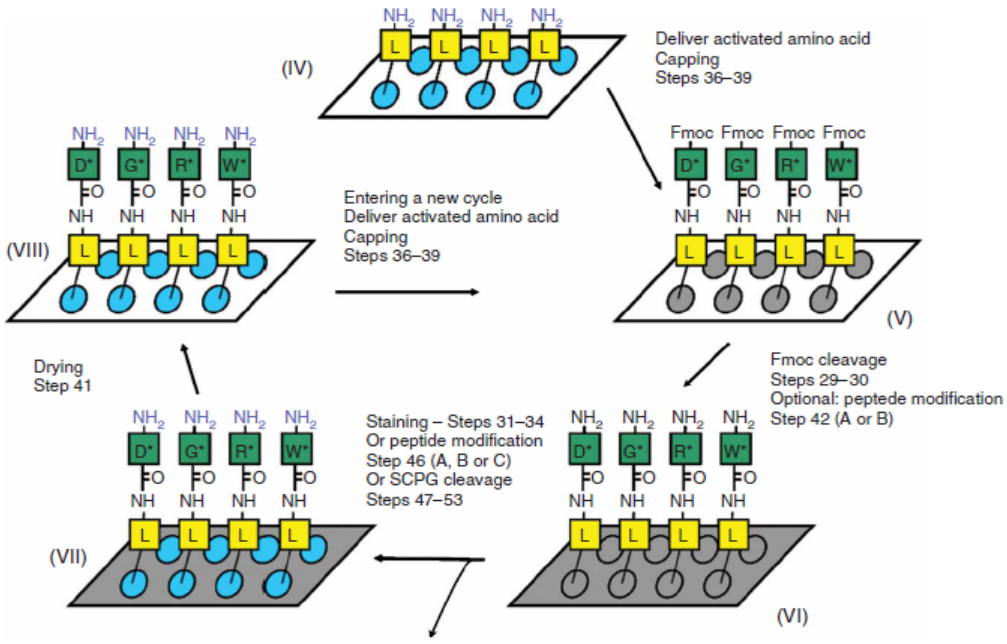

Figure 16. Amino acid coupling cycle for SPOT-synthesis.[110]

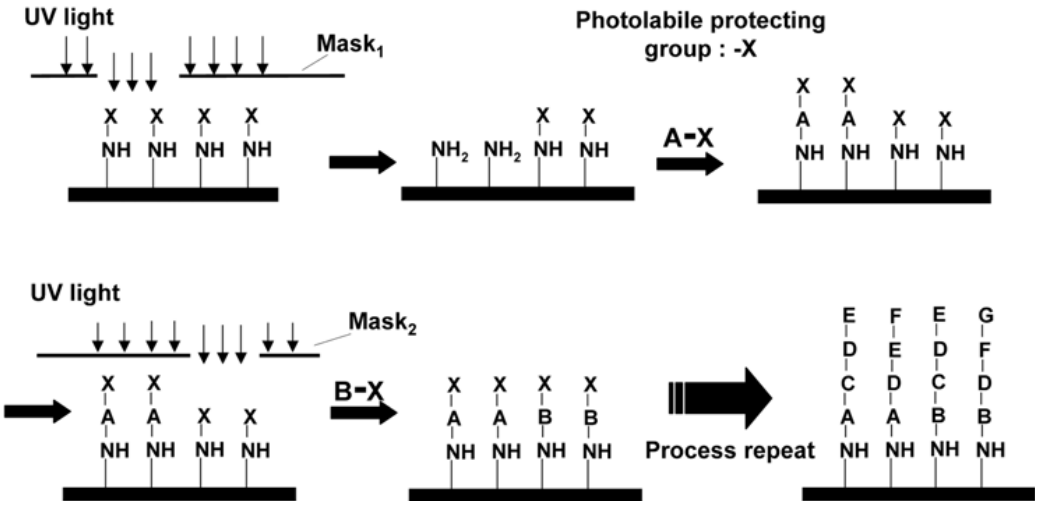

Figure 17. Light-directed, spatially-addressable peptide array synthesis.[108]

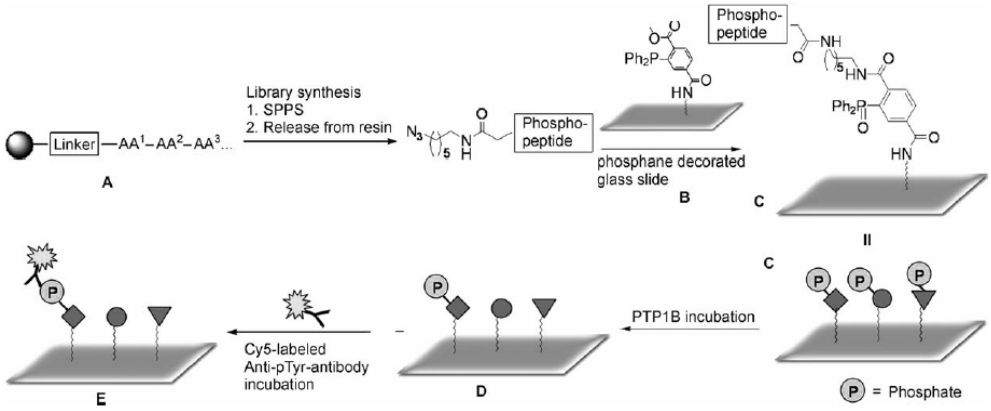

Figure 18. Mapping substrate specificity of protein tyrosine phosphatase on a phospho-peptide microarray.[112]

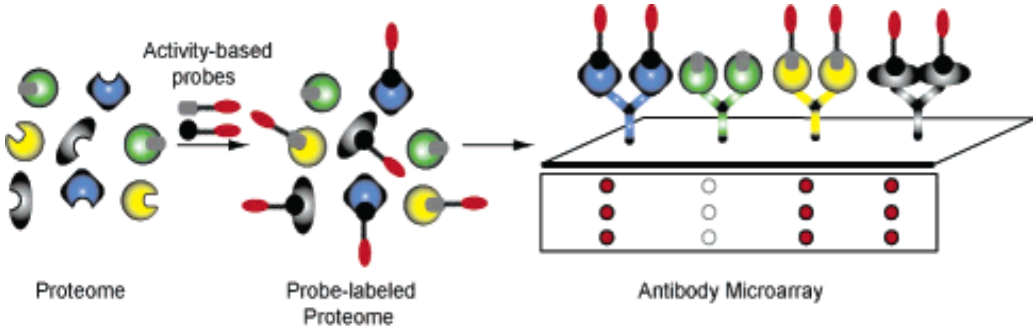

Figure 19. Antibody-based ABPP microarrays where proteomes are labelled, in solution, with fluorescent-based probes and then captured on glass slides with enzyme-specific antibodies.[114]

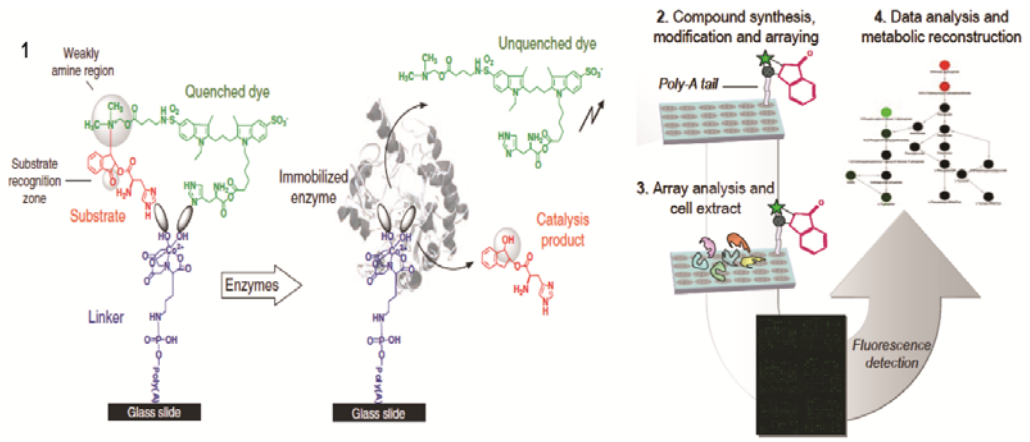

Figure 20. Reactome array for sensing the links between the metabolome and genome.[117]

In order to perform high-throughput enzyme activity screening, microwell[118] and microdroplet arrays[119-120] have been developed, which physically separate the array elements in such a way that the enzymatic reaction products remain localized at each feature and cannot diffuse between them. Another essential issue for screening catalytic reactions is maintaining enzyme activity on microarray surfaces. Hydrogels, which contain large quantities of solvent and behave as intermediates between dry and wet systems, are applied to peptide/protein arrays for this purpose.[121] In Figure 21, a semi-wet peptide/protein array is produced using a supermolecular hydrogel composed of glycosylated acetate which provides a suitable semi-wet reaction medium.[122] New technologies are under development to improve the microarray platform for protein interaction and enzyme activity profiling, including self-assembling protein arrays[123] and label-free enzyme arrays detected by mass spectrometry[124] or surface plasmon resonance.[125]

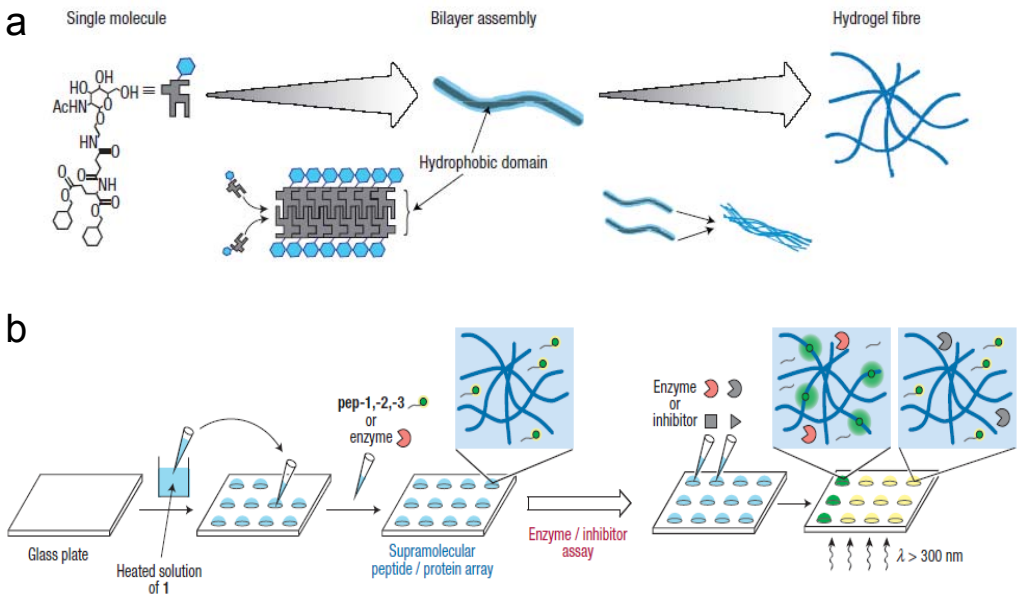

Figure 21. A semi-wet peptide/protein array using supermolecular hydrogel. (a) Gel formation and (b) enzymatic assay in the hydrogel.[122]

In this dissertation, a new method for identifying modulators of enzyme function is described that involves screening arrays of 10,000 defined and addressable peptides on a polymer-coated glass slides, for the ability to interact with enzymes and change their activity. In Chapter 2, I will discuss the coating of enzyme-bound microarrays with viscous poly vinyl alcohol (PVA) polymer, which allows the simultaneous monitoring of enzyme-peptide binding and peptide-bound enzyme activity. In Chapter 3, I will describe the discovery of peptides that modulate enzyme function, identified from performing enzyme assays on PVA-coated peptide arrays. In Chapter 4, an aggregation-based peptide inhibition mechanism is explored in detail. In Chapter 5, peptides selected from microarrays are used to modify surfaces for capturing enzymes with improved activity and stability. In Chapter 6, the commercialization of the above technology

will be discussed. Finally, in the last chapter, Chapter 7, I will discuss issues that have been faced during experimentation, and suggest some future improvements.

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CHAPTER 2: POLYMER-COATED MICROARRAY FOR MEASURING CATALYTIC ACTIVITY

ABSTRACT

A method is developed for screening catalytic activity on high-density peptide arrays. A polyvinyl alcohol (PVA) solution was applied to array surfaces to limit the diffusion of product molecules released from enzymatic reactions, allowing the simultaneous measurement of enzyme activity and binding at each peptide spot. Further development of PVA coating involved the modification of PVA using anti-product antibodies to slow down diffusion of the product, and the design of cascade reactions in the polymer layer for detecting the enzymes without a profluor substrate. Part of this work was published on the Journal of American Chemical Society.

INTRODUCTION

Enzyme regulation plays an important role in biological metabolism[1] and the ability to control enzyme activity through non-covalent interactions is central to therapeutics[2]. The modulation of enzymes is also important for industrial production of products and in enzyme-based assays.[3-4] Screening libraries of small molecules, peptides and nucleic acids has been used to identify ligands that bind to proteins and modulate their function.[5-6] Peptides are promising molecules for the modification of enzyme function because of the large chemical diversity available[7] and established methods for library synthesis[8]. In principle, assaying high-density microarrays of molecular libraries provides a high-throughput approach to screening for molecules that alter enzymatic function. Microarrays have been used for this purpose in the past[9-10], by constructing arrays of small molecules[5,11-12] or peptides[13-14], printing the enzyme substrate on

the surface[14-16] and activity-based protein profiling[17]. However, in general, the ability to measure enzyme activity on standard slide-based arrays is limited by diffusion of reaction products away from the sites of enzyme action. This problem is normally overcome by physically separating the array elements in such a way that enzymatic reaction products cannot diffuse between them, for example microwell[18] and microdroplet arrays[19-20]. Hydrogel, which contains large quantities of solvent and behaves as an intermediate between dry and wet systems, can maintain the activity of biomolecules, or even cells immobilized on it[21] , and has applications in many biological processes, such as protein[22] or cell immobilization[23], bioresponsive sensing[24-25] and biomedical applications[26-28]. Recently, hydrogels have been applied to protein arrays for assaying enzyme activity[29] and protein-ligand interactions[30].

A method for monitoring enzymatic reaction on the high-density microarray is described that involves screening an array of 10,000 defined and addressable peptides on a polymer-coated glass slide.[31] The slow diffusion of product molecules in the polyvinyl acohol coating layer makes it possible to resolve the enzyme activity in the spots with little cross contamination.

EXPERIMENTAL SECTION

Chemicals Amplex®Red, Fluorescein di-β-D-galactopyranoside (FDG), resorufin β-D-galactopyranoside (RBG), fluorescein diphosphate (FDP) and Alexa Fluor 647 were purchased from Invitrogen (Eugene, OR). Phenylethyl β-D-thiogalactoside (PETG), horseradish peroxidase (HRP), β-galactosidase (β-Gal, E.coli), alkaline phosphatase (APase), alcohol dehydrogenase, glucose dehydrogenase, poly vinyl alcohol (PVA, M.W.: 124,000~186,000), 4-nitrophenyl phosphate (PNPP), phenazine methosulfate (PMS), phosphate buffered saline

(PBS) and tris buffered saline (TBS) were obtained from Sigma (St. Louis, MO). Polyclonal anti-fluorescein antibody was purchased from Abcam (Cambridge, MA). A 4 mg/mL stock solution of β-Gal was prepared in 10 mM potassium phosphate buffer with 0.1 mM $MgCl_2$ at pH 7.4. A 1.2 mg/mL stock solution of APase was prepared in 0.1 M Tris containing 50 mM NaCl, 10 mM $MgCl_2$ and 0.1 mM $ZnCl_2$ at pH 8.2. 2.5 mg/mL HRP stock solution was prepared in pH 6, 10 mM sodium acetate.

Photobleaching experiment The diffusion coefficient of fluorescein was measured by Fluorescence Recovery after Photobleaching (FRAP). FRAP experiments were conducted on a Zeiss LSM 510 Meta confocal microscope (Jena, Germany) with a 40 ×, 1.3 NA oil-immersion objective using the 489 nm laser line. Fluorescence emission was collected using 515 nm LP and 505-550 nm BP filters. The fluorescein concentration was 50 μM. FRAP experiments were carried out by first bleaching a circular region (Diameter, 34 μm) at high laser power setting (100 mW), then scanning the bleached region with low laser power setting (0.5 mW) to monitor the fluorescence recovery process. The laser power setting listed above is the input laser power to the microscope. The actual laser power at sample is much lower after passing through the lens system and the filters. The diffusion coefficient D can be determined from the $\tau_{1/2}$ (fluorescence recovery half-time) according to the following equation[37],

$$D = (w^2 / 4\tau_{1/2}) r_D , \qquad (1)$$

where w is the actual radius of the bleached region, $\tau_{1/2}$ corresponds to the time when the fluorescence intensity reaches 50% of complete recovery, r_D=0.88 for circular beams.

Microarray fabrication Peptide microarrays were generated using the established, in-house printing method in the Center for Innovations in Medicine.[32] Each microarray was prepared by robotically spotting approximately 10,000 distinct polypeptide sequences, in duplicate, on a glass slide possessing an amino-silane surface coating. Synthesized peptides (70% purity) were purchased from Alta Biosciences Ltd (Birmingham, UK). Each polypeptide was 20 residues in length and the 17 amino-terminal positions were randomly chosen from 19 amino acids (excluding cysteine) using a pseudo-random computational process. The last three carboxy-terminal positions of each peptide constituted a glycine-serine-cysteine (GSC) linker, used for conjugating the peptides to amino-silane surfaces through the C-terminal cysteine via a maleimide linker, Sulfo-SMCC (Pierce, Rockford, IL). A Telechem Nanoprint60 was used to spot approximately 500 pL of 1 mg/mL peptide per feature on glass slides with 48 Telechem series SMP2 style 946 titanium pins.

Enzyme assays on PVA-coated arrays As shown in Figure 22, a microarray containing 10,000 20-mer, random-sequence peptides was first incubated with a solution containing dye-labeled enzyme (Alexa^{TM} 647), allowing the enzyme to bind with peptides on the array surface (Figure 22a). Unbound enzyme was washed off, and a substrate analogue (fluorescent-based) was mixed with a 5 % (*%w/w*) PVA buffer solution and spin-coated onto the array surface to form a ~ 50 μm layer (Figure 22b). The PVA-coated array was then incubated in a constant humidity chamber to allow the enzymatic reaction to occur. The substrate molecules in the PVA layer were converted to products by the enzymes bound to specific peptides on the array surface (Figure 22c), and remained localized at the peptide spot because of the PVA viscosity. For each of

the 10,000 peptides in the array, both the relative binding level of Alexa^{TM} 647-labeled enzyme and the relative amount of fluorescein produced during the incubation period were determined by dual color scanning (Figure 22d). Each array experiment was repeated at least three times under the same conditions for statistical analysis. HRP[33], APase[34] and β-Gal[35] were chosen as representative enzymes due to the availability of substrate analogues (Amplex Red, FDG and FDP in Figure 22e) and the wealth of structural and mechanistic information available for these enzymes.

Microarray data analysis Array images were first processed with GenePix Pro 6.0 (Molecular Devices, Sunnyvale, CA), and then microarray data was imported into GeneSpring 7.2 (Agilent, Foster, CA) for statistical analysis. To enable statistical comparisons between experiments, each slide was median-normalized: the raw data was normalized to the median signal of each array. Because enzyme activity sometimes appeared artificially low at the edge of the array due to insufficient PVA coating, peptides in these regions were not selected as candidates for further analysis.

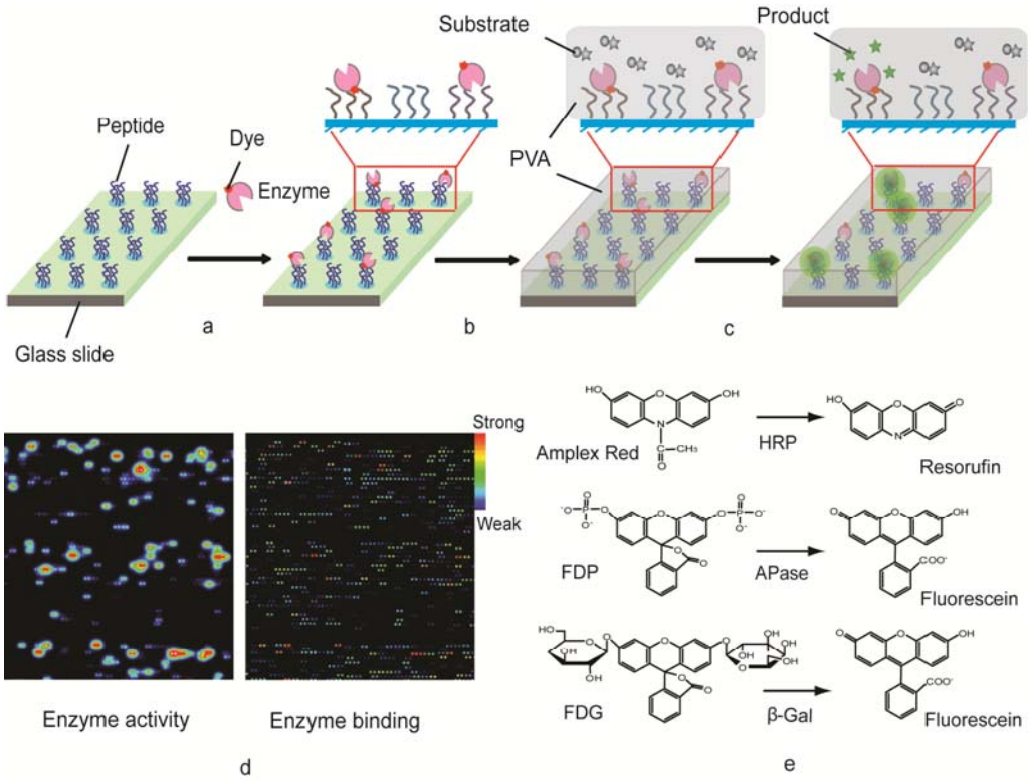

Figure 22. The overall process for screening peptide/enzyme interactions using peptide arrays. (a) Enzyme incubation. (b) PVA coating on the array surface. (c) The enzymatic reaction takes place in the PVA layer. (d) Fluorescent scanning images (a representative region) of binding (Alexa 647) and activity (fluorescein) for β-Gal on the PVA-coated array. Conditions: β-Gal, 5 nM; incubation time, 2 hours at room temperature; substrate, 50 μM FDG; PVA concentration, 5%; reaction time, 3 mins at room temperature. (e) Substrate analogues Amplex Red, FDP and FDG used for evaluating activity of HRP, APase and β-Gal, respectively.

RESULTS AND DISCUSSION

Limiting Diffusion using a Polyvinyl Alcohol Layer In general, the ability to measure enzyme activity on standard slide-based arrays is limited by diffusion of reaction products away from the sites of enzyme action. As shown in Figure 23a, serious cross-contamination between nearby spots was observed when incubating the enzyme bound array in buffer solution. In order to limit the diffusion of the products so that they remained in the immediate vicinity of the bound enzyme on the array (Figure 23b), the enzyme substrate was applied in a thin coating of Polyvinyl Alchohol (PVA).

PVA is non-fluorescent, optically transparent, water soluble and highly viscous and has applications in many biological assays (Figure 24).[36-37] The diffusion coefficient of fluorescein in a layer of 5% PVA in phosphate buffer was measured via fluorescence recovery after photobleaching (FRAP)[38] and found to be ~ 50 $\mu m^2/s$, roughly 6-fold slower than in phosphate buffer without PVA (Figure 25). As shown in Figure 22d, it was relatively easy to resolve the enzyme activity in the spots with little cross contamination. This is because of the spacing of the spots, the viscosity chosen for the PVA and the time allow for reaction. In addition, each peptide is duplicated side-by-side producing a distinctive oval around the active spot.

Enzyme activity on PVA-coated microarrays In Figure 26, the ability of enzymes to function in the PVA medium was demonstrated by real-time imaging of bound β-Gal activity on PVA-coated peptide arrays. One can see that the fluorescent products continued to accumulate over the entire time of the measurement, indicating that the enzyme remained active during that period. This assay was also used to determine the best period of time to run the enzyme

reactions for subsequent analyses. The greatest resolution between spots was observed after the first few minutes of incubation at the substrate concentrations used here.

In general, cross contamination is only an issue for peptides that bind the enzyme very weakly and those peptides are normally of little interest. In the typical strong enzyme-binding spot analyzed in Figure 27 a and b, the product molecule diffused ~160 μm (in one direction) away from the original spot and resulted in 10% of the signal intensity from one spot at the neighboring spot. The average distance between two spots is ~ 100 μm (from edge to edge). This only represents a significant problem when a spot showing very weak activity resides next to one with very strong activity (Figure 27c and d). Less cross contamination can be obtained by either increasing the space between spots or using more viscous PVA. To eliminate 'false positives' as much as possible, the candidates were selected from the top 200 strong binders.

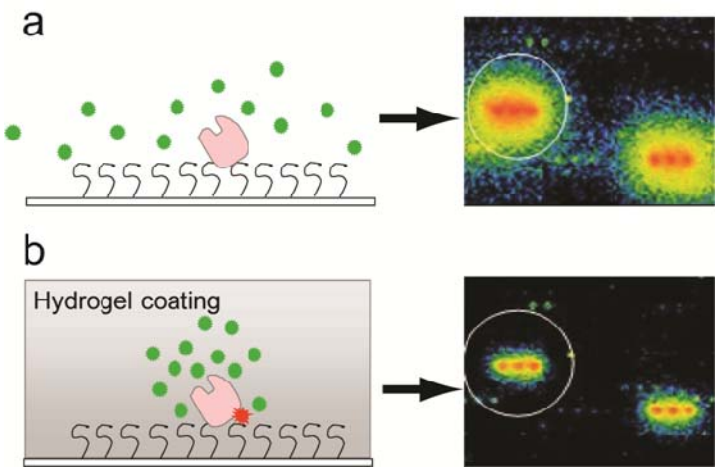

Figure 23. Product diffusion on the microarray surfaces coated with (a) phosphate buffer solution and (b) 5% polyvinyl alcohol. β-Gal was bound to the array surface for catalyzing the hydrolysis of FDG to fluorescein.

Figure 24. Structure of PVA, PVA solid and viscous solution.

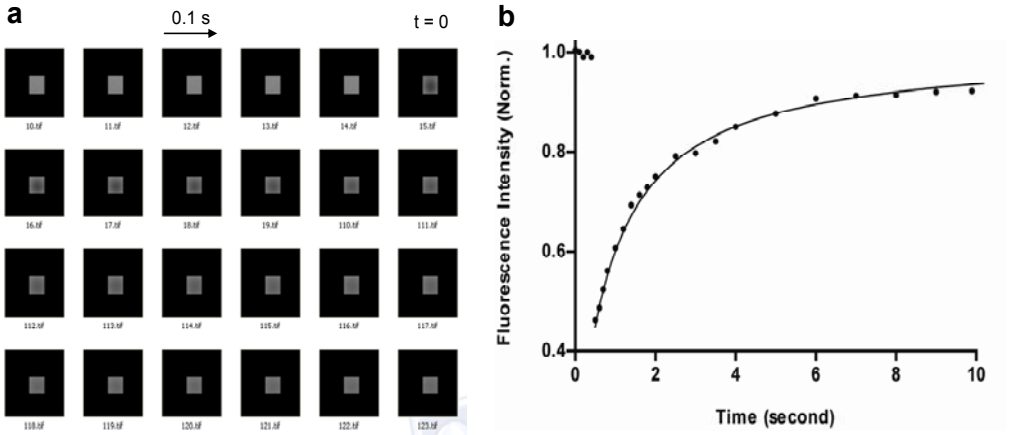

Figure 25. FRAP of fluorescein in 5% PVA. (a) A series of images of the fluorescence recovery process using 50 μM fluorescein. Photobleaching started at t = 0 s, duration of the laser bleaching, ~ 50 ms; scanning interval, ~ 100 ms. (b) Data analysis of the fluorescence recovery process. $\tau_{1/2}$ = 1.3 s.

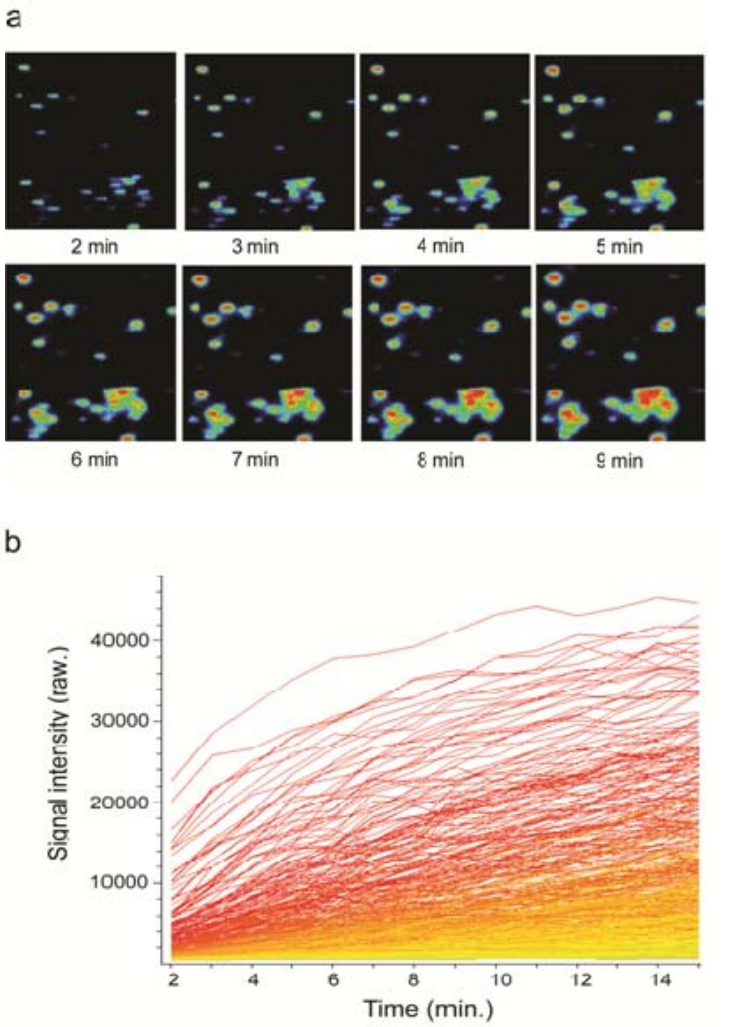

Figure 26. The ability of enzymes to function in PVA polymer. (a) Real-time imaging of β-Gal activity on PVA-coated peptide arrays. Fluorescence is generated due to the production of resorufin released from RBG hydrolysis. A 10 mm × 20 mm region of the slide containing ~ 1000 spotted peptides was monitored (only a very small part of that area is shown). Conditions: substrate, 10 μM RBG; PVA concentration, 5%; Scanning interval, 1 min. (b) Kinetics measurements of RBG hydrolysis catalyzed by bound β-Gal on peptide arrays. Each trace represents the kinetics at a particular spot (a particular attachment peptide) on the array.

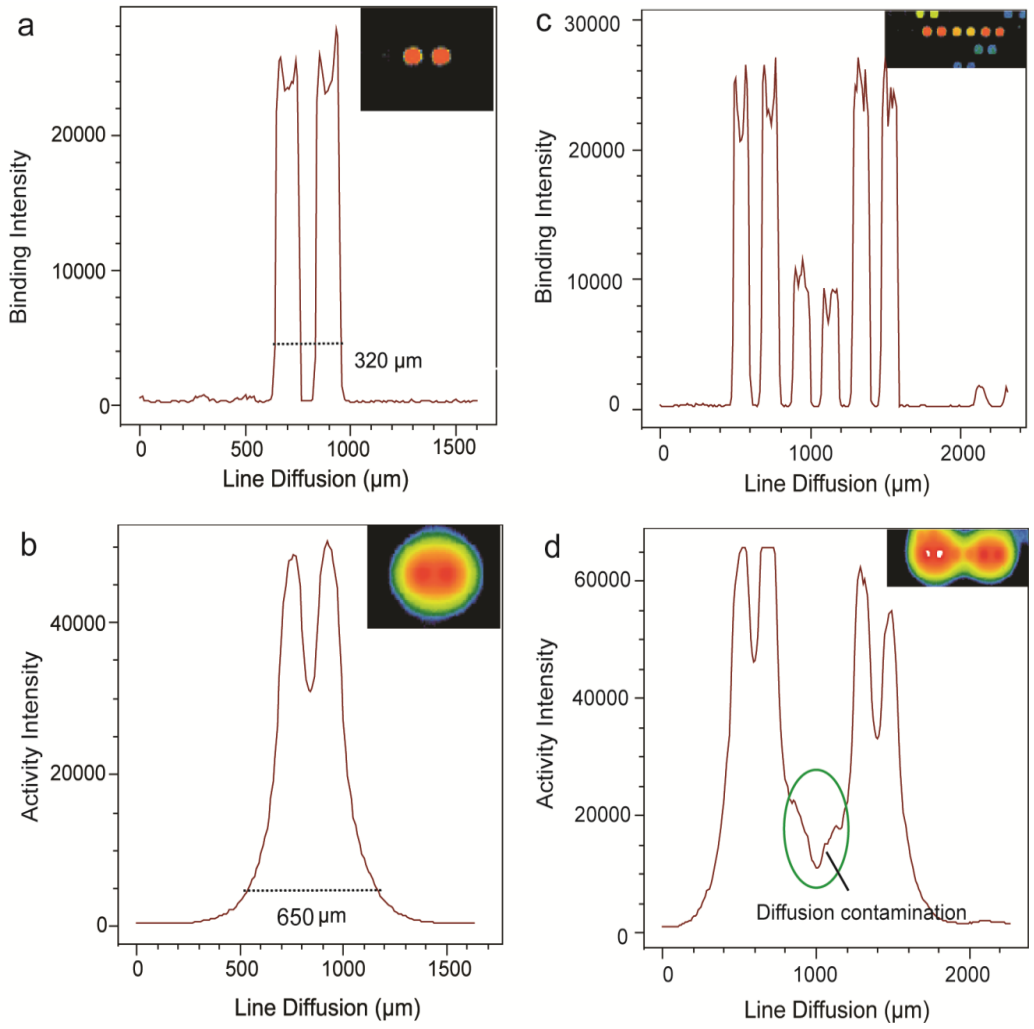

Figure 27. Diffusion of fluorescent products between spots in the PVA layer. Single-line scanning analysis of (a) enzyme binding intensity and (b) bound enzyme activity was used to evaluate how diffusion affects the measurements of specific activity on the surface. The amount of product that has diffused between spots can be determined by comparing the peak width of enzyme activity (which is subject to diffusion) to the peak width of enzyme binding (no diffusion). (c) and (d) are example spots that contaminate each other.

Ultra-slow product diffusion in an antibody-modified PVA layer In the above discussion, the diffusion contamination between spots was reduced to a low level if the reaction was incubated for a few minutes. However the diffusion of product molecules will still become more serious with increasing reaction time, even in a PVA layer. It complicates the application of the technology to slow catalytic reactions. To overcome this problem, anti-product antibodies were used to capture product molecules in the PVA medium and slow down their diffusion rate (Figure 28). Antibodies are big molecules of ~ 150 kDa, diffusing much more slowly than small molecules. If the product molecules released from an enzymatic reaction are captured by antibodies, the product-antibody complex will exhibit a much slower diffusion rate compared to free product molecules in the PVA layer due to the larger molecular size. To test the concept, an FITC-labeled antibody was applied in the 5% PVA layer to mimic the diffusion properties of a flurorescein-bound antibody (Figure 29). The diffusion coefficient of FITC-labeled antibody was measured to be ~ 2.8 μm^2 /s in 5 % PVA, which was much slower than the fluorescein diffusion rate of ~ 50 μm^2 /s in the same PVA medium.

An anti-fluorescein antibody was added to the PVA layer to capture the fluroescein molecules released from the β-Gal enzymatic reaction on the array surface. As shown in Figure 28b and c, the fluorescein molecules were restricted to the vicinity of the enzyme-bound spots catalyzing them by adding the antibodies in the PVA layer, while the molecules diffused further away from the reaction sites and contaminated nearby spots in the blank PVA layer. Figure 30 shows that the enzyme-bound spots were resolved better for the arrays coated with antibody-modified PVA compared to the array coated with blank PVA.

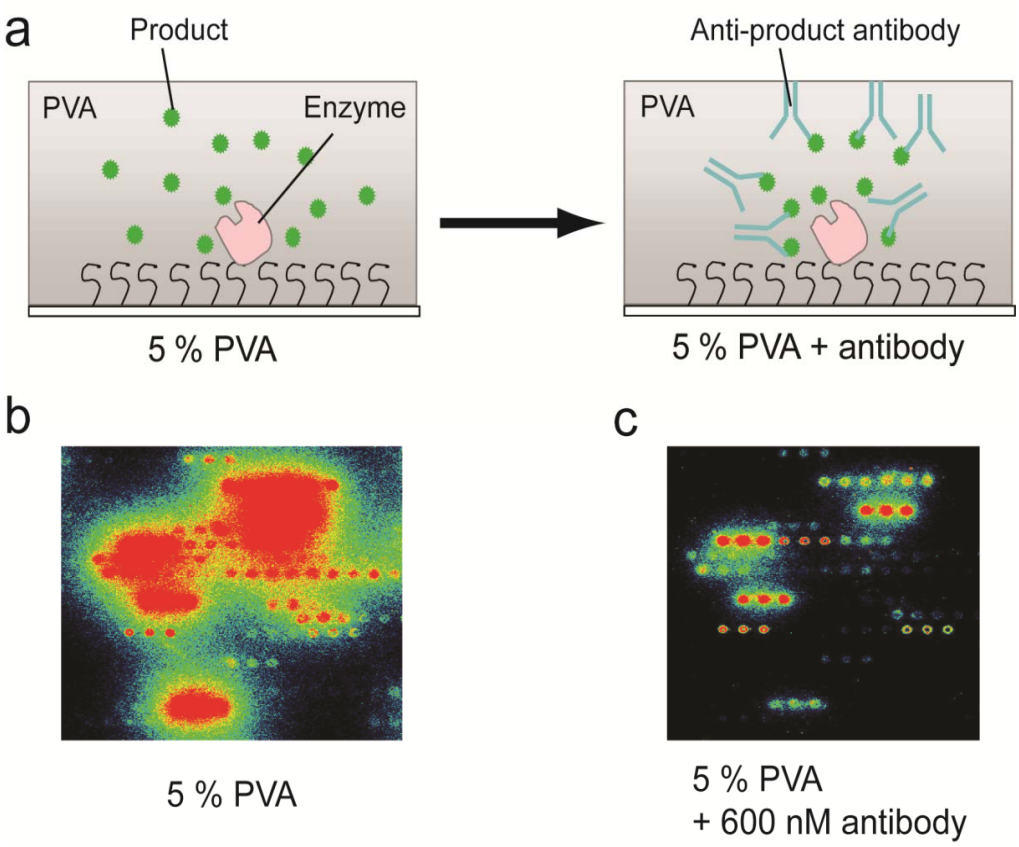

Figure 28. Ultra-slow product diffusion in PVA by (a) adding anti-product antibodies which will capture product molecules and form big complex diffuse slowly in PVA medium. Fluorescent images of (b) the long-period fluorescein diffusion in blank 5% PVA and (c) the fluorescein diffusion in 5% PVA mixed with 500 nM anti-fluorescein antibody. β-Gal catalyzed the conversion of fluorescein from FDG and the reaction on array was incubated for 30 minutes with 500 nM FDG in the PVA layer.

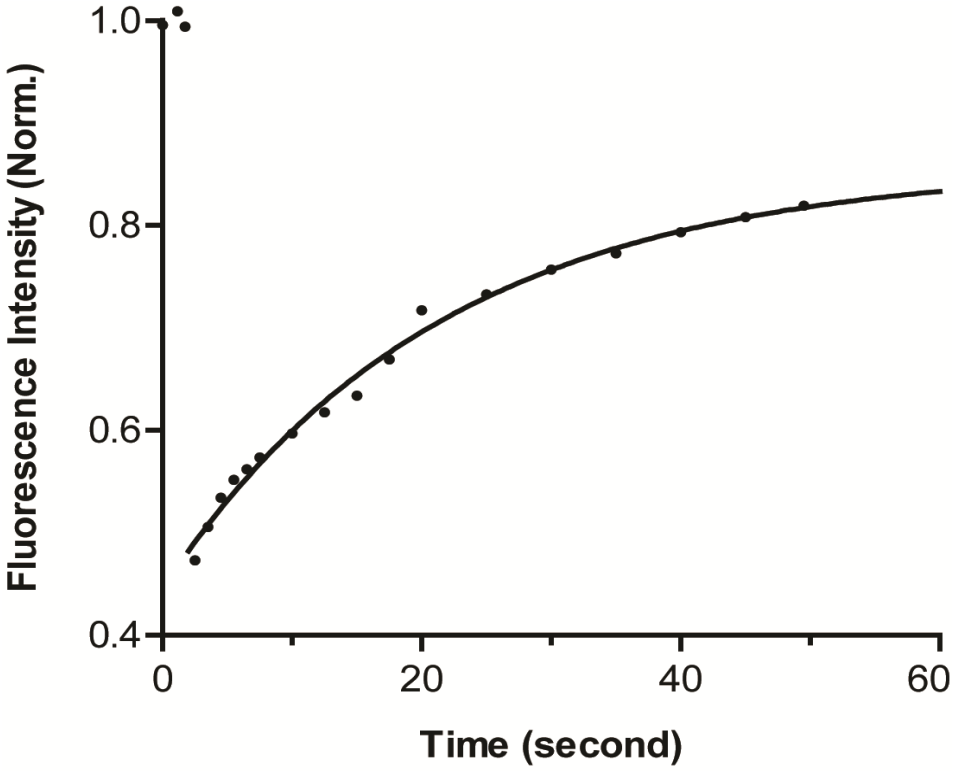

Figure 29. FRAP of FITC-labeled antibody in 5% PVA. Photobleaching started at t = 3 s; duration of the laser bleaching, ~ 100 ms; scanning interval, ~ 400 ms; Antibody concentration, 2 μM; $\tau_{1/2}$ = 23 s; D=2.8 μm^2/s.

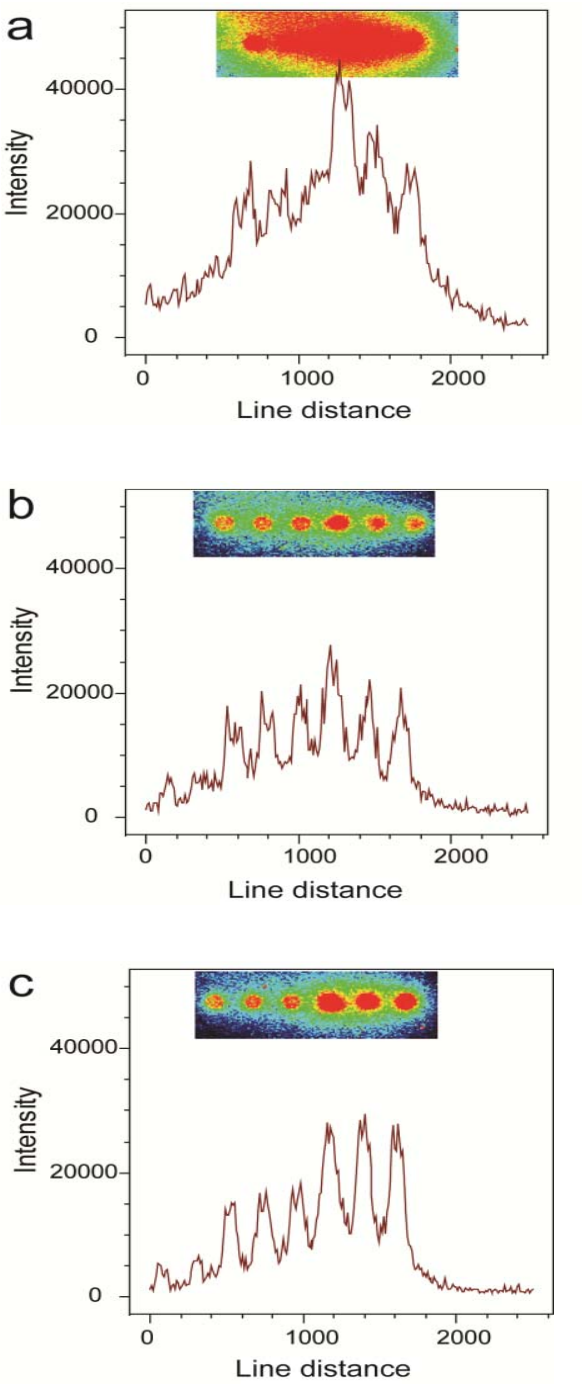

Figure 30 Diffusion of fluorescent products between spots in the PVA layers with or without antibody. Single-line scanning analysis of the arrays coated with (a) blank 5% PVA; (b) 5 % PVA mixed with 200 nM anti-fluorescein antibody and (c) 5% PVA mixed with 500 nM anti-fluorescein antibody.

Design of a cascade reaction in a PVA layer to measure dehydrogenase activity The methods discussed above use a profluor as a substrate for the enzymes assayed. This limits the application of the technology because many enzymes do not have fluorescent substrate analogues available. To solve this difficulty, approaches were developed to use a nonfluorescent product to generate a fluorescent signal with designed enzyme-linked cascade reaction in the PVA coated array. In the enzyme-linked cascade reaction, the nonfluorescent product of the enzyme under study acts as a substrate for a second enzyme/catalyst in the PVA layer and thereby is converted to fluorescence signal for monitoring. To explore this concept, dehydrogenases were chosen as the target enzymes to study. These enzymes oxidize a substrate by transferring one or more hydrides (H^-) to an acceptor, usually NAD^+/$NADP^+$. Dehydrogenases represent a class of important enzymes that are prevalent in biological metabolism. Some of these enzymes are drug targets or are used as catalysts for chemical syntheses in biocatalysis industry.

The reaction product NAD(P)H has weak fluorescence at 460 nm with maximal excitation absorbance at 340 nm, which is not appropriate for standard microarray fluorescent imaging. A cascade reaction was designed to use NAD(P)H as the substrate to convert nonfluorescent resazurin to strongly-fluorescent resorufin (emission ~ 585 nm), catalyzing by phenazine methosulfate (PMS) in Figure 31. Alcohol dehydrogenase (ADH, *Yeast*; ~ 141 kDa, tetrameric protein) and glucose dehydrogenase (GDH, *Thermoplasma acidophilum*; ~ 155 kDa, tetrameric protein) were used as model enzymes in this study due to the wealth of structural and mechanistic information available for these enzyme systems. In Figure 32, an enzyme-bound peptide array was coated with a PVA

layer containing ethanol, NAD+, PMS and resazurin, which allowed the cascade process of first converting NAD+ to NADH by surface-bound dehydrogenase, and then generating strongly fluorescent resorufin by the PMS-catalyzed reduction of resazurin. The activities of dehydrogenase bound to the microarray were monitored using regular fluorescence imaging and the activity of enzyme-bound spots could be resolved. The fluorescent molecules diffused farther away from the original spot for the cascade reaction than for the single-substrate reaction, due to the two diffusion steps of NADH and resorufin involved in the cascade reaction. A further optimization may be achieved by conjugating NAD^+ to PVA polymer (120 ~ 180 kDa) to slow down the diffusion of the molecules.

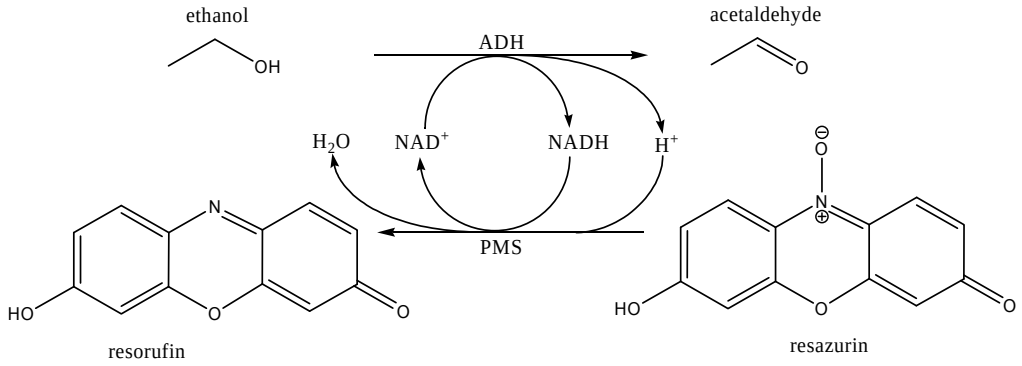

Figure 31. A cascade reaction that uses NAD(P)H genreated by dehydrogenase to convert nonfluorescent resazurin to fluorescent resorufin. Phenazine methosulfate (PMS) was used to catalyze the cascade reaction.

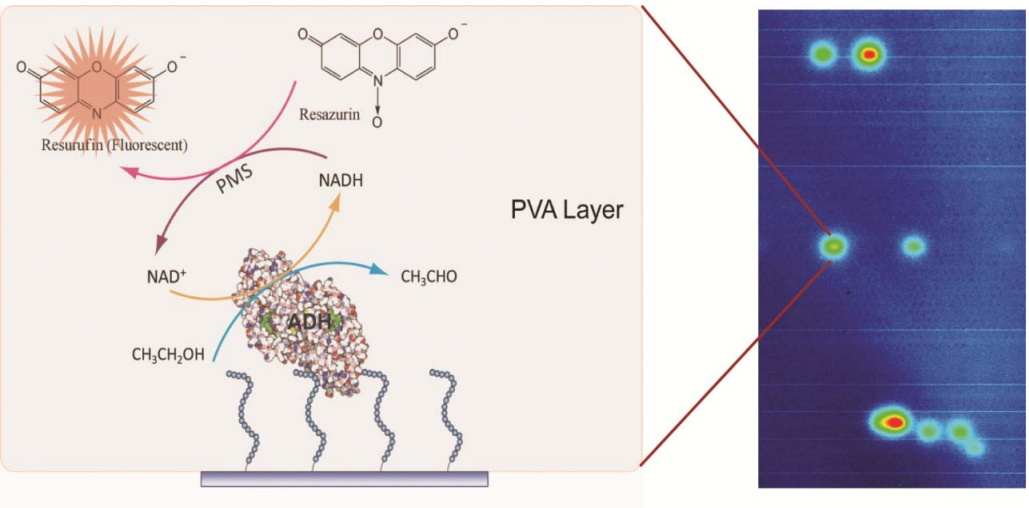

Figure 32. Monitoring of dehydrogenase activity on peptide microarrays through a designed cascade reaction in the PVA layer. Conditions: 10 nM Alexa647-labled ADH first bound to peptide array for 2 hours at room temperature in pH7.3 buffer containing 1 X PBS and Tween 20 (0.05%(*%v/v*)); PVA coating: 5% (*%w/w*) PVA containing 3% *(%v/v)* ethanol concentration, 1 mM NAD^+; 50 μM PMS and 100 μM resazurin; reaction time, 3 mins at room temperature.

CONCLUSION

The approach described above represents a surprisingly simple and general method of screening enzyme activity on high-density microarrays. The peptide slides can be printed inexpensively and rapidly analyzed given an appropriate enzyme assay. PVA were simply prepared with various reaction buffers and coated onto the array surface to limit the diffusion of product molecules. As a result, many enzyme/peptide pairs can be processed in parallel under almost any set of desirable conditions. Much slower diffusion of product molecules on the microarray surface was achieved by modifying PVA with anti-product antibodies, allowing longer periods for monitoring the catalytic reaction and accumulating product. The further development of enzyme-linked cascade

reaction in the PVA layer made it possible to detect enzymes without using a profluor as substrate. As a demonstration, the activity of a peptide-bound dehydrogenase was measured on the microarray surface. Finally, this approach is not limited to peptides; any small molecule that is arrayable could also be searched in this format for enzyme modulators.

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SUPPLEMENTARY INFORMATION

Detailed Protocol of Enzyme assay on the PVA coated arrays β-Gal was labeled with Alexa Fluor 647 (Invitrogen) and diluted to 5 nM in bovine serum albumin (BSA) buffer (1× PBS with 3% (v/v) BSA and 0.05% Tween 20). The activity of the labeled β-Gal was ~ 75% of that of the wild type in solution. The peptide array was first prewashed with surface cleaning solvent (7.33% (v/v) acetonitrile, 37% isopropyl alcohol and 0.55% trifluoroacetic acid in water) for 5 minutes to reduce non-covalent bonding peptides on the array surface. The array was then treated to block any active SMCC (Sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate) linker remaining from peptide spotting by applying 330 μL capping buffer (3% (v/v) BSA, 0.02% (v/v)

mercaptohexanol, 0.05% (v/v) Tween20 in 1×PBS) and incubating for one hour in a humidity chamber (Stain Tray Slide System, Fisher Scientific, Pittsburgh, PA). The array was then washed once with pH 7.4 TBST (1 × Tris-Buffered Saline and 0.05% (v/v) Tween 20) and at least twice with water, and dried by centrifuging at 1,500 rpm for 5 minutes. After the blocking step, 330 µL 5 nM Alexa 647-labeled β-Gal was applied to the array. The array with the enzyme solution was sealed using an AbGene Frame (Fisher Scientific) and slide cover, and incubated for two hours at room temperature in a humidity chamber in the dark as shown in Figure 22a. After two hours, the slide cover was removed and the array was washed three times with 1 × TBST, 5 minutes each wash. This was followed by three washes with pH 7.4 10 mM potassium phosphate and 100 µM $MgCl_2$ buffer, 5 minutes each wash.

The array was then spin-coated with a 5% PVA solution that included substrate analogues FDG or RBG, forming a thin PVA layer on the array surface (Figure 22b) using a commercial spin coater (WS-400B-6NPP/LITE, Laurell, North Wales, PA). The PVA solution was prepared by adding PVA powder into phosphate buffer and then heating the solution in a microwave. The heating process was repeated several times until all of the PVA powder was dissolved. The enzyme substrate (or substrate analogue) was then added into the PVA solution to the desired substrate concentration. About 700 µL of PVA solution was added onto the array surface and the array was fist spun at 300 rpm for 10 seconds, and then spun at 2,000 rpm for 15 seconds to make the polymer spread evenly on the array surface. The final PVA layer thickness was ~50 µm as measured by scanning confocal microscopy (DNAscope™ , Biomedical Photometrics Inc., Waterloo, Canada).

The PVA-coated peptide array was immediately incubated in a humidity chamber allowing the enzyme reaction to take place. The substrate molecules in the PVA layer were converted to fluorescent product by the enzyme molecules bound to specific peptides in the array. The product molecules diffused slowly in the viscous PVA polymer layer and remained near the spot where the reaction took place. The reaction incubation time was optimized by using a real-time scanning fluorescence imager and observing the accumulation of product molecule during the reaction (Figure 26). In this work, the incubation time for enzyme reaction was 3 mins for β-Gal , HRP and APase. The slides were then dried rapidly in a vacuum desiccator to stop the enzymatic reaction. The array was then read by a standard array reader (PerkinElmer, Waltham, MA) with dual color scanning using 488 and 647 nm laser lines. The AbGene frame and Open Frame DNAscope™ were used for real time imaging of the β-Gal activity on the array surface. Each array experiment was repeated at least three times under the same conditions for statistical analysis.

The array activity assays for APase and HRP used procedures very similar to that used for β-Gal. In the case of APase, FDP was used as the substrate analogue and a Tris buffer (1×Tris-Buffered Saline) was used instead of a phosphate buffer. The array activity assay for HRP was performed by using 50 μM Amplex®Red and 1 mM H_2O_2 as substrates mixed with 5% PVA prepared in 10 mM, pH 6 sodium acetate. The Amplex®Red was converted to resorufin (emission at ~585nm) by HRP in the presence of H_2O_2.

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CHAPTER 3: EXPLORING PEPTIDE SPACE FOR ENZYME MODULATORS

ABSTRACT

A method is presented for screening high-density arrays to discover peptides that bind and modulate enzyme activity. Simultaneous measurement of enzyme activity and binding at each peptide spot were performed on the polyvinyl alcohol-coated array. For proof of concept, it was possible to identify peptides that bound to horseradish peroxidase, alkaline phosphatase and β-galactosidase and substantially altered enzyme activity by comparing the binding level of peptide to enzyme and bound enzyme activity. This basic technique can be used to screen many enzymes in parallel under many set of desirable conditions, and may be generally applicable to find peptides or other small molecules that modify enzyme activity. The work has been published on the Journal of American Chemical Society.

INTRODUCTION

Enzyme modulation is crucial for living systems and plays important roles in regulating metabolic function.[1] Searching for small-molecule ligands with the ability to control enzyme activity is central to therapeutics[2]. The modulation of enzymes is also of great interest to industrial production of products and in enzyme-based assays.[3-4] Screening libraries of small molecules, peptides and nucleic acids has been used to identify ligands that bind to proteins and modulate their function.[5-6] Peptides are promising molecules for the modification of enzyme function because of the large chemical diversity available[7] and established methods for library synthesis[8]. In principle, assaying high-density microarrays of molecular libraries provides a high-throughput approach to screening for molecules that alter enzymatic function. Microarrays have been used for this

purpose in the past[9-10], by constructing arrays of small molecules [5,11-12] or peptides[13-14], printing the enzyme substrate on the surface[14-16] and activity-based protein profiling[17]. The recent development of polymer-coated microarrays makes it possible to simultaneous screen enzyme activity and binding on high-density array surfaces and identifies peptide modulators that bind to the enzyme with substantial alteration of its function.[18]

In this chapter, a method for identifying modulators of enzyme function is described that involves screening an array of 10,000 defined and addressable peptides on a polymer-coated glass slide for the ability to interact with an enzyme and change its activity. This is performed by simultaneously monitoring both the binding and activity of the enzyme at each peptide spot on the microarray surface.

EXPERIMENTAL SECTION

Chemicals and **Microarray data analysis** have been described in Chapter 2.

Solution-based enzyme assays Peptides selected from microarrays were synthesized and purified for use in solution-based enzyme assays, which were performed on a SpectraMax M5 96 well plate reader (Molecular Device, Sunnyvale, CA). Peptides were first incubated with enzyme for half an hour, and then the substrate was added to the wells to measure the enzyme activity. At least three replicates were tested parallel. The IC_{50} of each inhibitor was determined by fitting the concentration vs. inhibition curve to the function 'Fit LogIC50' in the GraphPad program using the fitting equation "Y=Bottom+(Top-Bottom)/(1+10^(X-LogIC50))". The "Bottom" term was constrained to 1, which represents the maximal inhibition of 100%. The "Top" term was constrained to 0,

which represents the minimal inhibition of 0%. Each data point is the average of at least 3 replicates.

RESULTS AND DISCUSSION

In Chapter 2, a method of monitoring enzymatic reactions on high-density peptide microarrays was developed, which involved the coating of the array surface with a viscous PVA polymer. This allowed the simultaneous measurement of enzyme activity and binding at each peptide spot, and made it possible to identify peptides that bind to the enzyme and substantially modulate its activity. Three enzymes were tested on the peptide microarray in Figure 33: HRP, APase and β-Gal which were monomeric, dimeric and tetrameic proteins, respectively.

The binding level and activity of HRP on the peptide array for the 1000 top binders are shown in Figure 33a. As expected, the total activity generally increases with the amount of enzyme bound. Peptides exhibiting weak binding and lower enzyme activities are mainly distributed in Region (i) (lower left). Peptides that show both strong binding and enzyme activities are distributed in Region (ii) (upper right). The peptides that appear to bind and inhibit enzyme activity are distributed in Region (iii), showing relatively weak enzyme activity compared to the level of enzyme binding. The surface-specific activity of HRP was calculated for each of the spots, in Figure 33a, by dividing the total bound enzyme activity by the total binding intensity (Figure 33d). The median-normalized specific activities ranged from 0.33 to 11, suggesting that the nature of the interactions between the enzyme and the peptides on the surface was affecting enzyme activity. Higher than median activities for particular peptides

could be due to favorable orientation of the enzyme by the peptide or peptide stabilization of a more active conformation (Figure 34a).

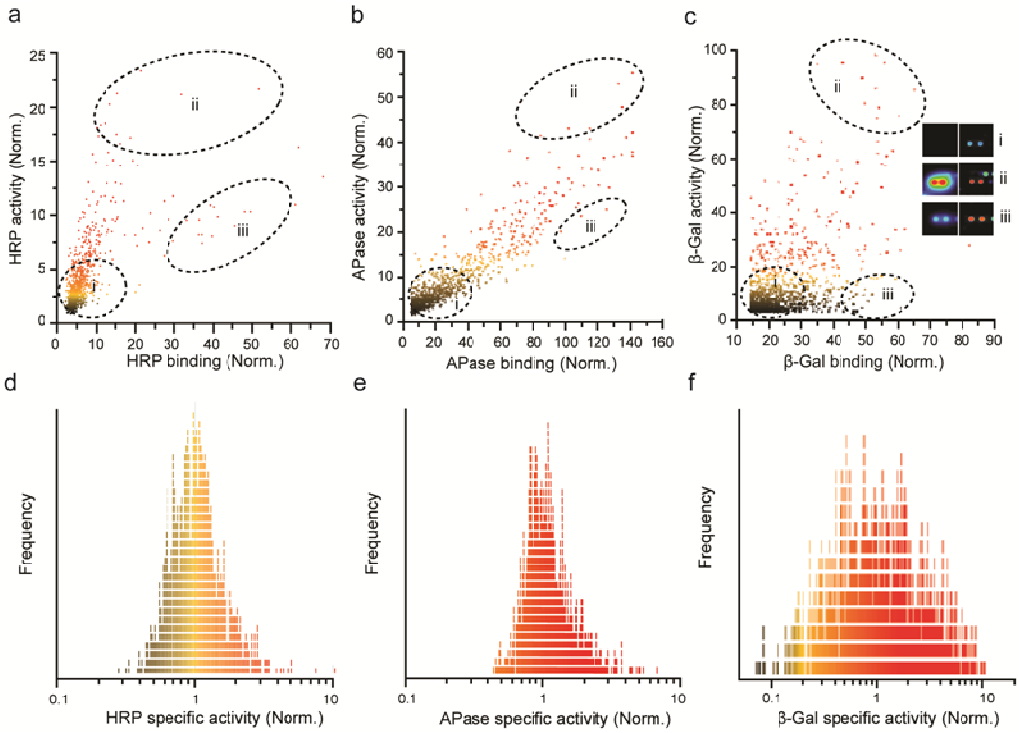


Figure 33. The median-normalized activity of bound (a) HRP, (b) APase and (c) β-Gal on the microarrays as a function of the amount of enzyme bound to a particular peptide on the array for the 1000 strongest binding peptides. Frequency distribution of surface specific activity of (d) HRP, (e) APase and (f) β-Gal. Examples of raw fluorescence images associated with specific classes of peptides in the array are shown as an inset of (c). (i) Weak enzyme activity with weak enzyme binding intensity, (ii) strong enzyme activity with strong enzyme binding intensity, (iii) weak enzyme activity with strong enzyme binding intensity.

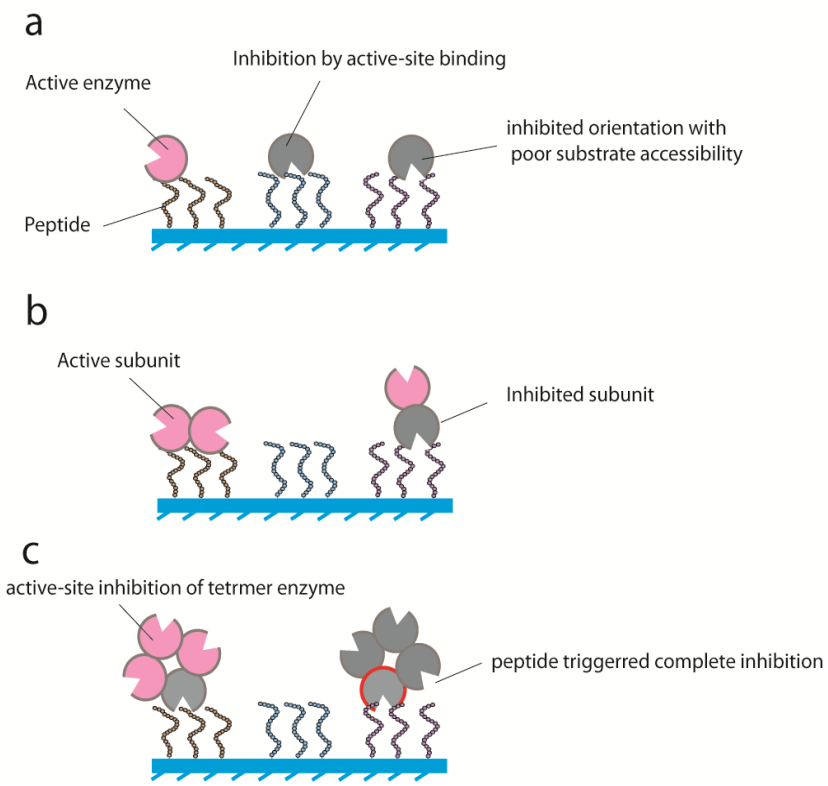


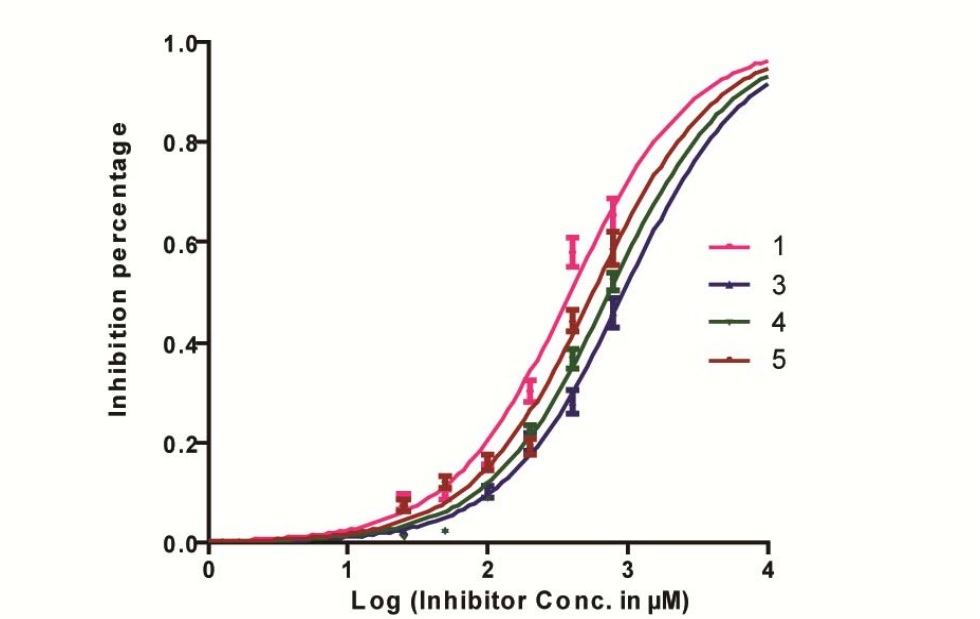
Figure 34. The predicted inhibition of monomeric, dimeric and tetrameric enzymes on a peptide microarray surface. (a) For monomeric enzymes (e.g. HRP), the inhibition on the microarray may be due to direct active-site interaction or an inhibitory orientation with poor substrate accessibility. (b) For dimeric enzymes with two structural independent subunits (e.g. APase), one subunit of the two interacts with peptides and is inhibited, leaving the other subunit still active. (c) For tetrameric enzyme, one would expect to see partial inhibition on the array surface since there will be one or two subunits that do not bind to peptides and are open to substrates. Complete inhibition of enzyme will be observed only if the peptide can trigger a conformational change of the whole tetramer enzyme via induced allosteric inhibition.

Figure 33b shows the binding level and activity of APase on the peptide array for the 1000 top binders. As shown, the increase of total activity with the amount of enzyme bound forms a significantly tighter correlation compared to HRP, with the median-normalized specific activity ranging from 0.42 to 6.6 (Figure 33e). APase (E.coli) is a homo-dimeric enzyme which possesses an unshared active site in each subunit[19] (structure independent). Therefore, it may be that only one of the two subunits interacts with a surface peptide at any given time and, thus, only the activity of that subunit is modulated (Figure 34b). This idea is consistent with the fact that the lowest activities were about half of the median surface-specific activities of the enzyme. The seven peptides with the lowest surface-specific activities were selected, re-synthesized and tested in solution. Four of them were able to inhibit the enzyme in solution as well as on the surface, with IC_{50} values (concentration of 50% inhibition) between 400 μM and 900 μM (Figure 35).

In contrast to APase, β-Gal (E.coli) is a tetramer with the active site on the interface of two subunits and has known allosteric inhibition and activation[20-21]. As shown in Figure 33c, there is much more variation in β-Gal surface specific activity than APase activity as a function of binding to different peptides. Examples of the raw fluorescence images associated with each region of the activity vs. binding plot are shown in the right panel of Figure 33c. Strong inhibition was seen in Region (iii) with the median-normalized specific activity lower than 0.2. If the peptides were acting as simple active site inhibitors, one might expect that β-Gal would never have less than about one half to three fourths of the median activity of the enzyme due to its tetrameric nature and the likely ability of peptides on the surface to interact with only one or two subunits at

a time. However, the strong inhibition in Region(iii) of Figure 33c suggests that some peptides may trigger conformational changes in the entire tetramer and inhibit the whole enzyme (Figure 34c). Consistent with this scenario, activities as low as 0.07 of the median surface specific activity are observed (Figure 33f). These results suggest that the simple ratio assay described here may provide a general approach for directly detecting peptides that allostericly inhibit particular enzymes.

10 peptides inhibiting β-Gal (in Region (iii) of Figure 33c) and resulting in low surface-specific activities on the array were synthesized and purified for solution-based enzyme inhibition assays. 8 Peptides (1-8) were found to inhibit β-Gal activity in solution with a range of IC_{50} values from 1.2 μM to 30 μM (Table 1 and Figure 36). As controls, several peptides from Region (ii) of Figure 33c (strong binding and high activity, e.g. peptide 11) and from Region (i) (weak binding and weak activity, e.g. peptides 12 and 13) were also synthesized and tested for the inhibition of β-Gal in solution. These peptides showed much higher IC_{50} values (> 300 μM) than the selected peptide inhibitors. These results imply that modulation of enzyme activity via surface-bound peptides corresponds, in most of cases, to the effects of those peptides in solution. As an indication of the specificity of the selected peptide inhibitors, they were also tested for their effects on APase activity. Most showed much weaker inhibition of APase than β-Gal (>20 fold higher IC_{50}).



Peptide	Sequence	Enzyme activity (Norm.)	Enzyme binding (Norm.)	Specific activity (Norm.)	IC50 (µM)
1	YLQTAYPNPQKLFHMRSGSC	12 ± 3	79 ± 11	0.5	380 ± 70
2	WFHLKRPGAFGIAANIHGSC	14 ± 6	99 ± 15	0.5	insoluble
3	FQMKRHTRRYRENIIGFGSC	9 ± 2	63 ± 6	0.4	940 ± 60
4	GERIKYMQFAVRPSHSRGSC	5.8 ± 0.6	42 ± 4	0.4	750 ± 70
5	MRTVYRDSYHIMMFRKMGSC	4.2 ± 0.7	29 ± 7	0.5	560 ± 70
6	VPFGLLAAIVFAAAPMPGSC	3.3 ± 0.9	23 ± 2	0.5	> 1000
7	IGMTSFHSGYTPMPRQYGSC	3.3 ± 0.3	24 ± 4	0.5	insoluble

Figure 35. Solution test of selected peptides inhibiting alkaline phosphatase activity. Peptides 1-7 are the inhibitors selected that have the lowest surface specific activity (normalized to the median of the array). The IC_{50} of the peptide inhibition is measured at a substrate concentration of 200 µM PNPP and an alkaline phosphatase concentration of 200 µg/L; Temperature, 25 ^{o}C. All data is the average of at least 3 replicates. Inhibition percentage = (Activity $_{uninhibited}$ – Activity $_{inhibited}$) / Activity $_{uninhibited}$.

Table 1. Solution test of selected peptides inhibiting β-Gal activity [*].

Peptide	Sequence	Enzyme activity (Norm.)	Enzyme binding (Norm.)	Surface specific activity (Norm.)	IC_{50} (μM) (β-Gal)	IC_{50} (μM) (APase)
1	RVFKRYKRWLHVSRYYFGSC	0.9 ± 0.4	50 ± 10	0.08	1.7 ± 0.2	80 ± 5
2	KFHHFWKWHWRWHHRPFGSC	1.9 ± 1.9	49 ± 10	0.18	1.2 ± 0.2	> 250
3	PASMFSYFKKQGYYYKLGSC	2.3 ± 2.5	64 ± 7	0.16	13 ± 2	> 200
4	LGRMFAYRWRLKIKHRLGSC	2.6 ± 1.5	47 ± 11	0.25	10 ± 1.2	> 175
5	FLMRKYNKQRVFYIAFRGSC	0.8 ± 0.6	48 ± 9	0.07	10 ± 1.4	> 150
6	FNAPIWWYIYPRHVRHAGSC	0.8 ± 0.5	42 ± 5	0.09	6 ± 1	> 75
7	FRNFPVPVIFRYLNPWPGSC	2.3 ± 1.1	52 ± 8	0.20	7 ± 2	> 200
8	GVFPRRFGYVWVHLTEKGSC	0.8 ± 0.3	51 ± 2	0.07	30 ± 3	> 100
9	HIPWWWQNYPSWYPYRLGSC	1.5 ± 0.9	43 ± 6	0.16	insoluble	-
10	SYMLYHHFIWFKTHYSQGSC	2.4 ± 1.2	47 ± 8	0.22	> 120	> 120
11	YHNNPGFRVMQQNKLHHGSC	92 ± 13	38 ± 6	11	> 500	> 500
12	EFSNPTAQVFPDFWMSDGSC	0.7 ± 0.3	0.4 ± 0.3	-	> 1000	> 400
13	ESVPTDLPMDTMEGKNWGSC	1.2 ± 0.5	0.5 ± 0.4	-	350 ± 30	> 400
14	Phenylethyl β-D-thiogalactoside				35 ± 3	> 1000

[*] Peptides 1-10 are selected inhibitors, Peptides 11-13 are the negative control peptides and 14 is the competitive inhibitor of β-Gal. The IC_{50} of the peptide inhibition is measured at 25 ^{o}C with a substrate concentration of 100 μM RBG (resorufin β-D-galactopyranoside) and a β-Gal concentration of 150 μg/L. APase concentration, 200 ug/L, PNPP (4-nitrophenyl phosphate) 200 μM. '-' unavailable data due to poor solubility or weak inhibition.

In addition to inhibitors, peptides that enhanced the surface-specific activities of β-Gal were also found (e.g. Region (ii) in Figure 33c) which suggests that peptides can stabilize the active conformations of each enzyme (or alternatively optimize its orientation and function on a surface). In room-temperature solution tests, some peptides in that region enhanced β-Gal activity by about 50% but did not show better activation of β-Gal in solution than negative control peptides (weak binder peptide 12 and 13), possibly because β-Gal is stable and nearly at its maximum possible activity in solution at room temperature. However, this result suggests that it might be possible to discover peptides that enhance stability of the enzyme on the surface or under other conditions that might favor inactive conformations of the enzyme (e.g. high

temperature, pH). To test this, the enzymes were bound to peptides on microarrays at room temperature and then the arrays were incubated at higher temperatures (61° C for APase and 55 ° C for β-Gal, both enzymes lose activity at the high temperatures, Figure 37) for one hour and assayed for activity at room temperature (HRP was not tested in this way). As shown in Figure 38, most of the peptide-bound enzymes lose activity after incubation at high temperature (compared to Figure 33). However, there are a few peptide-bound enzymes that remain stable after this treatment (Table 2 and 3). For Apase, up to a 14-fold improvement in remaining activity over the median level is observed after extended exposure to a temperature of 61 °C and for β-Gal up to 31-fold improvement in remaining activity over the median after exposure to 55 °C is observed. These results suggest that both enzymes can be stabilized by binding to particular peptides.

CONCLUSION

In this chapter, the PVA-coated microarray was demonstrated to discover modulators of enzyme activity by performing parallel measurements of activity and binding for the entire array. HRP, APase and β-Gal tested in this work demonstrated a wide variation in binding to the 10,000 spotted peptides (median-normalized binding levels from 0.2 to 70 for HRP, from 0.1 to 150 for APase and from 0.3 to 85 for β-Gal). A >10-fold variation over the array was found in the surface specific activity for HRP and APase and >100-fold for β-Gal. In most cases tested, enzyme inhibition observed on the surface was also demonstrated in solution-based measurements. Not only was it possible to rapidly and easily discover enzyme inhibitors in this fashion, enzyme stabilizing peptides were also found; some of the peptides were able to promote maintenance of enzyme

activity on the surface even after prolonged exposure to high temperatures. Such peptides might be useful in enzyme immobilization applications, resulting in improved enzyme activity and stability.

Why can peptides have such diversities in modulating enzyme function? In solution, proteins are quickly switched between different conformations, some conformation may favorite catalytic reactions, and some may induce inhibition. It is possible that a peptide prefers to bind to a specific conformation of the enzyme, and thereby switch the balance between different protein conformations. This can either make the protein more stable, or damage its activity. It will be very interesting if peptides can be used to differentiate the conformations of the target proteins.

The PVA-coated peptide slides can be rapidly analyzed given an appropriate enzyme assay and assayed at many conditions (like temperature, pH and solvent). It may be a generally method to discover molecules that modulate enzyme activity at desired conditions.

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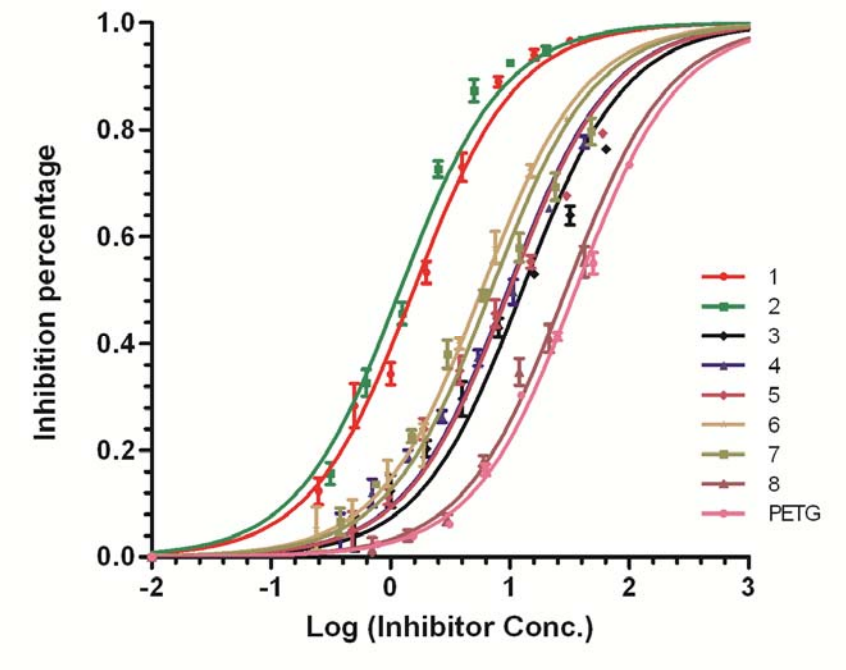

Figure 36. Curve fit determining the IC_{50} of peptides inhibiting β-Gal using the data presented in Table 1. GraphPad Prism 5 was used for enzyme kinetics fitting to the equation: Y=Bottom + (Top-Bottom)/(1+10^(X-LogIC50)). Here, Bottom is constrained to 1 which represents the maximal inhibition of 100% and Top is constrained to 0 which represents the minimal inhibition of 0%. All data is the average of at least 3 replicates. Inhibition percentage = (Activity $_{uninhibited}$ – Activity $_{inhibited}$) / Activity $_{uninhibited}$.

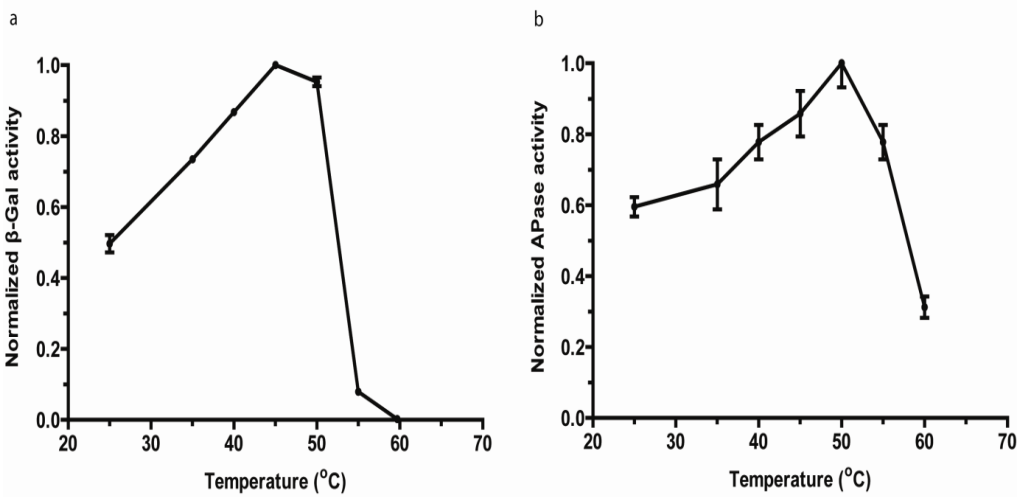

Figure 37. Solution thermal stability of (a) β-Gal (E.Coli) and (b) APase (bovine). The native enzyme (not bound to a peptide) is first incubated at the test temperature for one hour, and then the activity is determined by adding substrate. In the thermal stability graph, β-Gal loses most of its activity at temperatures higher than 55 °C and Apase loses activity at temperatures higher than 60°C. The activities at different temperatures are normalized to the value of highest activity and all values are the average of three replicates.

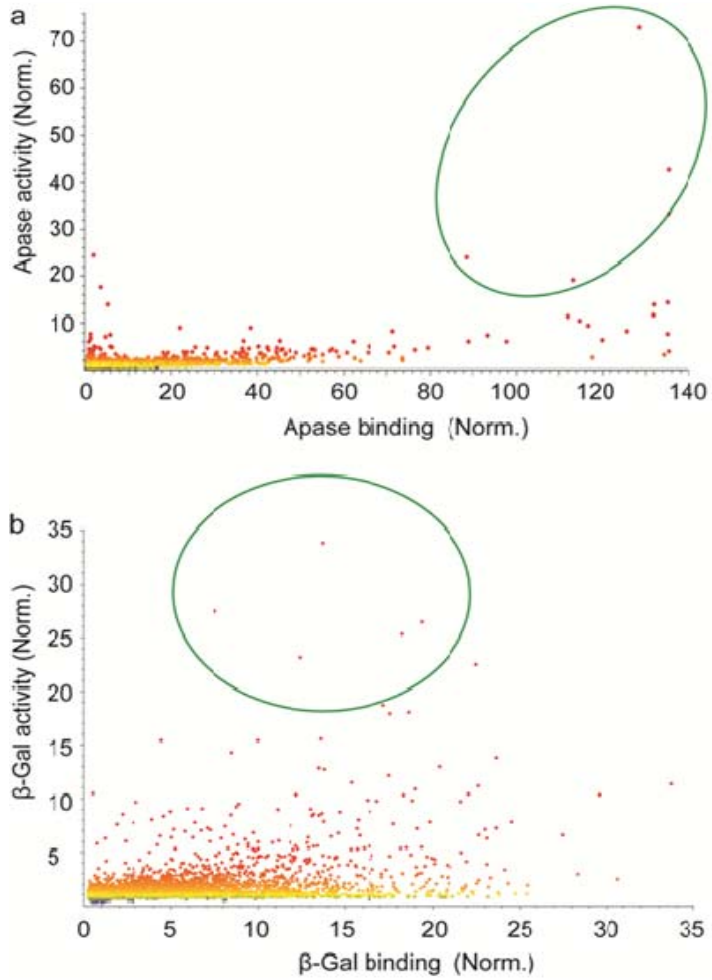

Figure 38. Thermal-stability test of APase and β-Gal on a peptide microarray. (a) APase was first bound to a peptide microarray at room temperature, and then incubated in Tris buffer at 61 °C for one hour. The activity was measured by coating the APase bound slides with 50 μM FDP and incubating for 3 minutes at room temperature. (b) β-Gal was first bound to microarray at room temperature, and then incubated in phosphate buffer at 55 °C for one hour. The activity was measured by coating the β-Gal bound slides with 50 μM FDG and incubating for 3 minutes at room temperature. The selected regions (circled) contain peptides binding to the enzyme with the highest specific activity after incubating at high temperature.

Table 2 Selected peptides for enhancing thermalstability of APase [*].

	Sequence	Apase Microarray (incubate at 61 °C		
		Enzyme activity (Norm.)	Enzyme binding (Norm.)	Surface specific activity (Norm.)
1	HWKRRHKHKWPKRHPHKGSC	73	128	14
2	HRKHWRKRHKKHWKKRKGSC	9	22	10
3	HFRKWHKRRWKHHKKWKGSC	43	135	8
4	WKKKRKHRHKKHWHPWRGSC	24	88	6
5	HWHHRWWHHWKHPHHWRGSC	34	135	6

* APase was first bound to a peptide microarray at room temperature, then incubated in Tris buffer at 61 °C for one hour. The activity was measured by coating the APase bound slides with 50 μM FDP and incubating for 3 minutes at room temperature. All data is median normalized and the average of at least three parallel slides.

Table 3 Selected peptides for enhancing thermalstability of β-Gal [*].

	Sequence	β-Gal Microarray incubate at 55 °C		
		Enzyme activity (Norm.)	Enzyme binding (Norm.)	Surface specific activity (Norm.)
1	YHNNPGFRVMQQNKLHHGSC	23	3.1	31
2	IWHPHEGKRKWSHSYNLGSC	12	3.8	13
3	MWGLDRNKQVQRHIYHQGSC	14	4.9	12
4	AQHYQSFRLMTPHEVHKGSC	14	5.5	11
5	YLQTAYPNPQKLFHMRSGSC	12	7.9	6

* β-Gal was first bound to the microarray at room temperature, and then incubated in phosphate buffer at 55 °C for one hour. The activity was measured by coating the β-Gal bound slides with 50 μM FDG and incubating for 3 minutes at room temperature. All data is median normalized and the average of at least three parallel slides.

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CHAPTER 4: ENZYME INHIBITORS SELECTED FROM PEPTIDE MICROARRAYS

ABSTRACT

In this chapter, peptides screened from high-density microarrays were evaluated for their ability to inhibit β-galactosidase in solution. Peptide inhibitors acted through a noncompetitive inhibition mechanism which was sensitive to enzyme and detergent concentrations. Dynamic light-scattering showed that the aggregation of enzymes with peptides was involved in the inhibition of enzyme activity. Detailed sequence analysis of peptides revealed that positive residues, such as K and R, played a critical role in peptides inhibiting enzymes. Further, it was possible to select additional peptides which either enhanced the inhibitory activity of these peptides or neutralized it. It may start with an enzyme in an inhibited form in solution and turn it on via a secondary peptide interaction.

INTRODUCTION

Small molecule-modulators that regulate enzyme activity play an important role in many biological functions, and are crucial for drug discovery.[1-2] Screening libraries of small molecules, peptides and nucleic acids has been widely used to discover ligands that bind to proteins and modulate their functions.[3-4] Peptides represent a promising class of potential enzyme modulators[5] due to their large chemical diversity[6] and the existence of well-established methods for library synthesis[7]. Peptides and their derivatives are found to inhibit many important enzymes[8], like dehydrogenases[9], protein kinases[10] and proteases.[11] Cell-permeable peptides are becoming more and more useful in blocking cellular signaling pathways.[12-13] High-density microarrays containing peptide libraries or enzyme substrates synthesized or printed directly

on array surfaces, provide a high-throughput approach to screening for peptides that alter enzymatic function.[14-16] Recently, hydrogel-coated microarrays have been used as a means of screening for enzyme activity modulated by specific protein-peptide interactions, which has made it possible to perform activity assays using high density microarrays.[17]

It is important to understand the unique mechanisms of peptide-modulated enzyme inhibition in order to explore potential applications of peptide-based molecules to therapeutics and the biocatalysis industry. In this chapter, peptides screened from microarrays were evaluated for their ability to inhibit β-galactosidase (β-Gal) which revealed the aggregation of peptide-inhibited enzyme complexes. Previously, many lead compounds selected from high-throughput screening inhibit enzymes noncompetitively with poor specificity and act by aggregating them into colloidal particles.[18-20] As what will be described below, it is possible to find peptides that specifically aggregate or dissociate with the enzyme only in the presence of another particular peptide. It may switch the enzyme between an aggregated, inhibited state and an active state by using two or more selected peptides.

EXPERIMENTAL SECTION

Chemicals. Fluorescein di-β-D-galactopyranoside (FDG), resorufin β-D-galactopyranoside (RBG) and Alexa Fluor 647 (Alexa 647) were purchased from Invitrogen (Eugene, OR). Phenylethyl β-D-thiogalactoside (PETG), β-galactosidase (β-Gal), poly vinyl alcohol (PVA, M.W.: 124,000~186,000), phosphate buffered saline (PBS) and tris buffered saline (TBS) were obtained from Sigma (St. Louis, MO). A 4 mg/mL stock solution of β-Gal was prepared in 10 mM potassium phosphate buffer with 0.1 mM $MgCl_2$ at pH 7.4. A 2 mg/mL

stock solution of peptide was first prepared in pure water, and then diluted in phosphate buffer to the desired concentration.

Microarrays. Microarray production and associated assay protocols were described in previously chapters. GenePix Pro 6.0 (Molecular Devices, Sunnyvale, CA) and GeneSpring 7.2 (Agilent, Foster, CA) were used for microarray data analysis, also described previously.[17,21] To enable statistical comparisons between experiments, each slide was median-normalized: the raw fluorescence intensities from each peptide spot were normalized to the median fluorescence signal for each array.

Solution-based enzyme assays. Solution-based enzyme assays were performed on SpectraMax M5 96 well plate readers (Molecular Device, Sunnyvale, CA) as described previously.[17] Briefly, peptides were first incubated with enzyme for 20 minutes, and then the substrate was added into the wells to measure the enzyme activity, including at least three replicates per peptide. The IC_{50} of each inhibitor was determined by fitting the concentration vs. inhibition curve to the function 'Fit LogIC50' as defined in the program GraphPad using the fitting equation "Y=Bottom+(Top-Bottom)/(1+10^(X-LogIC50))". The "Bottom" term was constrained to 1, which represents the maximal inhibition of 100%. The "Top" term was constrained to 0, which represents the minimal inhibition of 0%. The inhibition percentage of peptide-inhibited enzyme was calculated using the equation of "Inhibition Percentage = (Activity $_{uninhibited}$ – Activity $_{inhibited}$)/ Activity $_{uninhibited}$ × 100%". K_m and V_{max} (and thus k_{cat}, when combined with the total enzyme concentration used) of β-Gal were determined by fitting the activity vs. substrate concentration curves in the GraphPad program using the fitting equation of "Y=V_{max}*X/(K_m+X)".

Dynamic Light Scattering (DLS). A Zetasizer Nano S (Malvern Instruments) was used for the DLS study. The stock peptide and enzyme samples were first passed through 0.2 μm filters to remove any dust particles. 1X TBS with 0.1 mM $MgCl_2$ was filtered using a 0.2 μm filter and used as the buffer in the DLS experiments. The peptide and enzyme samples were diluted down to desired concentration using filtered buffer.

Fluorescence Correlation Spectroscopy (FCS). FCS measurements were conducted using a confocal microscope (ECLIPSE TE2000-U, Nikon) with continuous wave laser excitation at 532 nm (Millennia Xs, Spectra-Physics). The fluorescence was collected after passing through a filter designed for the fluorescent dye, Alexa 555 (emission ~ 580 nm), and then split into two parts with equal intensity and directed to two avalanche photodiodes, as described previously[22]. Correlation curves were measured using a dual-channel digital correlator with a sample time of 12.5 ns (Flex2k-12x2, Correlator, Bridgewater, NJ) and the vendor's software. The analysis was performed using home-written software based on LabView (version 7.1, National Instruments). For all measurements, the dye-labeled β-Gal concentrations were in the low nanomolar range.

RESULTS AND DISCUSSION

In the previous chapters, an approach was described for screening high-density arrays to identify peptides that bind to enzymes and modulate their activity.[17] In Table 4, 7 peptides, selected from microarrays, were found to inhibit β-Gal activity in solution with IC_{50} values ranging from 1.0 μM to 13 μM. Substrate titration studies suggested that most peptides are noncompetitive inhibitors (except peptide 1), generating more reduction in K_{cat} with less change

on K_m (Figure 39). As controls, the Michaelis constants for PETG-inhibited β-Gal (a known competitive inhibitor)[23] were also measured, which showed that the competitive inhibition of β-Gal generated a reduction in K_m with little effect *on* K_{cat} compared to uninhibited enzyme, as expected. The peptides were also tested for their effects on APase activity as an indication of the specificity of the peptide inhibitions. Most showed much weaker inhibition of APase than β-Gal (>20 fold higher IC_{50}).

Table 4 β-Gal inhibitory peptides selected from microarrays[a].

Peptide	Sequence	pI	Solution Test		
			IC_{50} (μM) (β-Gal)	K_m(μM) *	K_{cat} (s^{-1})*
1	RVFKRYKRWLHVSRYYFGSC	10.6	1.0 ± 0.2	250 ± 45	35 ± 4
2	PASMFSYFKKQGYYYKLGSC	9.4	13 ± 2	154 ± 13	33 ± 2
3	KFHHFWKWHWRWHHRPFGSC	11	1.2 ± 0.2	166 ± 26	39 ± 4
4	LGRMFAYRWRLKIKHRLGSC	11.8	10 ± 1.2	159 ± 26	34 ± 3
5	FLMRKYNKQRVFYIAFRGSC	10.5	10 ± 1.4	149 ± 16	31 ± 2
6	FNAPIWWYIYPRHVRHAGSC	9.3	6 ± 1	128 ± 22	35 ± 3
7	FRNFPVPVIFRYLNPWPGSC	9.5	7 ± 2	123 ± 10	34 ± 2
8	Phenylethyl β-D-thiogalactoside	-	35 ± 3	256 ± 19	54 ± 3
Enzyme	β-galactosidase (150 μg/L)	-		134 ± 15	63 ± 4

[a] 1-7 are selected peptide inhibitors, 8 is a known competitive inhibitor of β-Gal. The IC_{50} of peptide inhibition was measured at 25 °C with a substrate concentration of 100 μM RBG (resorufin β-D-galactopyranoside) and a β-Gal concentration of 150 μg/L. *K_m and K_{cat} are measured at a peptide concentration approximately equal to the IC_{50}.

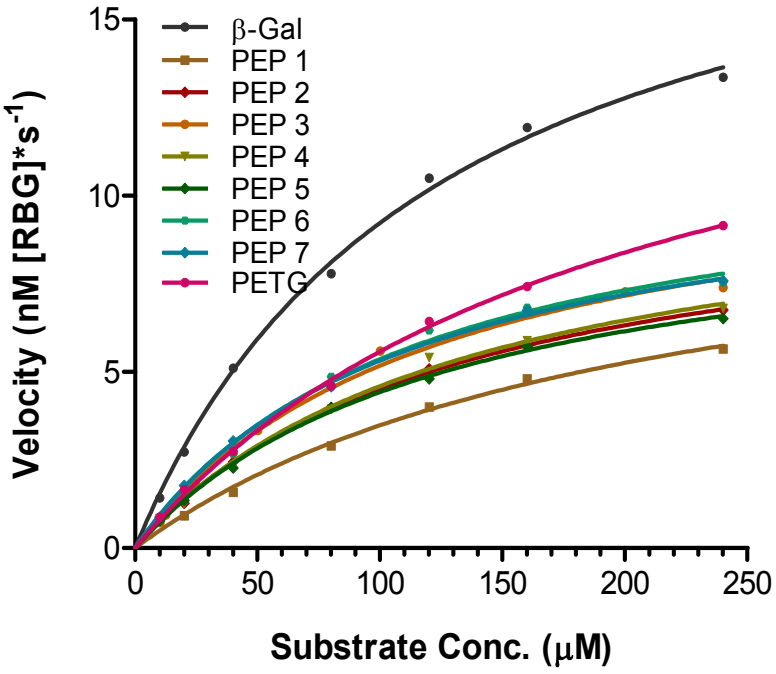


Figure 39. Curve fit used to determine the apparent K_m and k_{cat} values for β-Gal inhibited with various peptides in this study; the resulting values are given in Table 4. GraphPad Prism 5 was used for enzyme kinetics fitting to the equation: $Y=V_{max}*X / (K_m+X)$. The enzyme concentration was 150 μg/L (~ 0.33 nM); the RBG substrate was titrated from 10 μM to 240 μM. All data is the average of at least 3 replicates.

Titration studies of enzyme and detergent concentration were performed to further explore the inhibition of β-Gal by peptides. In Figure 40a, each of the peptides inhibiting β-Gal showed a ~2-fold or greater decrease in inhibition with increasing enzyme concentration between 1 and 1000 nM enzyme. Given that the peptide concentration was held at 20 μM, the strong dependence on enzyme concentration for inhibition suggests that the stoichiometric ratio of peptides to enzyme is quite high for effective inhibition of β-Gal (apparently on the order of 100 in most cases). In Figure 40b, peptide inhibition also shows sensitivity to the

detergent concentration, with the inhibition of β-Gal becoming weaker with increasing detergent, at constant peptide concentration. The degree of this effect was rather variable from one peptide to another (e.g., very small effect on peptide 1, but a dramatic effect on peptide 2), but in all cases some affect on the ability of the peptide to inhibit was observed. In contrast, the enzyme and detergent concentrations had little effect on the competitive inhibition of β-Gal using PETG. These results are consistent with peptide induced aggregation of the enzyme as a mechanism of inhibition. This generally requires a high inhibitor to enzyme ratio and is sensitive to detergents that disrupt aggregation.[18]

Dynamic light scattering (DLS) was used to further explore the role of aggregation in the inhibition mechanism of the peptides. Three peptides (peptides 1-3) were tested using DLS because these peptides exhibited good solubility and strong inhibition. As shown in Figure 41a, DLS of 30 μM peptide 1 alone showed a major peak with a hydrodynamic diameter of 3 ~ 6 nm, which corresponds to the size of the 20-mer linear peptide. β-Gal in solution at 50 nM showed a peak at ~ 15 nm, which is consistent with the diameter of the enzyme. When peptide 1 and β-Gal were mixed at the same final concentrations, enzyme activity was strongly inhibited (inhibition percentage > 90%), and large particles with diameters > 200 nm were observed by DLS. Apparently, multiple Peptide 1 molecules associate with the enzyme, creating large aggregates. This inhibition only occurs when both the peptide and the enzyme are present. A similar effect was also observed for peptide 2 (Figure 41b), which aggregated with β-Gal and inhibited enzyme activity. For peptide 3, DLS showed that peptide 3 aggregated at inhibitory concentrations even in the absence of the enzyme (Figure 41c). Presumably inhibition in the case of peptide 3 occurs via an interaction between

the peptide aggregates and the enzyme, as has been observed in previous studies.[18, 24]

Dynamic light scattering gave clear evidence for aggregation and for two different modes of aggregation-associated inhibition (enzyme induced aggregation or interaction between the enzyme and pre-existing peptide aggregates). However, because of the concentration limitations of that technique, it was necessary to take data at rather high enzyme concentrations (> 50 nM). Fluorescence correlation spectroscopy (FCS) was used to study the aggregation of peptide with β-Gal at low nanomolar enzyme concentrations. Figure 42a shows the autocorrelation curves derived from monitoring the fluctuations in dye molecules moving in and out of a small volume of liquid defined by the confocal optical system used to take these measurements.[22] Small molecules like free Alexa 555 dyes move in and out of the volume rapidly and thus their autocorrelation decays completely within 2 ms (there resident time in the small volume was less than 2 ms). In contrast, the Alexa 555-labeled β-Gal (3 nM enzyme) gave rise to an autocorrelation trace that extended out to roughly 100 ms, which was consistent with its much larger size. When 20 μM peptide 1 was added to the enzyme solution, causing the strong inhibition of β-Gal, the correlation time increased dramatically (it is difficult to measure, but was on the order of 60 s), indicating the formation of very large particles with long resident times in the confocal volume. This is shown dramatically in Figure 42b, right panel, where one can see large, long-lived fluctuations in the fluorescence from the confocal volume as a function of time, representing the slow movement of large particles in and out of the volume. The dye molecules and labeled enzymes themselves do not show this behavior. Clearly, even at nanomolar

enzyme concentrations, the peptide is inducing aggregation under conditions that give rise to inhibition.

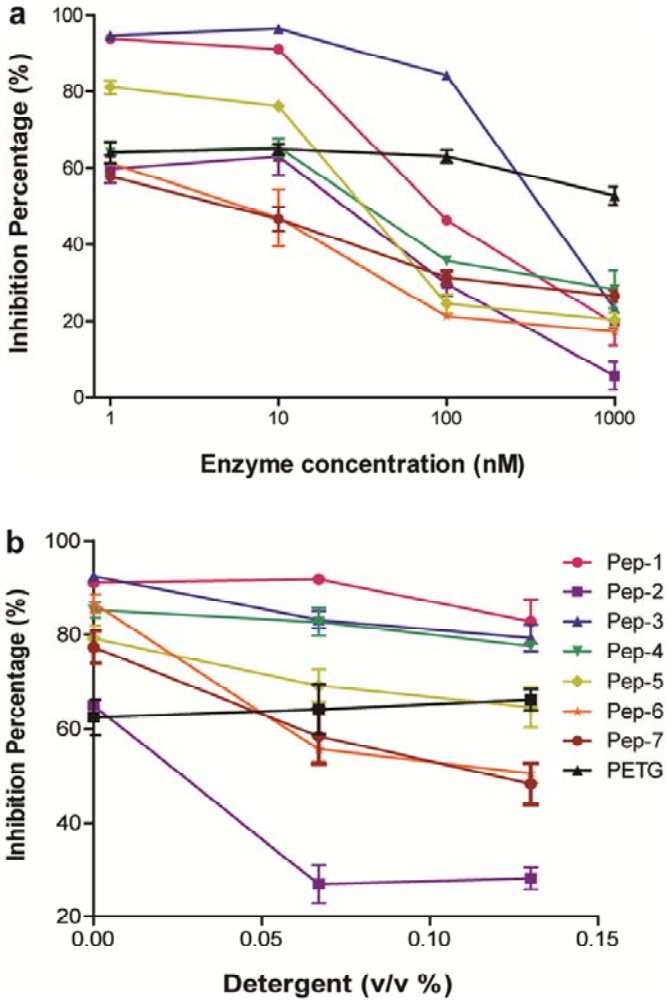


Figure 40. Titration of enzyme and detergent concentrations with respect to peptide inhibition. (a) Peptide inhibition as a function of β-gal concentration ranging from 1 to 1000 nM. The peptides are ~ 20 μM, and PETG is ~ 50 μM. Enzyme activity is assayed using 50 μM FDG. (b) Peptide inhibition as a function of detergent concentration (Tween 20). PETG is used as a control as it inhibits β-gal competitively. β-gal concentration: ~ 1 nM. *Inhibition Percentage* = *(Activity* $_{uninhibited}$ *– Activity* $_{inhibited}$*)/ Activity* $_{uninhibited}$ *× 100%.*

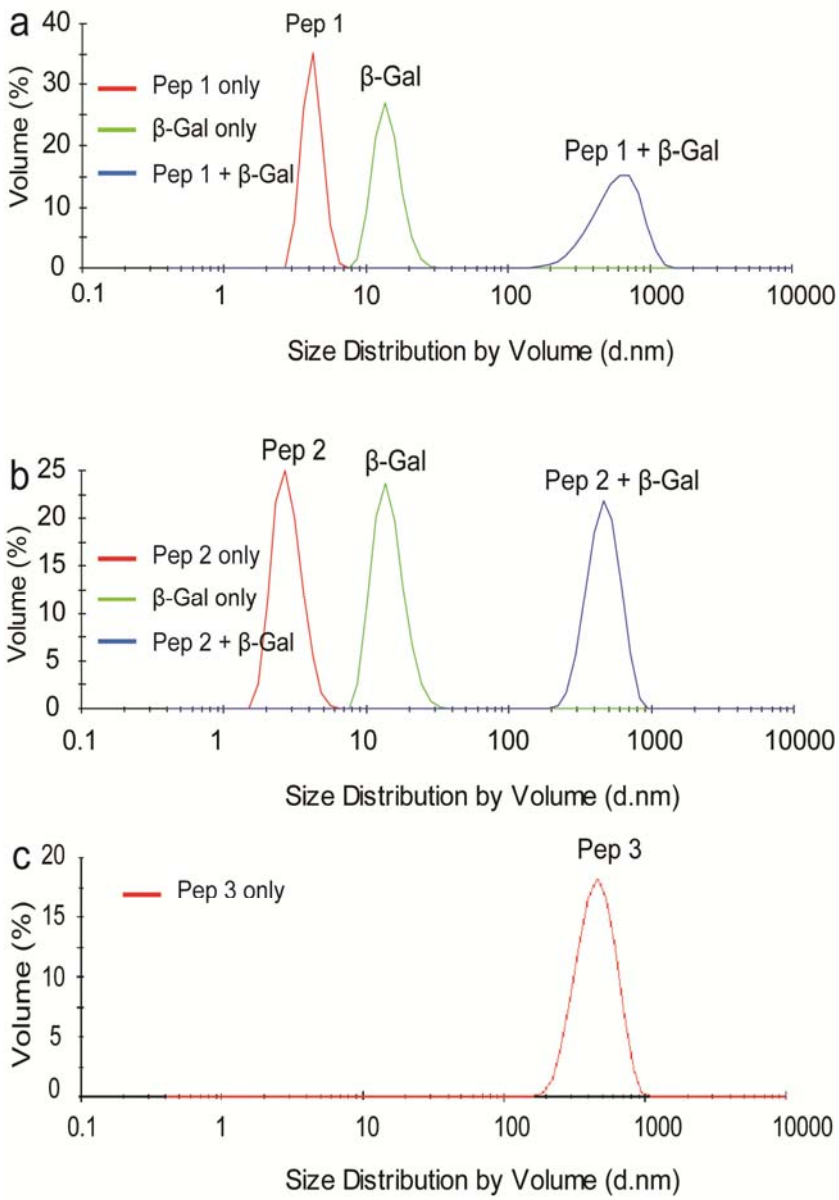


Figure 41. Dynamic light scattering studies of peptides inhibiting β-Gal. (a) The particle distributions of 30 μM peptide 1, 50 nM β-Gal and the mixture of peptide 1 with the enzyme were labeled with red, green and blue color, respectively. (b) The particle distributions of 150 μM peptide 2, 50 nM β-Gal and the mixture of peptide 2 with the enzyme were labeled with red, green and blue color, respectively. (c) The particle distributions of 100 μM peptide 3 solution. For all the mixtures of peptides with enzymes, β-Gal was inhibited by more than 90%.

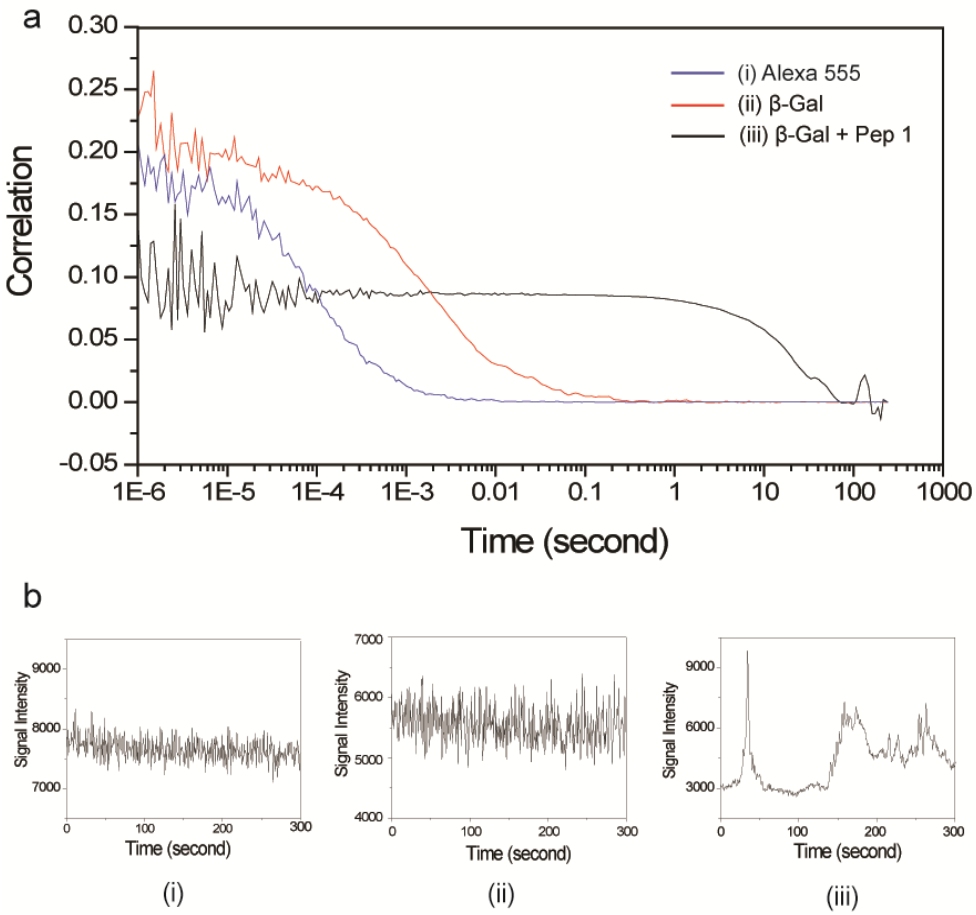


Figure 42. Fluorescence correlation study of β-Gal/peptide aggregation. (a) Fluorescence correlation decay of (i) Free Alexa 555 (Blue line), (ii) β-Gal (Red line) and (iii) β-Gal/peptide 1 complexes (Black line). (b) Time-dependent signals from monitoring fluorescence fluctuations in the confocal volume of a microscope set up for single molecule detection (i) Free Alexa 555, (ii) β-Gal and (iii) β-Gal/peptide 1 complexes. Alexa 555, 10 nM; β-gal, 3 nM; peptide 1, 20 μM. Inhibtion Percentage, > 90%.

Alanine scanning and truncations of peptides 1 and 2 were used to examine the dependence of peptide inhibition upon the specific peptide sequences in solution. For peptide 1, an alanine scan showed that the positively-charged residues at positions 4 (K), 5 (R), 8 (R) and 14 (R) played important roles in inhibiting β-Gal activity (Figure 43a(i)). Substitutions of those residues with alanine decreased the ability of the peptide to inhibit the enzyme by 5-10 fold. It had been also observed that some substitutions made the peptide inhibit the enzyme stronger, for example, alanine substitutions at positions of 2 (V), 9(W) and 12 (V). The truncation analysis of peptide 1 revealed that a 12-mer positively-charged peptide, RVFKRYKRWGSC (pI 11.4), was able to inhibit the enzyme with nearly the same IC_{50} as the 20-mer peptide 1, as shown in Figure 43b (i). This 12-mer peptide can be further reduced to a 10-mer peptide, FKRYKRWGSC (pI 10.2), with an IC_{50} of ~ 3 μM, which also maintains the ability to inhibit β-Gal. More comprehensive point-variant screening[25-26] was applied to the shorter peptide, FKRYKERWGSC, to study the specific residue contributions to inhibition of β-Gal. In Figure 44, 49 single-point variants, containing all substitutions of the amino acid set { S, Y, E, L, W, Q, and R } in each of the 7 randomized positions (FKRYKER – the GSC C-terminal linker was held constant for all peptides and the W residue was not altered), were synthesized, and tested for inhibition of β-Gal in solution. The results revealed that the FKRYKERWGSC sequence was nearly optimized; any amino acid substitution, except the replacement of R with K at position 5, resulted in decreasing the inhibition of β-Gal.

In Figure 43a (ii), similar effects were observed for peptide 2 in that positively-charged lysine residues at positions 10 (K) and 16 (K) were most

important for inhibiting β-Gal activity. The enhancement of peptide inhibition was also observed for the alanine substitution at the position of 5 (F) with ~ 2-fold stronger inhibition of the enzyme. For the peptide, a 12-mer positively-charged peptide, KKQGYYYKLGSC (pI 9.7), was the critical fragment for inhibiting β-Gal. Clearly positively-charged residues have a great deal to do with the ability to inhibit.

Stronger inhibition of β-Gal was observed by covalently crosslinking the inhibitory peptides with enzymes. As shown in Figure 45, 20 μM PEP 1 was incubated with 30 nM β-Gal for 20 minutes first, then 0.5% formaldehyde was added to the peptide/enzyme mixture and incubated for another 20 mins inorder to crosslink the peptide with enzyme. The whole mixture was diluted by 100-fold in buffer in such a way that the free peptide concentration will be as low as 20 nM. For the uncrosslinked peptide/enzyme mixture, the enzyme activity was recovered to ~ 70% activity of the uninhibited enzyme solution. However, for the crosslinked peptide/enzyme mixture, it was still strongly inhibited with the activity was as low as ~ 6% of the uninhibited enzyme solution even though the peptide concentration was much lower than its IC_{50} (50-fold lower). The effect of formaldehyde crosslinking on enzyme activity was also studied as a control showing that enzyme still maintained ~50% activity. It suggested β-Gal was almost irreversible inhibited by crosslinking the inhibitory peptides with enzyme.

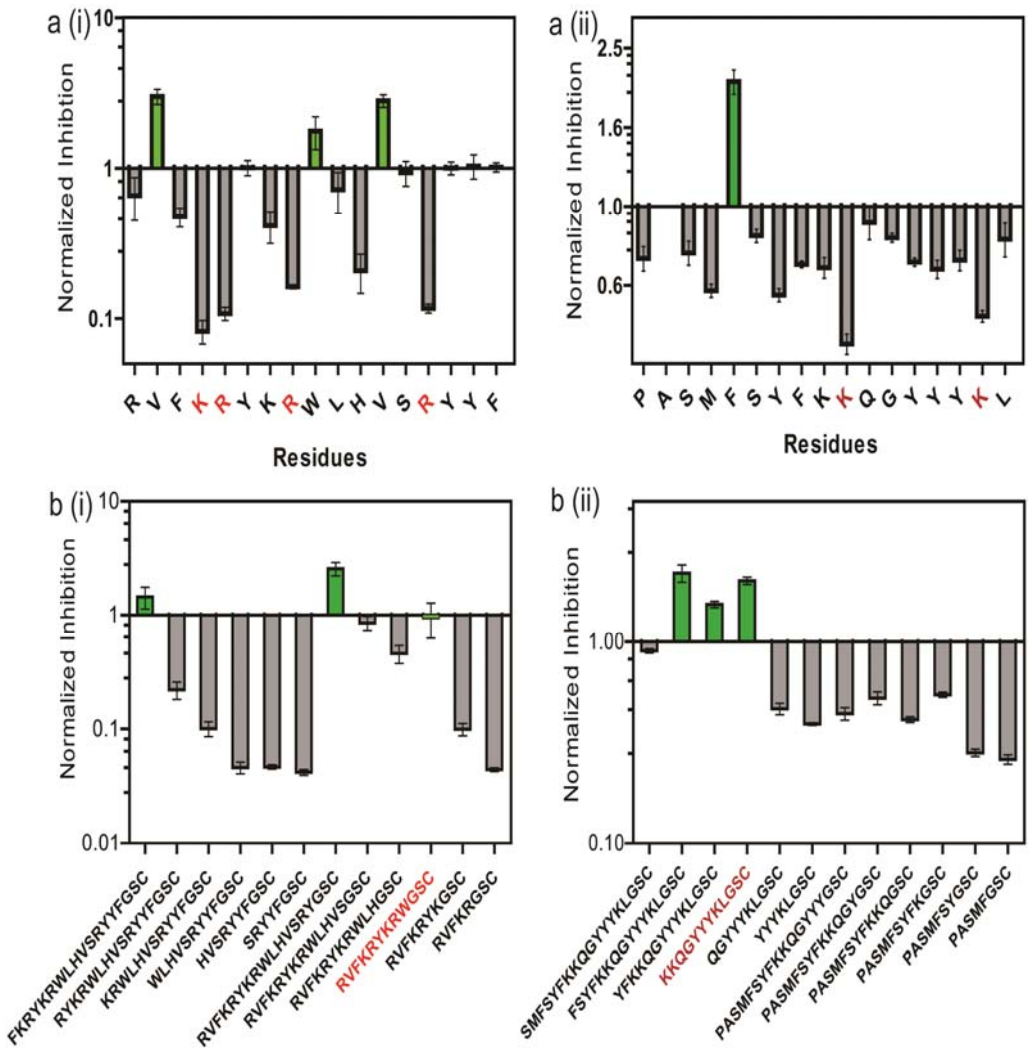


Figure 43. Alanine scans and sequential truncation measurements of peptide 1 and 2 inhibitory activity. (a) An alanine scan of peptide 1 (i) and peptide 2 (ii) with respect to inhibition of β-Gal. (b) A truncation scan of peptide 1 (i) and peptide 2 with respect to inhibition of β-Gal. All inhibitions of alanine-substituted peptides were normalized to that of peptide 1 and peptide 2, respectively.

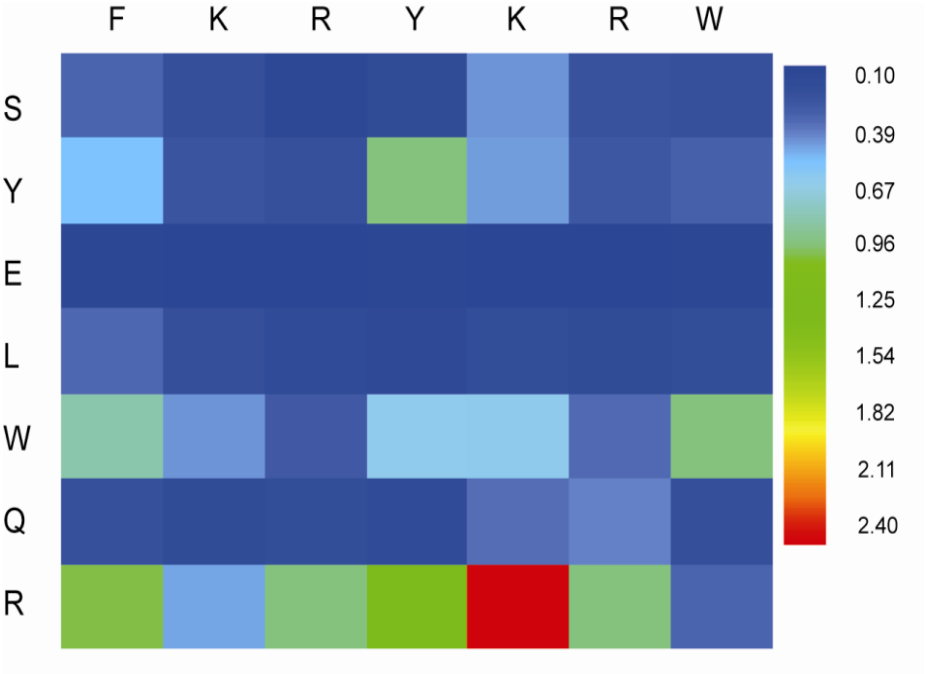


Figure 44. Point-variant screening of the peptide FKRYKRWGSC at each of the 7 N-terminal positions including substitutions of residues {S, Y, E, L, W, Q, and R}. The inhibition of β-Gal by variants was normalized to that of the lead peptide, FKRYKRWGSC.

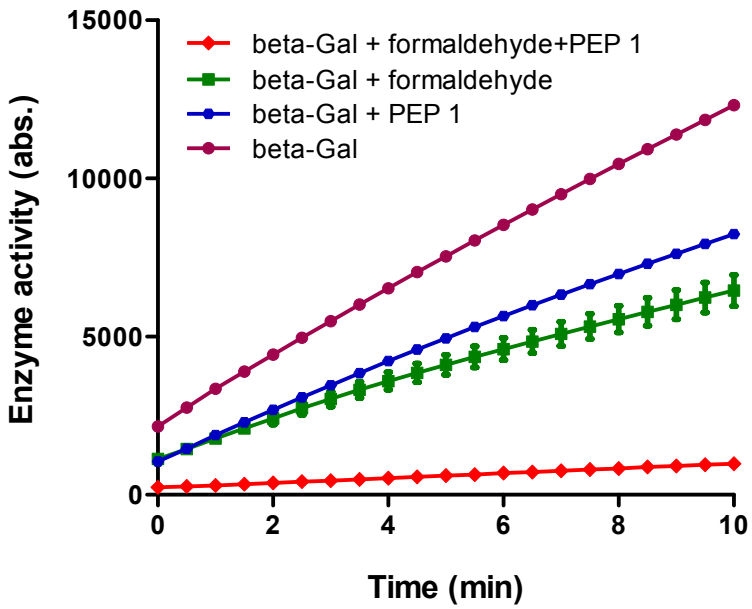


Figure 45. Enhancement of peptide-inhibiting β-Gal by corsslinking the inhibitory peptide with enzyme using 0.5 % formaldehyde. Final enzyme conc.: 0.3 nM; PEP 1 conc.: 20 nM. 50 μM RBG was used for assaying enzyme activity.

Multiple peptides were observed that bound to the aggregated and inhibited β-Gal complex. In Figure 46, PEP1/ β-Gal complex were applied to the peptide microarrays, several peptides showed higher signal intensity for binding to aggregated β-Gal complex than β-Gal free solution. Four peptides were selected that bound to the PEP1/ β-Gal complex and resulted in at least a 30-fold increase in enzyme inhibition relative to the PEP 1/enzyme complex alone (Table 5, NEW 1 - 4). Solution tests showed that two of the selected peptides, NEW 3 and NEW 4, enhanced the inhibition of PEP1/β-Gal complex by a factor of ~ 3 when added to the solution, as shown in Figure 47. The other two peptides also enhanced the inhibition, but not as strongly.

In contrast to inhibition reinforcement, two negatively-charged peptides, NEG 1 (EFSNPTAQVFPDFWMSDGSC, pI 3.4) and NEG 2

(ESVPTDLPMDTMEGKNWGSC, pI 3.9), were found to recover the activity of PEP1/ β-Gal complex. In Figure 48a, the activity of PEP1/β-Gal complex was measured as a function of NEG 1 concentration. The activity of inhibited enzyme was increased from less than 10% of the uninhibited enzyme to nearly 40 % by adding NEG 1, with a dynamic range of nearly 9-fold at most. A similar result was also observed for NEG 2-triggered recovery of inhibited β-Gal activity. Because the positive residues of PEP1 contributed greatly to the inhibition of β-Gal (Figure 43 and 44), it is likely that these negatively charged peptides bind to the aggregates and destabilize the PEP 1/β-Gal complexes. The recovery percentage of the inhibited enzyme was related to initial PEP 1 concentration; the higher the PEP1 concentration, the lower the recovery percentage. It may be that either PEP1 can partially induce the permanent inhibition of β-Gal, or there are some very stable aggregated peptide/enzyme complexes, or perhaps partly inhibited core complexes, that cannot be completely destabilize by the negatively-charged peptides.

Table 5 Four selected peptides that showed increased binding to the PEP 1/ β-Gal mixture, as screened using peptide microarrays.

Peptide	Sequence	β-Gal Binding [a]	β-Gal Binding [b]
NEW 1	GVSHLHWIKMLNETTVMGSC	480 ± 80	27400 ± 4900
NEW 2	HISPQHMMAYSPKAFDYGSC	300 ± 50	21400 ± 4000
NEW 3	YDTLHRNRQMMDWQFEPGSC	330 ± 40	25600 ± 2400
NEW 4	MHNHAFNDNHGRGPTAWGSC	1200 ± 180	30400 ± 4000

[a] The microarrays were incubated with 5 nM Alexa 647-labeled β-Gal.

[b] The peptide microarrays were incubated with a solution containing 5 nM Alexa 647-labeled β-Gal and 10 μM PEP 1.

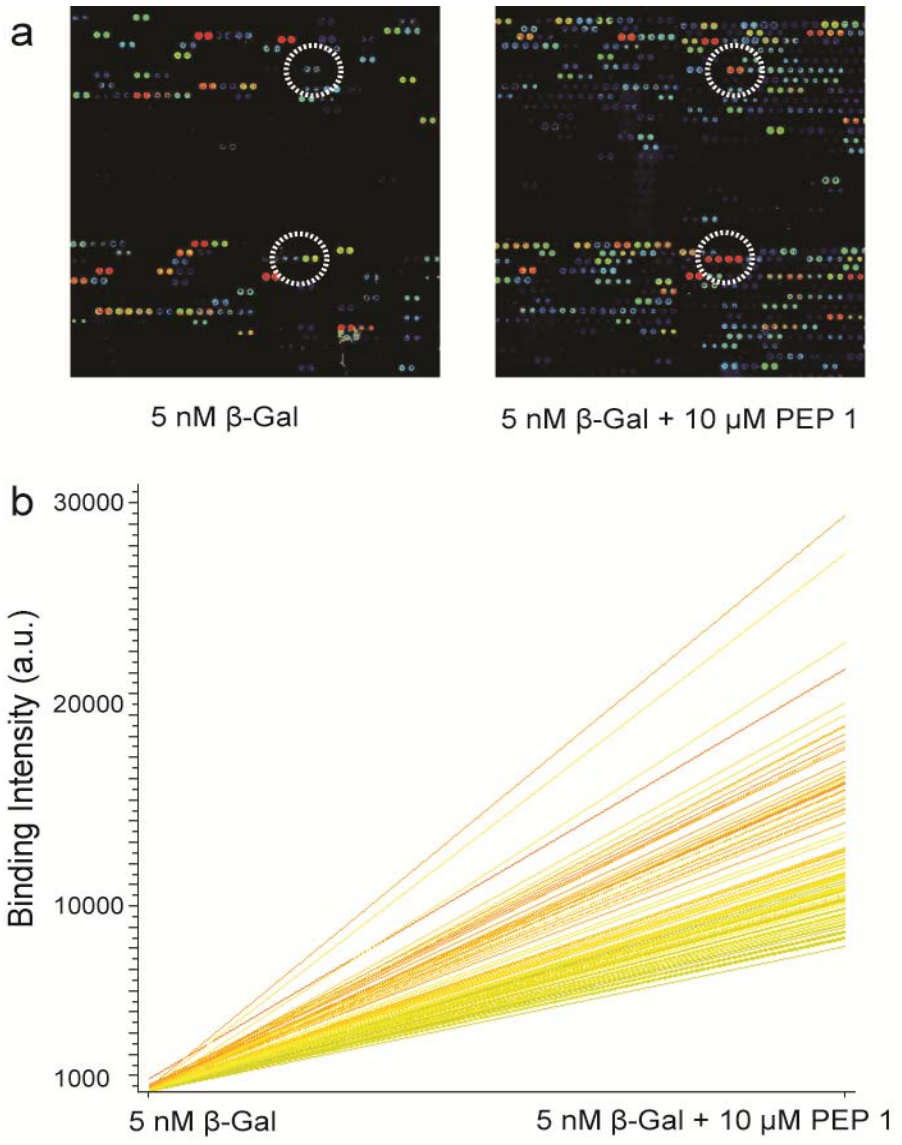


Figure 46. Peptides binding to the aggregated PEP1/β-Gal complexes found through screening on peptide microarrays. (a) Fluorescent scanning images (a representative region) of enzyme binding for 5 nM β-Gal (left) and the PEP1/β-Gal mixture (right), respectively. β-Gal was labeled with Alexa 647. Conditions: β-Gal, 5 nM; PEP1, 10 μM; incubation time, 2 h at room temperature. (b) The peptides that show increased binding to the enzyme when incubated with the β-Gal and PEP1 mixture.

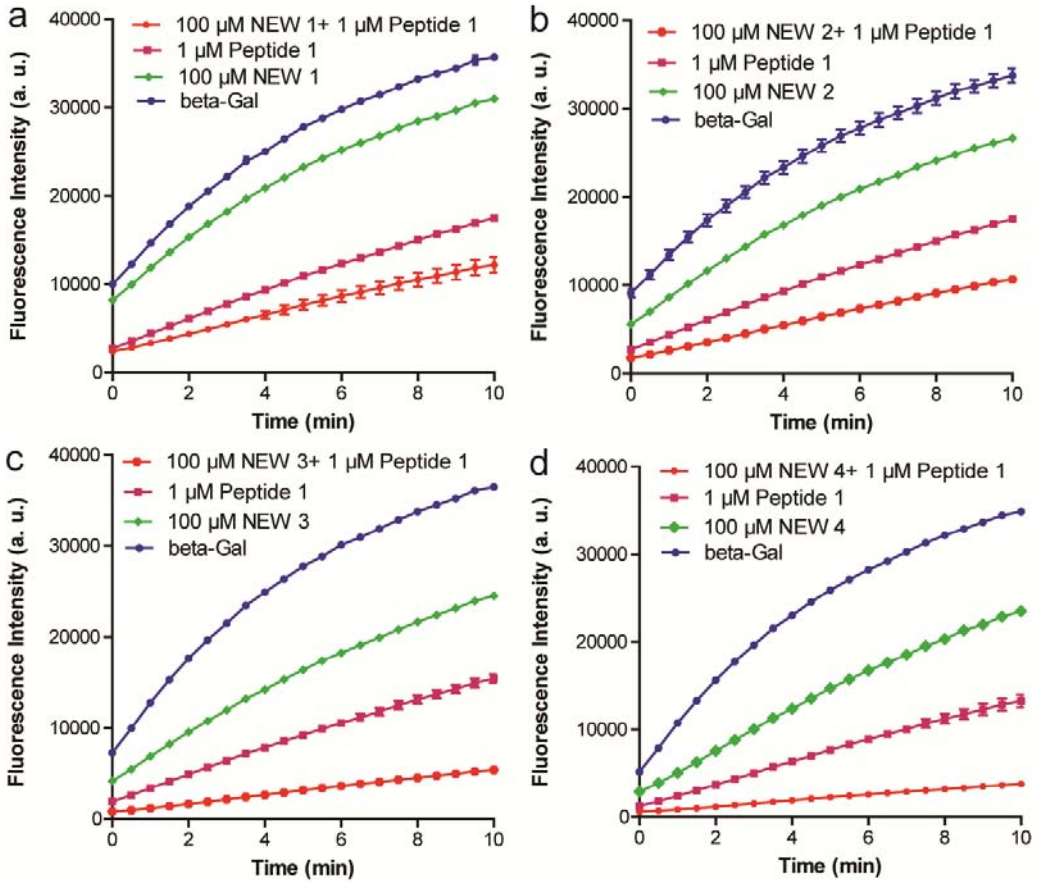


Figure 47. Peptides that inhibit β-Gal cooperatively with peptide 1. (a) NEW 1; (b) NEW 2; (c) NEW 3 and (d) NEW 4. β-Gal was first incubated with peptides for 20 mins and then activity was assayed using 100 μM RBG at 25 ºC.

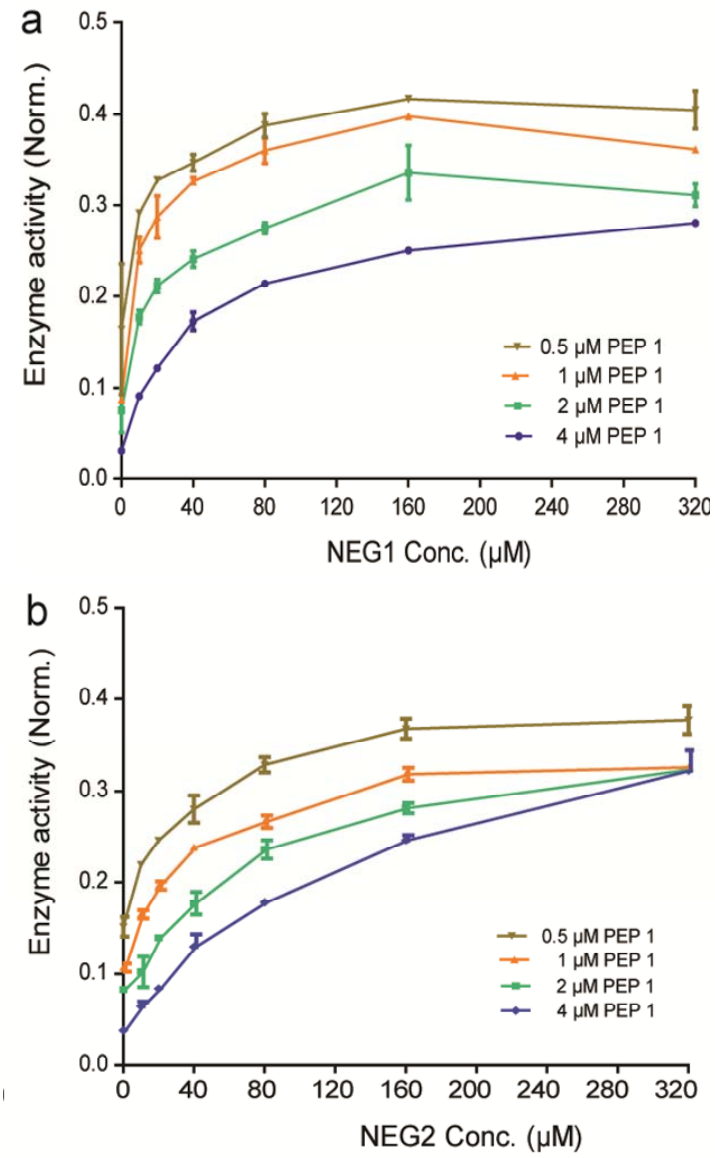


Figure 48. Activity recovery of inhibited PEP1/β-Gal complex using peptides (a) NEG 1 and (b) NEG 2. All enzyme activities are normalized to that of uninhibited enzyme. For the recovery tests, β-Gal was first incubated with peptide 1 for 20 minutes, then the negatively-charged peptides (NEG 1 and NEG 2) were added into the mixture and incubated for another 20 minutes before enzyme activity was tested. Enzyme activity was assayed using 100 μM RBG.

There are some further considerations about the mechanism of peptide inhibiting enzyme. First, what is the possible kinetics process for peptide-inducing β-Gal aggregation and inhibition? As we know, enzymes are quickly switching between different conformations in solution. In Figure 49, enzymes prefer more to the active conformation with less aggregation in physiological conditions. But the inhibitory peptides may bind more tightly to the inactive conformation of the enzyme, and switch the balance more to the right. As a result, more and more enzymes bind to peptides with their inactive and sticky conformation, and finally most of the enzymes are inhibited and aggregated.

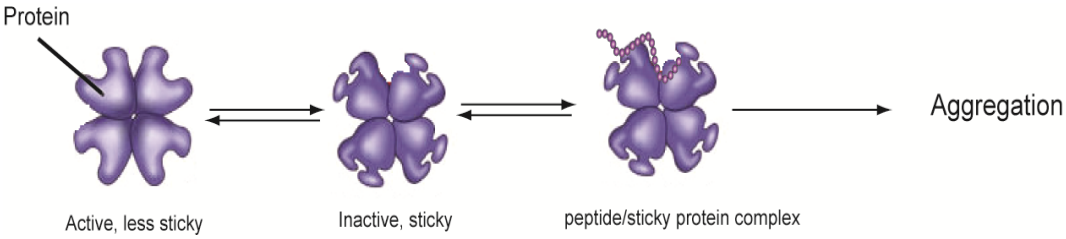


Figure 49. Kinetics model of peptide-inducing β-Gal aggregation and inhibition.

Another question is why we have not seen the classically competitive inhibitors of β-Gal for the peptides selected from microarray? As we discussed in Chapter 3, β-Gal is a tetrameric enzyme. If there was a peptide binding to the active site of β-Gal, it would only inhibit one subunit, and the enzyme can still maintain ¾ of the uninhibited activity. The inhibition is not significant on the microarray and difficult to be selected. As we predicted in Chapter 3, the strong inhibition of β-Gal observed on microarray cannot be caused by the active-site inhibition, it should be resulted from some nonclassical inhibitions, like noncompeptive inhibition, in such a way that the entire enzyme inhibition can be induced by peptide binding to one or two subunits of the tetramer.

How can we improve the assay methods to select classically competitive inhibitors? One method is to utilize the known competitive inhibitors of target enzymes to block the binding between peptides with enzymes on the microarray. As shown in Figure 50, peptides that bind to the target enzyme are identified by incubating the enzyme solution with peptide arrays. Then the similar assay is run again with the competitive inhibitor-bound enzyme solution. The peptides that specially bind to the active site of the enzyme may be blocked by the competitive inhibitor and thereby show the decrease in binding to the enzyme.

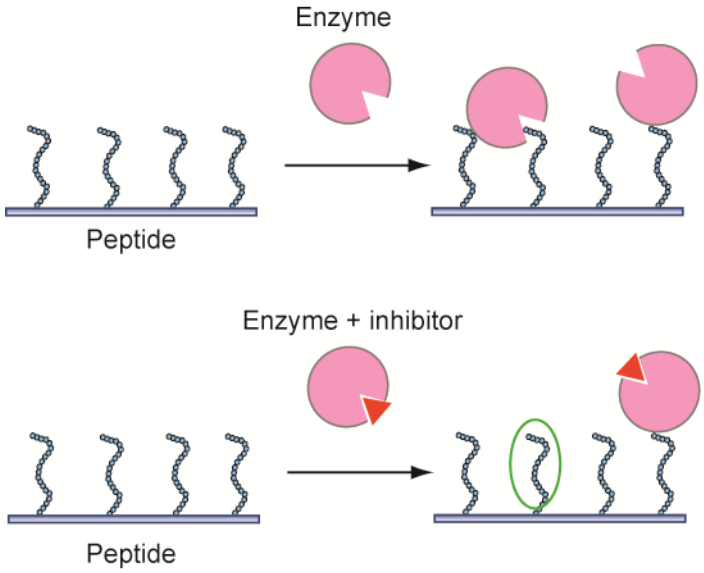
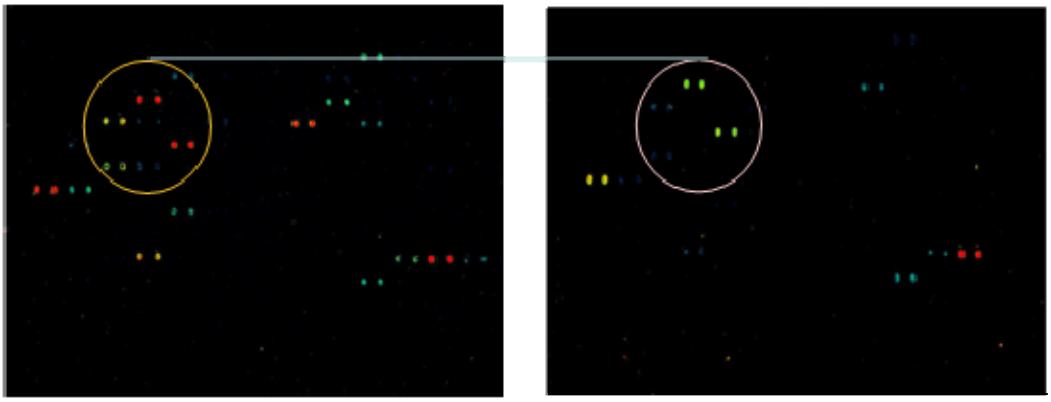


Figure 50. Identification of peptides that bind to the active site of the target enzyme by blocking the peptide-enzyme interactions with known active-site binding inhibitor.

In Figure 51, some peptides binding with dehydrofolate reductase (DHFR) were blocked by methotrexate (MTX) which is a competitive inhibitor of DHFR. This initial test shown that some peptides did bind to the region close to the active site and thereby the bindings were affected by the active-site binding

competitive inhibitors. The selection will be more efficient if the ligand blocking experiment is combined with the enzyme activity screening together.



Peptide binding with 100 nM Alexa555-labeled DHFR

Peptide binding with 100 nM DHFR + 1 μM MTX

Figure 51. Methotrexate (MTX) blocking the binding of peptides with DHFR. Peptide binding with DHFR is shown in the left figure, and the blocking test with MTX is shown in the right figure.

CONCLUSION

In summary, a unique aggregation-mediated mechanism for inhibiting β-Gal using peptides selected from microarrays has been demonstrated. Aggregated complexes were formed even at low nanomolar enzyme concentrations. Further study revealed that positively-charged peptide residues played important roles in inhibiting enzyme activity. Stronger and irreversible inhibition of enzyme activity was observed by crosslinking the peptides with β-Gal. Moreover, multiple peptides were found to further regulate the activity of inhibited peptide/ β-Gal complex, either stabilizing the aggregated complex and resulting in stronger inhibition or destabilizing the aggregated complex and thereby

restoring the enzyme activity. It should be possible to design switchable enzyme systems in this way, using pairs of selected peptides.

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CHAPTER 5: PEPTIDE-MODIFIED SURFACES FOR ENZYME IMMOBILIZATION

ABSTRACT

A method is presented for utilizing peptide ligands to immobilize enzymes on surfaces with improved enzyme activity and stability. Ligands, selected from peptide microarrays and optimized through point-variant screening, were covalently attached to surfaces for the purpose of capturing target enzymes. Compared to conventional methods, enzymes immobilized on peptide-modified surfaces exhibited higher specific activity and stability, which might be generally applicable to immobilizing enzymes with optimized orientation, location and performance.

INTRODUCTION

Surface-immobilized enzymes play an important role in many biocatalytic processes and industrial applications.[1-2] The activity, stability and selectivity of enzymes can be improved if they are immobilized properly on surfaces.[1,3] Many conventional protein immobilization methods[1], which rely on nonspecific absorption of proteins to solid supports or chemical coupling of reactive groups within proteins, have inherent difficulties, such as protein denaturation, poor stability due to nonspecific absorption[4-5] and the inability to control protein orientation[1,5]. New strategies for enzyme immobilization are needed which allow precise control over orientation and position and thereby provide optimized activity. Peptides represent a promising class of potential protein-anchoring/modulating molecules due to their large chemical diversity[6] and the existence of well-established methods for library synthesis[7]. Peptide or small molecule ligands that bind to a unique region of a protein can be used for

orienting the protein and modulating its activity through specific ligand-protein interactions on a solid support.[8-10] In this chapter, I will present a method for creating peptide-modified surfaces that immobilize a target enzyme with optimized orientation and activity.

EXPERIMENTAL SECTION

Chemicals Resorufin β-D-galactopyranoside (RBG) and Alexa Fluor 647 were purchased from Invitrogen (Eugene, OR). β-galactosidase (β-Gal, E.coli), polyvinyl alcohol (PVA, M.W.: 124,000~186,000), 4-nitrophenyl phosphate (PNPP), Phosphate Buffered Saline (PBS) and Tris Buffered Saline (TBS) were obtained from Sigma (St. Louis, MO). BS^3 (Bis[sulfosuccinimidyl] suberate) , alkaline phosphatase-conjugated strepavidin and iodoacetyl resin were purchased from Pierce (Rockford, IL). Sulfo succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC) was purchased from bioWORLD (Dublin, OH). Aminated microwell plates were ordered from Corning. A 4 mg/mL stock solution of β-Gal was prepared in 10 mM potassium phosphate buffer with 0.1 mM $MgCl_2$ at pH 7.4.

Enzyme immobilization on modified microwells Peptides were conjugated to aminated microwell surfaces through the specific reaction between C-terminal cysteines and the maleimide-activated surfaces, as shown in Figure 52. 10 mM SMCC was prepared in 1X PBS buffer, pH 7.4. Next, 30 µL of SMCC was added into each aminated microwell and incubated for one hour at room temperature. The microwell plate was then briefly washed with pure water three times. Then, 30 µL of a 300 µM peptide solution, prepared in 1X PBS pH 7.4 plus 1 mM TCEP, was thn added to the appropriate SMCC-activated microwells. The reaction was incubated for 4 hours at room temperature, in the dark. After the

conjugation reaction was complete, the microwells were washed for 5 minutes in 1X TBST, three times, followed by three washes in water. To immobilize the enzyme on peptide-modified surfaces, 30 µL of 25 nM biotin-labeled β-Gal was incubated in the peptide-modified microwells for two hours in 10 mM phosphate buffer, pH 7.3 with 100 µM $MgCl_2$ and 0.05% Tween 20 (v/v%), at room temperature. The microwells were washed for 5 minutes in 1X TBST, three times, followed by three washes in phosphate buffer. At this point, the β-Gal-bound microwells were ready for testing. β-Gal was labeled with biotin using EZ-Link Sulfo-NHS-Biotinylation Kit purchased from Pierce (labeling ratio: ~ two biotin per enzyme molecule). Figures 53-55 show the detailed optimization procedures for peptide-modified surfaces.

Covalent attachment of β-Gal to NHS (*N*-Hydroxysuccinimide)-activated surfaces was performed using BS^3 homogeneous amine-reactive cross-linker, as recommended by the manufacturer. First, 30 µL of 2 mg/mL BS^3 prepared in 1X PBS, pH 7.4 was incubated with the aminated microwells for half an hour. Then, the microwells were briefly washed with nanopure water, three times, to remove unreacted BS^3 molecules. Finally, 30 µL of biotin-labeled β-Gal was incubated with the microwells for one hour, which were then washed three times in 1 X TBST, followed by three washes in phosphate buffer.

The activity assay of surface-bound β-Gal was performed on a SpectraMax M5 96-well plate reader (Molecular Device, Sunnyvale, CA) by adding 100 µL of 100 µM RBG into the wells. The relative amount of surface-bound β-Gal was measured using an enzyme linked immunosorbant assay (ELISA). β-Gal was first labeled with biotin. Alkaline phosphatase-conjugated strepavidin (0.4 mg/ml) was diluted at 1:1000 in 1X PBS, 0.05% (v/v) Tween 20.

Next, 30 µL of streptavidin solution was added to the β-Gal-bound wells and incubated for one hour at room temperature. The streptavidin solution was then removed and the plate was washed three times with TBST buffer and three times with TBS buffer. Then, 200 µL of 1 mM PNPP was added to each well. The alkaline phosphatase activity was subsequently measured by reading the absorbance increase at 405 nm on the M5 plate reader. The β-Gal binding level was determined from the activity of alkaline phosphatase-conjugated strepavidin bound to the wells.

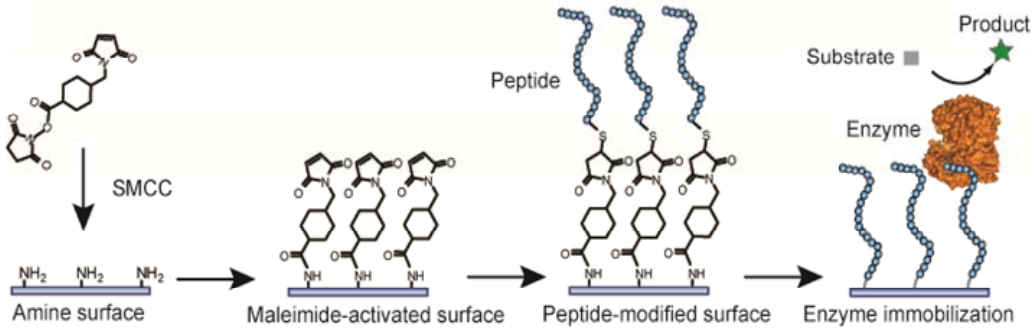
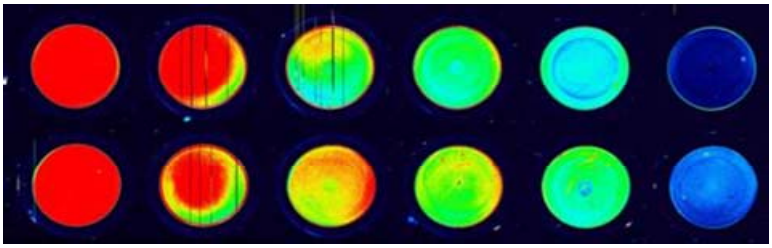


Figure 52. The overall process for conjugating peptides to aminated microwells through specific reactions between C-terminal cysteines and maleimide-activated surfaces.

a



b

Sample	Raw intensity (Alexa647)	Conjugation Yield
Amine surface	30757	0.00
0.675 mM SMCC	19035	0.38
1.25 mM SMCC	16116	0.48
2.5 mM SMCC	12008	0.61
5 mM SMCC	7813	0.75
10 mM SMCC	3025	0.90
20 mM SMCC	1356	0.96

c

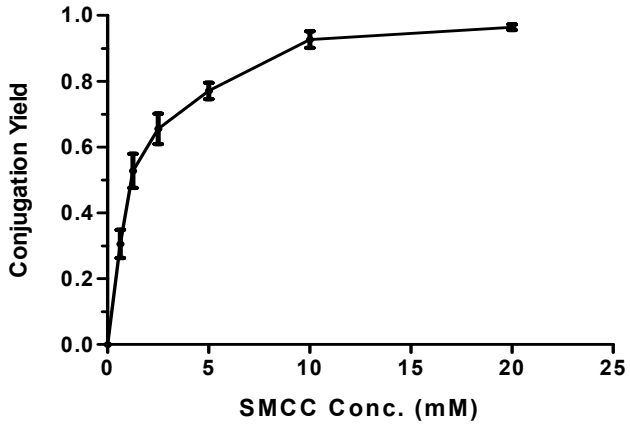


Figure 53. Optimization of SMCC conjugation: (a) raw fluorescence images of Alexa-647-labeled aminated microwells with SMCC capping at different concentrations. SMCC will conjugate to the amine groups on the microwells and prevent the labeling of Alexa-647 dye molecules. The higher the conjugation yield, the less the fluorescence from the labeled fluorophores; (b) Fluorescence intensities of Alexa-647-labeled microwells with different concentrations of SMCC capping; (c) surface conjugation yields of SMCC at different concentrations. The result shows that a 10 mM SMCC solution can be used to achieve a surface conjugation yield of more than 90%.

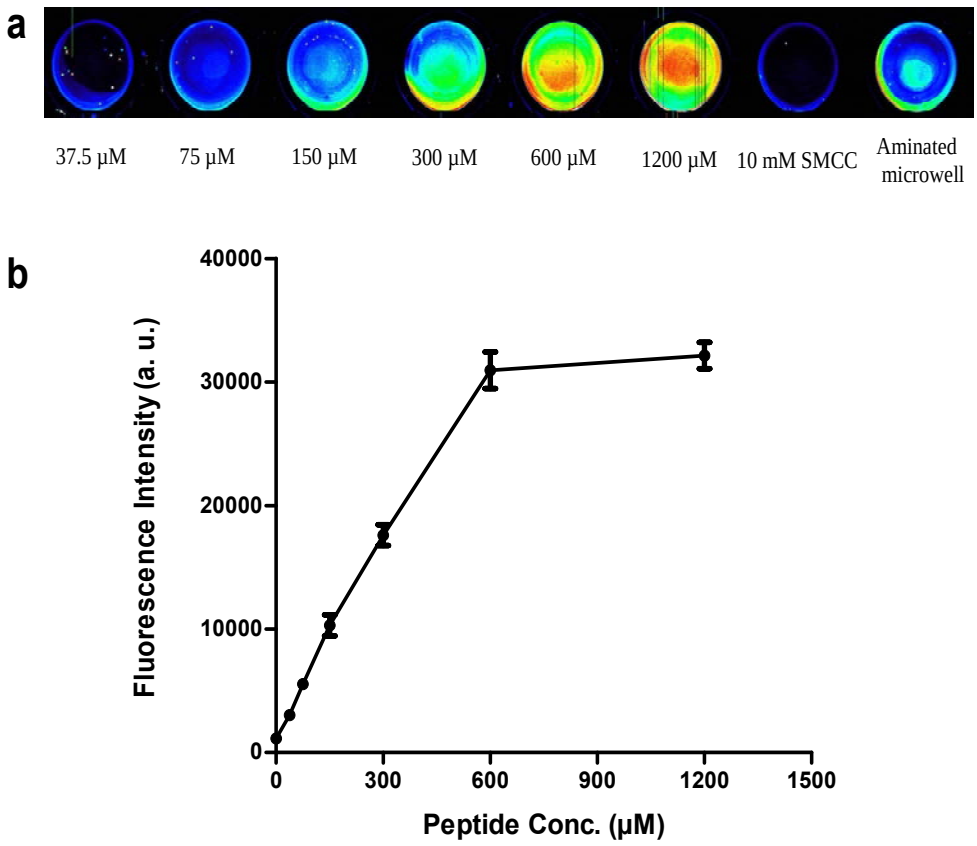


Figure 54. Optimization of peptide concentration for surface conjugation: (a) raw fluorescence images of Alexa-647-labeled microwells conjugated with different concentrations of peptide solution. 10 mM SMCC was first used to activate the aminated microwells, and then peptides were conjugated to SMCC-activated surfaces through specific reactions between the C-terminal cysteine and maleimide. Surface peptide densities were measured by labeling the microwells with amine-reactive Alexa 647. The more peptide on surface, the stronger the labeled fluorescence intensity due to the reaction between the dye and the peptide amine groups. (b) Surface fluorescence intensity as a function of peptide concentration used for conjugation. The peptide used is YHNN with a sequence of “YHNNPGFRVMQQNKLHHGSC”.

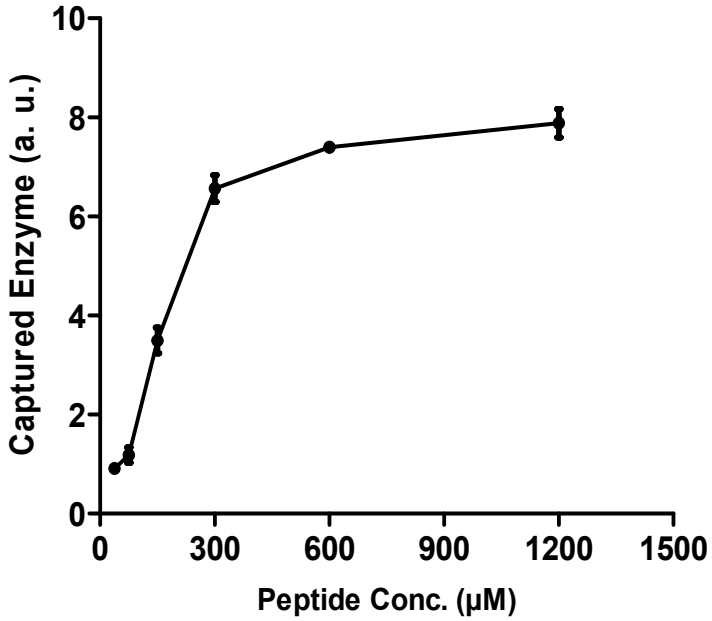


Figure 55. Optimization of peptide density for capturing β-Gal on peptide-modified microwells. Different densities of surface-immobilized peptide were created by varying the concentrations of peptide solution used for immobilization. The amount of immobilized β-Gal started to get saturation when surface was modified with 300 μM or higher concentrations of peptide solution. The peptide used for optimization was YHNN.

Determining Michaelis constants of immobilized β-Gal The determination of the enzyme kinetic constants (K_M and k_{cat}) of immobilized β-Gal was performed on peptide-modified iodoacetyl polyacrylamide resin (UltraLink, Pierce, 50-80 μm diameter). To modify the bead surface with peptide, peptide solutions were incubated with iodoacetyl resin for one hour in 50 mM Tris buffer, 5 mM EDTA, pH 8.5. The unreacted iodacetyl groups were then capped with 50 mM L-cysteine. The amount of peptide immobilized on a bead surface was determined by comparing the peptide concentration of the unbound fraction (the remaining free peptide concentration after binding to the surface) to the starting

concentration through absorbance changes at 280 nm. β-Gal was captured on the peptide-modified beads using the same protocol which immobilized the enzyme in the microwells, above. The amount of bead-immobilized β-Gal was measured by comparing the protein concentration of the unbound fraction to the starting protein concentration, determined at 280 nm. K_M and V_{max} (and thus k_{cat}, using the total enzyme concentration) of β-Gal immobilized on peptide-modified beads were determined by fitting the activity vs. substrate concentration curves in the GraphPad program using the fitting equation of "$Y=V_{max}*X/(K_m+X)$".

Peptide mapping to β-Gal The specific regions at which the peptides YHNN and QYHH bind to β-gal were determined by reversible formaldehyde cross-linking, as described previously.[8,12] 200 µL of a 150 µM peptide solution was first conjugated to 100 µL of UltraLink iodoacetyl resin using the method described above. To promote cross-linking, the peptide-modified resin was incubated with 200 µL of 500 nM β-Gal for two hours. 200 µL of 1% formaldehyde (v/v), prepared in 1X PBS, was added to the enzyme-bound resin for 10 mins. Then, the formaldehyde solution was removed quickly by centrifugation. The resin was washed three times with 1 mM Glycine, pH 2.5 to remove enzyme that did not undergo cross-linking. Proteolytic digestion was performed by incubating the enzyme-bound resin with 34 nM Glu-c in ammonium bicarbonate buffer, pH 8.5, overnight at 37 °C. Then, the resin was washed again with Glycine, pH 2.5 to remove Glu-c and any fragments that did not undergo cross-linking. The formaldehyde cross-linking was reversed by incubating the resin with 20 µL nanopure water at 70°C overnight. Following cross-link reversal, 100 µL of nanopure water was added to the resin to dissolve the free Glu-c-digested peptide fragments. The solution was spun to the bottom of the spin-

column and then dried, by evaporation, in a vacuum centrifuge. The dried sample was re-dissolved with 10 μL of 1:1 acetonitrile:H_2O containing 0.1% trifluoroacetic acid and saturated R-cyano-4-hydroxycinammic acid matrix. The sample was spotted on a standard MALDI-MS (Matrix-assisted laser desorption/ionization mass spectrometry) target plate, and analyzed using a Bruker Microflex MALDI-MS.

RESULTS AND DISCUSSION

In previous chapters, an approach for screening high-density peptide arrays was described to identify specific peptide sequences that anchor enzymes to surfaces and modulate their activity.[10] To demonstrate the utility of this approach more generally for optimized enzyme immobilization, two 20-mer peptides, YHNNPGFRVMQQNKLHHGSC (referred to as YHNN) and QYHHFMNLKRQGRAQAYGSC (referred to as QYHH) were selected from a microarray of 10,000 peptides based on their ability to bind β-Gal and optimize its surface-immobilized activity (Table 6). These peptides were then synthesized and covalently conjugated to aminated microwells, modifying the surface and mediating the binding of β-Gal through specific peptide-enzyme interactions (Figure 52). As controls, two inhibitory peptides, RVFKRYKRWLHVSRYYFGSC (RVFK) and PASMFSYFKKQGYYYKLGSC (PASM), and one weak-binding peptide, EFSNPTAQVFPDFWMSDGSC (EFSN), were also used to modify aminated microwells (Table 7).

Table 6 Microarray data corresponding to selected peptides used for surface conjugation [a].

Peptide	Sequence	pI	Enzyme activity (Norm.)	Enzyme binding (Norm.)	Surface specific activity (Norm.)
1	YHNNPGFRVMQQNKLHHGSC	9.3	92.3	37.8	10.9
2	QYHHFMNLKRQGRAQAYGSC	9.8	90.4	42.4	9.5
3	RVFKRYKRWLHVSRYYFGSC	10.3	0.9	50.3	0.08
4	PASMFSYFKKQGYYYKLGSC	9.4	2.3	63.9	0.16
5	EFSNPTAQVFPDFWMSDGSC	3.5	0.7	0.4	-

[a] Peptides 1 and 2 were selected to promote strong activity when bound to β-Gal. Peptides 3 and 4 bind to β-Gal but result in very low enzyme activity. Peptide 5 does not bind to β-Gal.

Table 7 Normalized activity and affinity of β-Gal immobilized on modified surfaces [a].

	Surface for protein immobilization	Binding affinity (Norm.)	Activity (Norm.)	Specific activity (Norm.)
1	YHNNPGFRVMQQNKLHHGSC	0.9 ± 0.03	2.1 ± 0.1	2.2 ± 0.1
2	QYHHFMNLKRQGRAQAYGSC	0.9 ± 0.02	2.3 ± 0.1	2.4 ± 0.2
3	RVFKRYKRWLHVSRYYFGSC	0.9 ± 0.05	0.1 ± 0.01	0.1 ± 0.01
4	PASMFSYFKKQGYYYKLGSC	0.5 ± 0.1	0.3 ± 0.06	0.5 ± 0.1
5	EFSNPTAQVFPDFWMSDGSC	0.1 ± 0.01	0.05 ± 0.01	0.4 ± 0.1
6	SMCC	0.2 ± 0.02	0.03 ± 0.01	0.1 ± 0.1
7	NHS	0.8 ± 0.1	0.6 ± 0.1	0.8 ± 0.1
8	Amine	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1

[a] Types of surfaces: 1 and 2 are selected peptide-modified surfaces; 3 and 4 are control surfaces modified by inhibitory peptides; 5 is a control surface conjugated with a weak-binding peptide; 6-8 are the conventional surfaces used for covalent or noncovalent enzyme immobilization, defined as in Figure 52, legend. All of the data is normalized to that of the amine surface, 8.

β-Gal immobilized on YHNN- and QYHH-surfaces exhibited much higher activity than β-Gal immobilized on control peptide-modified surfaces (Figure 56).

The relative specific activity of immobilized β-Gal was calculated for each surface by dividing the total bound enzyme activity by the total binding intensity. Conventional surface immobilization approaches were also tested including SMCC-activated (SMCC 6) and NHS-activated (NHS 7) covalent attachment, as well as noncovalent amine-surface attachment (Amine 8). YHNN- and QYHH-modified surfaces resulted in a specific activity of bound enzyme that was ~ 2-fold greater than amine noncovalent binding and nearly 3-fold greater than NHS attachment. In addition, the YHNN- and QYHH-modified surfaces have the advantage of specifically associating with β-Gal in a protein mixture. This was shown by binding β-Gal in a solution containing 3% Bovine serum albumin (BSA). YHNN- and QYHH- modified surfaces showed 15-fold more bound enzyme activity than the amine surface and 20-fold more than the NHS surface (Figure 57).

In addition, YHNN- and QYHH-modified surfaces were also found to improve the thermal and pH stability of immobilized β-Gal. The thermal stability of bound β-Gal was ~ 16-fold greater on the peptide-modified surfaces than free enzyme in solution after incubating at 55 °C for one hour (Figure 58) and more than 2-fold better than enzyme immobilize to either the NHS or amine surfaces. Immobilization of β-Gal on YHNN- and QYHH-modified surfaces also shifted the pH optimum from pH 8 in free solution to 7 on the surface. Long-term enzyme stability to storage on surfaces was greatly improved on peptide-modified surfaces, particularly when peptide modification was combined with the use of a hydrogel (5% polyvinyl alcohol, PVA) coating. β-Gal immobilized in this way and stored dry for one week at room temperature retained ~35% of its original activity . In contrast, enzyme similarly immobilized and stored on amine surfaces

retained less than 5% activity and NHS surfaces retained ~14% (Figure 59). If one considers both the increased binding capacity of the peptide-modified surfaces and their increased stability to storage, there was 20-fold more enzyme activity per surface area after storage on the peptide-modified surfaces than either the amine surfaces or the NHS surfaces, a significant factor in the commercial immobilization and storage of enzymes.

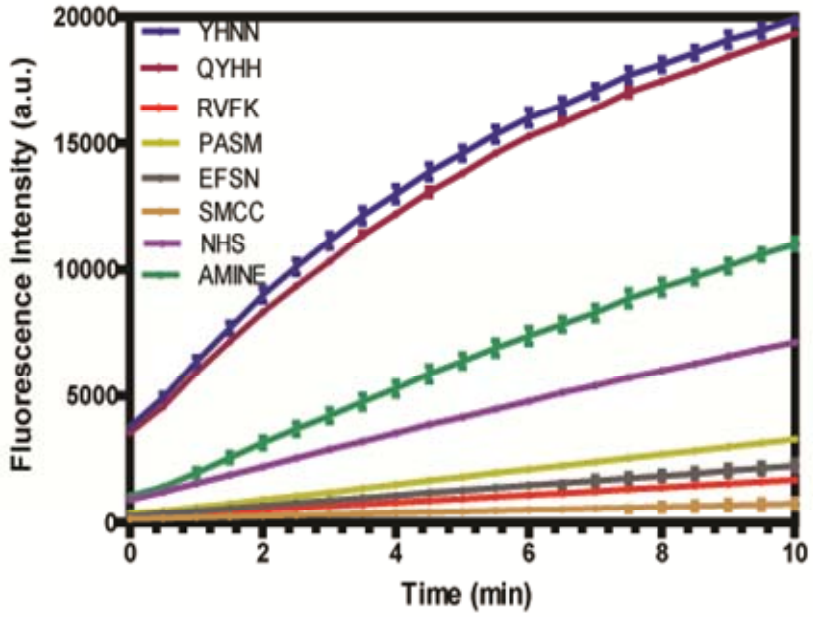


Figure 56. Activity of β-Gal immobilized on different surfaces. 25 nM β-Gal is first incubated with modified microwells for one hour and then enzyme activity is measured at 25 °C as a function of time using 100 μM Resorufin β-D-galactopyranoside as the substrate. YHNN, QYHH, RVFK, PASM and EFSN represent β-Gal bound to various peptide-modified surfaces (see text). SMCC and NHS represent enzyme covalently bound via thiol and amine conjugation, respectively. AMINE represents enzyme bound noncovalently to an aminated surface.

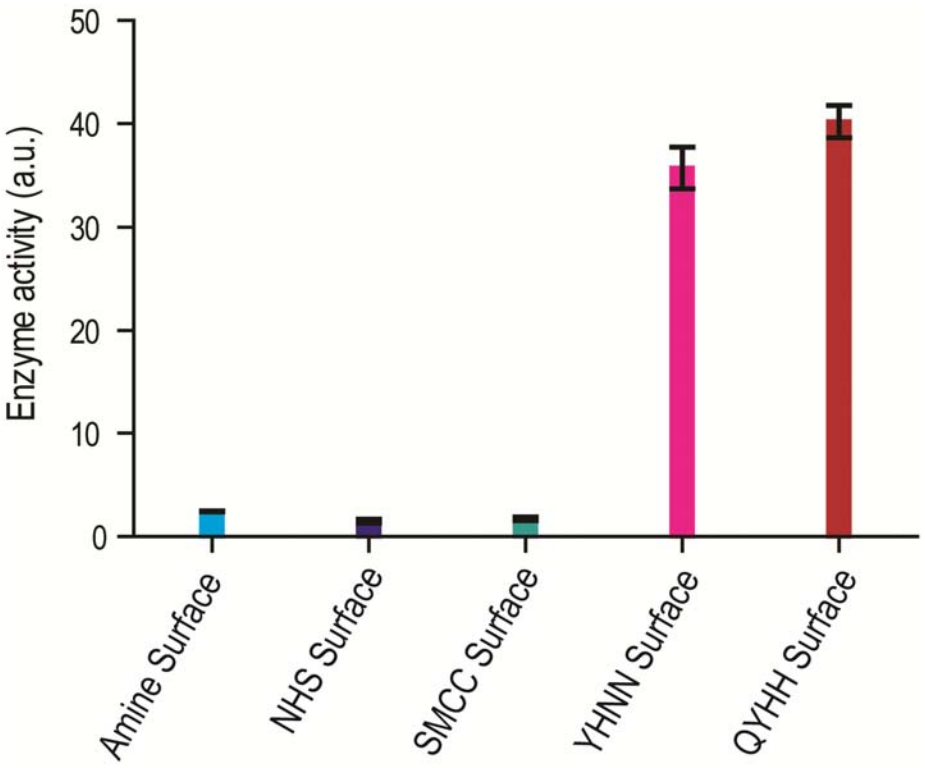


Figure 57. Specificity of different types of surfaces for capturing β-Gal in a protein mixture. 25 nM β-Gal was mixed with 3 % BSA and then applied to these surfaces. The activity of each enzyme-bound surface was measured by adding 100 μL of 100 μM RBG into each microwell.

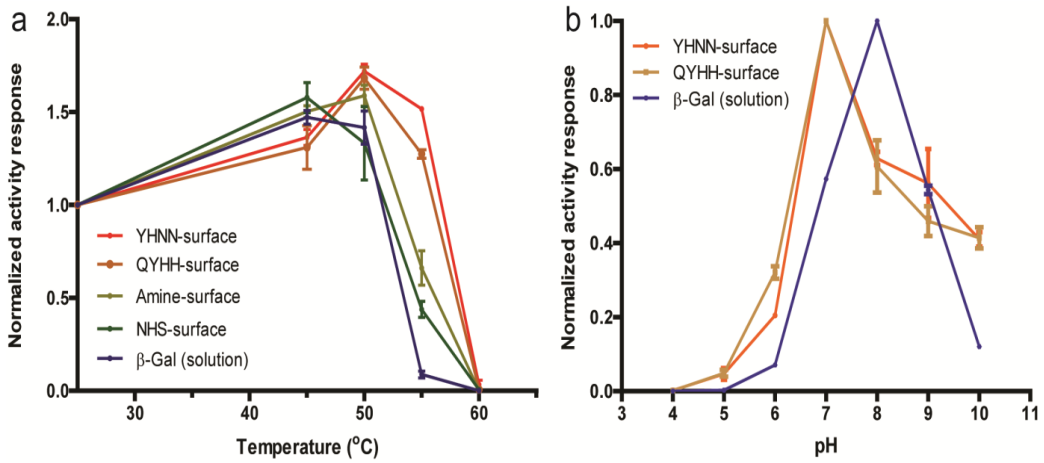


Figure 58. (a) Normalized thermal stability of immobilized β-Gal on different surfaces. The thermal stability of immobilized β-Gal was measured by incubating the enzyme at a specific temperature between 25 °C and 60 °C for one hour and then assaying its activity. Note that the activity of β –Gal after exposure to high temperature is substantially enhanced when bound to the YHNN and QYHH peptide-modified surfaces. The activities of β-Gal at different temperatures were normalized to that at 25°C. (b) Normalized activity of β-Gal immobilized on peptide-modified surfaces as a function of pH. The solution pH dependence of the activity is shown in dark blue for comparison. The activities of β-Gal at different pHs were normalized to the maximum activity at optimal pH.

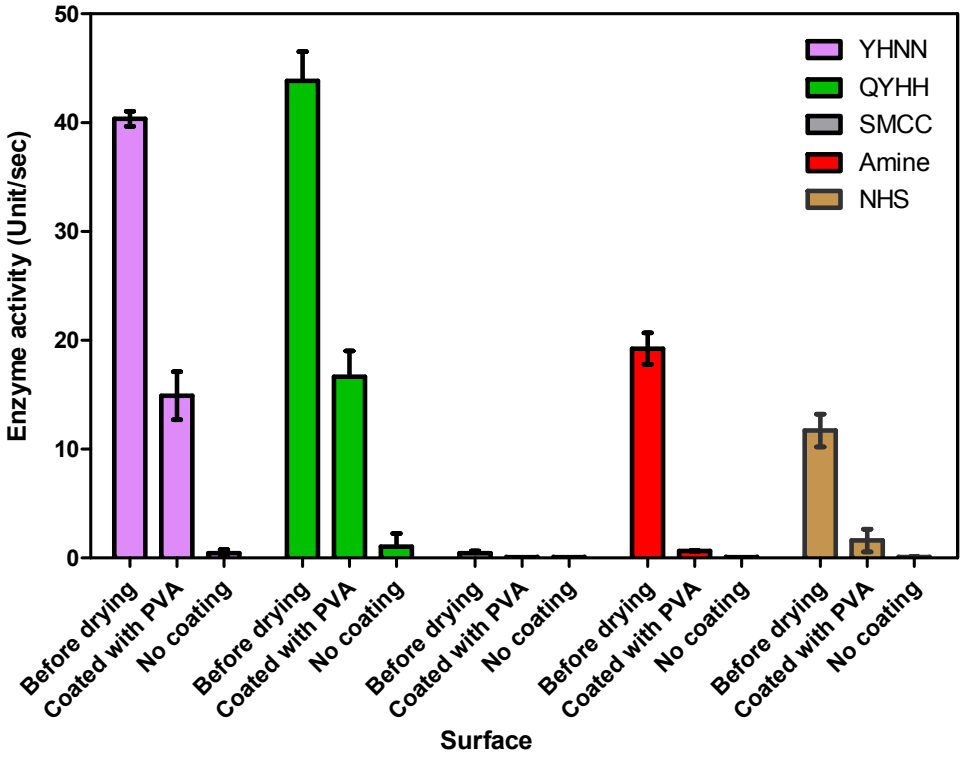


Figure 59. Long-term stability of β-Gal immobilized on different surfaces. The long-term stability of the enzyme was tested in two ways. One method used was to coat the enzyme-bound microwell with PVA, dry it and then store it at room temperature in this condition for a week (the second column for each sample). The other method used was to dry the enzyme-bound microwell without PVA coating and store it at room temperature for a week (the third column for each sample). The remaining enzyme activity after storage is assayed by adding 100 µL of 100 µM RBG into each well. As shown in the figure, the peptide surfaces combined with PVA coating greatly maintain the enzyme activity after storage compared to the other surfaces.

The apparent K_d values of the YHNN- and QYHH-modified surfaces were ~ 5 nM and ~ 4 nM for β-Gal, respectively (Figure 60). The apparent k_{cat} and K_m constants for immobilized β-Gal were measured on peptide-modified iodoacetyl

resin, which has a large binding capacity and allows for the quantification of the absolute amount of bound enzyme (Figure 61). k_{cat} values were ~ 46 s^{-1} for the YHNN- surface and ~ 53 s^{-1} for the QYHH- surface, similar to the k_{cat} of ~ 58 s^{-1} under the same conditions for the free enzyme. The apparent K_m values of β-Gal bound to the YHNN- and QYHH-modified surfaces were ~ 240 μM and 250 μM, respectively, compared ~ 130 μM for the free enzyme. The apparent increase in K_m for the surface-bound enzyme may be due to slow diffusion of substrate molecules to the surface and local substrate depletion.[11]

In Figure 62, peptide-protein binding sites for YHNN and QYHH were determined by proteolytic mapping using reversible formaldehyde cross-linking[12]. YHNN and QYHH both bound to the same protein fragments (419-447) at the subunit interface of β-Gal (Figure 63 and 64). β-Gal from *E. coli* is only active in its tetrameric form[13], and it may be that YHNN and QYHH enhance the activity and stability of β-Gal by stabilizing its tetrameric structure.

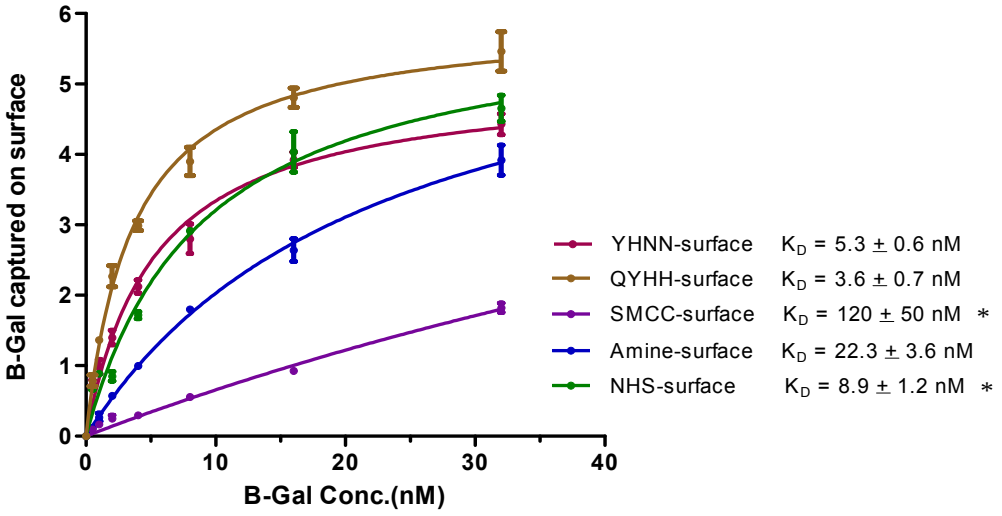


Figure 60. The apparent dissociation constants of different surfaces determined by ELISA. All data is fitted using the program GraphPad and the fitting equation: $Y=B_{max}*X/(Kd + X)$, where B_{max} is the theoretical maximum binding level of β-Gal. [*] For the SMCC- and NHS-surfaces, there is no real K_D since this is a covalent conjugation with no dissociation equilibrium. In that case, the apparent K_D is calculated from curve fitting and likely reflects the saturation of enzyme attachment to the NHS-surface.

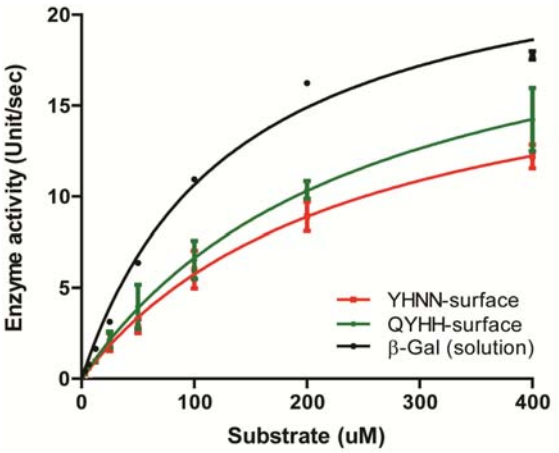


Figure 61. Colored curves: Curve fit used to determine the apparent K_M and k_{cat} of β-Gal immobilized on YHNN- and QYHH-modified beads using an amount of bound enzyme equivalent to a solution enzyme concentration of ~ 150 pM. Black curve: fit used to determine K_M and k_{cat} for the free enzyme at 150 pM. The enzyme activities are measured as a function of the concentration of the substrate RBG, between 3 μM and 400 μM, at 25 ^oC. All data is fitted using the program GraphPad and the fitting equation: $Y=V_{max}*X/(K_m+X)$.

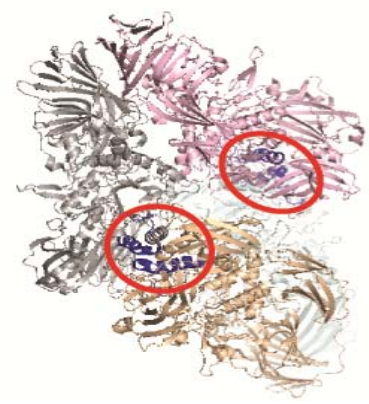
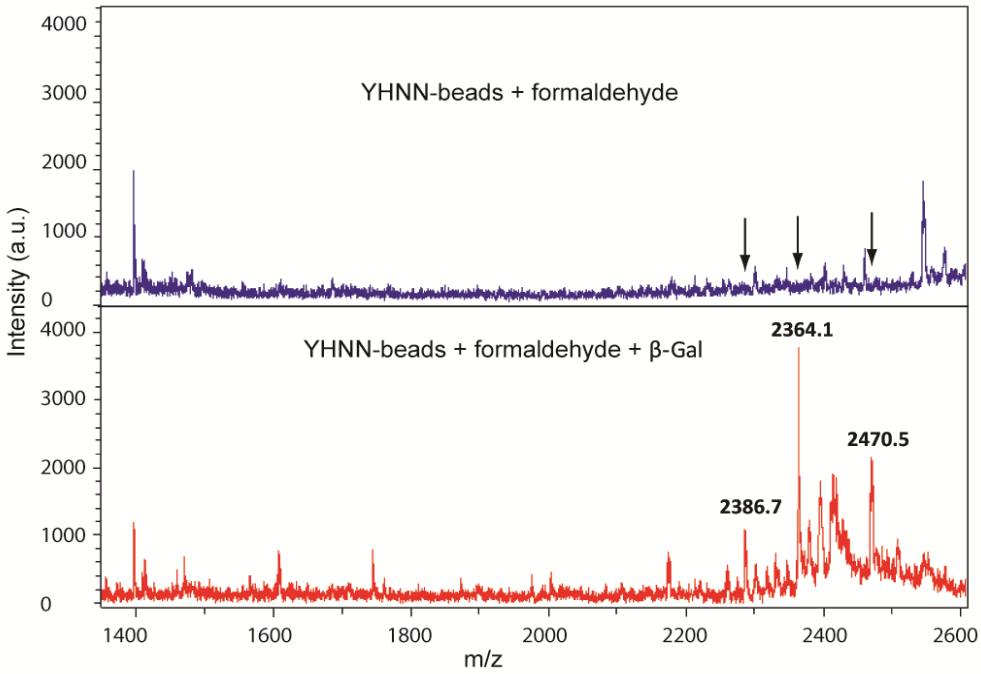
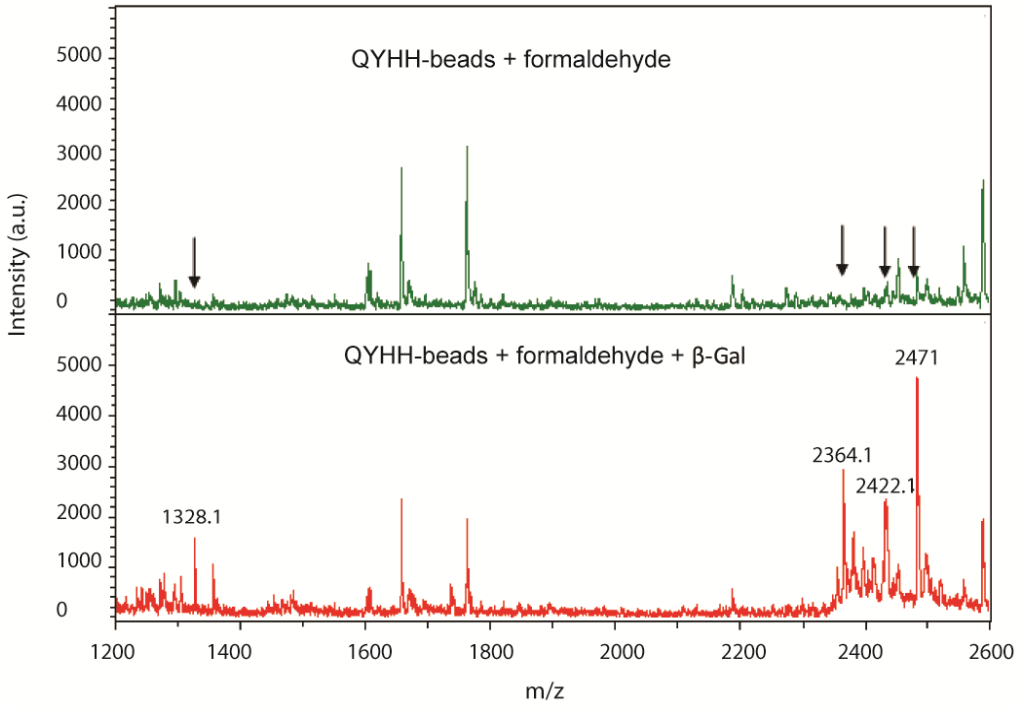


Figure 62. Proteolytic mapping of peptide binding to tetrameric β-Gal with binding regions circled. Each subunit is labeled with a unique color showing the symmetry of the β-Gal structure. The binding regions (amino acids 419-447) are highlighted in blue.



Observed Mass (Da)	β-Gal Fragment	Fragment Sequence	Oxidation
2260.2	430-447	PRWLPAMSERVTRMVQRD	2
2286.7	470-487	ALYRWIKSVDPSRPVQYE	5
2363.9	419-438	GMVPMNRLTDDPRWLPAMSE	3
2379.8	419-438	GMVPMNRLTDDPRWLPAMSE	4
2395.9	419-438	GMVPMNRLTDDPRWLPAMSE	5
2411.9	419-438	GMVPMNRLTDDPRWLPAMSE	6
2422.2	429-447	DPRWLPAMSERVTRMVQRD	5
2370.5	429-447	DPRWLPAMSERVTRMVQRD	8

Figure 63. MALDI-TOF analysis of β-Gal crosslinked to YHNN-modified beads after Glu-c digestion. The upper figure is a YHNN-bead blank control and the lower figure is β-Gal crosslinked to YHNN-beads. The protein fragment analysis indicates that the main binding region between the enzyme and the YHNN-beads is located near the amino acid residues 419-447 (bottom table). Many of the digest fragments observed are actually from the same stretch of amino acid sequence but have different degrees of oxidation as a result of exposure to formaldehyde.



Observed Mass (Da)	β-Gal Fragment	Fragment Sequence	Oxidation
1328.2	419-429	GMVPMNRLTDD	5
2355.6	430-447	PRWLPAMSERVTRMVQRD	8
2364.1	419-438	GMVPMNRLTDDPRWLPAMSE	3
2380.4	419-438	GMVPMNRLTDDPRWLPAMSE	4
2396.5	419-438	GMVPMNRLTDDPRWLPAMSE	5
2412.6	419-438	GMVPMNRLTDDPRWLPAMSE	6
2422.1	429-447	DPRWLPAMSERVTRMVQRD	5
2471	429-447	DPRWLPAMSERVTRMVQRD	8

Figure 64. MALDI-TOF analysis of β-Gal crosslinked to QYHH-modified beads after Glu-c digestion. The upper figure is a QYHH-bead blank control and the lower figure is β-Gal crosslinked to QYHH-beads. The mapping data shows that the binding region of the peptide QYHH with β-Gal is near amino acid residues 419-447, just as was observed for the peptide YHNN. This region of β-Gal is located near the interface between two subunits of the protein.

Point-variant screening[14,15] was applied to the YHNN peptide to improve both the affinity and activity of bound enzyme. 132 single-point variants, containing all substitutions of the amino acid set {Y, A, D, S, K, N, V, W} in each

of the 17 randomized positions, were synthesized, printed on a microarray and analyzed for affinity and activity[10]. Figure 65a shows the binding level vs. activity of β-Gal for each single-point variant, normalized to the YHNN- lead peptide. Several variants increased both binding level and activity, (region ii), including variant V9Y (YHNNPGFR**Y**MQQNKLHHGSC) which increased binding by 1.5-fold and specific activity by nearly 3-fold compared to the YHNN- lead peptide (Table 8). V9Y conjugated to an aminated microwell increased both the binding and the specific activity of immobilized β-Gal by ~2-fold compared to YHNN. This corresponds to a total bound enzymatic activity on the V9Y-modified surface that is ~ 12-fold greater than the NHS surface and more than 5-fold greater than the amine surface (Figure 66). Combining two advantageous point mutations into a single peptide (e.g.V9Y and N13Y, Table 8) resulted in an increase in the affinity of the peptide for binding to β-Gal but did not significantly enhance the specific activity of bound enzyme compared to single-point variants.

The library of single-point variants was also screened for enhanced thermal or pH stability of immobilized β-Gal. For thermal stability screening, enzyme was bound to microarrays containing the 132 single-point variants, at room temperature, and then the arrays were incubated at 55 °C for one hour and assayed for activity at room temperature. A few point variants improved the resulting activity of bound β-Gal by nearly 50% compared to the YHNN- lead peptide (Figure 65b, circled region, and Table 9). pH stability was screened by incubating enzyme-bound arrays in buffers ranging from pH 6 to pH 9 for one hour and then assaying activity at the pH used for incubation. In Figure 65c, some variants were found to significantly improve the specific activity of bound β-Gal at both low (pH 6) and high (pH 9) pH compared to the YHNN- lead peptide

(e.g. Q12A, YHNNPGFRVMQ**A**NKLHHGSC shows a 4.1-fold activity increase at pH 6 and a 2.8-fold increase at pH 9, Table 10).

CONCLUSIONS

A simple method for immobilizing enzymes through specific interactions with peptides anchored on surfaces has been developed. Peptides can be rapidly selected from microarrays and covalently conjugated to surfaces for capturing target proteins. Peptide-modified surfaces improve both the specific activity and stability of bound β-Gal compared to free enzyme or to conventional enzyme surface immobilization approaches. In addition, the affinity and activity of one of the peptide-modified surfaces was further improved by single-point variant screening. Variants were found that not only improved activity under normal conditions, but enhanced thermal stability, increased enzyme activity at extreme pH and improved the stability of the enzyme to storage in hydrogels. This approach should be applicable to the immobilization of a wide variety of enzymes on surfaces with optimized performance, and provides a potential mechanism for the patterned self-assembly of multiple enzymes on surfaces.

ACKNOWLEGEMENT This work was supported by Technology and Research Initiative Funds (TRIF) provided to Drs. Neal Woodbury and Stephen Johnston by ASU and the Edson Student Entrepreneur Initiative provided to Mr. Jinglin Fu at ASU. I thank John Lainson for the peptide array production; Donnie Shepard, and Loren Howell for the peptide synthesis and purification, Daniel Garry for the initial test on peptide-surface conjugation, Stephen Johnston for helpful discussions and Carole Flores for reading and editing the manuscript.

Figure 65. Point-variant screening of a lead peptide, YHNN. β-Gal was bound to a microarray containing 132 YHNN variants and its activity was measured. (a) The activity of bound β-Gal on microarrays as a function of the amount of enzyme bound to a particular variant feature at room temperature. (i) Variants with poor affinity and activity; (ii) Variants with stronger affinity and higher activity; (iii) Variants with stronger affinity but relative lower activity. All data is normalized to the binding and activity values for the lead peptide, YHNN. (b) Thermal-stability assay. β-Gal was bound to the microarray containing YHNN variants as in (a) at room temperature, followed by incubation in phosphate buffer at 55 °C for one hour. Enzyme activity was then assayed at room temperature. The selection region (circled) contains variants that bind to the enzyme with higher relative specific activity (the ratio of binding to activity) under thermal stress compared to YHNN (c) pH activity range assay. YHNN variant microarrays were bound to β-Gal in buffers with pHs ranging from 6 to 9 for one hour and then assayed for activity at the pH of incubation.

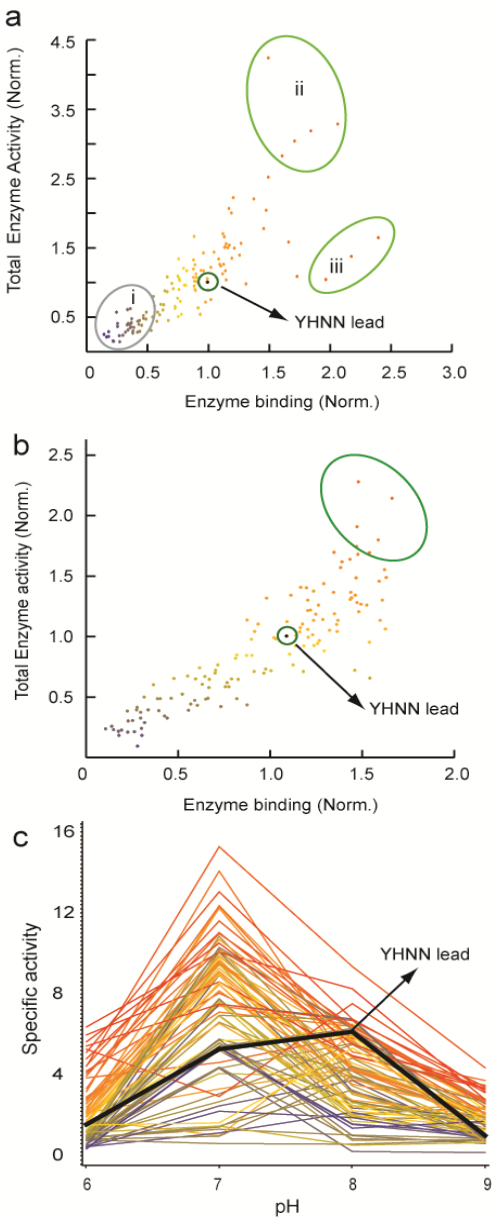


Table 8 Selected point variants that improve the binding affinity and activity of β-Gal on a microarray surface [a].

	Sequence	Binding	Total activity	Surface Specfic activity
1	YHNNPGFRVMQQNKLHHGSC	1.00	1.00	1.00
2	YHN**S**PGFRVMQQNKLHHGSC	1.22 ± 0.15	1.89 ± 0.19	1.55
3	YHNNPG**Y**RVMQQNKLHHGSC	1.77 ± 0.14	3.15 ± 0.29	1.78
4	YHNNPGF**Y**VMQQNKLHHGSC	0.93 ± 0.20	1.55 ± 0.22	1.67
5	YHNNPGFR**Y**MQQNKLHHGSC	1.50 ± 0.12	4.25 ± 0.18	2.82
6	YHNNPGFR**W**MQQNKLHHGSC	1.62 ± 0.13	2.83 ± 0.1	1.75
7	YHNNPGFRV**Y**QQNKLHHGSC	1.72 ± 0.15	3.04 ± 0.26	1.77
8	YHNNPGFRVMQQ**Y**KLHHGSC	1.50 ± 0.21	2.52 ± 0.40	1.68
9	YHNNPGFRVMQQNK**W**HHGSC	2.10 ± 0.15	3.29 ± 0.20	1.57
10	YHN**S**PGFR**Y**MQQNKLHHGSC	1.62 ± 0.16	3.53 ± 0.26	2.18
11	YHNNPG**Y**R**Y**MQQNKLHHGSC	2.35 ± 0.22	6.01 ± 0.40	2.56
12	YHNNPGF**Y**Y**Y**MQQNKLHHGSC	0.66 ± 0.11	0.91 ± 0.10	1.37
13	YHNNPGFR**Y**MQQ**Y**KLHHGSC	2.20 ± 0.20	4.78 ± 0.50	2.17
14	YHNNPGFR**Y**MQQNK**W**HHGSC	2.05 ± 0.26	5.72 ± 0.61	2.79

[a] Peptide 1 is the YHNN- lead peptide; Peptides 2-9 are selected single-point variants; Peptides 10-14 are combinations of two single-point variants. The combination of two selected single-point variants sometimes improves the affinity to β-Gal, but does little to enhance the specific activity of the bound enzyme. All data is normalized to the YHNN- lead peptide.

Table 9 Point variants that improve the thermal stability of bound β-Gal [a].

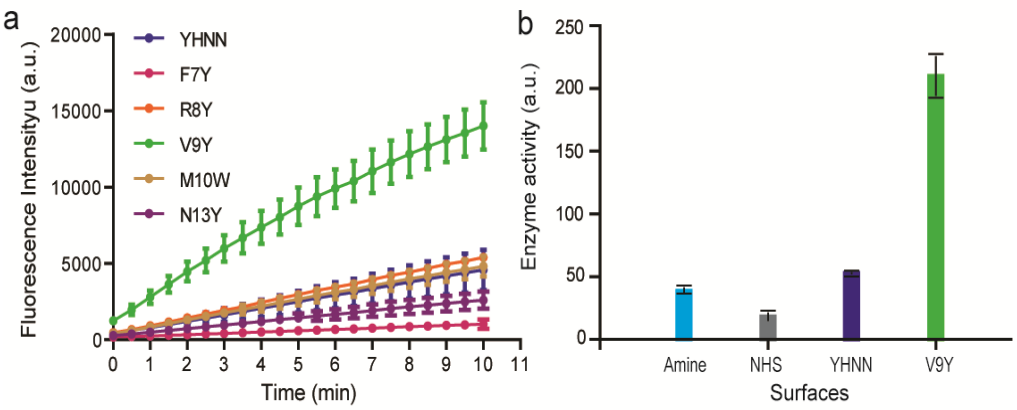
Sequence	Binding (Norm.) at 55 C	Specfic activity (Norm.) at 55 C
WHNNPGFRVMQQNKLHHGSC	1.28	1.45
YHSNPGFRVMQQNKLHHGSC	1.48	1.42
YHNNPGYRVMQQNKLHHGSC	1.77	1.38
YHNNPGWRVMQQNKLHHGSC	1.07	1.35
YHNNPGFRYMQQNKLHHGSC	1.5	1.86
YHNNPGFRVMQQNKLAHGSC	0.89	1.54
YHNNPGFRVMQQVKLHHGSC	1.67	1.47

[a] All data is normalized to the YHNN lead peptide.

Table 10 Point variants that modulate the optimal pH range of bound β-Gal [a].

Sequence	Specific activity at pH 6	Specific activity at pH 7	Specific activity at pH 8	Specific activity at pH 9
YHANPGFRVMQQNKLHHGSC	2.53	1.37	1.05	2.71
YHNSPGFRVMQQNKLHHGSC	3.08	1.58	0.95	3.30
YHNNPGYRVMQQNKLHHGSC	2.68	1.58	0.80	3.46
YHNNPGWRVMQQNKLHHGSC	2.13	1.82	0.84	3.70
YHNNPGFRYMQQNKLHHGSC	3.47	1.99	1.09	3.39
YHNNPGFRSMQQNKLHHGSC	3.61	1.47	0.90	3.29
YHNNPGFRVMAQNKLHHGSC	3.83	1.33	0.81	3.31
YHNNPGFRVMNQNKLHHGSC	2.04	1.34	0.69	1.89
YHNNPGFRVMQANKLHHGSC	4.08	1.91	1.35	2.76
YHNNPGFRVMQNNKLHHGSC	3.27	1.42	1.11	3.11
YHNNPGFRVMQQAKLHHGSC	2.85	1.53	1.02	2.55
YHNNPGFRVMQQNKNHHGSC	2.85	1.49	1.53	4.33

[a] All data is normalized to the YHNN lead peptide.



c

	Sequence	β-Gal Binding	Total β-Gal Activity	Surface Specific Activity
YHNN	YHNNPGFRVMQQNKLHHGSC	2.0 ± 0.3	6.2 ± 2.1	3.1
F7Y	YHNNPGYRVMQQNKLHHGSC	1.1 ± 0.2	1.5 ± 0.5	1.3
R8Y	YHNNPGFYVMQQNKLHHGSC	2.0 ± 0.3	8.8 ± 0.5	4.4
V9Y	YHNNPGFRYMQQNKLHHGSC	4.2 ± 0.2	27.2 ± 3.6	6.5
M10W	YHNNPGFRVWQQNKLHHGSC	1.9 ± 0.2	3.9 ± 0.9	2.1
N13Y	YHNNPGFRVMQQYKLHHGSC	1.9 ± 0.2	7.4± 0.8	3.9

Figure 66. Activity of β-Gal immobilized on different surfaces which were modified by the lead peptide (YHNN) and its point variants, respectively, as selected from microarray screening. (a) YHNN and 5 selected single-point variants were used to modify aminated microwell surfaces for capturing β-Gal. 3 variants, R8Y, V9Y and N13Y showed improvement of total bound enzyme activity. The variant V9Y demonstrated the best enzyme immobilization performance: a roughly 2-fold improvement both in surface affinity and relative specific activity of the bound enzyme, and nearly a 5-fold improvement in total activity of the bound enzyme. (b) Total enzyme activity immobilized on the YHNN- and V9Y-modified surfaces compared to amine and NHS surfaces. (c) Binding and activity of β-Gal immobilized on surfaces modified by peptides. The relative β-Gal binding amount is determined by measuring the activity of APase conjugated streptavidin bound to the biotinlyted β-Gal. The enzyme activity is evaluated by adding 100 μL of 100 μM RBG into each microwell.

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CHAPTER 6: COMMERCIALIZATION OF THE TECHNOLOGY

ABSTRACT

Efforts in commercializing the technology developed during the Ph.D study are described in this chapter. We have attempted to start a company named “NOVLOLATOR” to provide technology solutions for enzyme applications. The initial investigation of market and business strategy was performed to determine the appropriate commercial application of the technology.

INTRODUCTION

Commercial use of enzymes in the manufacturing processes is generally classified in terms of industrial use or specialty use. Industrial users are generally placed into three classes: technical, food and animal feed (Figure 67)[1] with technical using as the largest portion including: detergent, starch, textile, fuel alcohol, leather and paper manufacture. Specialty users are mainly from pharmaceutical companies, diagnostics, research and biotechnology organizations, or involved in specialized organic synthesis. It is estimated that the global market value for enzymes will reach almost $6 billion dollars by 2011, with an annual growth of almost 8 percent.[1] This growth will be driven by the increasing demand in the pharmaceutical, fine chemical, and bioethanol industries. Commercial enzymes are required to function in a variety of challenging conditions. Consideration of thermalstability, pH tolerance and manufacturing ease are examples of crucial components to enzyme performance and utility. The continued lowering of barriers to commercialization is essential for expanding the application of enzymes in commercial use. Examples of some of the main challenges to the successful commercial use of enzymes are:

- Inhibition of enzyme activity due to high concentrations of substrates and products.
- Functional instability in the presence of other ingredients (e.g. tolerance to organic solvents).
- Instability to temperature and non-native pH environments.
- Lack of sufficient, economical enzyme immobilization techniques that allow for industrial re-use of enzymes.[2]
- Lack of direct high throughput methods for the selection of catalytically active and stable enzymes.

Many enzyme based applications and processes could be improved if there were a means to increase the activity of an enzyme or stabilize and maintain its catalytic activity over time. Most commercial enzymes are found by screening natural organisms or created by improving existing enzymes. Directed evolution is the most widely-used tool to generate stable and active enzymes under desired conditions. But in many cases, directed enzyme evolution consumes considerable time and money to generate the optimized mutant enzyme satisfying the requirements. A combination of rational design and directed evolution may be a more powerful tool to create new enzymes with desired function. Small molecules that can modulate enzyme function are also used to optimize enzyme activity under a given set of conditions, which may expand the use of enzymes.

This chapter describes our efforts in exploring business opportunities in developing peptides or other small molecules that improve enzyme function for commercial use.

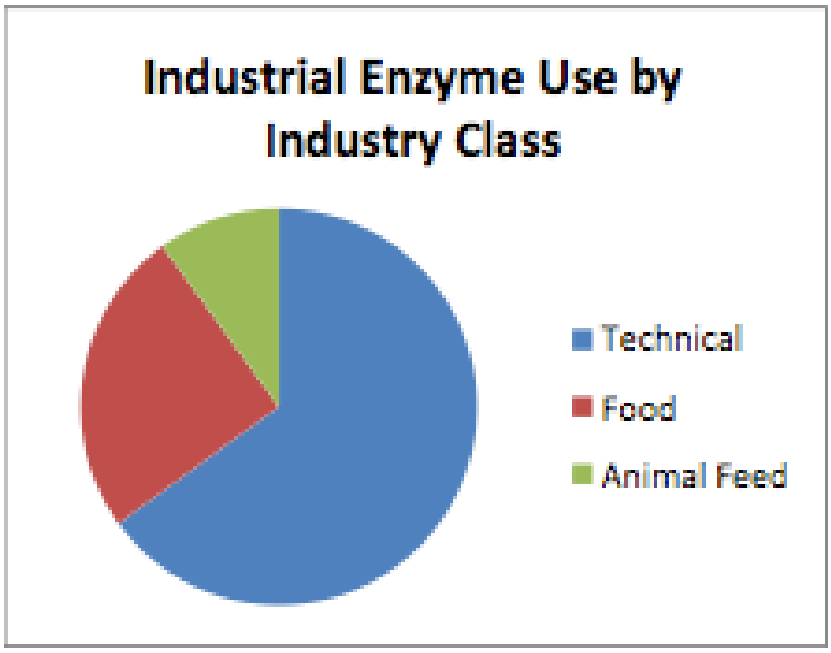

Figure 67. Enzyme use in Industry.[1]

BUSINESS CONCEPT/STRATEGY

Company Overview During my Ph.D study, I collaborated with another graduate student, Berea Williams, and have tried to start a small company named “NOVOLATORS” based on the technology described in the previous chapters. NOVOLATORS is a technology service company that provides solutions for enzyme applications. The company provides methods and solutions to improve the performance of enzymes under a large range of desired conditions (high temperature, low pH, etc.). Figure 68 is the logo design of the company.

Figure 68. The logo of the company.

Business Opportunity Many enzyme based applications and processes could be improved if there were methods to increase or decrease the activity of a specific enzyme under a particular set of conditions. In particular, being able to inhibit one enzyme in the presence of others, stabilizing and maintaining its catalytic activity over time, or enhancing its activity under nonnatural conditions (high temperature, low pH, etc.) could greatly increase the utility of enzymes for a variety of applications.

NOVOLATORS utilizes the technology described in the previous chapters to modulate enzyme under a wide range of desired conditions to improve the performance of an enzyme. Novel enzyme modulators have various applications in consumer products, biotechnology, the pharmaceutical industry and the medical diagnostic industry. They can be used to decrease amounts of enzymes needed, extend the range of enzymatic conditions, stabilize enzymes over time/temperature and modulate enzyme activity under desired conditions.

Distinctive Competence NOVOLATORS' technology has the advantages of rapid high throughput enzyme modification, extending the range of enzyme working conditions, applicability to many enzyme classes, lower R&D cost, shorter development time period and industrial compatibility.

Development Strategy NOVOLATORS will target many sectors of the enzyme industry including the detergent, fine chemical, bioethanol, and purification processes. These sectors make up a large portion of the enzyme market and have a very high demand for functional enzymes in harsh conditions. Furthermore, those sectors are not subjected to the Food and Drug Administration (FDA) guidelines, which will take years to process.

Funding We have received $ 18, 000 from the Edson Student Entrepreneur Initiative at Arizona State University for initialing the business.

MARKET ANALYSIS

Market Structure The global demand for enzymes is estimated to be a $6 billion dollar industry in 2011.[1] This growth will be driven by the increasing demand in the pharmaceutical, fine chemical, and bioethanol industries. The leading manufacturer of enzymes is Novozymes with 26% of the total enzyme market and 46% of the industrial enzyme market, and a net profit of ~$180 million in 2008. Other enzyme producers include Genencor, Danisco, Allergan, Roche, Genzyme, DSM and BASF, which share 36% of the market. Two main commercial consumers of enzymes are industrial and specialty markets. The industrial sector mainly uses enzymes in food, animal feed, and detergents. The specialty sector includes the pharmaceutical, fine chemical, and bioethanol industries, which use enzymes in disease detection, biotechnology research, organic synthesis, and purification processes.

Potential Customer We will provide service to enzyme-consuming fields, like detergent (Dial @ Henkel), biofuel, surface-based catalysis and chemical production. The pharmaceutical and research enzyme fields are also potential markets.

Market Competitor Enzyme Companies like Novozyme and Genencor, are the main providers for commercial enzymes. Nonspecific enzyme stabilizers (PEG, glycerol, cellulose, etc.) are commonly used in enzyme products for stabilization.

ENZYME TARGET

In collaboration with Technology Service Venture Group (TVSG) and Arizona Technology Enterprises (AzTE), we conducted a study of the commercial enzymes and identified some potential targets.

RESEARCH ENZYMES Taq DNA Polymerase was purified from the hot springs bacterium *Thermus aquaticus* around 1976, and became one of the most important discoveries in molecular biology. Currently, the world market for Taq polymerase is in the hundreds of millions of dollars[3]. There is a wide range of polymerase prices from the lowest of $0.1/unit to the highest of $1.0/unit. The average price for Taq polymerase used in academic research is ~ $ 0.3/unit.

The thermostability of Taq DNA polymerase is the critical feature that facilitated the development of Polymerase Chain Reaction. The thermalstability of the Taq polymerase is listed in Table 11. Special care has to be taken to avoid loss of activity at high temperature or protein denaturation happened at room temperature storage. Recent DNA sequencing technology that involves the use of polymerase requires the immobilized polymerase on surface with strong activity and good stability at room temperature. Thereby, the high quality enzyme with stable activity at both room temperature and high temperature will be more welcomed to academic and industrial research. If we develop peptide-modified beads for capturing polymerase with improved thermal stability, for example, extending the enzyme half life from 5 min to 50 min at 97.5 ºC, it will reduce the

amount of enzyme to ~ 10 - 20 % of the traditional use. It will use less polymerase for the same reactions than the traditional methods.

Taq Polymerase Half Life	Temperature
5 min	97.5°C
40 min	95°C
120 min	92.5°C

Table 11. The thermal stability of Taq polymerase

Reverse transcriptase is widely used in the detection of pathogen and disease diagnostics. The improvement on the thermal stability of the enzyme will reduce the secondary structure of mRNA and improve the detection accuracy. BioRed is trying to develop thermal-stable reverse transcriptase for its disease diagnostics. It is possible to use peptides to stabilize the enzymes at higher temperature and thereby improve the accuracy of mRNA reverse transcription assay.

Restriction enzymes are enzymes that cut a DNA molecule at a particular place. They are essential tools for recombinant DNA technology. The enzyme "scans" a DNA molecule, looking for a particular sequence of usually four to six nucleotides. Once it finds this recognition sequence, it stops and cuts the strands. Although half life values differ greatly among enzymes, it is common procedure to keep the enzymes cold to avoid activity loss. The use of restriction enzymes continues to increase in biological and biotechnical research. Potential problems for the restriction enzymes are: low enzyme activity under the conditions of high glycerol and high pH; activity sensitive to organic solvents; product inhibition at high concentration of substrate DNA and poor stability that many enzymes simply stop working even under the best conditions after half an hour.

INDUSTRIAL ENZYMES Cellulase is a class of enzymes that catalyze the cellulolysis (or hydrolysis) of cellulose. Demand for Cellulase has been increasing rapidly, and it will become one of the most important enzymes for sustainable development of biofuels. The reason for this is the high abundance of its substrate cellulose (100 billion dry tons/year produced), which could be used to produce biofuel. The potential cellulase market has been estimated to be as high as $400 million per year if cellulases are used for hydrolyzing the available corn stover in the midwestern United States.[4] This enzyme is relatively expensive and cost reduction must be achieved if it is to be used for commercial production. Genencor International and Novozymes Biotech claimed recently to greatly reduce the cost of cellulose by improving the production method and by improving enzyme activity. In order to have a sustainable technology, cellulases must be improved in several characteristics: higher catalytic efficiency on insoluble cellulosic substrates, increased stability at elevated temperature and certain pH levels, and higher tolerance to end-product inhibition.

TEAM MANAGEMENT

Berea Williams, Team Leader

Berea entered graduate school in 2005 and graduated with her PhD in chemistry from Arizona State University. Her graduate education has used a highly interdisciplinary approach to solving fundamental problems in biochemistry research. Berea is also the president of the Biodeisgn Graduate Student Organization (BGSO) and leads a seminar series called “Careers Outside the Lab”.

Jinglin Fu, Co-Team Leader

Jinglin is a fourth year PhD graduate student in the Department of Chemistry and

Biochemistry and the Biodesign Institute at Arizona State University. His successful research in developing novel enzyme modulators has resulted in two provisional patents. Jinglin has extensive knowledge in enzymology and high throughput screening. He is very interested in translating fundamental research ideas into commercial products.

MILESTONES FOR THE BUSINESS DEVELOPMENT

Milestone 1 (4/1/2009 – 7/1/2009) We have prepared the business plan for “NOVOLATOR” for the application to the Edson Student Entrepreneur Initiative.

Milestone 2 (7/1/2009 – 10/1/2009) We conducted the initial study of the market and suggested potential enzyme targets with high margin and which are used extensively, such as cellulase used in biofuels. We have contacted two potential investors/customers, Dial@Henkel and BioRed. Unfortunately, these investor/customers do not believe our product line is mature enough to invest in currently, however they do see potential in this field.

Milestone 3 (10/1/2009 – 1/1/2010) We surveyed the use of commercial enzymes with the collaboration of the Technology Venture Service Group at ASU and concluded that the best fit market for our technology was enzymes used on surfaces, including bead-based catalysis, well-based catalysis and electrocatalytic enzymes. However, most commercial enzyme information in these areas is protected by the company either as patent or trade secret.

Milestone 4 (1/1/2009-7/1/2010) We have decided to delay the start of the company, and perform additional experiments to demonstrate the technology, for example, improving the immobilized enzyme activity on peptide surfaces. It will make the technology more useful if we can combine the peptide selection

with the protein engineering. For example, it is possible to design new enzyme variants incorporated with peptide fusions selected from microarray. The variants may possess new properties that are modulated by the incorporated peptide minidomains.

CONCLUSION

We have decided to delay our effort to commercialize this technology until after the end of Edson student entrepreneur initiative project. There are a couple of issues:

Entering the Market We have tried to determine which enzymes either are of interest to industry or are used by companies. However, most commercial enzyme information is protected by the company either as patent or trade secret. We discussed this with a senior scientist at Dial@Henkel, and he suggested that we continue to demonstrate the technology using widely known enzymes (which may not have high commercial margins) and then start more detailed collaborations and discussions with enzyme companies.

Competing technology NOVOLATOR is based on a new technology using peptides to modulate enzyme function. The investor/customers (Dial) do not believe our product line is mature enough for investment, however they do see the potential for this field. The expensive cost of peptides is also a big limitation. There are established technologies for improving enzyme function, like directed evolution. We have not fully demonstrated the advantage of our technology over the conventional methods in the practical application. It will be more promising to industry if we can combine the selection of peptide ligands with protein engineering to create new enzyme mutants with optimized activity and stability (discussed more in the next chapter).

Funding We have received $ 18, 000 from the Edson Student Entrepreneur Initiative at Arizona State University for initialing the business. We have considered applying for SBIR/STTR grants, but there are limitations in terms of our student status. Also one of the founders, Berea William, decided to work at Dial after her graduation.

Patent Ownership After several discussions with AzTE and advisors from Law school, we have figured out that ASU owns the patents rather than the inventors themselves (ASU paid money for the patent application). If we want to start a company based the technology patented, the licensing of the technology from ASU is required.

Overall, it is an excellent experience for Ph.D students to think about commercializing the technology that they have been developed during their study. I appreciate the funding support from Edson program which made it possible for us to start the business investigation and trials. As an Edson fellow, I have received some basic entrepreneurial education in the Launch Prep Entrepreneur Course. The initial investigation involves market analysis, business strategy development, contact with potential customers, communication with business and law consultants, and exploring funding opportunities. I have benefited greatly from these experiences which have broadened the scope of my study beyond fundamental research.

ACKNOWLEGEMENT This work was supported by the Edson Student Entrepreneur Initiative Program provided to Berea Williams and Jinglin Fu. We are thankful for the help of Scott Perkofski as the coordinator for the Edson program, Tom Goodman and Jeremy Burdon from Arizona Technology Enterprise for their suggestions; Neal Woodbury and Mike Mobley as the advisors for our project and Peter He from Dial@Hekel for the helpful discussions.

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CHAPTER 7: CONCLUSIONS AND FUTURE CONSIDERATIONS

CONCLUSIONS

In this dissertation, a simple and general method of discovering enzyme modulators has been developed by performing parallel measurements of enzyme binding and activity on peptide microarrays. Chapter 1 introduced enzyme theory and the importance of discovering enzyme modulators for therapeutics and industrial applications. The microarray platform represents a powerful tool for high-throughput screening of enzyme binding to small molecules, and profiling enzyme activity.

Chapter 2 described the development of polymer-coated microarrays for monitoring enzymatic activity. Viscous poly vinyl alcohol (PVA) is an ideal medium to restrict molecule diffusion with a simpler fabrication process compared to microwell or microdroplet arrays. PVA has good properties for biological and imaging application, such as optical transparency and non-fluorescence, as well as low toxicity. It will generate a flat film if dried quickly and thereby stop all reactions at the same time. There are many other viscous hydrogels, like agarose or polyacrylamide, but they either have autofluorescence or shrink during the drying process. Slower diffusion was achieved by adding anti-product antibodies into the PVA layer, which formed large complexes with corresponding product molecules and diffused more slowly than product molecules alone. We realized that the using a fluorescent substrate analogue limited this technology from achieving broader application. To overcome that problem, I developed a cascade reaction system within the PVA layer that was able to convert nonfluorescent products into fluorescent signals. The screening of dehydrogenase activity was presented as a demonstration.

In Chapter 3, it was able to identify several peptides that modulated enzyme function, using the polymer-coated array methodology that I had developed. It was not only possible to identify enzyme inhibitors; peptides that enhanced enzyme activity were also identified. Enzymes can be assayed on microarrays under many conditions, such as pH, temperature and organic solvent in order to discover molecules that will promote or inhibit enzyme activity in varied environments. This approach was demonstrated by identifying peptides that improve the thermal stability of enzymes screened on microarrays at high temperatures. Some of the inhibitory peptides selected from microarrays were also able to inhibit enzymes in solution, exemplifying the transfer of function from surface to solution.

Chapter 4 described the mechanism of peptide inhibition of β-Gal in solution. Some peptides were able to inhibit β-Gal in solution with IC_{50} values ranging from 1 μM to 30 μM. These peptides exhibited noncompetitive kinetics in the apparent Michaelis constants, which meant that the peptides inhibited the enzyme not by binding to the active site. Next, it was found that the enzyme and detergent concentration affected the peptide inhibition of β-Gal, which suggested that peptides might aggregate with enzymes. Aggregation of peptides with enzymes was verified using dynamic light scattering and fluorescence correlation. It is also possible to design peptide pairs that can switch enzyme function based on stabilizing or destabilizing the aggregation of enzymes. It will be of therapeutic interest if the peptides can induce specific aggregation with target enzymes or disaggregate protein complexes.

Protein-ligand binding may be affected by the fluorophores used to label the protein. First, the fluorophore itself or the fluorophore-protein interface may

create a binding site for some peptides (e.g. hydrophobic sequences), thereby driving the whole protein binding with the peptides. These selected peptides generally do not bind to the non-labeled protein and result in false positives. In my experience, such sequences generally contain multiple continuous hydrophobic residues or positive-charged residues, such as WWW, KKK, RRR or HHH. Second, labeled fluorophores may dramatically change the surface pI of a protein. Many fluorophores contain negatively-charged groups, like carboxyl group. Most labeling molecules are conjugated to lysine residues which neutralize the positively-charged amine on the side chain. Those make protein more negatively-charged, driving the pI to be more acidic. Ideally, the non-labeling detection methods, like Surface Plasmon Resonance or Mass Spectrometry, will make microarray selection more efficient and accurate. An alternative approach is to use a secondary antibody for detection, or to label the protein with a low ratio of fluorophore (~ 1:1).

In chapter 4, positively-charged residues, such as K and R, were found to be crucial for inhibiting β-Gal through a detailed sequence analysis of two selected 20-mer peptide inhibitors and the point-variant screening of a 10-mer peptide. In chapter 5, point-variant screening of a 20-mer peptide that bound to β-Gal and enhanced its thermal activity were also performed to understand the contribution of amino acid residues. In Figure 69a, the heat map revealed that most substitutions at positions of Y1, F7, R8 and H17 resulted in an affinity decrease of peptide binding to β-Gal, and substitutions at H2 resulted in an increase in peptide binding to the enzyme. It suggested that the residues Y1, F7 and R8 were important for binding to β-Gal. Histidine (H) showed contradictory results for H2 and H17, but binding decreased for the substitutions at H17,

perhaps due to its close proximity to the C-terminal immobilized surface. It was also observed that substitutions of the YHNN lead peptide with K and D resulted in decreased peptide binding to the enzyme. Aspartate (D) is a negatively-charged residue; it has been observed by others that negatively- charged residues (D and E) may decrease the binding of a peptide with an enzyme.[1]

In Figure 69b, the heat map of specific activity revealed that most substitutions at positions F7, R8 and V10 resulted in an increase in bound enzyme activity. This suggested that those three residues inhibit the activity of the bound enzyme. The key role of R in inhibiting enzyme activity that has been observed here, was also consist with results obtained in Chapter 4. The substitutions at Y1 and H2 caused a decrease in enzyme activity, suggesting that Y1 and H2 were important for maintaining the activity of the bound enzyme. Interestingly, H2 showed a negative contribution to the binding of the peptide to the enzyme in Figure 69a, but a positive contribution to the activity of the bound enzyme. A similar contrary behavior between enzyme binding and activity was also observed for substitutions at F7 and R8.

From the above study, it is concluded that the hydrophobic residues F and W, the positive-charged residue R and the hydrophilic residue Y may generally increase the affinity of peptide binding to a protein. The negatively-charged residues D and E may generally decrease the affinity of peptide binding to a protein. For the activity of a peptide-bound enzyme, H, Y, W and A may be important for maintaining or improving enzyme activity. The positively-charged residues R and K often inhibit the activity of peptide-bound enzymes. It should be possible to select peptides with desired function (e.g.inhibit or promote enzyme activity from a more narrow amino acid set)

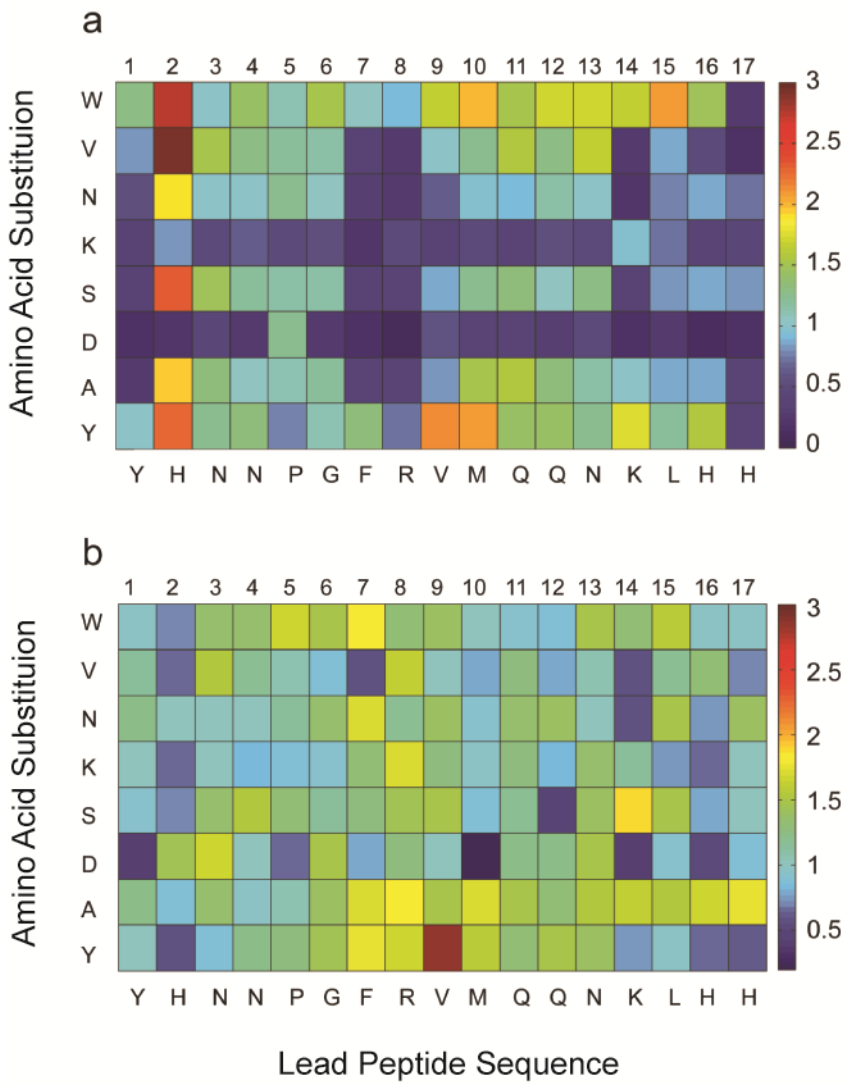

Figure 69. Heat map of single-point variant screening of YHNN for (a) binding to β-Gal and (b) specific activity of bound enzyme. All the single-point variants were printed on the microarray with three replicates of each sequence. A standard enzyme assay on the array was performed as described in Chapter 2. The specific activity of enzyme that bound to variants was calculated through dividing the total bound enzyme activity by enzyme binding intensity. All data was normalized to the binding and specific activity values of β-Gal bound to the lead peptide. Each data is the average of at least three runs

Chapter 5 described the use of selected peptides to immobilize enzymes on solid surfaces. There are several advantages of using peptides for surface-based catalysis. First, peptides generally have lower affinities in solution, with protein binding dissociation constants (K_D) ranging from 1 μM to more than 100 μM. The affinity of peptide-modified surfaces for a particular protein can be improved by more than 1000-fold with apparent K_D values of ~ a few nM, due to the avidity effect created by the high density of ligands. Second, peptides screened from microarrays exhibit the ability to modulate enzyme activity on the array surface. It is relatively easy to use this approach to select peptides that work as desired on a surface (even a surface somewhat different from the one that the original selection was performed on), compared to the process of selecting peptides on surfaces and then trying to use them in solution. Third, enzymes immobilized on peptide surfaces exhibited high activity and stability. In one example, it was possible to preserve enzyme activity for more than a week under dry conditions by immobilizing the enzyme on peptide surfaces coated with PVA polymer. This facilitates the transportation and storage of enzyme-related products. Last, there are well-established solid-phase peptide synthesis methods. Therefore, it is possible to produce peptide surfaces (like beads) in large quantities so that our peptide modulators may be combined with these synthesis technologies for commercial applications.

Chapter 6 described my effort in commercializing the technology in collaboration with another graduate student. After the initial market analysis and contact with potential customs, my collaborator and I determined that additional development and demonstration of the technology were required before entering the enzyme market.

Future Considerations

Screening enzyme activity on polymer-coated microarrays provides a simple, high-throughput and general method to discover enzyme modulators. Modifying the array-coating polymer with the ability to sense enzyme activity would simplify the technology and broaden the potential applications. In our current method, enzyme substrate is mixed with PVA for sensing enzyme activity. If the PVA or another coating polymer were to be modified with substrate (covalently linked), the product molecules generated from catalytic reactions would be trapped by the PVA with little diffusion. One possibility is to modify PVA or PVA derivatives with NAD+ for dehydrogenase detection. Poly acrylic acid, poly lysine, poly acrylamide[2] and DNA gel[3] are interesting polymers that can be modified with multi-functional groups or small molecules that will react with enzymes immobilized on array surfaces.

Another interesting future improvement is the design of enzyme variants that have the peptide fusions selected from microarrays incorporated genetically in their DNA sequences, creating a protein-peptide fusion. Some peptides have been shown to improve enzyme activity and stability under many conditions, for example, stabilizing an enzyme at high temperature or shifting the optimal pH. We also showed the enzyme was irreversibly inhibited by crosslinking the inhibitory peptide with enzyme in Chapter 4.

If the selected peptides can be engineered into either the N- or C- termini of wild-type enzymes, as fusions, the variants may possess new properties that are modulated by the incorporated peptide minidomains. In this way, it may be possible to create enzyme variants that are active under user-desired conditions

(e.g temperature, pH and organic solvent) by incorporating the fusion peptides selected from the microarray. This approach would be more interesting to the biocatalysis industry where they need more stable and active enzymes in their production process, but do not want to both produce an enzyme and a peptide.

Finally, it may be possible to utilize selected, enzyme-specific peptide ligands to immobilize multi-enzyme cascade systems on surfaces. It has been known that many enzymes involved in the cellular metabolism have highly controlled position and orientation within the cell membrane. This is thought to facilitate multistep reactions by enhancing substrate transfer efficiency between component enzymes.[4-6] It would be of great interest to design artificial catalysis systems that mimic cellular multi-enzyme cascades such that their catalytic efficiencies are maximized. Peptides have demonstrated the potential to anchor proteins on surfaces and control their orientations as well as optimize their activities and stabilities. If multiple peptide/enzyme pairs are selected, the peptides can be covalently attached to a surface or scaffold and serve as anchors for capturing target enzymes in controlled orientations. As such, wherever a particular binding peptide is placed, its enzyme partner will bind. This provides the basis for a self-assembling system that allows one to create enzyme cascades, on solid surfaces, that are optimized for maximal function under particular reaction conditions (e.g., temperature, pH, prolonged storage) and to catalyze reaction pathways by assembling several enzymes with defined spatial relationships (as shown in Figure 70).

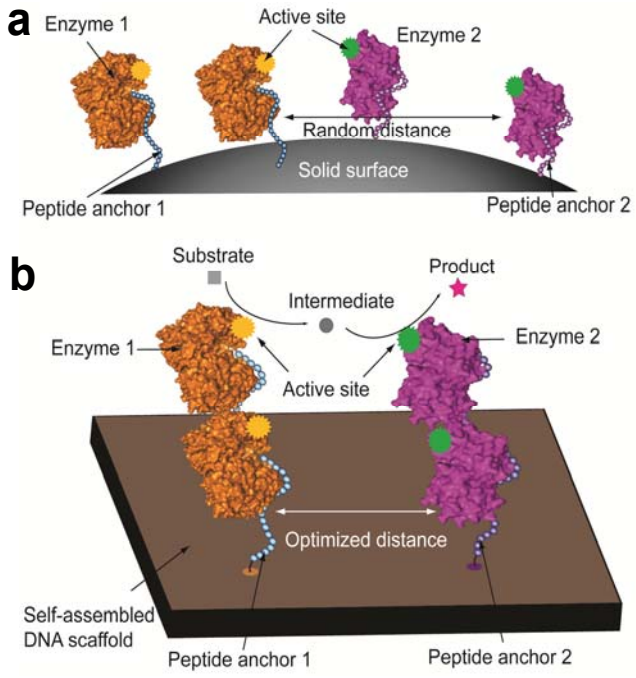

Figure 70. Self-assembly of (a) a multi-enzyme system on a solid surface with homogenous orientation but random distribution and (b) a multi-enzyme cascade on a DNA nanostructure with precisely-controlled orientation and position.

Personal Experience

I'd like to complete this story by telling about my graduate research experiences. Initially, I planned to perform two research projects, developing a peptide catalyst and selecting a peptide transformer (see below). In order to select candidates that catalyze hydrolysis reactions from a microarray containing 10,000 peptides, I had to think of a way to slow down molecular diffusion on surfaces to enable high-throughput screening of peptide libraries on the microarray platform. After several trials, I chose poly vinyl alcohol (PVA) as the coating to be used in my experiments. The model reaction was the hydrolytic reaction of Fluorescein di-β-D-galactopyranoside (FDG) which produces strong fluorescence after hydrolysis.

The objective of the peptide transformer project was to use peptides to mimic the binding of important biomolecules with proteins and replace expensive drug molecules with cheaper peptides (e.g. mimicking sugars). I attempted to use target molecules to block the binding of peptides with corresponding proteins on microarrays, hypothesizing that blocked peptides might bind to the same protein sites as the target molecules. Competitive inhibition of β-galactosidase (β-Gal) with phenylethyl thio-beta-D-galactoside (PETG) was one model system that I had planned to mimic. Both projects were in the very early stages of development, and Neal and Stephen gave me the freedom to try various ideas for troubleshooting.

After about half a year of research, I found it very hard to differentiate between the autofluorescence from the peptides themselves and the signals produced from the hydrolysis of the substrate. I needed a positive control to tell me what the real hydrolysis reaction would look like on the microarrays. I came up with an idea to perform β-Gal assays on PVA-coated microarrays since I knew that the enzyme would bind to many peptides on the arrays and that it could catalyze the hydrolysis of FDG. I discussed the idea with Neal and Stephen, and they both encouraged me to try it. Therefore, I did a very simple test to coat the β-Gal-bound array slides with PVA polymer containing FDG. Surprisingly, some enzyme-bound features exhibited strong fluorescence after the treatment. After several brainstorming discussions with Neal and Stephen, I felt that it would be more interesting to identify enzyme modulators by performing enzyme assays on the polymer-coated arrays. Over the next four years, I focused my research on developing polymer arrays for exploring peptide space to find enzyme modulators, as described in my dissertation.

Later, I realized how lucky I was during those initial trials. First, I was able to combine the two initial projects into a new and interesting idea. The peptide catalyst project provided a polymer coating method for monitoring catalysis. The peptide transformer project taught me a lot about enzymology and performing enzyme binding assays on peptide arrays. Second, β-Gal is a very stable and active enzyme with strongly fluorescent substrates. Early on, I washed the enzyme-bound microarrays with water, not buffer solution. It is very likely that I could not see much catalytic activity on the arrays because all of the enzymes were dead. But some peptide-bound enzymes survived the tough water washing procedure and drying steps, and still exhibited activity after coating with PVA polymer containing substrates. (My ‘mistake’ also demonstrated the power of peptide modulators to maintain enzyme activity in very harsh conditions).

It is also very important that my advisors, Neal and Stephen, gave me the freedom to try my own ideas and encouraged me to follow those thoughts that seemed promising. When I presented new ideas, they seldom made “YES” or “NO” judgments. Instead, they used their experience to help me define the possible problems and applications. I have benefitted greatly from those insightful and friendly discussions.

In the end, I’d like to say, SCIENCE always gives people surprises and gifts. It will convey, bear great fortune if one dares to try with careful observation, critical thinking and hard work.

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BIOGRAPHICAL SKETCH

Jinglin Fu was born in Jilin, a city located in the Northeast of China, in 1980. He received his B. A. degree in Chemistry from Zhejiang University in 2003, and then continued a master study at Institute of Micrototal Analysis System focused on microfluidic chip-based optical detection systems. He came to Arizona State University in 2006, and later joined Dr.Woodbury's lab for his Ph.D study. In collaboration with Dr.Johnston's lab, he developed a new approach of screening enzyme activity on polymer-coated microarray. The method was later applied to discovering peptides that altered enzyme function by binding to it. Mr.Fu was very interested in applying the lab technology to commercial use. During his Ph.D study, he was awarded $ 18,000 from Edson Student Entrepreneur Initiative for the idea of using peptides to improve the stability and activity of industrial enzymes. He also collaborated with Neal Woodbury and Hao Yan to write a proposal of "Enzymology of multi-enzyme systems on self-assembled surfaces" which was founded by National Science Foundation. Mr.Fu was a student member of American Chemical Society. He joined the summer program of "Science Policy at Washington D.C." organized by Center of Nanotechnology Society in 2009. He was also a mentor for SOLUR undergraduate research program at ASU, and had supervised six undergraduates and one high school students in the lab.

PUBLICATIONS:

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