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The search for a peptide ligand targeting the lipolytic enzyme cutinase

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Abstract

A constrained nonapeptide phage display library was evaluated as a potential source of affinity ligand(s) for purification of cutinase, a lipolytic enzyme. After seven cycles of biopanning, 500 clones were isolated and individually tested for their capability to interact with cutinase. Three out of six sequenced clones carrying a cutinase-specific constrained peptide showed the same insert sequence (CRLHH-WRYC). Sequences of two of the other clones highlighted a LXXW motif as a critical determinant in the make-up of a cutinase-specific sequence. Although the affinity of the most commonly found peptide for cutinase is low, we suggest that LXXW motif may be a suitable starting point in the development of affinity peptides suitable for use in the study and purification of cutinase.
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1. Introduction

Cutinase from *Fusarium solani pisi* is a small (22 kDa) carboxylic ester hydrolase that bridges functional properties between lipases and esterases [1]. Its natural function is to catalyze the hydrolysis of cutin, the insoluble lipid polyester matrix that covers plant surfaces. However, cutinase also catalyses triglyceride hydrolysis, esterification, inter- and intra-transesterification reactions efficiently. This activity profile gives it a number of important applications in industries ranging from those dealing with detergents to foods and chemicals [2]. For this reason, cutinase has been a subject of a number of studies related to its structure, function, purification and application [1–5]. Purification of the enzyme has often been done using non-selective separation techniques. In particular, purification by extraction in aqueous two-phase systems has been intensively studied [6,7]. In such an approach, an interaction of the enzyme with butyrate was found to improve the selectivity of extraction [7] indicating that a selective purification methodology can be achieved.

The availability of a cutinase-specific ligand would substantially facilitate the development of a chromatographic purification process. This study reports an attempt made at a search for such a cutinase-specific ligand using combinatorial selection methodology. Such technologies involve the use of large libraries of compounds made of molecularly interrelated building blocks, e.g. amino acids, which are combined randomly and offer a gigantic pool of potential binding candidates to target molecules of interest. There is currently an increasing interest in probing the combinatorial libraries based on a variety of chemical motifs for suitable affinity ligands [8–11]. Peptide libraries have been a commonly used source of potential binders [8,11,12]. Peptides offer a number of advantages as ligands, especially in terms of operational stability in contrast to protein ligands like antibodies. A few studies on using the selected peptides as affinity ligands, or even as affinity fusion tags binding to a predetermined ligand, have been reported [8,11,13].

In this work, a constrained nonapeptide phage display library was used as a source of potential ligand(s) binding to recombinant cutinase produced in *Saccharomyces cerevisiae*. This allowed us to identify a peptide motif that may

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serve as a starting point in the search for a peptide ligand suitable for purification purposes.

2. Materials and methods

2.1. Reagents

The M13 nonapeptide phage display library allowing display of peptides on phage protein III and *Escherichia coli* K91 were kind gifts from Dr. E. Ruoslahti (Cancer Research Center, La Jolla Cancer Research Foundation, La Jolla, CA, USA). Recombinant cutinase purified from culture supernatant of *S. cerevisiae* was kindly provided by Professor Maarten Egmond (Unilever Research Laboratories, Vlaardingen, The Netherlands). Sulfo-*N*-hydroxysuccinimide (S-NHS) and 1-ethyl-3-(dimethylaminopropyl)-carbodiimide (EDAC) were from Sigma-Aldrich (St. Louis, MO, USA). Synthetic peptides were prepared by Synpep (Dublin, CA, USA). Horseradish peroxidase (HRP)-labeled sheep anti-M13 immunoglobulin and 2′,2′-azino-bis(3-benzthiazoline-6-sulfonic acid) diammonium (ABTS) were from Detection Module Recombinant Phage Antibody System (Amersham Pharmacia, Uppsala, Sweden). All chemicals used were of analytical grade.

2.2. Immobilization of cutinase

Cutinase was immobilized in 96-well microtiter plates by two different modes. The first approach involved simple adsorption to plates with high binding capacity (Costar, Cambridge, MA, USA). Cutinase (5 μg/ml) in 0.1 M sodium phosphate buffer, pH 7.5 (100 μl/well) was incubated overnight at 5 °C on a rocking table. The wells were then washed twice with PBS (140 mM NaCl, 25 mM KCl, 20 mM $NaPO_4$, pH 7.5) containing 0.05% Tween 20 (PBS-T), and twice with PBS. The other method used was covalent immobilization of cutinase to CovaLink plates (Nunc, Roskilde, Denmark). One hundred microliters of aqueous solution containing 500 ng of cutinase and 0.2 mM S-NHS was added per well. The reaction was started by adding 50 μl of 6.2 mM EDAC, and was allowed to proceed overnight on a rocking table at 5 °C. The wells were subsequently washed three times with a solution containing 2 M NaCl, 80 mM $MgSO_4 \cdot 2H_2O$, 0.5% Tween 20 in PBS; the last wash was done on a rocking table for 10 min.

2.3. Phage selection

The selection of phages from M13 nonapeptide library was performed using covalently immobilized cutinase. One hundred microliters of solution containing 10^{12} plaque-forming units per ml (pfu/ml) in TBS-T buffer (0.5% Tween 20, 150 mM NaCl, 50 mM Tris–HCl buffer, pH 7.5) was added per well and incubated for 2 h at room temperature on a rocking table. The wells were then washed 10 times with TBS-T buffer and bound phages were eluted with 0.1 M glycine–HCl solution, pH 2.2. The eluates (100 μl) were neutralized with 75 μl of 1 M Tris–HCl, pH 9.1, and mixed with 200 μl of a fresh culture of *E. coli* K91. The mixture was incubated for 40 min at room temperature in order for the phage particles to infect the *E. coli* cells. The infected cells were grown overnight on a rotary shaker at 37 °C in Luria–Bertani (LB) medium containing 20 μg/ml tetracycline. The cells were subsequently removed by centrifugation and the phages purified by precipitation with 0.15 vol. of PEG/NaCl solution (16.7% (w/v) polyethylene glycol 8000 (PEG 8000) and 3.3 M NaCl). The selection procedure was repeated through six additional cycles of biopanning using the amplified eluate of the preceding selection as the input for the next round of selection.

2.4. Identification of cutinase-specific clones

The phage clones obtained during the seventh cycle of biopanning were plated out and 500 individual clones were cultivated in 250 μl LB medium containing tetracycline (20 μg/ml) in the wells of sterile 96-well microtiter plates (Nunc). After 12 h on a rotary shaker at 37 °C, the cells were spun down by centrifugation (at 3000 rpm for 20 min), 100 μl of the supernatant was added to wells with adsorbed cutinase while 100 μl was added to the plates with adsorbed BSA as control. The binding was allowed to proceed during 2 h on a rocking table at room temperature. The wells were then washed three times with PBS-T and twice with PBS, before measuring the bound phages by enzyme immunosorbent assay (EIA). The most promising 16 phage clones were amplified and selected again in triplicate by addition of 3.5×10^{12} pfu/ml to the wells with adsorbed cutinase as described above.

2.5. Enzyme immunosorbent assay

HRP-labeled monoclonal anti-M13 antibody diluted in PBS containing 1% non-fat milk powder (100 μl) was added to the plates containing the bound phages (see above), and incubated for 1 h on a rocking table at 22 °C. After washing with PBS-T and PBS, respectively, 100 μl of substrate solution (2.2 mg of ABTS in 10 ml of 50 mM citric acid, pH 4.0, containing 17 μl of 30% H_2O_2) was added. Absorbance at 405 nm was measured after 25–40 min of incubation.

2.6. Phage DNA sequencing

Single-stranded DNA from individual phages clones was purified using a QIAprep Spin M13 Kit (QIAGEN GmbH, Hilden, Germany). The purified DNA was amplified by polymerase chain reaction (PCR) using a primer (5′-CCCTCATAGTTAGCGTAACG-3′) annealing with the pIII gene sequence. The sequences were analyzed with a

373A DNA sequencer using Taq DyeDeoxy terminator cycle sequence kit (Applied Biosystems, Foster City, CA, USA).

2.7. Chromatography of cutinase on column with immobilized peptide

A peptide with the sequence corresponding to the deduced sequence of phage peptide clone D4 (CRLHHWRYC) was chemically synthesized and two molecules of aminohexanoic acid as a spacer were incorporated at the carboxy terminus of the peptide followed by a biotin residue as an affinity handle. The peptide was coupled to a Streptavidin–Sepharose matrix (prepared by Synpep), resulting in a peptide density of 2.3 mg/ml of the matrix. A 1.5 ml (2.3 cm × 0.9 cm) analytical column was loaded with the derivatized matrix and equilibrated first with 50 mM Tris–HCl buffer, pH 7.5, and then with the buffer containing 2 and 10% w/v PEG 8000, respectively. After loading 225 μg cutinase, the column was washed and elution was tried with sodium citrate buffer pH 4.0. Flow rate of 0.2 ml/min was used during the chromatography, fractions of 2 ml were collected, neutralized using 1 M Tris–HCl pH 9.0 wherever necessary, and tested for cutinase activity.

2.8. Assay of cutinase activity

Cutinase activity was determined by measuring the rate of hydrolysis of *p*-nitrophenyl butyrate at 20 °C, pH 8, according to a procedure described earlier [7]. The rate of absorbance change was recorded at 405 nm after addition of 20 μl enzyme sample to 980 μl substrate solution (prepared by adding 0.2 ml of 50 mM *p*-nitrophenyl butyrate (Sigma) to 10 ml solution of 50 mM taurodeoxycholic acid (Sigma), 10 mM Tris and 10 mM NaCl). A reference cuvette containing only the substrate solution was used to correct for background hydrolysis. One unit of enzyme activity was defined as the amount that degrades 1 μmol of the substrate per min under standard conditions.

3. Results

In an attempt to identify affinity ligands for cutinase, the enzyme was immobilized to microtiter plates for selection of binding phages from the library having cyclic nonapeptide peptides expressed on the pIII coat protein. The initial selection was done using covalently bound cutinase in order to minimize conformational changes that may occur on adsorption of the enzyme and also for the higher immobilization efficiency observed. The bound phages were recovered by non-selective elution at pH 2.2.

After seven cycles of selection, 500 clones were isolated and evaluated individually for binding to cutinase. This was preferably done using adsorbed cutinase so as to increase the specificity of phage binding due to the lower amount of enzyme immobilized to the Costar plates as compared to the CovaLink plates. Fig. 1 shows the relative binding strength of 16 promising clones judged from the enzyme immunosorbent assay. This result was the same irrespective of the enzyme immobilization method used. Six of the clones that interacted stronger than the other clones and the original library were sequenced in order to determine the amino acid sequences of the displayed peptides (Table 1). The three clones which showed the strongest interaction (B5, D4, E2) presented the same insert sequence (CRLHHWRYC), and two other clones, E7 and F6, showed a related sequence conserving a LXXW motif. The common feature of the peptides, including the one lacking the LXXW motif, seemed to be the presence of histidines and hydrophobic residues at specific locations (Table 1). In addition to these similarities, the peptide variant (H4) not carrying the LXXW motif also carried positive charges found in the most commonly selected sequence (Table 1), suggesting similarities in the recognition pattern of all selected peptides.

Table 1
Amino acid sequence of the selected clones

Clone	Sequence
B5	C R **L** H H **W** R Y C
D4	C R **L** H H **W** R Y C
E2	C R **L** H H **W** R Y C
E7	C F P **L** P W **W** H C
F6	C V P **L** P W **W** H C
H4	C K H T H Y G K C

Residues forming the LXXW motif are boxed.

Further studies were continued with the phage clone D4 that showed maximal interaction with immobilized cutinase. Phage solution (3.5×10^{12} pfu/ml) was added to wells containing different amounts of immobilized cutinase. Detection of the bound phages by EIA showed an increase in the signal of bound phages with increasing amount of immobilized cutinase, as well as a lack of signal in the absence of cutinase (Fig. 2). This confirmed that the binding was specific to the enzyme, and eliminated the possibility of unspecific binding of the phages displaying hydrophobic peptides to the plastic surface of the wells.

The peptide corresponding to the sequence of the clone D4 (Table 1) was synthesized. The peptide was seen to be insoluble in water and DMSO, and in mixtures of these solvents, but was soluble in citric and acetic acid solutions. Due to the strong inhibitory effect of these organic acids on cutinase, the direct effect of the isolated peptide on the enzyme activity assay could not be evaluated. The peptide was hence modified at the carboxyl terminal end with a spacer of two molecules of aminohexanoic acid and provided with a biotin residue for coupling to a Streptavidin–Sepharose matrix. The binding of cutinase to the peptide matrix was however too weak to be detectable.

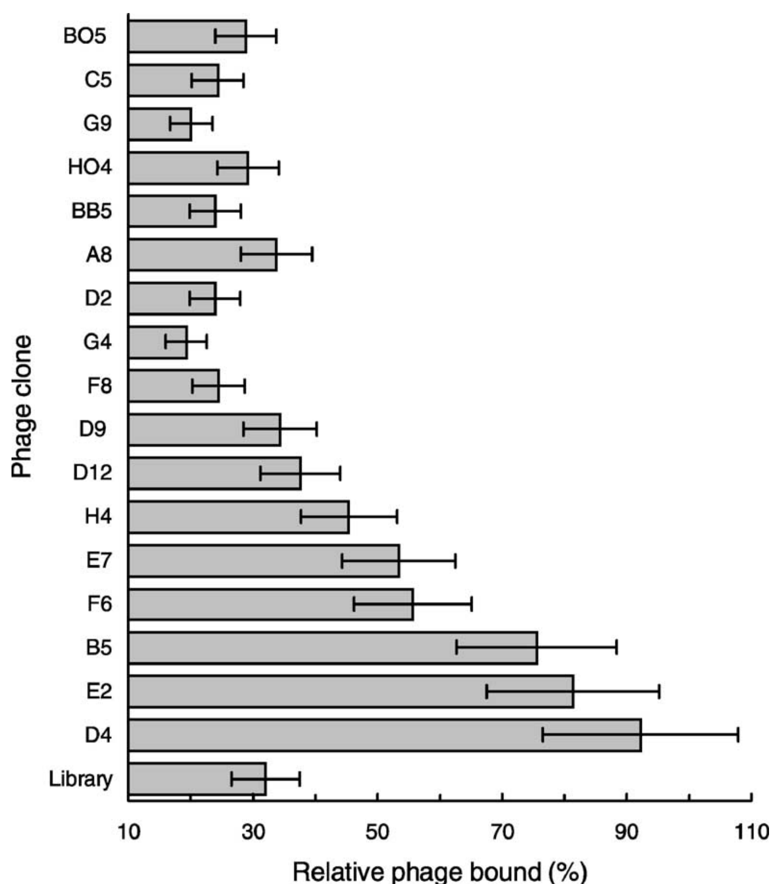

Fig. 1. Relative interaction between the 16 more promising clone phages and immobilized cutinase (by adsorption) measured by EIA.

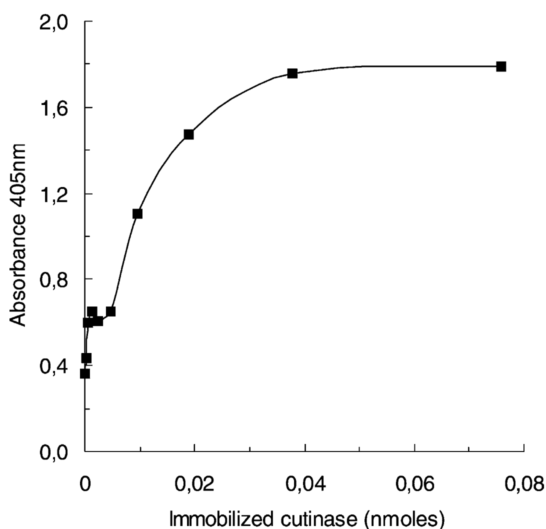

Fig. 2. Relative interaction between the clone D4 and different amounts of immobilized cutinase (by adsorption). Cutinase (0–1.5 μg/ml) was incubated in the wells of high binding capacity microtiter plates. The amount of immobilized cutinase was quantified as the difference between the specific activity added and that remaining in the supernatant after immobilization. One hundred microliters of clone D4 solution (3.5×10^{12} pfu/ml) was added per well and after 1 h incubation at room temperature on a rocking table; the wells were washed three times with PBS-T, and three times with PBS. The bound phages were measured by EIA.

4. Discussion

Selection of potential binders from a phage display peptide library is influenced by a variety of factors related to the library type and the selection conditions used. In an earlier study, peptides selected from a linear heptapeptide library, binding to a lipase from *Rhizomucor meihei*, exhibited no consensus sequence [14]. Selection using the lipase immobilized to polystyrene plates yielded "doublet" clones with two heptapeptide sequences separated by a stretch of amino acids, while immobilization to magnetic beads provided "monomeric" clones with a single heptapeptide sequence. Furthermore, no significant difference in the binding strength with the lipase was observed between the high binding clones from the two groups. The selected peptides on immobilization did not exhibit any binding to the soluble *R. meihei* lipase [14] confirming that peptides selected directly from a random peptide library by themselves often have a low affinity for their target.

In this study, a cyclic peptide library was used for selecting cutinase-specific peptides. Constrained peptides have been shown to bind more tightly to proteins as compared to linear unstructured peptides [11,15–17] that is ascribed to them having a structure in aqueous solution [18,19]. Libraries of such constrained peptides can also display

higher diversity, and their binding properties are less dependent on the context, i.e. between phage-bound and free peptide [18]. The selected peptides displayed on phage clones that showed the highest binding to cutinase (Fig. 1) had a common motif of leucine and tryptophane separated by two other residues (LXXW) (Table 1), and the binding of one such selected phage, D4, was found to be specific for cutinase (Fig. 2), although the synthetic peptide sequence by itself could not be shown to exhibit high affinity binding to the enzyme. Nevertheless, the conservation of the sequence strongly suggests that this structural feature is important for binding of a peptide to cutinase.

As the peptides are selected while they are the integral part of the phage coat protein, it is not very likely that binding to the target protein occurs through the displayed peptide only. Rather additional interactions may be provided by the phage coat protein, thus making the isolated peptide unable to mimic the displayed peptide fully. In fact, a cyclic peptide selected against mammalian serum albumin, when supplemented with a stretch of fixed viral sequence could bind the protein by a 10 times higher association constant, and could then be efficiently used for purification of serum albumin [11]. Furthermore, the interaction of peptide and enzyme in chromatography and in solution suffers, in comparison to the selection process and phage EIA, from a lack of avidity caused by multiple interactions between multivalently displayed peptide on phage and surface immobilized proteins. Such effects most likely contribute strongly to the inability of the D4 peptide to serve as a suitable cutinase-binding ligand in chromatography.

On the whole, there are thus only a limited number of reports concerning application of short peptides selected from phage display libraries as affinity ligands. Discontinuous and longer ligands isolated from phage display libraries have, in contrast, been shown to exhibit strong interactions and good performance in a chromatography format [20,21].

It seems that despite the higher diversity and economic advantages of the phage display peptide libraries as compared to the synthetic libraries, a limitation may lie in the clear-cut selection of binders useful as affinity ligands. There is thus a need for a strategy to convert phage-selected peptides into high-affinity reagents suitable for protein purification. We propose that new peptide libraries, either created by peptide synthesis on beads [22] or displayed on phage, and incorporating the LXXW motif would serve as an appropriate starting point to identify a high affinity peptide ligand-specific for cutinase. We furthermore propose that a change in the library technology used in this second step, that is a move from a phage-displayed to a bead-displayed peptide library would enhance the ability to select a high-affinity peptide carrying the LXXW motif that is not dependent on carrier effects like additional binding interactions provided by protein III, as proposed above. Such an approach may operate to resolve the difficulties in finding appropriate ligands specific for cutinase and in the end provide long-sought peptides suitable for affinity purification of this important enzyme.

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