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.

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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

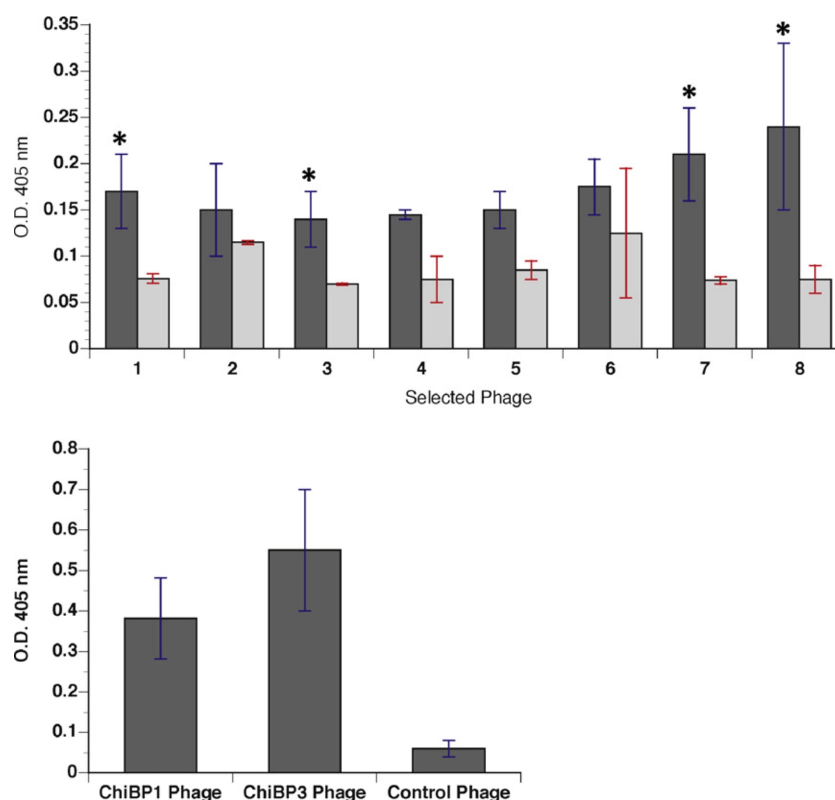

Fig. 1. Specific binding of phage to chitin. Top panel: eight phages from the third round of biopanning were picked and tested for their binding to chitin by Phage ELISA, as described in Section 2. Triplicate wells of microtiter plate were coated with chitin (dark bar) or skimmed milk (light bar) and incubated with the selected phage. Phage clones that showed a binding signal two times higher than the control are indicated by an asterisk, and were subjected to DNA sequence analysis. Average $OD_{405\,nm}$ values are shown with standard error. Bottom panel: confirmation of specific binding of ChiBP1 and ChiBP3 phage clones was demonstrated using chitin beads. The control phage was the phage that was selected from the same library. Binding of the phage to chitin beads was detected immunologically with an anti-phage antibody conjugated to horseradish peroxidase (HRP), followed by colorimetric reaction with ABTS substrate. Average OD values at 405 nm are shown for duplicate tubes, along with standard error.

by Fourier Transform Infrared (FTIR) spectroscopy. The measurement was performed at the Synchrotron Light Research Institute (SLRI), Nakhon Ratchasima, Thailand, using a Bruker Vertex 70 FTIR spectrometer (Bruker Optics Inc., Ettlingen, Germany) equipped with a nitrogen cooled MCT (HgCdTe) detector over a measurement range of 4000–600 cm^{-1}. The protein ChiBP3 solution was introduced in a sealed flow-through liquid cell AquaSpec (optical window: calcium fluoride, 4 mm thick) with 7 μm path length, including a mount for a thermostat controlled at 4 °C, 25 °C and 42 °C. FTIR spectra were recorded with a resolution of 4 cm^{-1} and 64 scans. The peptide secondary structure was analyzed from the shape of the amide I band by non-linear regression fitting of Lorenzian and Gaussian peaks to the original spectra. Before curve-fitting was performed on the amide I band, a straight baseline passing through the ordinates at 1760 and 1600 cm^{-1} was subtracted manually using scattering correction mode.

The best fit for decomposing the amide I band was obtained by Gaussian and Lorentzian components using OPUS 6.5 software (Bruker Optics Ltd., Ettlingen, Germany). The band position corresponding to the α-helix structure was fixed at 1656 cm^{-1}; the β-sheet structures were fixed at 1629 cm^{-1} and 1693 cm^{-1}; the β-turn structure was fixed at 1668 cm^{-1} and 1680 cm^{-1}; and the random coil structures were fixed at 1643 cm^{-1}. The curve fitting process was performed automatically until a satisfactory fit between the computed and experimental band was obtained. The percentages of these secondary structure components were calculated as the ratio of the corresponding peak areas to the total of the amide I peaks.

3. Results and discussion

3.1. *Affinity selection of chitin binding peptide*

Target immobilization on solid support is the key step for biopanning of a phage display library. In this research, the first challenge was to find a method to immobilize chitin onto the wells of a microtiter plate. Previous work by Bernard et al. [17] has shown that simultaneous treatment of microtiter plates with chitosan, a deacetylated form of chitin, and acetic anhydride can produce a surface-bound film of chitin. However, we were not able to isolate any specific phage clone using the amount of immobilized chitin described by this method. Therefore, we have optimized the immobilizing protocol by increasing the amount of chitosan and acetic anhydride per well to 6 μg and 35 μl, respectively, as well as by increasing the time of drying from 12 to 18 h, under laminar flow. This method yielded a sufficient amount of immobilized chitin for successful affinity selection of specific chitin binding phage after three rounds of biopanning. The Phage ELISA result for eight selected phage clones from the third round of biopanning is shown in Fig. 1 (top panel). Four phage clones that showed binding signals two times higher than the background were selected for DNA sequence analysis. The amino acid sequences of the four clones are shown in Table 1. Clone 3 and clone 7 were the same clones as they have an identical DNA sequence. Clones 1 and 3 were selected for further analysis because they shared certain similarities, i.e. both were rich in glycine and glutamic acid, as well as amino acids that can provide salt bridges, such as glutamic acid, aspartate, and lysine.

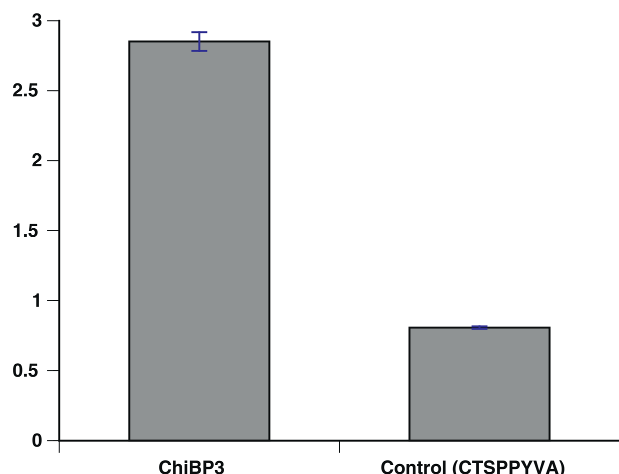

Fig. 2. Binding of free ChiBP3 peptide to chitin beads. Biotinylated ChiBP3 peptide or control peptide (CTSPPYVA) was incubated with chitin beads in a microcentrifuge tube at 4 °C, overnight. Bound peptide was detected by colorimetric assay using streptavidin–alkaline phosphatase (SA–AP) followed by para-nitrophenyl phophatase (pNPP) substrate. Average OD values at 405 nm are shown for duplicate tubes, along with standard error.

Table 1
Amino acid sequence of various peptides in this study.

ChiBP1	GEVGEQEKARVG
ChiBP3	EGKGVEAVGDGR[a]
ChiBP7	EGKGVEAVGDGR[a]
ChiBP8	AEPDATGWRSLG
mtCBP	EGKRVEAVGDGR
Control free peptide	CTSPPYVA
Control phage displayed peptide	EGVNKSGAQNIS

[a] These two clones are identical.

This is the first report on the selection of chitin binding peptides from a phage display peptide library. The reason for our success might be that a sufficient amount of chitin could be stably immobilized on a well of an ELISA plate, allowing simple biopanning on a microtiter plate, which is the most common and efficient method for performing affinity selection [13]. Immobilization of chitin as beads, which are not equal in size, tends to trap non-specific interaction, causing high background and retrieval of non-specific binders. Moreover, the main interaction between peptides and neutral sugars is one of hydrogen bonding, which is not a full-charge electrostatic interaction, and is therefore rather weak [18]. Previous biopanning attempts could only isolate peptide that interacted with chitotriose in an oxidized state. In that protocol, media including organic solvent were used in order to emphasize hydrogen bonding during the biopanning of a random 9-mer peptide library against chitotriose-agarose, packed in a column [18].

3.2. Specific binding of chitin binding peptide (ChiBP)

To confirm specific binding of selected phage clones to chitin, we performed binding experiments using chitin in different formats. Fig. 1 (lower panel) illustrates specific binding of phage displaying ChiBP1 and ChiBP3 to chitin beads when compared to the control, which was the non-binder phage selected from the same library against a non-related target (Table 1). The result clearly showed that only ChiBP1 and ChiBP3 phage clones could bind to chitin beads. In the next step, to demonstrate that the chitin binding activity of ChiBP is independent of the phage coating, we performed an experiment using a synthetic peptide. The ChiBP3 sequence was selected for this assay because it showed a higher binding signal and was selected twice from biopanning. As shown in Fig. 2, N-terminal biotinylated ChiBP3 showed specific binding to chitin beads when compared to a control peptide (CTSPPYVA), which was available in our laboratory. We would like to note that ChiBP3 is

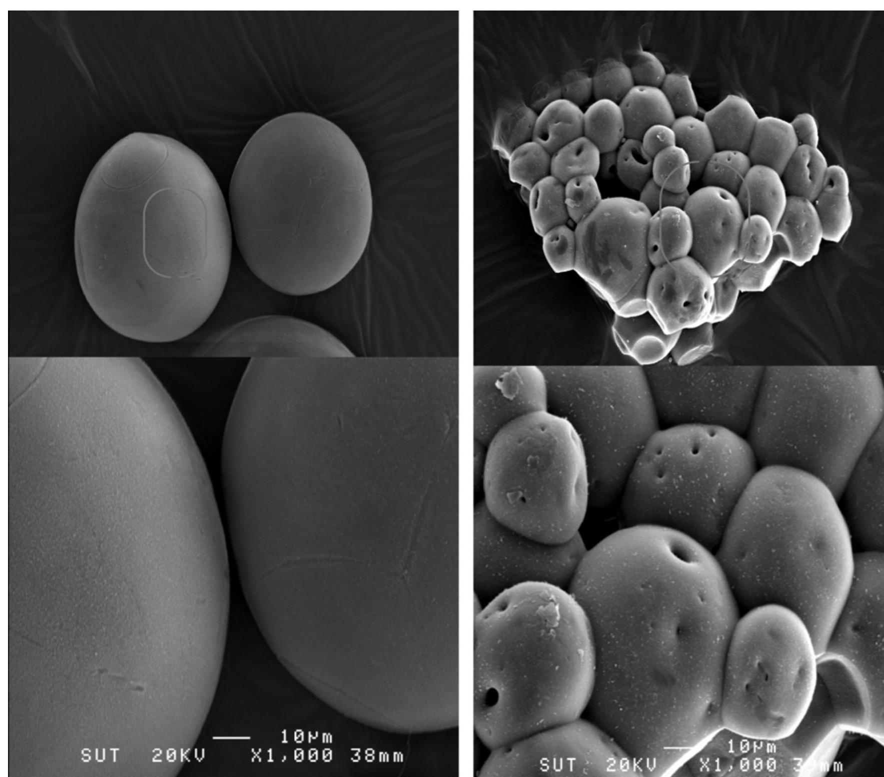

Fig. 3. Shape of chitin beads. Scanning electron microscopy (SEM) images of chitin beads in the absence (left panel) or presence (right panel) of 50 μg/ml of chitin binding peptide (ChiBP3). The upper panel shows images at 350× magnification and the lower panel shows images at 1000× magnification. Magnifications are also shown in bars.

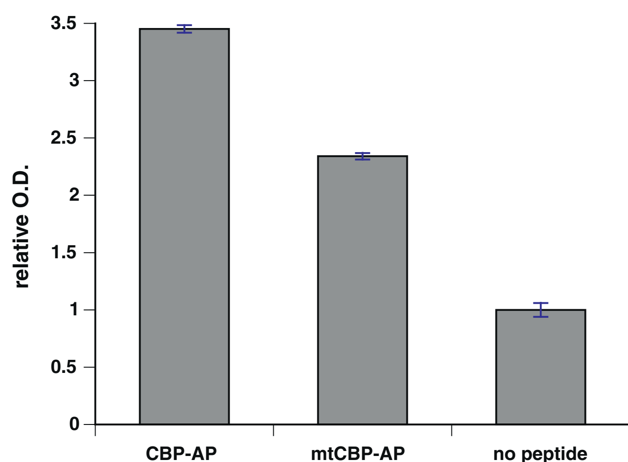

Fig. 4. Binding of alkaline phosphatase (AP)-fusion peptides. Crude *E. coli* lysate containing AP-fusions of chitin binding peptide ChiBP3 (CBP-AP) or G → R mutated peptide (mtCBP-AP) were incubated with chitin beads in a micro-centrifuge tube at 4 °C, overnight. Bound peptide was detected by colorimetric assay using para-nitrophenyl phophatase (pNPP) as substrate. Average OD values at 405 nm are shown for duplicate tubes, along with standard error.

hydrophilic and can be dissolved in water, whereas the control peptide had to be dissolved in dimethyl sulfoxide (DMSO); therefore, this experiment was performed in the presence of equal amounts of DMSO to rule out the possibility that DMSO could interfere with the binding.

To observe the effect of the peptide on chitin beads at a macromolecular scale, scanning electron microscopy (SEM) was carried out. The result displayed in Fig. 3 shows that the ChiBP peptides could aggregate the beads and change their shape. We suspect that peptide could cross-link the beads and glue them together. Moreover, it might be able to self-assemble, strengthening the network of the beads. We suspect that the binding of the peptides might have disrupted the chitin organization of the beads, creating holes and leakage of the fluid from inside, and consequently shrunk them. Nevertheless, the actual mode of this interaction remains to be explored.

3.3. Binding of chitin binding peptide–AP fusion

To confirm specific binding of ChiBP3 peptide and to demonstrate its application in the functionalization of chitin nanomaterial, the wild type ChiBP3 peptide was fused to the N-terminal of bacterial alkaline phosphatase (AP) by a genetic engineering technique (CBP-AP), as described in the Materials and Methods section. In addition, a mutant ChiBP3 peptide, of which one glycine (G) was replaced with arginine (R), was also generated (see Table 1). G was a small non-polar amino acid that is abundant in the ChiBP3 peptide; whereas R is a relatively long, basic amino acid, and is likely to interfere with the interaction of the peptide with chitin. The chitin binding activities of wild type and mutant peptide–AP fusions were demonstrated on chitin beads, as shown in Fig. 4. *E. coli* cell lysates containing peptide–AP fusion were used directly in the binding experiment, as has been previously reported [15,19,20]. The $OD_{405\,nm}$ of the AP fusion to wild type ChiBP3 peptide (CBP-AP) showed approximately 3.5 folds higher than the control (*E. coli* lysate expressing empty expression vector); whereas the binding signal of the G–R mutant, ChiBP3 (mtCBP-AP), decreased to ~30%. This result suggested that glycine plays a role in the interaction of ChiBP3 to chitin. In addition, it also indicated that this peptide could be used to functionalize chitin by fusion to various molecules.

Table 2
Effect of temperature on the secondary structure of ChiBP3 protein.

Conformational element	Wavenumber (cm^{-1})	Area (%)		
		4 °C	25 °C	42 °C
α-Helix	1656	16.1	17.3	18.2
β-Sheet	1629, 1693	27.5	26.0	23.6
Random coil	1643	15.6	14.7	13.4
β-Turn	1668, 1680	40.8	42.0	44.7

3.4. Analysis of chitin binding peptide

Amino acid sequence analysis of ChiBP3 revealed that the peptide has a molecular weight of 1173.25 and isoelectric point of 4.43. The peptide is acidic with a total charge of −1. To imagine the structure of this peptide, different programs were used, since there is no specific and precise program with high enough reliability to predict the secondary structure of such a short peptide. Analysis using Network Protein Sequence [21] indicated that both ends of the peptide have a coil structure, while its middle part has a sheet structure. By using information from this predicted secondary structure, an artistic impression of the peptide was drawn using ChemOffice and WebLab View software, as indicated in Fig. 5, left panel. This model was obtained after energy minimization and running a molecular dynamics (MD) simulation in an aqueous condition. We speculate that the acidic and basic charge residues at both ends of the peptide are responsible for electrostatic interaction with the chitin polymer. In addition, the acidic and basic residues might be able to make a salt bridge, which stabilizes the conformation of the peptide in solution; the peptide might therefore be able to self-assemble via the middle hydrophobic section [22,23].

In addition to computational analysis, secondary structure alteration of ChiBP3 protein under temperature change was determined by FTIR spectroscopy. The FTIR (amide I region) spectra and the Gaussian and Lorenzian curve-fitting of the amide I spectrum of the ChiBP3 protein at different temperature are shown in Fig. 5, right panel. The individual fitting bands are presented according to their assignments: α-helix, 1656 cm^{-1}; the β-sheet, 1629 cm^{-1}, and 1693 cm^{-1}; the β-turn, 1668 cm^{-1}, and 1680 cm^{-1}. The two extra bands (1709 and 1727 cm^{-1}) presented in the fitting curve can be ascribed to the C=O vibration of glutamic acid (two residues) and aspartic acid (one residue) in the peptide chain, and were thus not included in the protein secondary structure calculation. The result of this fit suggested that over 40% of ChiBP3 contains β-turn structure and ~27% of β-sheet, ~15% random coil, and approximately 16% of α-helix structure. This result was in accordance with the secondary structure predicted by Network Protein Sequence Analysis as described above, which indicated that the beta sheet was a dominant structure. The effect of temperature on the change of ChiBP3 secondary structure is presented in Table 2. The raising of α-helix structure was observed when the temperature was increased. The partial loosening of the β-sheet and random coil structure was detected when the temperature was increased from 4 °C to 42 °C. At 42 °C, the β-turn structure was increased significantly.

3.5. Peptide-induced surface coating of colloidal chitin

Specific interaction of ChiBP3 to chitin-coated wells and chitin beads from the previous experiments prompted us to test its activity on colloidal chitin, which is the cheapest form of chitin that can be dissolved in water [16]. We suspected that the peptide might be able to induce colloidal chitin to form a network, hence inducing it to form a coat over a specified surface. To test this hypothesis, a solution of 0.5% colloidal chitin in water was incubated overnight for 15 h at 42 °C with various concentrations of ChiBP3. Then, the tubes were laid down and the temperature was lowered to room

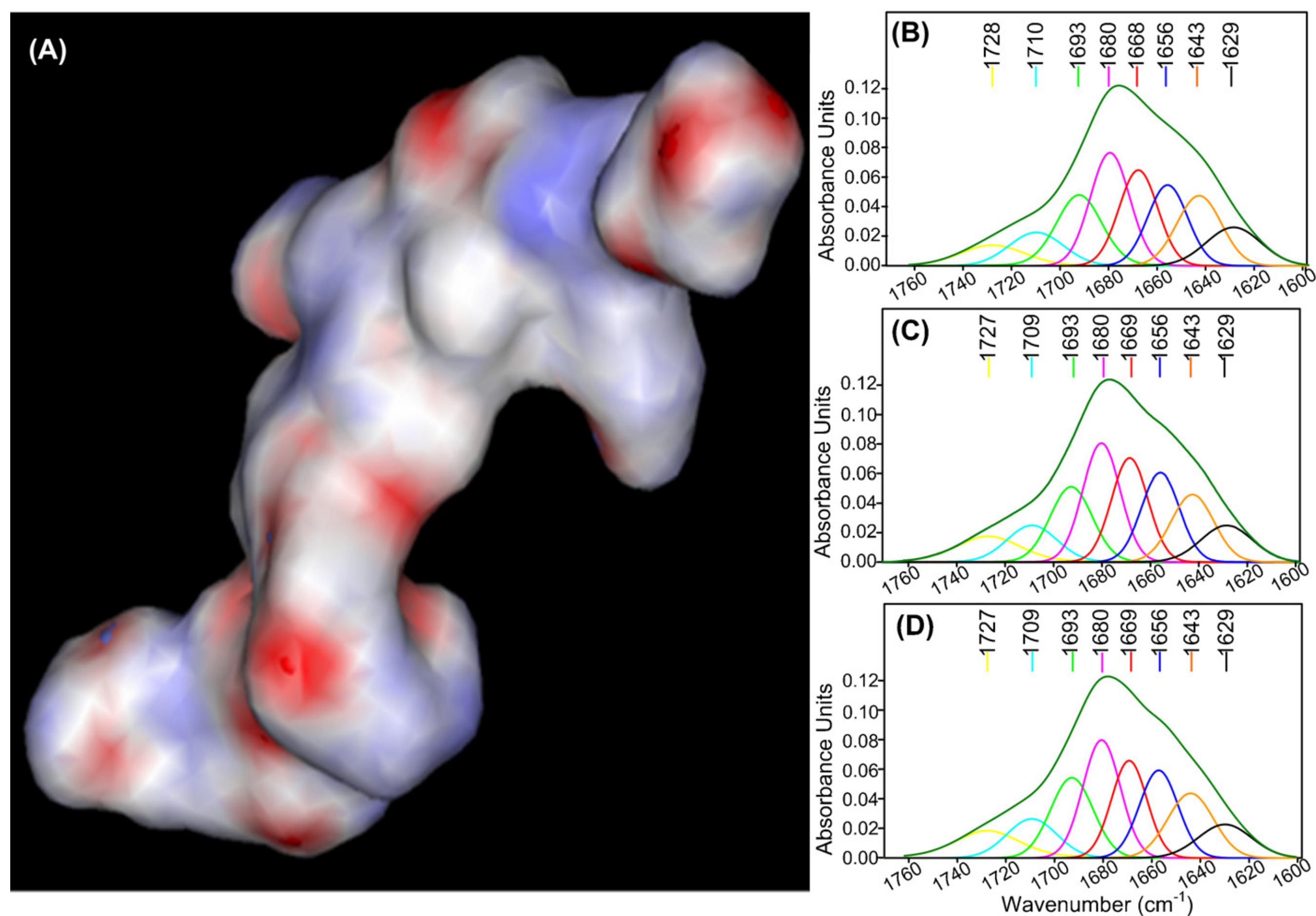

Fig. 5. Structure of chitin binding peptide ChiBP3. Left panel: the image of the ChiBP3 peptide was created using ChemOffice and WebLab View software [21]. The peptide comprises coil structures at both ends and a sheet structure in the middle. Charged residues are found at both ends, while its middle part mainly contains hydrophobic residues. Right panel: secondary structure analysis of ChiBP3 protein measured by FTIR at 4 °C (top), 25 °C (middle), and 35 °C (bottom) is shown. The original spectrum and the result of Lorenzian and Gaussian curve-fitting of the amide I spectrum of the ChiBP3 protein are presented in the coincident full line. The individual fitting bands are presented according to their assignments: 1656 cm^{-1}: α-helix; 1629 cm^{-1} and 1693 cm^{-1}: β-sheet; 1643 cm^{-1}: random coil; 1668 and 1680 cm^{-1}: β-turn.

temperature. During this time, we observed a patch of chitin only on the side of the tube with ≥50 μg/ml peptide (Fig. 6A). After discarding the contents inside the tubes and adding Congo red solution, the chitin coat on the side of the tube could be clearly observed, as shown in Fig. 6B. Investigation of this chitin coat using SEM revealed that it appeared as a tightly packed porous structure (Fig. 6C and D), which is attractive for tissue engineering [2,5,24]. This result suggested that the peptide could be used to induce the formation of chitin coat on any desired surface. This strategy is simple, versatile and easy to modify. More importantly, no special solvent is needed; therefore, it might be applicable for numerous surface-coating purposes.

The rationale behind the above-mentioned experiment is based on observation of the biological self-assembly system of various types of peptides [25,26]. Zhang and Altman [27] suggest that there are three types of self-assembling peptides that have been engineered. Type I peptides undergo intermolecular self-assembly; Type II peptides undergo self-assembly and disassembly, i.e. intermolecular and intramolecular self-assembly under the influence of various conditions. Type III peptides undergo self-assembly onto surfaces [27]. Therefore, we propose that the ChiBP3 peptide is a Type II peptide that undergoes self-assembly and disassembly, i.e. intermolecular and intramolecular self-assembly under the influence of different temperatures. The 42 °C temperature used in this study was arbitrary. We suspected that by alleviating the temperature, the peptide would possess a structure that favors interaction with colloidal chitin and, upon cooling down, a patch of peptide–chitin network could be formed via cross-linking or self-assembly of the peptides. In fact, we found that the peptide could not induce chitin coat formation if the tube was left at room temperature without prior heating (data not shown). The actual mechanism of peptide-aided coating of colloidal chitin and the optimal conditions for forming desired coats must be further explored.

3.6. Formation of peptide–chitohexaose macromolecular network

In addition to chitin polymer, we also investigated the activity of the peptide on chito-oligosaccharide. ChiBP3 peptide was mixed with chito-hexamer in a similar fashion, as had been peptide and colloidal chitin in the previous experiment. After an overnight incubation at 42 °C, a structure of the network formed by ChiBP and chitohexaose could be observed by SEM, as shown in Fig. 7. The network is porous but less dense than that of ChiBP3 and colloidal chitin. This biomolecular network is clearly induced by the peptide ChiBP3 because it could only be found in the presence of 50 μg/ml of the peptide (right panel). This result indicated that, in addition to chitin polymer, the ChiBP peptide could also interact with chito-oligosaccharide, and induce the formation of a biomacromolecular network. Since chitin and chito-oligosaccharide [28] are biodegradable, biocompatible and non-toxic, the porous structure formed by peptide-induced chitin or chitin derivatives could serve as a scaffold for tissue engineering or an attractive platform for targeted

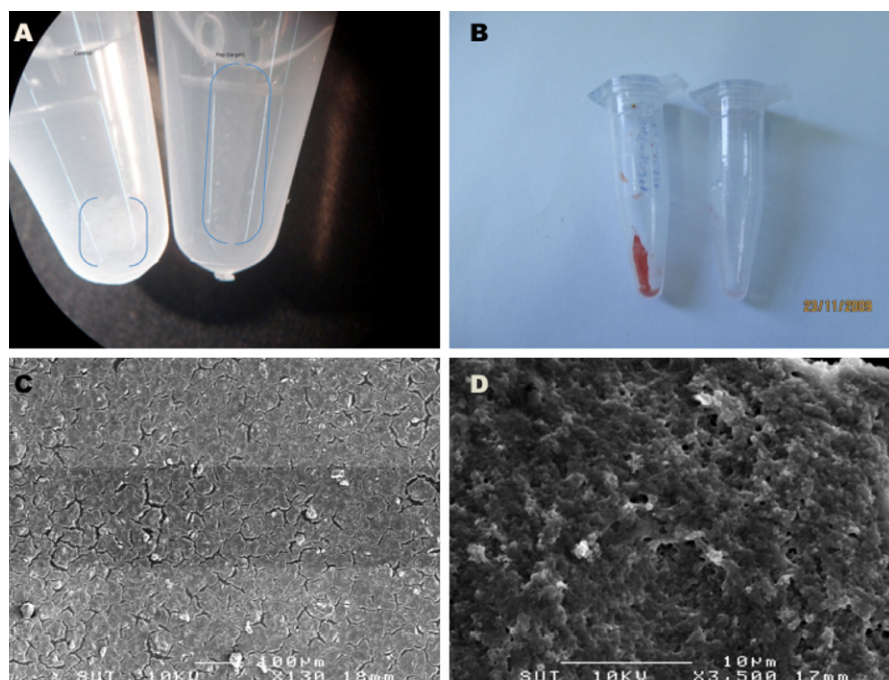

Fig. 6. Peptide-induced coating of colloidal chitin. A simple coating experiment was demonstrated in a microcentrifuge tube. (A) The left tube contained only 0.25% colloidal chitin, whereas the right tube contained both 0.25% chitin and 50 μg/ml (35 μM) of ChiBP3 peptide. A chitin patch could only be observed after an overnight incubation of colloidal chitin (in water) in the presence of ChiBP3 peptide at 42 °C. (B) After the content inside the tube was discarded, the chitin coat on the surface of the tube can be clearly seen as a red patch after staining with Congo red. (C) and (D) are SEM images of the chitin coat at 130× and 3500× magnification, respectively. Magnifications are also shown in bars. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

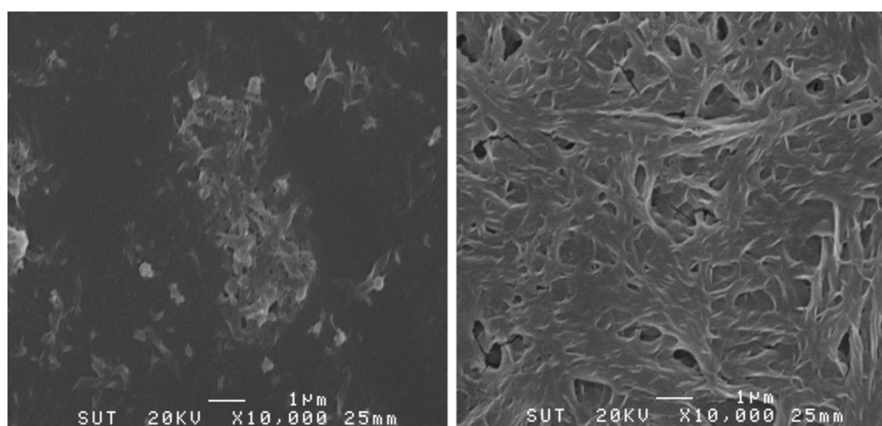

Fig. 7. Peptide-induced formation of chitohexaose network. SEM images of samples after incubating 5 μM chitohexose in the absence (left panel) or presence of 50 μg/ml (35 μM) ChiBP3 peptide (right panel) at 42 °C, overnight. Biomacromolecular network could only be seen in the sample that contained both peptide and chitohexaose.

drug delivery [5]. Further optimization of various factors such as temperature, time, pH, salt, and ratio between peptide and chitin or chitin-derivatives, needs to be undertaken in order to obtain a biomacromolecular network of desired properties for various applications.

4. Conclusions

This research reports an efficient technique for the identification of a novel short chitin binding peptide (ChiBP) by applying phage display technology. There have been previous reports on the identification and characterization of chitin binding domains (CBD) [29,30] or chitin binding proteins (CBP) [31,32] from different organisms. In addition, protein purification kits based on the interaction between CBD-tagged protein and chitin beads are available commercially [17]. However, the ChiBP peptide presented in this study is much smaller than these chitin-binding domains or proteins, and therefore its applications are different. In this work, peptide-aided formation of chitin-based nanomaterial was demonstrated for the first time. Since peptides are biocompatible and can be easily engineered to tag any interesting proteins, this finding could provide a new approach to chitin research and accelerate the development of chitin-based nanoparticles for numerous applications in nanobiotechnology in the future.

Acknowledgments

This work was financially supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission and Suranaree University of Technology (SUT) and National Research Council of Thailand (NRCT). We would like to thank Miss Suchintana

Chumseng for excellent technical assistance. FK thanks James Ketudat Cairns, Kenneth Haller and Kritsana Sagarik for their useful discussions.

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