International Journal of Biological Macromolecules 50 (2012) 1267–1274

Contents lists available at SciVerse ScienceDirect

International Journal of Biological Macromolecules

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

Formation of chitin-based nanomaterials using a chitin-binding peptide selected by phage-display

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ARTICLE INFO

Article history:
Received 21 January 2011
Received in revised form 21 March 2012
Accepted 22 March 2012
Available online 30 March 2012

Keywords:
Phage display
Peptide
Chitin
Nanomaterial
Nanobiotechnology

ABSTRACT

Targeting polymers with peptides is an efficient strategy to functionalize biomaterials. Phage display technology is one of the most powerful techniques for selecting specific peptides for a wide variety of targets. A method to select a chitin-binding peptide from a 12-mer random peptide library was successfully performed against chitin immobilized in wells of microtiter plates. The synthetic chitin binding peptide (ChiBP) could bind to chitin beads and disrupt their structure. This selected peptide was successfully used to immobilize alkaline phosphatase on chitin. In addition, the peptide could induce colloidal chitin in water to form a chitin coat on the surface of plastic tubes. Scanning electron microscopy (SEM) revealed that the peptide could induce colloidal chitin and chitohexaose to form networks when the temperature was raised to 42 °C.

1. Introduction

Chitin, a poly-β-1,4-*N*-acetylglucosamine (GlcNAc), is one of most abundant biopolymers in nature, because it is the main component of the exoskeleton of arthropods, such as insects, arachnids and crustaceans, and is a structural polysaccharide in fungal cell walls [1,2]. It is a cheap renewable biomaterial that is biocompatible, biodegradable and bio-absorbable with antibacterial, wound-healing and immuno-modulating activities; consequently there have been many reports on its biotechnological applications in various fields [3]. Recent research in nanomaterial sciences has suggested potential roles for chitin and its derivatives in the emerging field of nanobiotechnology, which relies on non-specific interactions with chitin [4–10]. Therefore, identifying a method to harness chitin properties, such as assembly, recognition and specificity, will greatly enhance the functionality of this cheap biopolymer. Peptides have been shown to be an effective means to functionalize biomaterials [11]. Selections of various combinatorial phage display peptide libraries have been used to identify peptides that bind to a wide range of inorganic materials and nanostructures [12]. However, so far, there have been no studies on the selection of short peptides that binds to chitin polymers. This is the first study on the identification of chitin binding peptides from affinity selection of a phage display 12-mer random peptide library. Biopanning procedures, the structure of chitin binding peptides (ChiBP) and their interactions with chitin are reported. In addition, we also demonstrated the potential of using this peptide as an efficient strategy to functionalize chitin, which could be developed in the future for use as nanoparticles for drug delivery, surface coating or scaffold for tissue engineering.

2. Materials and methods

2.1. Immobilization of chitin onto a 96-well plate

Three hundred milligrams of chitosan (product number 417963, ≥75% degree of deacetylation (DDA), Sigma–Aldrich) were dissolved in 50 ml of 0.1 M sodium acetate buffer (pH 3.0). Dissolution occurred slowly and was facilitated by putting the material in a capped plastic 50-ml centrifuge tube and rocking at low speed on a rotating platform overnight at room temperature. The resulting material was diluted 1:10 in 0.1 M acetic acid (pH 5.0), and 125-μl aliquots (6 μg) were added into each well of a 96-well microtiter plate (Nunc, Denmark). Following the addition of 35 μl of acetic anhydride, the plate was placed in a fume hood and allowed to dry overnight. The wells were then filled with 1x phosphate-buffered saline (PBS) (4 mM KCl, 1.76 mM KH_2PO_4, 0.14 M NaCl, 10 mM Na_2HPO_4, pH 7.2), which was replaced a few minutes later with 200 μl of the same buffer containing 2% skimmed milk at 37 °C for

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http://dx.doi.org/10.1016/j.ijbiomac.2012.03.016

1 h. This neutralized the surface and blocked nonspecific adsorption sites from remaining in the wells. Following removal of this solution, the plates were ready to use in the next step of the biopanning process. Three wells of three microtiter plates were treated in this way. Plate 1 was used in the first round of biopanning, while Plates 2 and 3 were used for the second and third round of biopanning, respectively.

2.2. Biopanning of chitin binding peptide

Three rounds of biopanning were undertaken with chitin that had been immobilized in wells of microtiter plates according to a previously published protocol [13]. Briefly, each well was washed three times with PBST (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.1% Tween 20) before adding 25 μl of the 12-amino acid-long random peptide library (SUT12, $\sim 10^{10}$ pfu) in 125 μl of PBST, and incubating at room temperature for 2 h. The wells were then washed five times with PBST, and the bound phages were eluted by adding 50 μl of 50 mM glycine–HCl, pH 2.0. Solutions were then neutralized with 200 mM $NaHPO_4$ (pH 7.5). The eluted phages were amplified by infecting a log-phase *Escherichia coli* K12F′, before being subjected to the second round of biopanning. The eluted phages from the second round of biopanning were used directly for the third round without overnight amplification. After three round of biopanning, individual phage clones were isolated to confirm their specific bindings using Phage ELISA. The phage display library of a random peptide was constructed by cloning DNA inserts assembled from synthetic degenerate oligonucleotides ($NN(G/T)_{12}$) into an M13 vector, such that the random peptides were expressed as N-terminal fusions to the M13 minor coat protein pIII. The complexity of the library is $\sim 10^9$ members [14].

2.3. Phage ELISA

Chitin was coated onto triplicate wells of microtiter plate as described in the previous section. The wells were washed three times with PBST, and then 200 μl of each culture supernatant containing individual phage clones was added into the appropriate wells. Following incubation at room temperature for 1 h, the wells were washed five times with PBST. To detect the bound phage, 100 μl of a 1:5000 dilution of horseradish peroxidase (HRP)–anti-M13 in PBST was added into each well and the plate was incubated at room temperature for 1 h. After that, the wells were washed five times with PBST, and then 100 μl of 2,2′-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) substrate containing 0.05% H_2O_2 was added into each well. After a 20-min incubation, the optical density (OD) at 405 nm on each well was measured with a microtiter plate reader (TECAN, Austria GmbH).

2.4. Binding assay on chitin beads

Two hundreds microliters of 50% slurry of chitin beads (catalog number S6651S, New England Biolab) in a microcentrifuge tube were centrifuged at 4000 rpm for 30 s, and the supernatant was discarded. The beads were then washed with de-ionized water five times before incubation, and with 2% skimmed milk for 1 h to block non-specific binding. After that, the beads were washed three times with 1x PBS (137 mM NaCl 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4), following which 200 μl of culture supernatant containing individual phage was added. After overnight incubation at 4 °C, the beads were washed five times with 1xPBS before the bound phages were detected by adding 200 μl of 1:5000 HRP–anti-M13 and incubated for 1 h at room temperature. After washing three times with PBST, 150 μl of ABTS–0.05% H_2O_2 was added to the tubes and incubated at room temperature for 20 min before the chitin beads were spun down and the supernatants were taken to measure an OD at 405 nm.

To determine the binding of free peptides, 100 μl of 0.5 mM N-terminal biotinylated peptides (ChiBP3 or Control peptide) was incubated with 100 μl of the chitin beads that had been blocked with 2% skimmed milk in a micro-centrifuge tube. After incubation for 2 h at room temperature, the beads were washed five times with TBST (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween). Bound peptides were detected by adding 100 μl of streptavidin–alkaline phosphates (SA–AP), followed by washing with TBST five times. Then, 100 μl of p-NPP substrate (Sigma Fast TM) was added and incubated at room temperature for 20 min before the chitin beads were spun down and the supernatants were taken to measure an OD at 405 nm.

2.5. Construction and binding of alkaline phosphatase (AP) fusion peptides

Synthetic oligonucleotides encoding wild type and G → R mutant ChiBP3 peptides were annealed to generate double strand DNA fragments containing appropriate 5′ overhangs for cloning into the pKP300deltaIII expression vector that was predigested with *MFe*I and *Sal*I restriction enzymes. The expression of peptide–AP fusions was under the control of a phoA promoter and could be induced by lowering the concentration of phosphate in the medium [15]. Integrity of the constructs was confirmed by automated DNA sequencing (Macrogen, Korea). AP-fusion peptides were collected from the cell lysate after induction for ~10 h. Bound AP fusion–peptides were detected by using para-nitrophenylphosphate (p-NPP) substrate as described above.

2.6. Formation of macroscopic structure

2.6.1. Colloidal chitin and ChiBP3

A solution of colloidal chitin at a concentration of 5 mg/ml in water was prepared according to a previously published method [16]. One hundred microliters of this solution was mixed with 100 μl of various concentrations of peptides, i.e., 1 μg/ml (0.7 μM), 100 μg/ml (70 μM), and 200 μg/ml (140 μM). They were placed in a thermomixer (Eppendorf), which had been set at 42 °C. After 15 h, the tubes containing the solution were taken and left at room temperature. The SEM images were taken after the samples were left at room temperature for 15 h.

2.6.2. Chitohexaose and ChiBP3

One hundred μl of 0.7 μM (100 μg/ml) ChiBP3 peptide in water and 100 μl of 5 μM chitohexaose (Seikagaku, Tokyo, Japan) in water were mixed and incubated at 42 °C for 15 h before being subjected to scanning electron microscopy (SEM) analysis.

2.7. Scanning electron microscopy (SEM)

Glutaraldehyde was added to the samples for about 2 h before being removed. Samples were first washed by phosphate buffer and then by increasing the concentration of alcohol (ethanol, 30, 50, 70, 90, 95, and 100%, respectively). After putting the samples in critical point drying machine (CPD, samdri-PVT-3B, TOASIMIS CO), they were then coated with gold (JOEL, JFC-1100) in preparation for photomicrography by SEM; JEOL, JSM-6400.

2.8. FTIR spectroscopy

The ChiBP3 peptide was dissolved in distilled water at 10 μg/ml. Its secondary structure was determined at different temperatures