Selection and characterization of lipase abzyme from phage displayed antibody libraries

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Abstract

Antibodies with enzymatic activity were named abzymes or catalytic antibodies. In the present study, the lipolytic abzymes were selected from the phage displayed antibody libraries against a transition state analog (TSA) of lipases/esterases. After three rounds of selection, four monoclonal phage particles capable of binding significantly with the TSA were obtained. The soluble scFv antibody fragments were further expressed and obtained using Escherichia coli strain HB2151. The binding capabilities and the apparent enzymatic activities of the purified antibody proteins were measured. The 3D structures of the expressed antibodies were also predicted through homology modeling and binding-site prediction algorithm. The present method demonstrates that selection from phage displayed antibody libraries is an efficient and convenient means to find new abzymes.

Keywords: Abzyme; Biopanning; Catalytic antibody; Lipase; Phage display antibody library

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are part of the family of hydrolases that act on carboxylic ester bonds. The natural substrates of lipases are triglycerides of long-chain fatty acids, but they also accept hydrophobic esters of primary and secondary alcohols. The versatile functions make it widely applied in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries [1]. Despite of these industrial applications, their uses still remain limited due to high production costs and low performance of some lipase-mediated processes. Previous crystallographic analysis of lipase revealed the presence of a typical α/β hydrolase scaffold comprised of a core of about five parallel β-sheets flanked by several α-helices [2]. Detailed mechanistic studies indicated that the enzymatic hydrolysis may occur with the assistance of a catalytic triad (Ser-His-Asp), which is structurally similar to serine proteases. Another important feature of the majority of lipases is the shielding of the catalytic site from the external environment by loops or lids that lie over the catalytic triad. The lids are displaced to different extent during the process of interfacial activation, allowing the lipid substrate to enter the active site. All lipase also share the consensus peptide GxSxG (x represents any amino acid) as part of the binding site responsible for interfacial activation [3].

Interactions of antigen and antibody resemble to some extents the recognition and specific binding between enzyme and substrate. Apart from its natural function, the immune system can generate a molecular imprint of virtually any natural or synthetic compound. The unique ability of antibodies to complement the input substance was exploited successfully for eliciting antibodies with tailored catalytic activities. Most of these abzymes were created by immunization with template molecules (the transition state analogs), which are stable mimics of rate-limiting transient states or intermediates of the chemical transformation [4].
Phage display technology has been developed and applied extensively for the past few years. Phage display allows specific screening based on binding affinity to a given target molecule by the in vivo selection process. The capabilities of direct coupling of phenotype and genotype making phage display techniques a very useful tool for the selections [5]. The phage displayed random peptide libraries have been successfully used to mimic epitopes of antigens (mimotopes) and induce humoral immune response in animals [6–8]. On the other hand, phage displayed antibody libraries displaying the single-chain variable regions (scFv) of the human antibodies have also been designed and constructed [9,10]. These libraries have been applied extensively in very diverse fields, such as to generate anti-carbohydrate antibodies to recognize the non-reducing terminal mannose residues [11], to select human antibodies as candidates for diagnostic imaging and for obtaining insight into targets displayed in atherosclerotic plaques [12]. The concept of internal image of idiotypic antibodies has provided the basis for eliciting catalytic antibodies. For example, a monoclonal IgM 9A8 that was obtained as an antibody to AE-2 mAb, a known inhibitor of acetylcholinesterase, displayed esterolytic activity [13]. Comparison of active sites of 9A8 and the 17E8 esterolytic enzyme produced against the transition state analog revealed structural similarity although the two antibodies were elicited by two different approaches [14].

Based on the concept, the human catalytic antibodies with glutathione peroxidase activity had been isolated from the phage displayed antibody library [15].

In the present work, we decided to explore the possibility of finding new abzymes with lipase activities. The potential lipolytic abzymes were selected from the phage displayed antibody libraries, the Tomlinson libraries I and J, using the TSA of lipases/esterases as the target. The binding capabilities to TSA and the apparent enzymatic activities of the selected scFv antibody proteins were measured. The molecular structures of the selected antibodies were also predicted and compared with the known lipase structures.

Materials and methods

Lipase activity assay. The commonly used procedure to investigate esterase and lipase activity makes use of p-nitrophenyl ester with aliphatic chains of various length and the release of p-nitrophenol is measured spectrophotometrically at 410 nm [16]. To perform the assay, the target molecules or the lipase (made from Mucor javanicus, Sigma, as the positive control) was dissolved in the lipase assay buffer (100 mM Tris–HCl, 150 mM NaCl, 0.3 mM CaCl₂·2H₂O and 0.1 mM MgCl₂·6H₂O, pH 7.5) and mixed with the substrate solution (1.6 mM 4-nitrophenyl butyrate, pH 9.5) at 4°C overnight. Next day, the tubes were washed three times with 2% MPBS (PBS with 2% skin milk) and blocked with 2% MBPS for 2 h at room temperature. The synthesized TSA (20 mM in DMSO) was diluted to 200 μM with H₂O and incubated in the coated immunotubes for 1 h at room temperature, washed five times with PBS. Then the phage libraries (100 μL, approximately 5 × 10¹⁰ pfu in 2% MBPS) were reacted with the TSA in the tubes for 2 h at room temperature. The unbound phages were washed 10 times (round 1) or 20 times (round 2 and 3) with PBS. The bound phage particles were eluted with 500 μL trypsin (1 mg/mL) after 10 min incubation. The phage particles were used to determine the titer, the further rounds of selection, screening for binding capability by polyclonal phage ELISA or monoclonal phage ELISA, and for the production of soluble scFv antibody fragments.

ELISA binding assay of the polyclonal or monoclonal phage. Binding capabilities of the selected phage protein with TSA were measured by ELISA. Each well was coated with 60 μL avidin (100 μg/mL) at 4°C overnight or 2 h at room temperature. Then the wells were washed three times with PBS and blocked with 2% MBPS for 2 h at room temperature. The selected phages (10¹⁰–10¹³ pfu/mL) were mixed with TSA (100 μM for the experiments, or 0 mM as the control) in 2% MBPS for 1 h at room temperature. Then discarded the phage solution and washed the well three times with PBST (0.1% Tween-20 in PBS) before adding 100 μL of HRP-conjugated anti-M13 antibody (1/2500 dilution in 2% PBST). After incubating for 1 h at room temperature, the wells were washed with PBST for three times and 100 μL substrate solution (100 μg/mL TMB (3,3’,5,5’-tetramethylbenzidine) in 100 mM sodium acetate, pH 6, with 10 μL of 30% hydrogen peroxide added to 50 mL of this solution directly before use) were added to each well and reacted for 2–5 min before the addition of 50 μL 1 M sulfuric acid to stop the reaction. OD₄₅₀ and OD₆₅₀ readings were measured using the ELISA reader to estimate the binding affinity.

Production of soluble scFv antibody fragments. The selected phage was inoculated into E. coli strain HB2151 (a non-suppressor strain). Individual colonies were picked into 2× TY solution (16 g tryptone, 10 g yeast extract, 5 g NaCl in 1 L aqueous solution) with 100 μg/mL ampicillin and 1% glucose and grown until OD₆₀₀ = 0.9. Expression of the scFv was induced by the addition of IPTG (isopropyl β-D-thiogalactoside, 1 mM final concentration) and grown at 30°C overnight. The cells were harvested and pelleted at 4°C and 10,000g for 10 min. Then the cells were resuspended in 40 mL PBS containing 5 mM EDTA and 0.1% Triton X-100, sonicated and centrifuged at 25,000g for 20 min. The supernatant was applied to a Ni-NTA agarose column (1.0 by 10.0 cm) in the presence of 50 mM sodium phosphate, pH 8.0, 0.3 mM sodium chloride and 250 mM imid-
azole. After extensive washing with 100 mL of 50 mM sodium phosphate, pH 8.0, 0.3 mM sodium chloride and 20 mM imidazole, the bound proteins were eluted with 100 mL of 50 mM sodium phosphate, pH 8.0, 0.3 mM sodium chloride and 250 mM imidazole, followed by extensive dialysis against PBS. Purity of the eluted proteins was evaluated by 12% SDS–PAGE [9,19].

DNA sequencing and sequence analysis. The single strand DNAs of the selected monoclonal phages were isolated and the nucleotide sequences were determined by the dideoxynucleotide chain-termination method [20] using a T7 Sequenase DNA sequencing kit (Amersham, USA). Two sequencing primers were used: forward primer 5'-CGA CCC GCC ACC GCC GCT G-3' and reverse primer 5'-CTA TGC GGC CCC ATT CA-3'. The nucleotide sequences were translated and compared using Vector NTI and tools on the SDSC Biology Workbench (http://workbench.sdsc.edu).

Molecular modeling. The amino acids of each of the selected scFv were submitted to Swiss-Model (http://swissmodel.expasy.org) for homology modeling and visualized through the Swiss-PDB Viewer (http://us.expasy.org/spdbv/). Molecular modeling calculations were also carried out using Insight II package (Accelrys, San Diego, CA). The templates in homology modeling were search using Blast algorithm from NCBI server to build a homology model, which was type set by CHARMM force field [21]. The initial model was quickly optimized using the robust steepest descent (SD) algorithm without solvent effect to exonerate initial bad contacts within the system. To account for the screening effect of solvent, the generalized Born with a simple switching (GBSW) model [22] was used as implicit solvent system. To warrant better starting protein geometry for subsequent molecule dynamics calculations, a second optimization was carried out using the Adopted Basis Newton–Raphson (ABNR) method. The system was then gradually heated to 300 K to equilibrate the system. Finally, a lengthy dynamics simulation at constant temperature and temperature (NVT) [23] was performed. To guarantee the system stability, the dynamics trajectories were carefully analyzed and the variations in temperature, total energy, potential energy, kinetic energy, and electrostatic potentials with time were scrutinized. The program DiscoveryStudio (Accelrys, San Diego, CA) was employed to search the cavities and characterize the putative binding sites of the built homology models by placing the system of interest into three-dimensional grid points and selecting a group of contiguous grid points that are within certain distance range from the edge of protein as a binding pocket [24].

Statistical assay. The Student’s t-test was used for statistical analysis. All of the measurements were performed in triplicate and the data were shown as means ± SD. Significance was defined as $P < 0.05$.

Fig. 1. Synthesis of the TSA of lipase, 5-(2-oxo-hexahydro-thieno[3,4-d]imidazol-6-yl)-pentanoic acid 11-[ethoxy-(4-nitro-phenoxy)-phosphoryl]-undecyl ester.
Results and discussion

The present study used the biotinylated suicide-inhibitor of lipase as the selection target [17]. As shown in Fig. 1, the synthesis started with the protection of 11-bromo-undecan-1-ol (compound 1) to avoid side reactions of the terminal hydroxyl group. Then the Michaelis–Arbuzov reaction of trimethylsilyl-protected (11-bromo-undecyloxy)-trimethylsilane (compound 2) with triethyl phosphite yielded the phosphonate, (11-trimethylsilylxyloxy-undecyl)-phosphonic acid diethyl ester (compound 3). Deprotection of compound 3 using sulfuric acid/acetone gave the (11-hydroxy-undecyl)-phosphonic acid diethyl ester (compound 4). Compound 4 was treated with acetic anhydride, produced an acetate end-group, which was then transformed into the corresponding ethylphosphonochloridate, yielded acetic acid 11-(diethoxy-phosphoryl)-undecyl ester (compound 5). It was then esterified with 4-nitrophenol to the phosphonate inhibitor, and yielded acetic acid 11-[ethoxy-(4-nitro-phenoxyl)-phosphoryl]-undecyl ester (compound 6). Compound 6 was treated with p-toluenesulfonic acid, yielded (11-hydroxy-undecyl)-phosphonic acid ethyl ester 4-nitro-phenyl ester (compound 7). Finally, compound 7 was coupled with biotin to give the biotinylated TSA, (11-{1-[4-(2-oxohyaxhydro-thieno[3,4-d]imidazol-4-yl)-butyl]-vinloxy}-undecyl)-phosphonic acid ethyl ester 4-nitro-phenyl ester (compound 8, final yield 0.20 g). The binding capability of the TSA with lipase was confirmed by ELISA using 1/2500 dilution of the biotin-conjugated goat anti-rabbit IgG as to check its linking with avidin coated in the wells (data not shown). The potential inhibitory effects of the suicide inhibitor on the lipase activity were also examined prior to the biopanning process. It was observed that approximately 8 µM TSA can completely inhibit the lipase activity (50 U/mL) in the assay medium.

The synthesized TSA was used as the baits for the panning process. After three rounds of panning process, 36 monoclonal phage clones were picked for binding assay. As shown in Fig. 2A, the primary screening results indicate that the phage supernatants A14, A15, B23, and B24 exhibit relatively higher binding capabilities toward the TSA molecules. The selected phage clones were then inoculated into E. coli strain HB2151 to produce the soluble antibody scFv fragments. The expressed proteins were purified through a Ni–NTA agarose column and the purity of the eluted proteins was evaluated by 12% SDS–PAGE. All

![Fig. 2. ELISA binding assays and lipase activity assays. (A) The primary screening using the ten-fold dilution of the supernatant solution after phage amplification. The shaded columns represent the binding of 20 selected phage clones detected by the HRP-conjugated anti-M13 antibody. The blanks represent the control experiments without coating TSA in the wells. (B) The relative binding capabilities of the antibody scFv proteins. The bindings of the scFv antibodies were detected by the HRP-protein A. (C) The relative lipase activities of the selected scFv proteins.](image-url)
four preparations yielded a single band of 36 kDa except that the yields of B23 and B24 were much less (data not shown). The relative binding capabilities of the antibody scFv proteins were assayed and detected by the HRP-protein A. As shown in Fig. 2B, the protein A14 binds stronger with the TSA. The relative lipase activities of the selected antibody scFv proteins were also measured. As shown in Fig. 2C, the enzyme activities of B23 and B24 proteins are surprisingly higher than that of A14 and A15. The results reveal that the tight binding with TSA may be not necessarily correlated with higher enzyme activity. The discrepancies may also be explained by the fact that although the lipase TSA was used as the target for the selection of abzyme, the water-soluble 4-nitrophenyl butyrate was used as the substrate (instead of using triglyceride) in an aqueous medium for simplifying the enzyme activity assay. Indeed, previous studies indicated that though lipases are water-soluble enzymes, they react efficiently with esters of fatty acids in the aggregated state, but act slowly on monomers of these esters in an aqueous system [25].

The amino acid sequences of the selected scFv (A14, A15, B23, and B24) were deduced and aligned (Fig. 3). The conserved catalytic triad Ser-His-Asp (3) was not found, however, the consensus peptide GxSxG appeared on the Gly-Ser linker regions of the four selected scFv (3, 9). The amino acid sequences of the selected scFv were submitted to Swiss-Model (http://swissmodel.expasy.org) for homology modeling. All of these scFv share more than 70% sequence identity and more than 80% sequence similarity with the chain B of 2GHW [26]. The homology models of these scFv proteins have similar structures, in which a domain with seven β-sheets and a domain with eight β-sheets are linked by a flexible loop as illustrated in Fig. 4A. The putative binding pocket of B24 (as an example) is crowned by six β-sheets and capped by the loop composed of residues Val34, Leu40, Leu42, Arg60, Glu68, Val70, Ser85, Val86, PHE90, Leu103, Met105, Leu108, Arg109, Glu111, Asp112, Thr113, Ala114, Tyr115, Val134, Thr135, and Val136. The pocket is buried deep under the surface of B24 protein with a narrow opening formed by residues Glu68, Ser85, Asp112, and Thr113 as depicted in Fig. 4B. The size of the pocket is more than 120 Å x 13.5 Å, that may accommodate very bulky molecules. The abundance of residues Val, Leu, Met, and Ala in the pocket suggests that the bottom is hydrophobic in nature, but may not give rise to good specificity. On the other hand, the hydrogen-bond interactions may be important at the opening of binding pocket as manifested by the appearance of residues Glu and Asp, resulting in enhanced binding affinity and specificity with small molecules [26].

<table>
<thead>
<tr>
<th>ScFv</th>
<th>Sequence</th>
<th>Structure</th>
<th>Identity</th>
<th>Similarity</th>
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<td>A14</td>
<td>MKYLLP---TAAGG---LLLAAQPMAMEVLLESGGLLVQPGGLRLSACASGFT 50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B24</td>
<td>MKYLLP---TAAGG---LLLAAQPMAMEVLLESGGLLVQPGGLRLSACASGFT 50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B23</td>
<td>MKYLLP---TAAGG---LLLAAQPMAMEVLLESGGLLVQPGGLRLSACASGFT 50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A15</td>
<td>MQLFSGSENEIPAYGSKRLLLLAAQPMAMEVLLESGGLLVQPGGLRLSACASGFT 60</td>
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<td></td>
</tr>
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Fig. 3. Amino acid sequence alignments of the selected scFv.
Similar structural features can also be found from other scFv proteins. Natural enzymes may not be suitable for biotechnological applications. The strategy of creating libraries of mutants and selecting for interesting mutants is increasingly being followed. The success of this strategy depends on the size, quality and diversity of the libraries and, crucially, on the sensitivity, efficiency and discriminating power of the selection technique available [27]. Using phage display techniques, the artificial libraries of antibodies can be used to derive binders to almost any target molecules. The selected antibodies can be used for all the same applications as conventional monoclonal antibodies, but were isolated in a relatively short time and without the need for animal immunization [9]. The present study demonstrates that selection from phage displayed antibody libraries against a TSA can be an efficient and convenient means to find new abzymes. Of course, using the antibodies and the antibody libraries originally generated against a specific TSA can further enrich the population of the binders with higher binding affinity with the TSAs and has a higher opportunity to isolate abzymes of higher activities.

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References


