การจำแนกและวิเคราะห์คุณสมบัติของเปปไทด์ที่จับกับไคติน จากคลังลูกผสมของฟาจ

นายเฟเซล ควูช้อบ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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IDENTIFICATION AND CHARACTERIZATION OF CHITIN BINDING PEPTIDE FROM PHAGE DISPLAY COMBINATORIAL LIBRARY

Feisal Khoushab

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Science in Biotechnology

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IDENTIFICATION AND CHARACTERIZATION OF CHITIN BINDING PEPTIDE FROM PHAGE DISPLAY COMBINATORIAL LIBRARY

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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เฟเซล ควูช้อบ : การจำแนกและวิเคราะห์คุณสมบัติของเปปไทด์ที่จับกับไคตินจากคลังลูกผสมของฟาจ (IDENTIFICATION AND CHARACTERIZATION OF CHITIN BINDING PEPTIDE FROM PHAGE DISPLAY COMBINATORIAL LIBRARY) อาจารย์ที่ปรึกษา รองศาสตราจารย์ ดร.มณฑารพ ยมาภัย, 72 หน้า.

ไคตินเป็นหนึ่งในสารโพลิเมอร์ที่พบเป็นจำนวนสูงมากในธรรมชาติ ซึ่งสามารถถูกย่อยสลายได้ มีคุณสมบัติในการต่อต้านเชื้อจุลินทรีย์ และมีความสามารถต่ำในการกระตุ้นระบบภูมิคุ้มกันของร่างกาย เทคโนโลยีการแสดงโปรตีนบนผิวเฟจสามารถนำมาใช้ในการศึกษาอันตรกริยาระหว่างเปปไทด์และโปรตีน ต่อวัสดุชนิดต่าง ๆ ได้อย่างกว้างขวาง ส่วนเปปไทด์สามารถนำมาใช้ในการรวมตัวกับวัสดุหลายชนิดเพื่อให้เกิดเป็นโครงสร้างโมเลกุลขนาดใหญ่ ด้วยแนวทางการรวมตัววัสดุแบบจากเล็กไปหาใหญ่ได้ ในงานวิจัยนี้ วิธีการในคัดเลือกเปปไทด์ที่มีความจำเพาะต่อไคตินได้ถูกพัฒนาขึ้นและสามารถนำไปใช้ในการคัดเลือกเปปไทด์หลายชนิด ที่มีความจำเพาะเจาะจงต่อไคตินจากคลังเปบไทด์หลากหลายบนผิวเฟจ ความจำเพาะในการจับของเฟจที่ได้รับการคัดเลือกมาสามารถแสดงได้โดยการวิเคราะห์บนจานพลาสติกหลายหลุม หนึ่งในเปปไทด์ไคติน (ChiBP) ที่คัดเลือกมาได้คือเปบไทด์ ChiBP3 นั้น สามารถชักนำให้ไคตินที่เป็นที่มีความยาว 6 หน่วย รวมตัวกันเป็นโครงสร้างเป็นแผ่นเยื่อที่มีรูพรุน นอกจากนั้นแล้ว ยังสามารถชักนำให้คอลลอยดอลไคติน ซึ่งละลายอยู่ในน้ำให้ รวมกันเป็นโครงสร้างแบบแผ่นเคลือบบนพื้นผิวได้ ภาพถ่ายจากกล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่านและแบบครอบคลุม แสดงว่าเปปไทด์ไคตินที่มีความยาว 12 กรดอะมิโน ซึ่งมีคุณสมบัติเป็นกรด สามารถชักนำ อนุพันธ์ไคตินชนิดต่างๆ เช่น เอ็น-อะซิติล-ดี-กลูโคซามีน, โอลิโกเมอร์ความยาว 2, 3, และ 6 หน่วย รวมทั้ง คอลลอยดอลไคติน ให้รวมกันเป็นโครงสร้างที่เป็นระเบียบลักษณะต่างๆ ผลการวิเคราะห์คุณสมบัติของเปปไทด์นี้ พบว่าในส่วนกลางของเปปไทด์มีโครงสร้างไม่ชอบน้ำ ส่วนที่ปลายด้านคาร์บอนและไนโตรเจน มีคุณสมบัติชอบน้ำ ด้วยความจำเพาะของเปบไทด์นี้ต่อไคติน และอนุพันธ์ต่างๆ จึงทำให้มีศักยภาพในการนำไปประยุกต์ใช้ในการสร้างโครงสร้างลักษณะต่างๆ เพื่อประโยชน์อย่างหลากหลาย อาทิเช่น งานด้าน นาโนเทคโนโลยี ด้านวิศวกรรมเนื้อเยื่อ และในระบบการส่งผ่านยา เป็นต้น

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2552 ลายมือชื่ออาจารย์ที่ปรึกษา_____

KUNTALEE RANGNOI : SELECTION OF MONOCLONAL ANTIBODIES SPECIFIC TO AFLATOXIN FROM PHAGE DISPLAY ANTIBODY LIBRARY. THESIS ADVISOR : ASSOC. PROF. MONTAROP YAMABHAI, Ph.D., 83 PP.

PHAGE DISPLAY/SCFV/ ANTIBODY/ NAÏVE /BIO-PANNING/MONOCLONAL /RECOMBINANT /AFLATOXIN/ALKALINE PHOSPHATASE FUSION

Aflatoxins are one of the major mycotoxins that contaminate several agricultural products and human food in many areas of the world. Several methods for aflatoxin determination have been developed, including thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), and immunological method. Among these, the most cost-effective method is immunological analysis that uses monoclonal antibodies as detection reagents. Phage display technology is a powerful alternative method for the production of monoclonal antibody. A specific antibody to aflatoxin B_1 (AFB_1), the most toxic and prevalence isoform, could be isolated by bio-panning from Phage-displayed human single-chain-variable-fragment (scFv) antibody libraries. After three rounds of affinity selection, ninety-six positive clones were induced for the production of soluble scFv antibody fragments by using *Escherichia coli* non-suppressor strain (HB2151), and tested for their ability to bind soluble AFB_1 by competitive enzyme-linked immunosorbent assay (ELISA). Amino acid sequence analysis of the selected clones was performed using information obtained from automated DNA sequencing. ScFv antibodies that could be inhibited by soluble AFB_1 were purified to analyze their structure by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis, and confirm their specific binding to AFB_1 by competitive ELISA. In addition, selected scFv clones were engineered to create scFv- alkaline phosphatase (scFv-AP) fusions and used as

reagents for one-step detection in ELISA format. The scFv-AP fusions showed 3-4 fold improved binding property when compared to soluble scFv form. The IC_{50} of scFv-AP by AFB_1 varied between 0.035-0.02 µg/ml and the limit of detection was approximately 0.006-0.03 µg/ml (6-30 ppb). The selected antibodies were specific to AFB_1 and could cross-react with AFG_1, but not to other aflatoxins, namely AFB_2, AFG_1, AFG_2, AFM_1. These results indicated that phage display technology could be used to obtain a specific antibody against aflatoxin, and that the scFv-AP fusion was an efficient detection reagent that could be further developed to generate a cost-effective diagnostic kit or biosensor for the detection of aflatoxin contamination in agricultural commodities and products in the future.

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Kuntalee Rangnoi

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LIST OF ABBREVIATIONS

°C	degree Celsius
µg	microgram
µl	microlitre
bp	base pair
DNA	deoxyribonucleic acid
et al.	Et alia (and other)
g	gram
h	hour
Kda	Kilo daltal
l	litre
mg	milligram
min	minute
ml	milliliter
mM	millimolar
ng	nanogram
pmol	picomol
rpm	revolution per minute
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume

CHAPTER I

INTRODUCTION

1.1 Introduction

Chitin, a poly-β-1, 4-N-acetylglucosamine (GlcNAc), is one of the most abundant polymers in nature (Cohen-Kupiec and Chet, 1998; Khor, 2002). It is the main component of Arthropod exoskeleton; a group of creatures that includes the insects, arachnids, crustaceans, etc., as well as cephalopods eyes (Herring, 1979), their tendons, and the linings of their respiratory, excretory, and digestive systems (Clark and Smith, 1936). Chitin is also a structural polysaccharide in fungal cell walls. It is biocompatible, biodegradable and bio-absorbable material with low immunogenicity properties and many potential applications, Figure 1, such as coating implantable devices, in tissue engineering, stem cell research, drug delivery etc.

Figure 1. Diverse potential applications of chitin and its derivatives.

The phage display technique was first described by G.P.Smith in 1985 (Smith, 1985). It is a powerful molecular biological tool and is based on the expression of random peptides or proteins on to the surface of a bacteriophage (filamentous bacterial virus) appended to a recombinant viral structural protein (Steingroewer et al., 2007). Phage particles, which are propagated in *E. coli*, are isolated by "panning" against a target bound to a solid-phase support (Mersich and Jungbauer, 2008). Phage display is used for almost any kind of problem that involves the interaction of peptides and proteins with other materials (Chow et al., 2008; Fukusaki et al., 2004; Szardenings et al., 1997). So far, many peptide ligands that can bind to antibodies, enzymes and receptors have been found using phage display library screening. This technique has also been used to identify novel

peptides for therapeutic protein and neuronal targeting (Fukusaki et al., 2004; James K. Liu et al., 2005).

The tremendous potential of applications of chitin is hindered by lack of material development at the micro- and nano-level. Furthermore, using chitin directly, without any changes also helps to take advantage of its mechanical strength when it is needed. Peptides are one of the tools, which offer the potential for harnessing chitin behavior.

In this thesis, phage display technology was applied to find peptide(s) with high affinity and specificity toward chitin. Chitin was immobilized on a solid surface based on a Michael P. Bernard et al, 2004 (Bernard et al., 2004) experiment, in which they have approved that chitosan can be acetylated in the presence of acetic anhydride to form chitin. This research took advantage of this finding to conduct experiments through which the following results have been obtained. In addition, a review on chitin and its applications along with principles of phage display and electron microscopy will be presented.

1.2 Research objectives

1. To identify chitin binding peptide from phage display combinatorial library.
2. To functionalize chitin using identified peptide.

1.3 Research hypothesis

1. By immobilizing chitin on a surface it may be possible to apply phage display technology to find peptide that can specifically by to chitin.
2. The found peptide may be applicable to functionalize chitin.

CHAPTER II

LITERATURE REVIEWS

2.1 Chitin's History and Etymology

It is an accepted fact that chitin was discovered by Henri Bracconot a French chemist, in 1811. Nevertheless, Professor Charles Jeuniaux, in his presentation at the 1st International Conference of the European Chitin Society held in Brest in 1995, pointed out that Hachett, an English scientist, discovered this biopolymer already in 1795. Hachett only pointed out the presence of an organic material in the cuticle of an arthropod, which was resistant to the usual chemical materials and treatment. Whilst, Braconnot found out that this material, in present of hot acid, produce acetic acid and he announced it as a new material (Roberts, 2006).

Henri Bracconot's name for chitin was *fungine*. In 1823 Odier (Dennell, 1946) found this same material in insects and plants and named it *chitine* (Roberts, 2006). This French word possibly has Greek roots (the Greek word chiton means mollusk) and means tunic or envelope. Other possible connections to the modern word chitin include the Akkadian word *kitu* which means flax, and the Summerian word *gada* (2000) and the Pahlavi (Old Persian) word *gadha*, all of which mean envelope or intestine envelope.

2.2 Source of chitin

Chitin, a poly-beta-1,4-*N*-acetylglucosamine (GlcNAc), is the main component of arthropod exoskeletons, and the reflective material both in the epidermis and the eyes of

cephalopods (phylum: *Mollusca*) (Herring, 1979), their tendons, and the linings of their respiratory, excretory, and digestive systems (Clark and Smith, 1936). It also exists in the cell wall of fungi (Blumenthal and Roseman, 1957). Moreover, it has been discovered that the epidermal cuticle of a vertebrate named *Paralipophrys trigloides* (fish) is chitinous (Wagner et al., 1993). So chitin is not only available in invertebrates but also vertebrates. Unlike cellulose, chitin can be a source of nitrogen as well as carbon (C:N = 8:1) (Struszczyk, 2006).

2.3 Structure of chitin

Chitin contains 6-7% nitrogen and in its deacetylated form, chitosan, contains 7-9.5% nitrogen. In chitosan, between 60 to 80% of the acetyl groups available in chitin are removed (Mathur and Narang, 1990). If the acetamido group of chitin at C-2 position is replaced by the hydroxyl group, then chitin's analog ,cellulose, (Ravi and Majeti, 2000) is produced (Figure2).

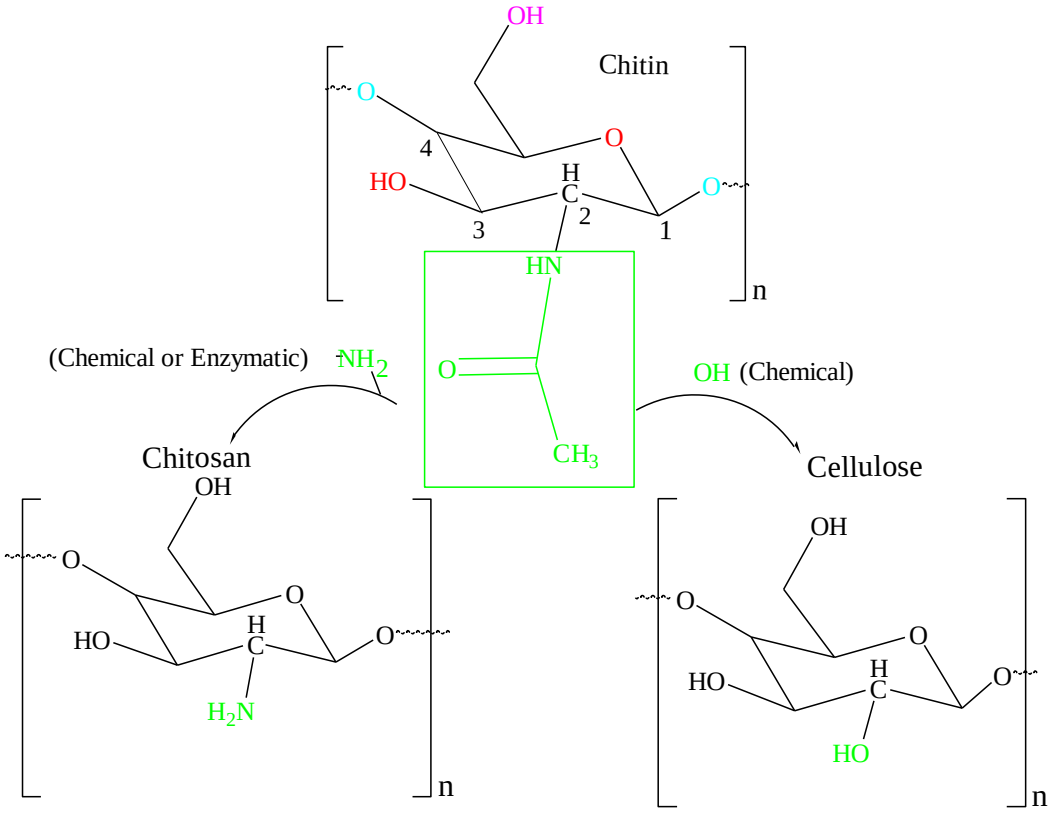


Figure 2. The difference between chitin, chitosan, and cellulose: Green indicates reactive groups that are different in chitin, chitosan, and cellulose; red indicates residues that are important for hydrophilic interaction; blue is the position where the polymer is extended, pink is the position where grafting co-polymers are produced as well as the site for hydrophilic interaction. (Chemdraw software)

There are three forms of chitin: α, β, and γ chitin. The α-form, which is mainly obtained from crab and shrimp shells, is widely distributed. It's the only form that is commercially available. α-chitin chains are aligned in anti-parallel fashion. The anti-parallel arrangement in α-chitin gives rise to strong hydrogen bonding and consequently makes it more stable. The β-form mainly obtained from mollusks such as squid, is arranged in parallel, and the γ-form, which has been proposed to be a mixture of α- and β-form, contains parallel and anti-parallel forms or a variant of α-form (Atkins, 1985). Finding γ-form in live organisms is hard. It mainly obtained in low amount at after treating other

forms of chitin with chemicals. Conversion from the β form to the α-form is possible, but not the reverse (Lavall et al., 2007; Mazeau et al., 2002; Schiffman and Schauer, 2009).

Chitin is insoluble in water due to its intermolecular hydrogen bonds (Minke and Blackwell, 1978). But water-soluble chitin-based derivatives such as chitosan or carboxymethyl chitin can be obtained. One of their most important features is the ability (flexibility) to be shaped into different forms such as fibers, hydrogels, beads, sponges, and membranes (Mano et al., 2007). The origin of chitin affects its crystallinity, purity, polymer chain arrangement, and dictates its properties (Rinaudo, 2006). In comparison with its derivatives, chitin itself has higher compatibility with blood due to less macrophage activation (Freier et al., 2005).

2.4 Applications of chitin

2.4.1 Immunology

The key property of chitin-derived products for application in various biomedical applications is the immuno-modulating effect, which is under critical review in the accompanying article of this special issue. Various mechanisms of immuno-enhancement activity of chitin and its derivatives have been reported. For example, chitosan exhibited the ability to boost nitric oxide (NO) production from macrophages in the presence of Interferon- γ (IFN- γ) through the NF-κB signaling pathway (Jeong et al., 2000). In addition, Saburo Minami et al, (1998) found that chitin and chitosan affected C3 and C5 components of complement system and concluded that complement system is activated by chitin and chitosan through the alternative pathway. After activating the complement, C5a is produced followed by an increase in migration of polymorphonuclears (PMNs) to the injured tissue. This is normally an inflammatory reaction but in the presence of chitin and chitosan the reaction does not have inflammatory consequences, such as

erythema, temperature elevation and abscesses formation (Minami et al., 1998). This makes chitin and chitosan highly attractive for inflammation treatment.

2.4.2 Hemostasis and wound healing

Hemostasis through blood coagulation is an important step for wound healing. The main cellular components in blood coagulation are platelets. It has been shown that chitosan has an hemostatic effect (Klokkevold et al., 1999). Okamoto Y *et al*, reported that chitin is an effective agent for hemostasis maintenance through aggregating platelets, and suggested that the effect of chitin and chitosan is due to both physical and chemical properties of these biopolymers, especially their amino groups (Okamoto et al., 2003). Shelma *et al,* (2008) developed a composite film by using chitosan and chitin. They used chitin nano-fiber to improve the composite's tensile strength and elasticity (Shelma R et al., 2008). Mi *et al,* designed an asymmetric chitosan membrane with the ability to protect skin by preventing bacterial invasion and halting the avaporation of the skin's water, two important factors for dressing wounded skin (Mi et al., 2001; Shin et al., 2008). A Chitosan acetate bandage has been shown to have good antibacterial activity when applied to burned skin which is contaminated with *Pseudomonas aeruginos* (Tianhong et al., 2009).

2.4.3 Scaffold for the regeneration of natural tissue

Chitin and its derivatives are being used as scaffold for bone and other natural tissue regeneration (Brandl et al., 2007), structures by which three-dimensional formation of tissues are supported (Tsioptsias et al., 2009). While looking for a good material for a good scaffold, there are at least four important factors that should be taken into account: 1) ability to form temporary matrix, 2) ability to form porous structure for tissue to grow, 3) biodegradability, and finally, and 4) non-toxic byproducts from the digestion (Drury and Mooney, 2003a; Khor and Lim, 2003). Thus, neither the physical nor biological properties of such biomaterials should be ignored (Brandl et al., 2007). Chitin and its derivatives have been shown to possess these criteria.

2.4.4 Neuro-tubes promoting nerve regeneration

Based on the fact that chitin has high mechanical strength under physiological condition (low for chitosan) chitin has the potential to be a good nerve guidance channel. Ferier et al. used this fact and made chitin tubes that could support nerve cell adhesion and neurite outgrowth (Freier et al., 2005). In a research related to nerve regeneration, it was shown that rabbits with the crushed common peroneal nerve exhibit better improvement in peripheral nerve regeneration in the presence of chitooligosaccharide. As result, chitooligosaccharide can be used as neuroprotective material with an ability to improve injured peripheral nerve regeneration (Yanpei et al., 2009).

2.4.5 Blood cholesterol control

Obesity is among the major public health issues in many countries. Hence, finding way(s) to prevent gaining weight or to reduce weight, or to induce weight loss is profoundly on demand nowadays. Chitin and chitosan are among the candidates battle obesity. They can reduce the amount of cholesterol in rats (Razdan and Pettersson, 1994). Several mechanisms have been proposed to explain this phenomenon. One is through electrostatic interaction between lipids and aminopolysaccharides (Furda, 1983). Chitin binds to lipid (cholesterol) micelles and inhibits their absorption. Another proposed mechanism is increasing the excretion of bile acid by which the amount of fecal fat increases (Gallaher et al., 2000). Feeding mice with chitosan had hypocholesterolaemic effect. It seems that the mechanism of chelestrol-lowering effect of chitosan is through suppression of food intake (Burton-Freeman, 2000). The hypocholesterolaemic effect of chitosan has also been found in humans. During a period of time chitosan were regularly, in a certain and constant amount, ingested by volunteers. Total amount of serum cholesterol decreased but after stopping the ingestion, the total value returned to the level before the ingestion (Yuji et al., 1993).

2.4.6 Drug delivery carriers

It is important for a drug delivery carrier to be removed after delivering drugs. In other words, it must not accumulate in the body nor must it be toxic (Dev et al., 2009). Chitin derivatives such as *N*-succinyl-chitosan (Kamiyama et al., 1999), carboxymethyl chitin (Dev et al., 2009), chitosan hydrogel (Ishihara et al., 2006), and hydroxyethyl chitin (Zhao et al., 2006) have been shown to possess such characteristics.

There are several methods by which colloidal chitosan is formed and then entrap macromolecules such as ionic crosslinking, desolvation, or ionic complexation (Janes et al., 2001). Nano-liposomes have been coated with chitosan which enhanced the efficiency of drug encapsulation of the liposomes (Haidar et al., 2008). Nanoparticles made of chitosan in association with polyethylene oxide have been used as protein carrier (Calvo et al., 1997). Oral delivery system has been developed by using chitosan and tripolyphosphate. In this system micro- and nano-particles were entrapped in beads made from chitosan in solution of tripolyphosphate (Bodmeier et al., 1989).

2.4.7 Antioxidant

The balance between oxidant formation and antioxidant defense in biological systems is important in order to protect biomolecules oxidation. The higher oxidation activity in cells, the higher potential for cell injury, which leads to cancer, arthritis, neurodegenerative, and aging (Calabrese et al., 2005). Chitin and its derivatives have been reported to have antioxidant properties (Ngo et al., 2009).

Chitin was chemically modified to obtain aminoethyl-chitin and it showed antioxidant activity against free radicals such as 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl, superoxide, and peroxyl (Je and Kim, 2006). It has been reported that antioxidant activity of hetro-chitosan is depend on its deacetylation degree and concentration (Park et al., 2004). It seems that free amino groups play a major role in antioxidant activity of chitnious materials (Je and Kim, 2006). The proposed mechanism behind this activity is

that the free radicals react with NH_2 groups and these groups absorb hydrogen ion from the solution and produce ammonium groups (Xie et al., 2001).

2.4.8 Antifungal and antimicrobial activity

Antifungal activity of chitinous products has been demonstrated in several articles. For example, Liu Jun-ang et al. reported the antifungal activity of chitosan against *Colletotrichum gloeosporioides* (*Melanconiales, Melanconidiaceae*), which causes *Camellia* anthracnose in *Camellia oleifera*, tea and *camellia* (Liu et al., 2009). Chitosan has been reported to possess the antibacterial ability against *E.coli* through cross-linking between chitosan (as cation) and anions over the surface of *E.coli* (Tsai and Su, 1999). It has been shown that *Bacillus subtilis* in presence of chitin exhibit better antibacterial activity which means this microorganism has the potential for being used as a bio-control agent (San-Lang et al., 2002). In addition, it was shown to inhibit the growth of *Xanthomonas* sp., which is pathogenic to *Euphorbia pulcherrima* (Li et al., 2008).

The mechanism of antifungal and antimicrobial activity of chitin and its derivatives has yet to be totally uncovered. In fact there are several proposed mechanisms. One of them is the ability of chitin and its derivatives to activate defense mechanisms of the host organisms (El Ghaouth et al., 1992a) such as inducing the chitinase accumulation (El Ghaouth et al., 1992b). Another one is leakage in the cell wall of bacteria due to the interaction between positively charged chitosan molecules and the negatively charged surface of the bacteria (Young et al., 1982).

2.4.9 Gene therapy

Being able to carry a large piece of DNA plays an important role in gene therapy. The carrier must be safe with low immunogenicity. One of the carriers being used - viral vectors - may not be safe enough for targeting cells (De Smedt et al., 2000). Cationic derivatives of chitin are being used to serve this purpose (Je et al., 2006). Galactosylated Chitosan has been grafted to dextran to make liver-specific DNA carrier (Park et al., 2000).

Another group has synthesized nanosphere delivery vehicle by the complex coacervation of DNA with chitosan (Leong et al., 1998). Chitosan can condense DNA and forms small discrete particles in particular condition that is why it has many applications or/and potential applications for gene delivery (Erbacher et al., 1998).

2.4.10 Food Technology

Chemical preservatives can be replaced with chitinous-based ones. There are advantages in that: 1) chitinous materials are safer, and 2) with their antimicrobial activity, it can protect food products against microbial invasion. Chitosan-based films -have been developed to serve this purpose (Bégin and Van Calsteren, 1999). It also has also been applied to improve the preservation of vacuum-packaged processed meat and it could delay the growth of *Entrobacteriaceae*, which is an indigenous bacteria in the food products (Ouattara et al., 2000) . It seems this antibacterial activity is due to the positive charge of C2 in glucosamine, monomer of chitosan. This positive charge interacts with negatively charged microbial cell membrane and gives rise to leakage of the intracellular constituents of microorganisms (Dutta et al., 2009).

2.4.11 Agriculture

Chitin oligosaccharides have been shown to play an important role in defense mechanism of plants against microbial invasion (Day et al., 2001). They could also help carrot somatic embryos survival (De Jong et al., 1993). Chitin fragments can desensitize the perception system of tomato, which can lead to improvement of the defense mechanism in tomato cells (Felix et al., 1998). Chitin in the form of lipo-chitin can induce the formation of nodule in soybean root (Minami et al., 1996). Rice is another important plantin which chitin fragments have been shown to boost the plant’s defense system (Yuki et al., 1997).

2.4.12 Bionanotechnology

Bionanotechnology, a marriage between biology and nanotechnology, is an emerging field. Through biomimetic approaches and strategies, many micro/nano-systems

can be produced. Chitosan has been used to immobilize and pattern biomolecules on microfabricated surfaces (Koev et al., 2006). Photolithographic method have been applied to integrate chitosan to micro- and nano-structures, which is an important step toward the fabrication of bioinspired micro-electromechanical systems (Cheng and Pisano, 2008). Nanoimprinting lithography was used to micro- and nano-pattern chitosan (Park et al., 2007). This micro/nanopatterning enables researchers to use chitosan for bionanotechnology applications such as nanobiodevices. Chitiosan has recently been in the preparation of graphitic carbon nanocapsules, tungsten carbide and tungsten carbides/graphitic carbon composites. In this system, after preparation of the precursors of chitosan and metal ions, they were carbonized. The system can be regulated by changing the type and/or ratio of the metal (Baoli and et al., 2010). Chitin whisker has been used to reinforce nanocoposites. It seems this ability is mainly depends on the ability of chitin whiskers to form 3D network. Any modification by which this network is disrupted gives rise to lose this ability or lower it (Gopalan Nair and Dufresne, 2003a; Gopalan Nair and Dufresne, 2003b; Gopalan Nair et al., 2003).

2.4.13 Capacitor and electrolyte

Electric double layer capacitors are being used as memory back-up tools and energy storage technology. Electrolytes used in these capacitors should have low internal resistance and high capacitance. There are two type of electrolyte; aqueous and nonaqueous. Aqueous electrolytes have high electrical conductivity (Tanahashi et al., 1990). KOH and H_2SO_4, which are being used to make aqueous electrolytes, are strong base and acid, thus are hazardous and difficult to handle. It is important to have less toxic or non-toxic aqueous electrolytes that are stable and exhibit good conductivity and low resistance. Yamazaki *et al*, made a gel using a mixture of cellulose, chitin, and $H_2SO_{4.,}$ which has all the mentioned features as well as a high charge-discharge ability (Yamazaki et al., 2009).

2.4.14 Wastewater treatment (Heavy metals and other pollutants removal)

The wide usage of heavy metals in industry has caused and continues to cause serious and widespread health problems. It is important to remove these metals from the environment. Several studies have shown the ability of chitin and chitosan to perform this task. For instance, absorbing Cu(II) and Cr(VI) (R Schmuhl et al., 2001), Fe (Franco et al., 2004), and Pb(II) ions in an aqueous environment (Jianlong et al., 2001). Chitin phosphate absorbs uranium in the presence of sodium carbonate solution (Sakaguchi et al., 1981). Chitosan-based chelating resins have been developed to absorb mercury (Hakim et al., 2008a), Ti(IV), V(V), Mo(VI) (Hakim et al., 2008b),W(VI), U(VI) (Oshita et al., 2008), Ag in aquatic environment (Hosoba et al., 2009), Cd, Ni, V, Ga, Sc, In, and Th (Hakim et al., 2007). Chitin and chitosan have been shown to have copper removal capability, which could help to obtain more stable diesel oil (Peiselt da Silva and Pais da silva, 2004). They also have been successfully tested for the adsorption of the organic pesticide, 2,4-dichlorophenoxyacetate (2,4-D) (El Gaini et al., 2009).

2.4.15 Intelligent materials or composites

Chitinous materials have been used in creating smart or intelligent materials or composites. These systems can respond to environmental changes. They show their functionality with the addition and removal of stimulation. These smart or intelligent materials are preferred to be integrated systems or mixed composite of materials. Shape memory materials are one of such materials (Wei et al., 1998). As it is obvious from the name, shape memory materials can remember and re-gain their original shape after the removal of the stimulus. This phenomenon is due to their being equipped with proper stimulus sensitive molecular switches. Among these shape memory materials, polyurethanes are gaining more attention. This is because of their good shape memory effect at room temperature as well as their low cost. But shape memory polyurethanes cannot bear repeated changes in the shape memory, and retention will decrease by

increasing the number of cycles of shape memory; consequently, chitin-based polyurethane shape memory have been developed to overcome these problems (Zia et al., 2009).

2.4.16 Energy production: an emerging application

Insects are widely distributed on earth, comprising 80% of species (Zhang et al., 2007). Robots with the ability to hunt and digest insects and obtain energy from them can serve humanity by performing missions in dangerous situations. A robot that contains a microbial fuel cell was created to digest chitin and metabolizes it by bacteria. This process produces electrons that act as horsepower of the system (Ioannis Ieropoulos et al., 2004). This system can take advantage of the wide distribution of arthropods and mollusks because chitin is available in both phyla. Since *Arthropoda* and *Mollusca* rank first and second in species diversity in all animal phyla (Giribet et al., 2006) and are a major source of chitin, this system can be highly applicable in both land and marine environment. Chitin has also been used to produce hydrogen. Chitin is utilized by *Clostridium paraputrificum* M-21 to produce hydrogen gas. This gas is considered to be a potential source of alternative energy (Evvyernie et al., 2000; Morimoto et al., 2005). The advantage of using chitin in this way is that most chitin sources are waste materials, such as shrimp shell; hence, since it is non-food material and there is no need to be concerned about pressure on food supplies.

2.4.17 Chito-oligosaccharides and their applications

Recent trends in the field of chitin research have focused on oligosaccharides, which are more soluble and have several attractive biological effects. N-acetylchitooligosaccharide and chitooligosaccharide (COS) originate from chitin and chitosan, respectively. Oligomers of chitin and chtitosan can be obtained both chemically and enzymatically. Their degree of polymerization (DP) is usually <20.

It has been shown that chitooligosaccharide accelerates the wound- healing effects of Poly vinyl alcohol (PVA) if it is used in the early stages of the healing process (You et al., 2004). It could inhibit the growth of *Actinobacillus actinomycetemcomitans*,

indicating that it has antimicrobial activity (Choi et al., 2001). *N*-acetylchitooligosaccharide causes an increase in biophotons emission from suspension-cultured rice cells. Biophotons are very weak light emitted from biological processes/systems. They are produced when energy of a system drops from an excited state to a stable ground state. It seems in this emission process *N*-acetylchitooligosaccharide indirectly generates a reactive oxygen species (ROS) in which NADPH oxidase and H_2O_2 are involved. This process gives rise to photon emission (Kageyama et al., 2006). Chitooligosaccharide has shown inhibitory effects on tumor growth and metastasis of lung cancer in mice (Shen et al., 2009). N-acetylchitohexaose and chitohexaose can also induce production of interleukins 1 and 2 and, consequently, help to improve the function of macrophages, natural killers, cytotoxic T cells, and polymorphonuclear leukocytes, in defense mechanisms. The anti-metastatic activity of Acetylchitohexaose against Lewis lung carcinoma in mice (Keiko et al., 1990), has been demonstrated. There are many reports indicating antitumor activity of chitin-based materials (Wang et al., 2008). It has been shown that oligosaccharides that contain *N*-acetylglucoseamin plays an important role in the interaction between HIV and T-helper during pathogenesis of this virus (Mizuochi and Nakata, 1999). *N*-acetyl-chitooligosaccharide was used as analogue to study lysozyme (Chipman et al., 1967). Thus, these materials can be used to study protein-carbohydrate interaction and associated enzymes activities.

2.5 Phage Display Technology

2.5.1 Biology of filamentous bacteriophage

It is estimated that total number of phages on earth is about 10^{30} to 10^{32}. Historically, it is considered that modern phage research started based on an article published by Frederick W. Twort in 1915. Twort only reported a phenomenon so called glassy transformation, which was later recognized to be the first report on the discovery of

bacteriophages. However, most of work primarily was done by Felix d'Herelle. The word bacteriophage was coined by Felix (Kutter and Sulakvelidze, 2005).

Among the bacteriophages are the filamentous bacteriophages or Ff. They belong to the *Inovirus* genus. The members of the Ff class of the filamentous bactriophages are f1, fd, M13 (Barbas et al., 2001) which have been used to develop phage display technology. They can only infect *Escherichia coli* via F'pili. To do so, a coat protein of phage so called pIII attaches to the F'pilus of *E.coli* follows by the injection of the genome of the phage into the cell. This genome is (+) single stranded DNA. By using the host machinery, complementary (-) DNA strand is synthesized. This (-) form is used for transcription. As for replication of the phage genome, the (+) strand plays role as primer and the (-) strand as template. By the end of the replication and the translation processes, phage particles are assembled and secreted out of the bacterial membrane (Figure 3) (Mullen et al., 2006).

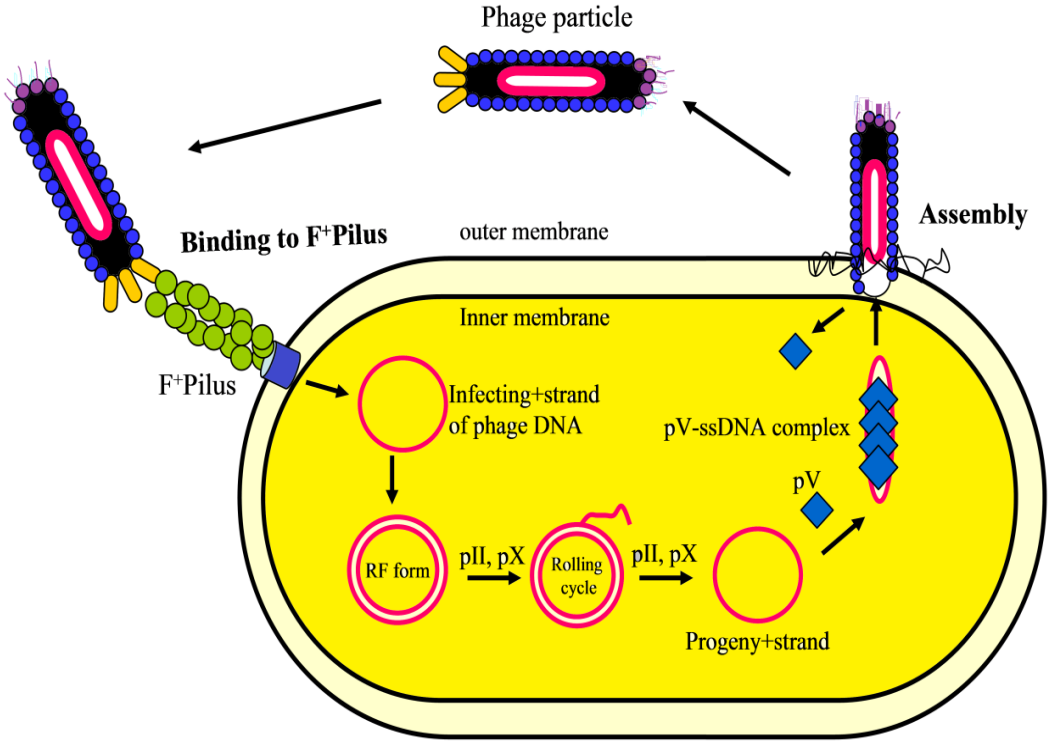


Figure 3. Filamentous Bacteriophage Life Cycle.

Their circular single-stranded DNA genome consists of 11genes (Barbas et al., 2001), and based on the function in the life cycle of phage these genes can be grouped into three groups:

1. Genes that encode protein involve in the replication (II, V, and X)
2. Another group of genes produce the capsid proteins (VII, VIII, III, and VI)
3. And the last group produce proteins by which phage is assembled (I, XI, and IV)

M13, as a member of Ff family, has been used in phage display technology since the dawn of this technology in late 1980. Genome of M13 is confined in thousands of copies of major coat proteins along with a few copies of four minor coat proteins at both ends (Figure 4).

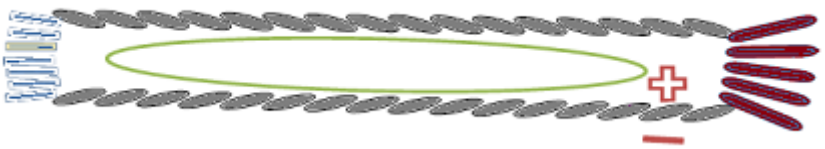


Figure 4. Simplified structure of M13.

2.5.2 Phage display technique

Phage display technique first describe by G.P. Smith, 1985 (Smith, 1985). It is a powerful molecular biology tool and based on the expression of random peptides or proteins on the surface of a bacteriophage (filamentous bacterial virus) appended to a recombinant viral structural protein. Desired phage particles, which are propagated in *E. coli*, can be isolated by "panning" against a target bound to a solid-phase support (Figure 5) (Mersich and Jungbauer, 2008; Steingroewer et al., 2007).

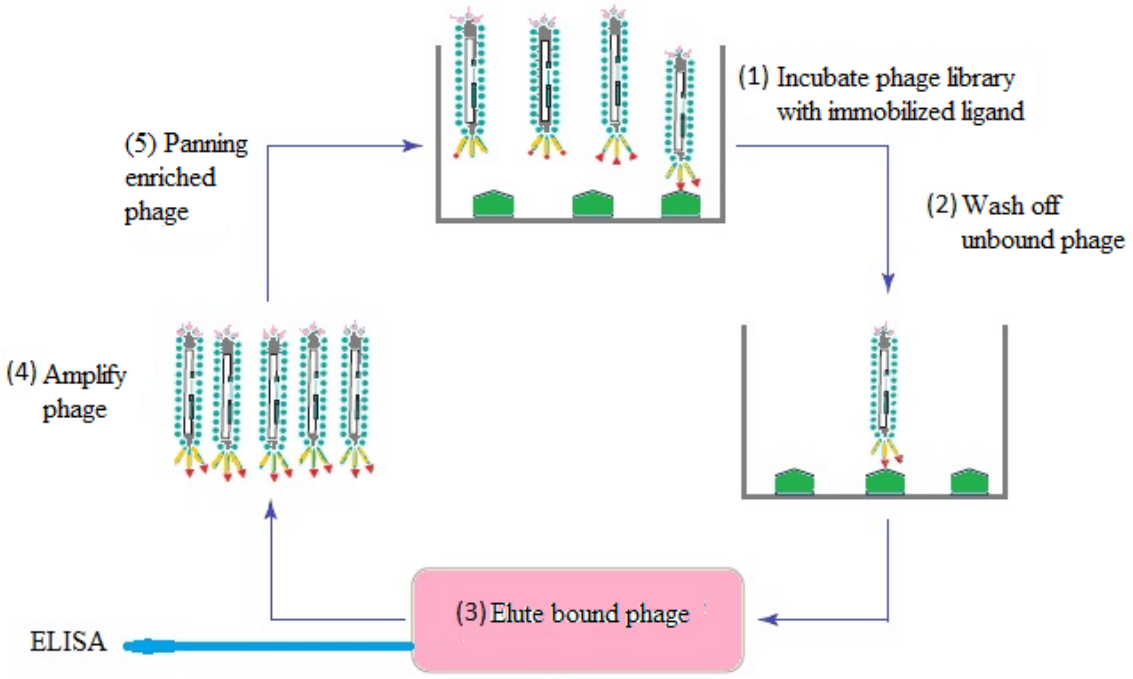


Figure 5. Biopanning, modified from (Mullen et al., 2006).

2.5.3 Phage displayed- Peptide

In general, the affinity selection (Figure 5) of ligands from phage display random peptide libraries involves 5 fundamental steps:

1) Preparation of a primary library or amplification of an existing library,
2) Exposure of the phage particles to a target (immobilized protein/cell surface protein/vascular endothelium) for which specific ligands are planned to be identified,
3) Removal of non-specific binders (washing/perfusion),
4) Recovery of the target bound phage by elution or direct bacterial infection and amplification of the recovered phage.
5) Back to step one about two to four times,

This “bio-panning” procedure can be repeated several times until the best binders are enriched. Phage display is in use for almost any kind of problem that involves

the interaction of peptides and proteins with other materials (Szardenings et al., 1997). So far many peptides ligand that can bind antibodies, enzymes and receptors have been found using phage display peptide library screening. It has also been used to identify novel peptide for therapeutic protein and neuronal targeting (James K. Liu et al., 2005), generating short peptides ligands for silver nano-wires (Priscilla et al., 2006), preparation of protein microarray (Qun et al., 2007), selection of tumor-binding ligands in cancer patients (David et al., 2006), cloning allergen (Rhynera et al., 2004), mapping epitope of monoclonal antibody (Siegel, 2002), and generating aluminum binding peptide and mild steel binding peptide (Zuo et al., 2005).

2.6 Electron Microscopy

Transmission Electron Microscope (TEM) and Scanning Electron microscope (SEM) are two basic instruments in electron microscopy. TEM was invented a little bit earlier than SEM. These two are different in their usages. While in TEM image is produced by electrons which pass through specimen and make two-dimensional image on florescent screen, in SEM, specimen is scanned by electrons generated from electron gun and secondary electrons emitted from the specimen are detected with a sensor and image is then produced (John and Russell, 1999). Figure 6 compares SEM and TEM with each other and with light microscope in more details.

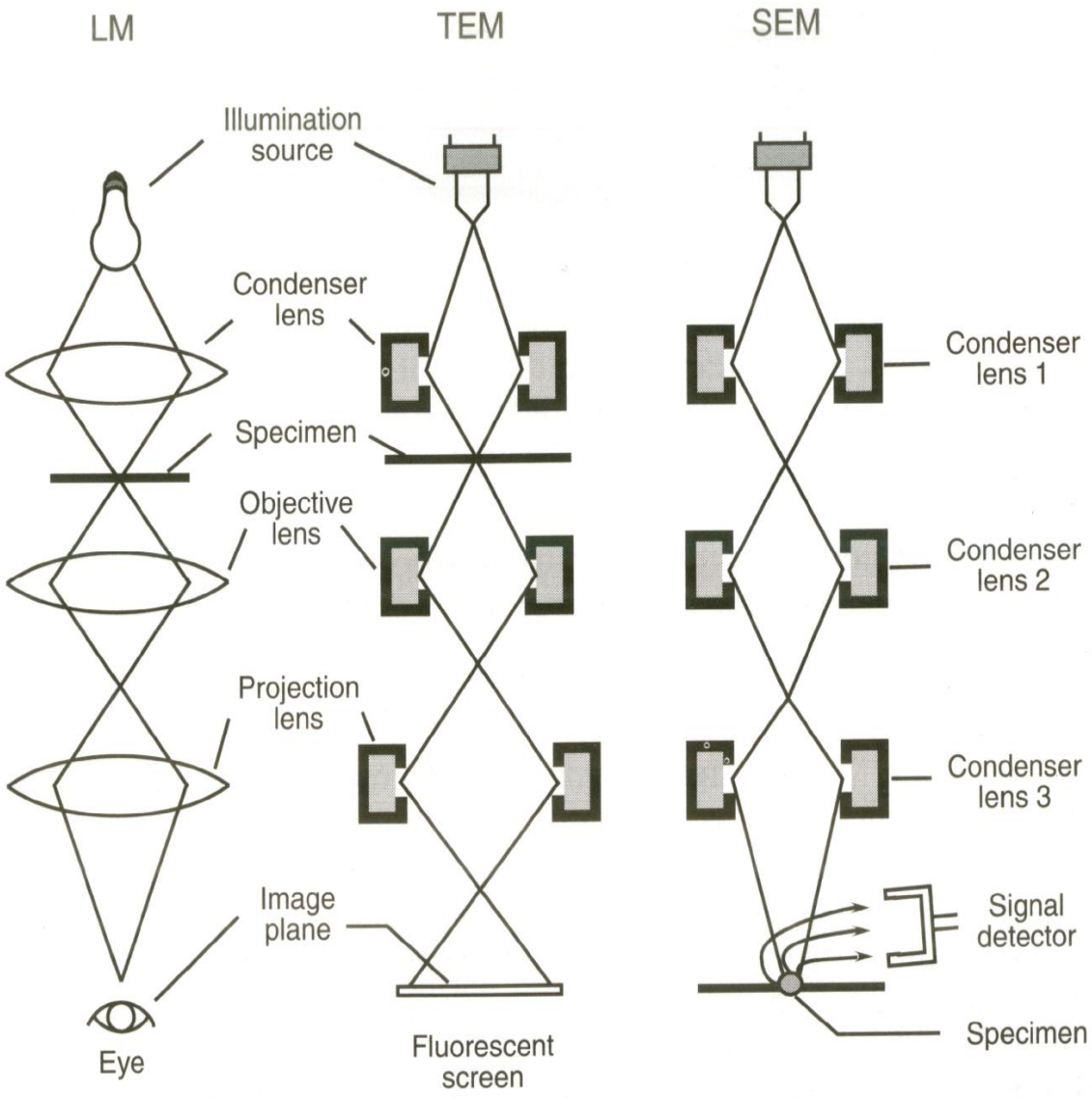


Figure 6. A comparison between principles of light microscopy vs TEM vs SEM.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

Phage library:	SUT12 peptide library (synthetic),
Bacteria:	*E.coli* K12F', *E.coli* Top10
Synthesized peptide:	Synthesized-biotinylated peptide from EZBiolab Co, USA
Chitosan:	FLUKA, Japan
Chitin monomer, trimer, hexamer:	Seikagaku Corporation, Japan
Chitin beads:	Biolab Co., USA
Microscopy	Transmission Electron Microscope, JEOL JEM-2010 Scanning Electron Microscope, JEOL, JSM-6400, Japan Olympus BX51 light microscope, Japan, Olympus CX31, Japan NIKON SMZ-U, Japan
Critical point drying (CPD):	Samdri-PVT-3B, TOASIMIS CO
Gold coater:	JOEL, JFC-1100
Softwares:	Chem draw Office (Ultra and Pro), WebLab view, BioEdit
Autoclave:	Hiclave HA-3000MIV, Hirayama, Japan
Balance:	Precisa 205A, Precisa Instruments, Switzerland Precisa 3000C, Precisa Instruments, Switzerland

Centrifuge machine:	Sorvall RC5C plus, Kendro laboratory Products, USA
	Eppendrof centrifuge 5810 R, Eppendrof, USA
Deep freezer -70 °C:	Heto, Ultra Freeze, Denmark.
ELISA reader:	Sunrise, TECAN, Austria
Freezer -20 °C:	Heto, HLLF 370, Denmark.
	MyBio LFT420, DAIREI, Denmark
Heat Box:	HB1, Wealtee Corp., USA
Incubator shaker:	C24 Incubator shaker, New Brunswick Scientific, USA
Incubator:	Memmert, BE 500, WTB Binder BD115,
	Shel-Lab 2020 Low Temperature Incubator, Sheidon, USA
Laminar hood:	Holten LaminAir HBB 2448, Denmark.
	BH2000 Series ClassII Biological Safety Cabinets,
	BHA120 & BHA180, Clyde-Apac,
Microcentrifuge:	Mini spin plus, Eppendrof, USA
	Eppendorf 54154, Eppendorf, Germany
pH meter:	Ultra Basic pH meter, Denver Instruments, Germany
Rotator:	Certomat TCC, B. Braun Biotech International, Germany
Shaker:	Innova 2300 platform shaker, New Brunswick Scientific, UK
	Certomat TC2, B. Braun Biotech International, Germany
Sonicator :	Waken GE100 Ultrasonic processor, Japan
Stirrer:	Variomag Electronicrührer Poly 15, Germany

	Magnetic stirrer MSH300, USA
Thermomixer:	Thermomixer compact, Eppendrof, USA
Vortex:	Vortex-Genie 2 G506, Scientific Industries, USA
Dry keeper:	Sanplatec Corp., Japan

3.2 Methods

3.2.1 Immobilization of chitin onto 96-well plate

300 mg chitosan was dissolved in 50 ml 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 it at low speed on a rotating platform overnight at room temperature. The resulting material was diluted 1:10 with 0.1 M acetic acid (pH 5.0) and aliquots (6 μg) were added to each well of a 96-well microtiter plate. 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 10× phosphate-buffered saline solution (40 mM KCl, 1.76 mM KH_2PO_4, 1.4 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 1 h. This neutralized the surface and blocked nonspecific adsorption sites remaining in the wells. Following removal of this solution, the plates were used. Three 96-plates were treated in this way.

3.2.2 Preparation of chitin beads

200 μl of chitin beads (BioLab) solution were centrifuged at 4000 xg for 30 seconds and the supernatant was discarded. The beads were then washed with DI water 5 times.

3.2.3 Biopanning (or Affinity selection) of chitin binding peptide

One of the prepared 96-plate wells was washed 3 times with PBST. Following the addition of 25 µl of the 12-amino acid long random peptide library (SUT-12) in 125 µl of PBST, the wells were sealed and incubated at room temperature for 2 hours. The wells were then washed with DI water 5 times, and after 15 minutes of incubation at room temperature, the bound phages were eluted by adding 50 µl of 50 mM glycine-HCl, pH 2.0. Solutions were neutralized by adding a neutralization solution. 100 µl of the eluted phages were added into an overnight culture of *E. coli* K12F' in 2XYT (1:100 dilution) and were incubated at 37°C. The amplified phages were collected by centrifuging cells at 4000xg at 4 °C for 10 minutes and the phage supernatant transferred to new tubes. 200µl of this supernatant was added to a second plate, which had already been washed 3 times with PBST. The wells were sealed and the plate was incubated at room temperature for 2 hours. The wells were then washed and filled with 50 µl of 50 mM glycine-HCl pH 2.0. After 15 minutes of incubation at room temperature, 50 µl of neutralization fluid was added and then the eluted phages were collected.

For the third round of biopanning, a third plate was picked and its coated wells were washed 3 times with PBST; 100 µl of solution from the previous step was added to each well, the wells were sealed and then incubated at room temperature for 1 hour. The wells were then washed 5 times with PBST, then washed and filled with 50 µl of 50 mM glycine-HCl pH 2.0. After 15 minutes of incubation at room temperature, 50µl of neutralization fluid was added and then the eluted phages were collected. 10-fold-serial dilution was performed. It was prepared from the eluted phage of the last step. In parallel top agar was prepared by mixing 4ml of 0.8% top agar (molten) with 40 µl of 2% X-gal and 100mM of IPTG. Four ml of this mixture was added to a tube containing 200 µl of *E.coli* K12F', and then was poured onto a 2XYT plate.

Then 5µl of each phage dilution from the 10-fold-serial dilutions was spotted onto hardened top agar. The plate was incubated at 37°C overnight. The 10^{-2} phage dilution yielded separated individual plaques, so these were used to pick affinity-selected phage clones. 20µl of this dilution was added to a tube containing 200µl of an overnight culture of *E.coli* K12F'. In parallel top agar was prepared by mixing 4ml of 0.8% top agar (molten) with 40µl of 2% X-gal and 100mM of IPGT. 4ml of this mixture was added to a tube containing 200µl of *E.coli* K12F' and the appropriate phage dilution, and then was poured onto a 2XYT plate. It was inverted and incubated at 37°C overnight. Individual blue plaques were picked and inoculated into 3 ml of *E.coli* K12F' diluted 1:100 in 2XYT at 37°C for 8 hours while agitated. Amplified phages were collected by centrifuging at 4000 xg at 4°C for 10 minutes. Supernatant was separated for phage ELISA (Kay et al., 2001).

3.2.4 Phage ELISA

The 96-well plate was coated with chitin as described in the previous sections and the coated and control wells were washed with PBST. After adding 150 µl blocking solution into each well all the wells were sealed and incubated at 4°C overnight. Then the wells were washed 3 times with PBST and 200 µl of each supernatant containing individual phage clones (obtained from the previous step) were added to the wells. Following incubation at room temperature for 1 hour, the wells were washed 5 times with PBST. 100 µl of 1:5000 dilution of horseradish peroxidase (HPR)-anti M13 in phosphate buffer saline with tween (PBST) was added to each well and the plate was incubated at room temperature for 1 hour. Afterward, the wells were washed 5 times with PBST, and then100µl of 2, 2 A-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) di- ammonium (ABTS) substrate before adding 0.05% H_2O_2 to each well. After 20 minutes incubation, the OD at 405 nm of each well was measured with a microtiter plate reader (TECAN Austria GmbH).

3.2.5 Plasmid purification

It was done by using QIAprep spin miniprep kit (QIAGEN Co). First, bacterial pellet cells were resuspended in 250 µl buffer P1 and, then, transferred to the centrifuge tube. 250 µl buffer P2 was added to this tube and the tube was thoroughly mixed by inverting it 6 times. Then, 350 µl buffer N3 was added. The mixture 6 times was immediately and thoroughly inverted and it was centrifuged at 13000 xg for 10 minutes. Supernatant from this step was added to QIAprep spin column and centrifuged for 45 seconds. The column was washed by adding 0.5 ml buffer PB and centrifuged for 45 seconds. The column was washed with 0.75 ml of another buffer, which was PE and centrifuged for 45 seconds. After discarding the flow-through, another round of centrifugation was carried out for 1 minute to remove residual was buffer. QIAprep then was moved to a 1.5-ml centrifuge tube. DNA was eluted and obtained by adding 50 µl buffer EB to the center of QIAprep spin column. Plasmids were sent for sequencing using universal primers.

3.2.6 Modelong

After sequencing, using tools from different websites: pga.mgh.harvard.edu, NCBI, ExPASy and softwares such as Chemdrawoffice, WebLab view, CLAWSTAL W, the sequences were analyzed and used to predict their structures.

3.2.7 Chitin beads and phage supernatant experiment

Tube was first blocked with skim milk (2%). After preparation of chitin beads; they were used as substrate for phage particles to find out whether or not selected clone can bind to chitin. Blocking solution was added to the chitin beads and incubated for 1 hour, and then were washed 3 times with PBS. 200 µl of selected phage's supernatant was added to 200 µl of chitin beads. It was incubated overnight at 4℃ and was then washed 5 times with PBS. 200 µl of 1:5000 HRP-antiM13 was added and it was incubated for 1 hour at

room temperature. It was then washed followed by addition of 150 µl ABTS-0.05 H_2O_2 (30%) to the tubes. After 20 minutes OD was measured at 405nm.

3.2.8 Chitin binding of Synthesized-biotinylated peptide vs CTS (Negative control)

After preparation of chitin beads according to above mentioned methed, 100 µl of the beads were added to the tube. Tube had been blocked with skim milk (2%). 100 µl of each synthesized peptide and CTS (a colon cancer binding peptide) was added to onto the tube containing the beads and they were incubated for 2 hours at room temperature. They were then washed 5 times with TBS-tween. 100 µl of streptavidin-alkaline phosphates (SA-AP) was added. Following by washing the tubes with TBS-tween (5 times), 100µl of p-nitrophenyl phosphate (p-NPP) was added. After 3 minutes OD was measure at 405 nm.

3.2.9 Macroscopic structure forming procedure

Chitin derivatives were used to figure out if obtained peptide could form any structure.

3.2.9.1 N-Acetyl-Glucoseamin(NAcGlu) and chitotrios

1 mg/ml solution of each was prepared. 100 µl of this solution was taken and mixed with 100 µl of 1 mg/ml (0.7 mM) the synthesized peptide solution. After 2 hour incubation at room temperature 10 µl of mixture was taken to be observed under microscope. And after 15 hour incubation at 42^{o}C, 20 µl of the mixture was taken again to be observed under microscope.

3.2.9.2 Chitohexaose and the peptide

Chitohexaose was prepared for gel-formation analysis. One hundred µl of each peptide (0.7 mM) and chito-hexaose (5mM) were mixed and incubated at room temperature for 2 hours before sampling for transmission electron microscopy (TEM)

analysis, and at 42^{o}C for 15 hours for scanning electron microscopy (SEM) analysis, respectively.

3.2.9.3 Chitohexaose, $CaCO_3$ and the peptide

A solution of 5 mg/ml of $CaCO_3$ was prepared and 100 µl of this solution was added to 100 µl of chitihexaose (5 mM) followed by addition of 100 µl of the synthesized peptide (0.7 mM). This mixture was incubated at 42^{o}C with shaking at 350 rpm to be used for SEM photography (Certomat TC2, Germany).

3.2.9.4 Colloidal chitin and the peptide

A solution of colloidal chitin with the concentration of 5 mg/ml was prepared. 100 µl of this solution was added to 100 µl of peptide solution with different concentrations (1mg/ml, 100mg/ml, 200 mg/ml). Then it was put in thermomixer, which had been set at 42 degree Celsius. After 15 hours the solution was taken and left in room temperature in which the structure gradually appeared. The EM photography was carried out after 15 hour being left at room temperature.

3.2.10 Electron Microscopy

3.2.10.1 *TEM*

Regarding the fact that the samples used for TEM had no seeable structure, thus, to avoid any interference of foreign particles to the structures inside the solutions, each sample was directly added (10 µl) to grid, dried, and used for photography.

3.2.10.2 *SEM*

Glutaraldehyde was added to the samples for about 2 hours before being removed. Samples were first washed by phosphate buffer and then by alcohol series (ethanol, 30, 50, 70, 90, 95, and 100% respectively). Critical point drying (CPD, samdri-PVT-3B, OASIMIS CO) was the next step. Samples were then coated with gold (JOEL, JFC-1100) in preparation for photomicrography by EM (SEM; JEOL, JSM-6400).

CHAPTER IV

RESULTS AND DISCUSSIONS

The main interaction between peptides and saccharides is hydrogen bonding, which is not a full-charge electrostatic interaction. As well, immobilizing chitin on solid surfaces is difficult, thus, only a few reports have so far been made on phage display peptide library screening against neutral saccharides (Fukusaki et al., 2004).

First challenge was to find a way to immobilize chitin. Fortunately an article had been published by Bernard, M.P., et al., in which they acetylated chitosan using acetic anhydride. On other hand, they produced chitin from chitosan. This addition of acetic anhydride gives rise to two reactions simultaneously; one is acetylation of chitosan and the other is the immobilizing the acetylated form to the surface. However, they used chitosan at nanoscale while in phage display technique it must be at macroscale. To reach this goal the system was optimized and the amount of chitosan and acetic anhydride necessary for experiment was found to be 6 μg and 35 μl/ well, respectively. Another factor that had to be considered was the time for drying out the wells. It was found that the best time was 18 hours under laminar flow.

After optimizing the system for immobilization, chitin was immobilized and phage display combinatorial library was applied. After three rounds of selection, phage ELISA was performed and 4 clones, 1, 3, 7 and 8, showed better signal so they were selected for sequencing (Figure 7). Plasmids were purified using QIAprep Kit and the purified plasmids were sent to Mcarogen Co., for sequencing.

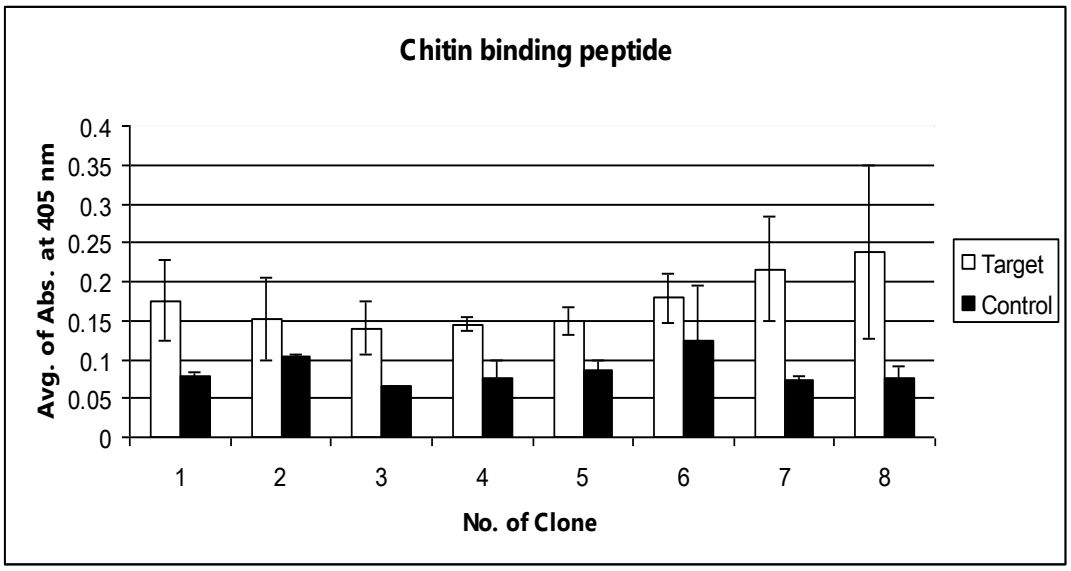


Figure 7. Phage ELISA of 8 selected clones from affinity selection experiment, in which chitin was immobilized onto 96-well plate. Clones number 1, 3, 7, and 8 were selected for further investigation. OD was measured at 405nm. Blue and red colors indicate target and control (2% skim milk), respectively.

Results of sequencing of DNA insert between restriction enzymes (*Xho*1 and *Xba*1) and 42 bp-sequence of each peptide was obtained as follow:

CBP1:

1 TCGAGGGGGG AGGTGGGTGA GCAGGAGAAG GCTAGGGTTG GG

CBP3:

1 TCGAGGGAGG GGAAGGGTGT GGAGGCGGTG GGGGATGGGA GG

CBP7:

1 TCGAGGGAGG GGAAGGGTGT GGAGGCGGTG GGGGATGGGA GG

CBP8:

1 TCGAGGGCGG AGCCTGATGC GTATGGTTGG AGGTCGTTGG GG

It was revealed that 2 of the clones, so called number 3 and 7, are identical. Then, these sequences were translated using NCBI tools. Below are the translations of the sequences:

CBP#1: GEVGEQEKARVG

CBP#3: EGKGVEAVGDGR

CBP#7: EGKGVEAVGDGR

CBP#8: AEPDATGWRSLG

These short peptides are considered to be chitin binder material. As result, their sequences were compared with Rebers-Riddiford (RR) consensus sequence, which is a conserved sequence in proteins of arthropods cuticle and it is responsible for their chitin binding property (Inoue et al., 2001). As it is shown in Figure 8, the R.R consensus sequence has 9 essential residues, 5 of which are identical with the reversed sequence of clones 3 and 7, whilst 4 residues are identical with reversed sequence of clone 1. Clone number 8 did not show similarity with other 3 clones.

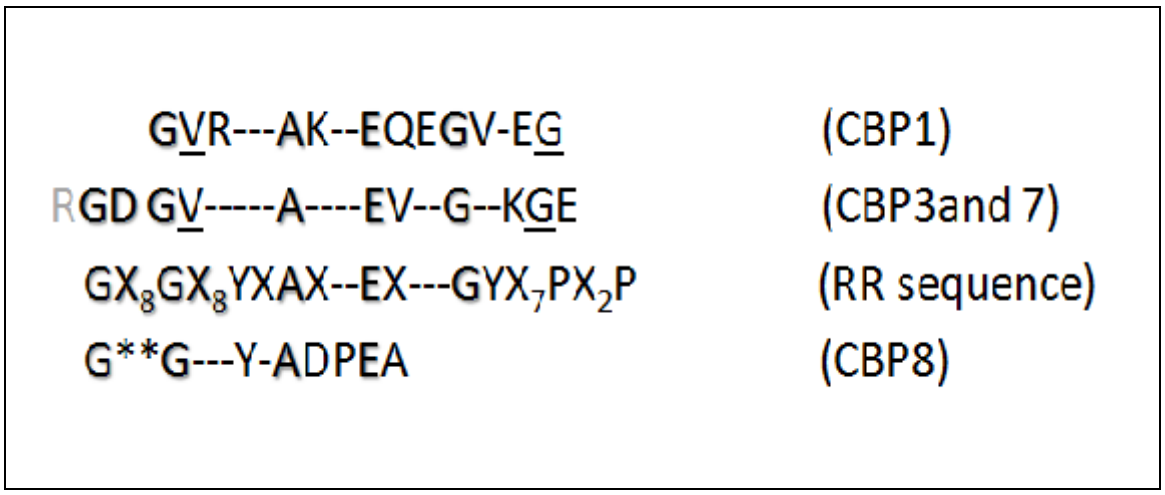


Figure 8. Alignment of selected clones with RR consensus sequence. Underlined residues show residues which are similar between CBP1 with CBP3 and 7, shadowed part show similar residues between CBP1,3 and 7 with RR consensus sequences.- indicates space. **is substitute of 4 residues of LSRW of CBP8. RR and CBP stand for Rebers-Riddiford consensus sequence and Chitin Binding Peptide, respectively. R in gray with its 2 neighboring residues (GD) makes up a motif, which is considered as a cell surface binding motif. CBP1, CBP3and7 are clones number 1, 3 and 7 respectively. The amino acid sequences of these three peptides were ordered from C-terminus to N-terminus to emphasize the similarity with RR motif.

Another attractive characteristic of this sequence is that it has RGD motif. RGD is considered to be a cell surface binding motif (Chow et al., 2008).

Another experiment was specifically designed to compare the binding activity of clone 1, 3 and 7 (sequences of clones 3 and 7 are identical; hence, we called their representative clone 3). In this experiment, chitin beads were used as the target. The results (Fig. 9) showed that clone 3 (representative of clones 3 and 7) has higher activity than clone 1. This is in accordance with its higher similarity to RR consensus sequence.

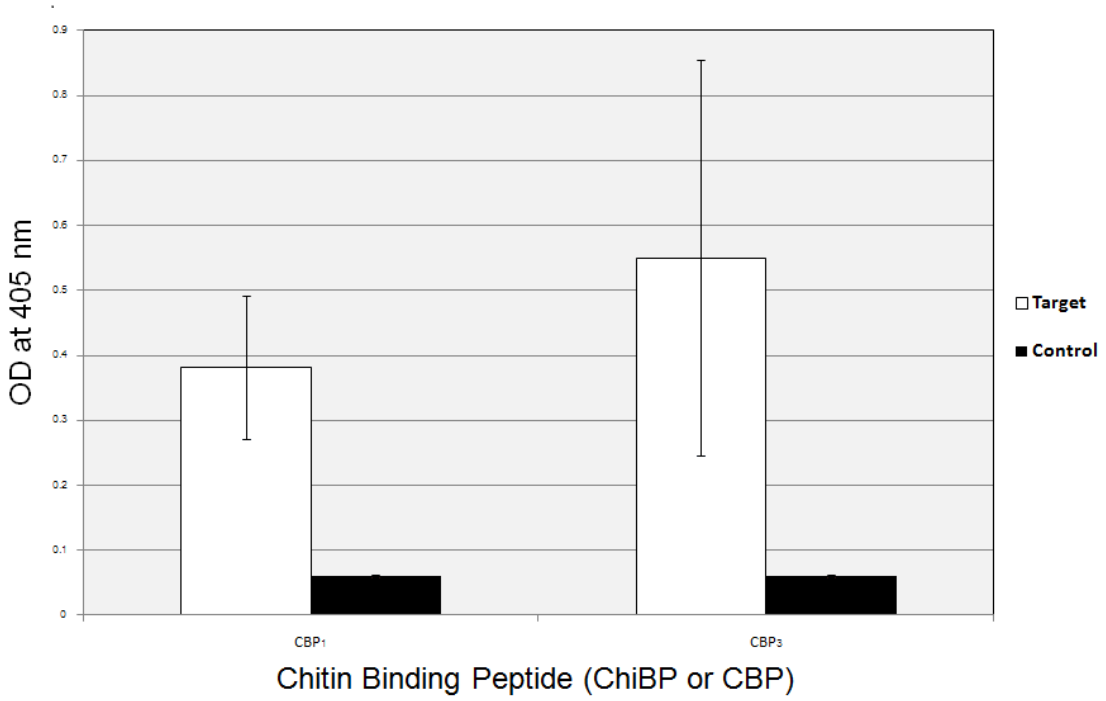


Figure 9. The results of phage ELISA in which chitin beads were used. ChiBP3, which is a representative of ChiBP 3 and 7, showed higher activity,control contains all material as target wells except the peptide.

After figuring out that ChiBP3 showed the higher activity toward chitin it was selected for further investigation. Using ExPASy tools its Isoelectric Point, Molecular Weight, Chemical formula, as well as its hydrophobicity and hydrophilicity were obtained (Figure 10).

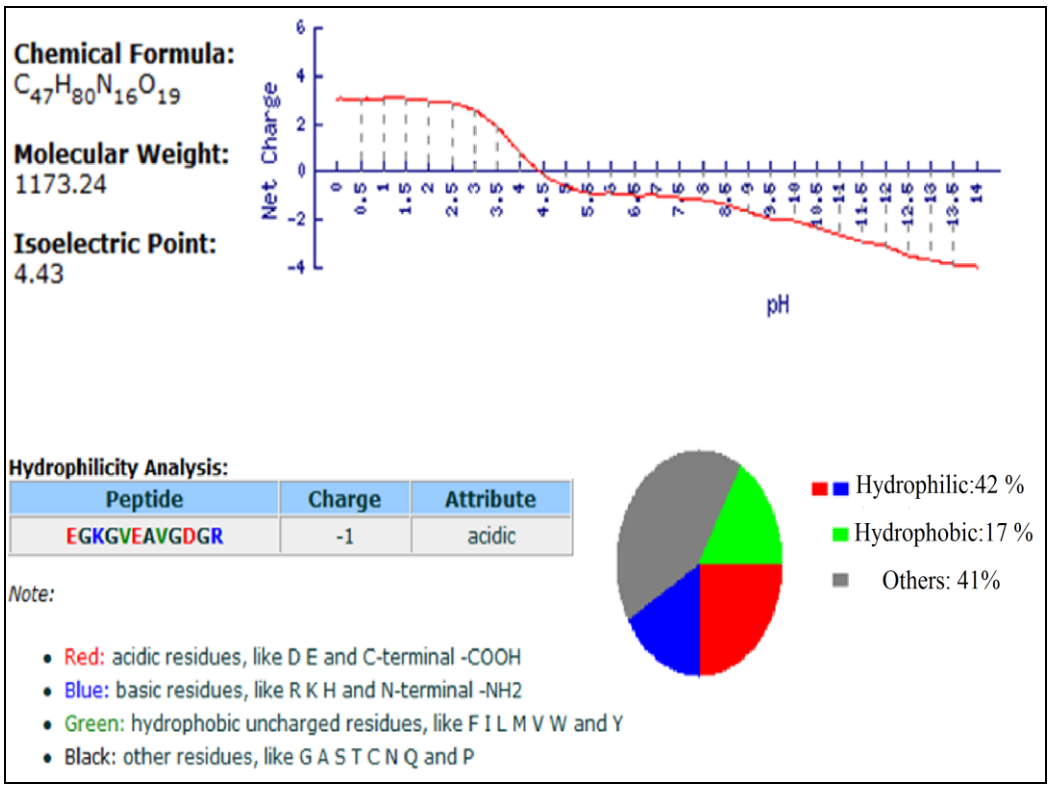


Figure 10. Analysis of ChiBP3 sequence. Top left: chemical formula, MW, and Isoelectric Point (pI), top right: a graph showing Isoelectric Point, lower left: shows the hydophilicity of the sequence along with its net charge of the sequence which indicates the peptide is acidic, Lower right: shows percentage of hydrophilic and hydrophobic residues of the sequence.

The analysis of Figure 10 showed that the selected sequence (ChiBP3 or CBP3) had Molecular Weight of 1173.25 and Isoelectric Point of 4.43. It is acidic with the charge of -1, and hydrophilic peptide sequence. As result, it was soluble in water. Thus, the peptide solution was prepared by dissolving it in water.

After synthesizing the peptide, another experiment was conducted to gauge the influence of phage particles on the binding activity of the peptide. Chitin beads were prepared according to the above-mentioned protocol. In this experiment CTS peptide was used as negative control due to the fact that it is not a chitin binder. In this case, ChiBP3

was dissolved in DMSO so that its activity could be compared with the activity of CTS, which was soluble in DMSO. The result, Figure 11, showed our peptide had a nearly 9 fold greater chitin binding activity in comparison with CTS. Thus, this experiment confirmed that ChiBP3 is specific for chitin. As well, DMSO could not disrupt its binding activity.

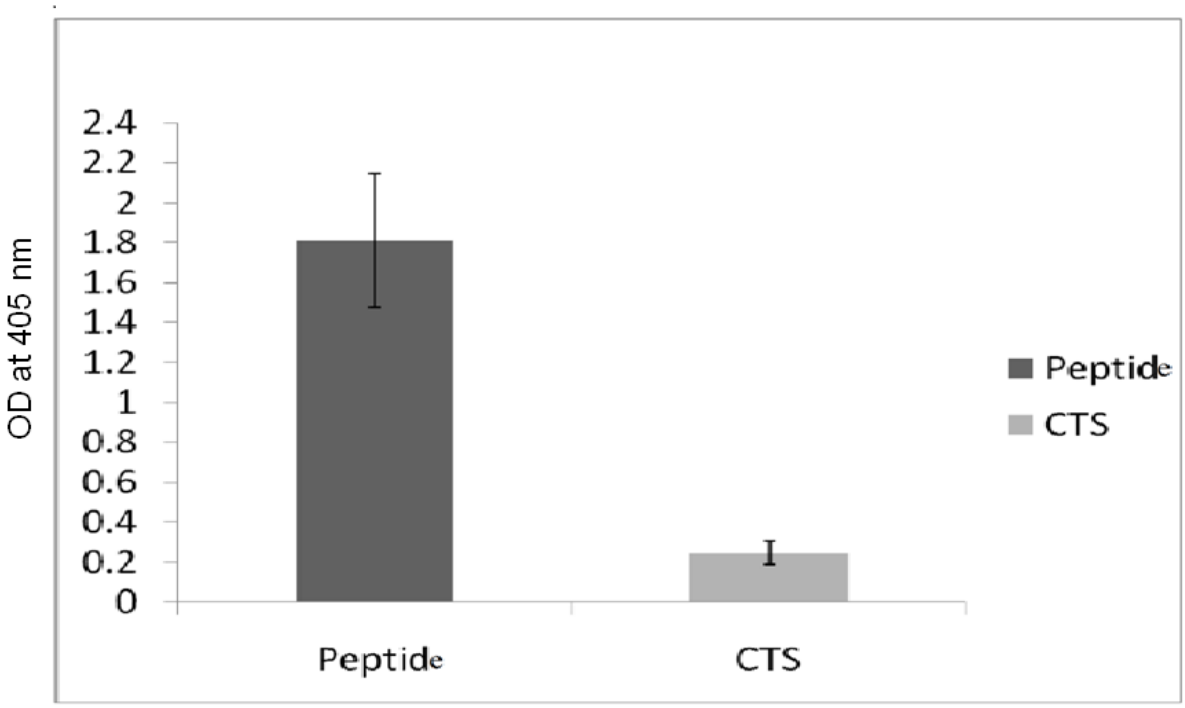


Figure 11. Chitin binding activity of ChiBP3, after synthesizing, vs CTS as control peptide. Both were dissolved in DMSO.

As well, this indicates that the binding activity, which was experimentally tested in the presence of phage particles, comes from the peptide itself and not the phage particles. As result of this experiment, it can be pointed out that phage particles have no or very little effect on the binding activity and ability of the peptides.

Above experiment all were quantitative, so some qualitative experiments were also performed to visualize the biomacromolecular formation. Scanning Electron Microscopy (SEM) was carried out to determine the effect of peptide material on the chitin beads and

prove the specific binding. The results, Figure 12, showed that the peptides bound to the chitin beads have aggregated them and changed their shapes. These effects could be due to the acidic characteristics of the peptide.

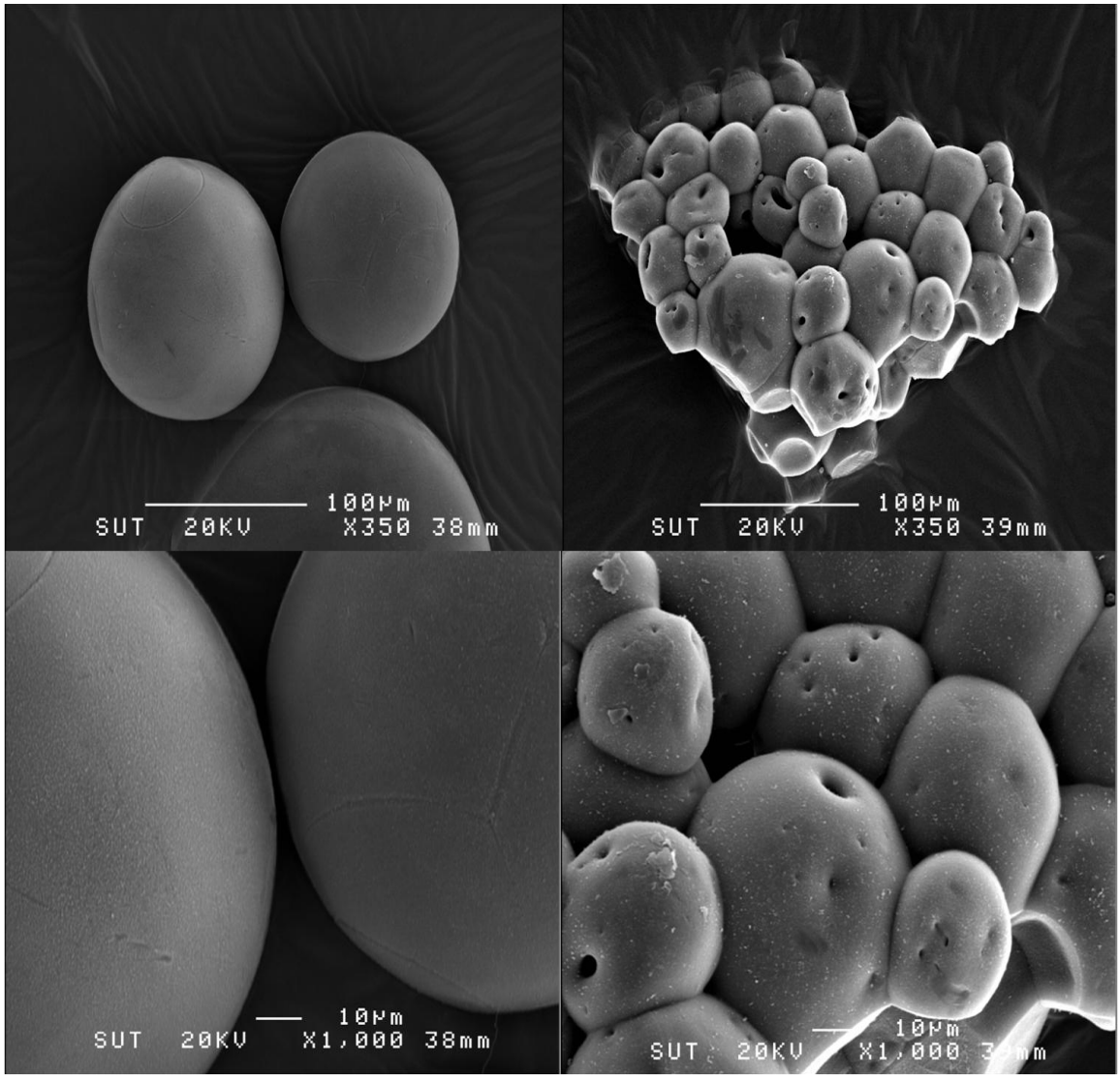


Figure 12. SEM photographs of peptide, in absence of phage, which bound to chitin beads. Upper and lower left are photographs of control, and top and lower right are peptide-attached beads with different magnifications.

These photos showing that the peptide caused the beads to be shrunk while aggregating. It may be due to acidic characteristic of the peptide. Aggregation may come from the attractive force between the peptides entities.

Predicting the structure of large peptide and proteins is difficult, especially when it comes to a short peptide with only 12 residues. As far as we are concerned, there is no specific and precise computer program with high enough reliability in predicting the secondary structure of such short peptides. Most importantly, bound and unbound peptide could possess totally different structure. Nevertheless, clone 3 (ChiBP3) was selected for predicting its secondary structure. A range of programs was used in order to obtain a more reliable predictive secondary structure of 12-mer peptide. Network Protein Sequence Analysis, ExPasy website (Figure 13) (Combet et al., 2000) could give some indications about secondary structure of our selected peptide.

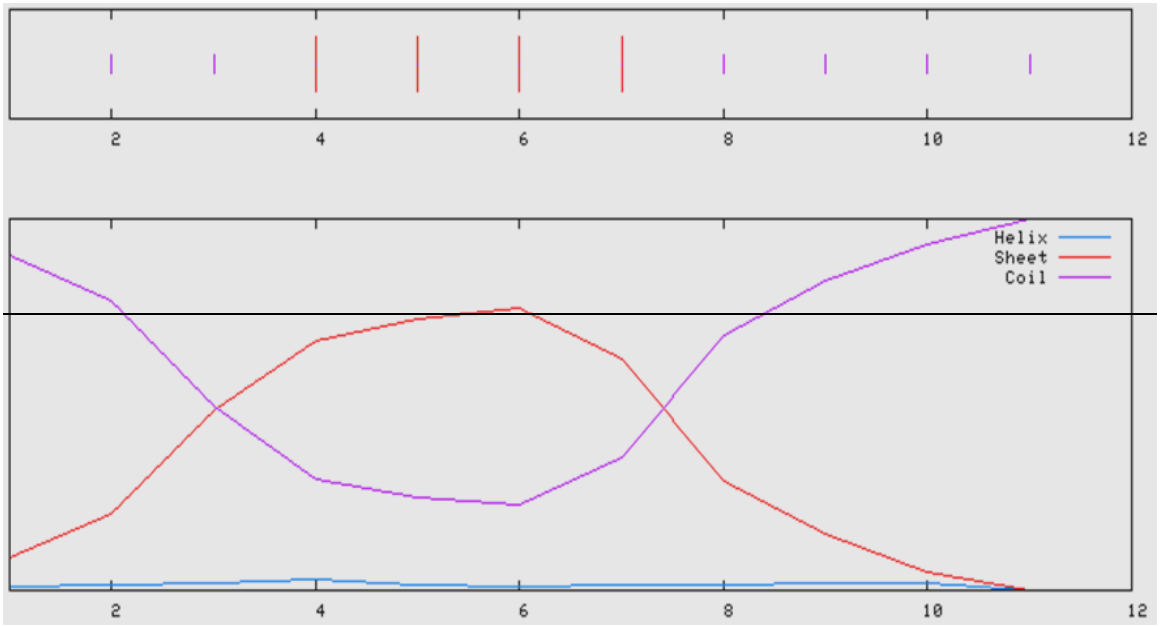


Figure 13. Prediction of secondary structure of selected sequence by using Network Protein Sequence Analysis. It shows both ends have coil structures and in middle has sheet structure.(Combet, C., Blanchet, C. et al. 2000)

According to this program both ends of peptide have a coil structure while its middle part is sheet-like (Fig.13).

Taking advantage of structural information obtained from Figure 13, the decision was made to build a 3D model of the selected peptide sequence by using ChemOffice and WebLab View softwares; the constructed model is shown in Figure 14.

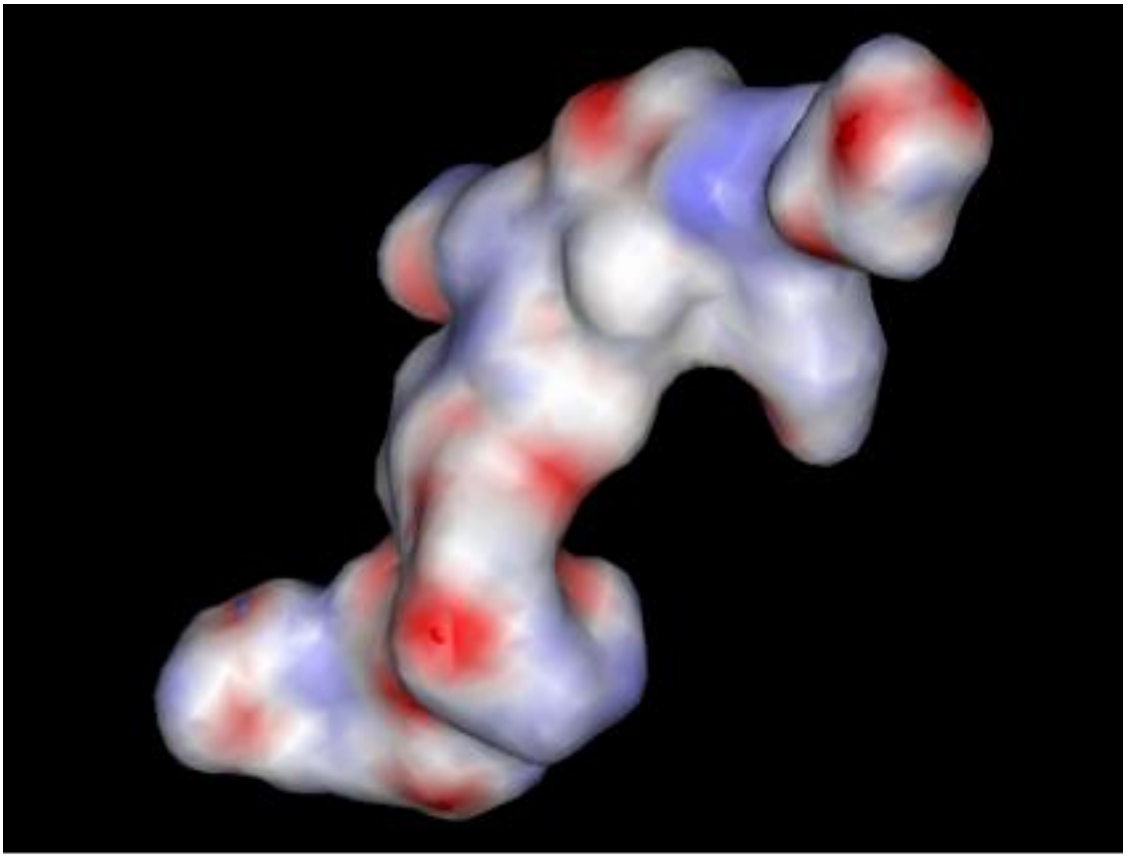


Figure 14. 3D model of CBP3. ChemOffice and WebLab View softwares were used to construct this model. In its ends it has charged residues, while its middle part mainly contains hydrophobic residues. Blue: basic and red: acidic, white: neutral.

This model was obtained after energy minimization and running MD simulation in an aqueous condition but other elements of this system are not shown here so that the model's expected structure can be seen more clearly. Since this sequence has basic, acidic and hydrophobic residues we speculate that acidic and basic residues can make salt bridge, which stabilize the conformation of peptide in solution while the hydrophobic part acts as the main binding mechanism (Abeln and Frenkel, 2008; Marqusee and Baldwin, 1987; Sivaramakrishnan et al., 2008; Smith, 1985).

To figure out whether or not the selected peptide can bind to the monomer and/or oligmer of chitin, N-acetyl-glucoseamin (NAcGlu), chitotriose, and chitohexaose were examined. Figure15 shows the result of TEM photography of NAcGlu after two hours of incubation at room temperature. It seems that the peptide changed the appearance of the structure formed by NAcGlu. Ball-like structures can be easily seen in B-F while the surface of the control (A) has no such structures. Some other ball-like structures were seen, which are shown in C, D, E and F. It is speculated that these are mixture of chitin-monomer and the peptide.

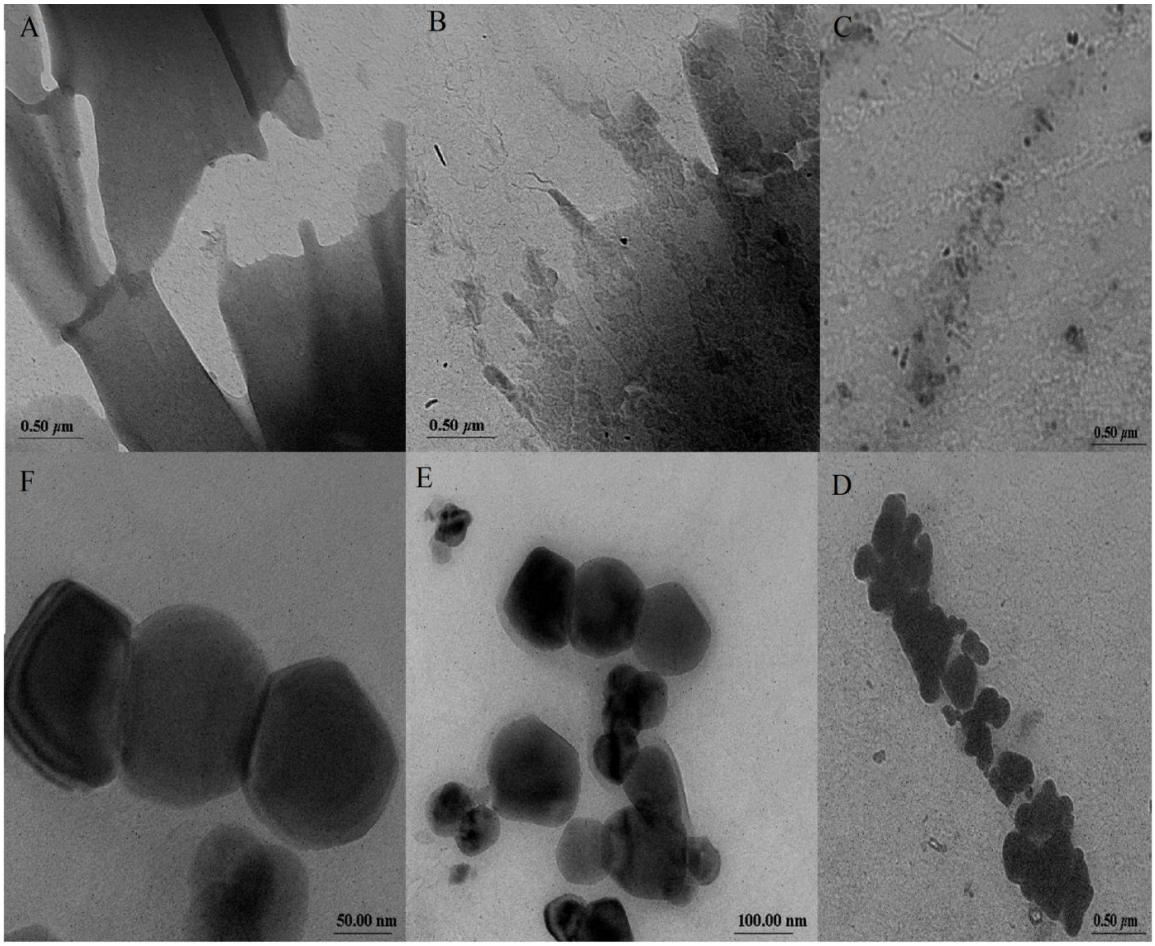


Figure 15. TEM photographs of NAcGlu. A is control (contains only NAcGlu dissolved in water); B, C, D, E, and F are TEM photographs of the peptide and NAcGlu with different magnifications. The photos were taken after 2 hours incubation at room temperature.

With the same condition as NAcGlu, chitobiose was also applied. For unknown reason nothing was seen in control, however an interesting phenomenon was seen in the TEM photographs of chitobiose as shown in Figure 16. The ball-like structure seen in NAcGlu photos (Figure 15) were clearly seen in TEM photographs of chitobiose-peptide sample (Figure 16). This may be due to the bigger size of chitobiose molecules in comparison to NAcGlu molecules, thus, the bigger structure could be made.

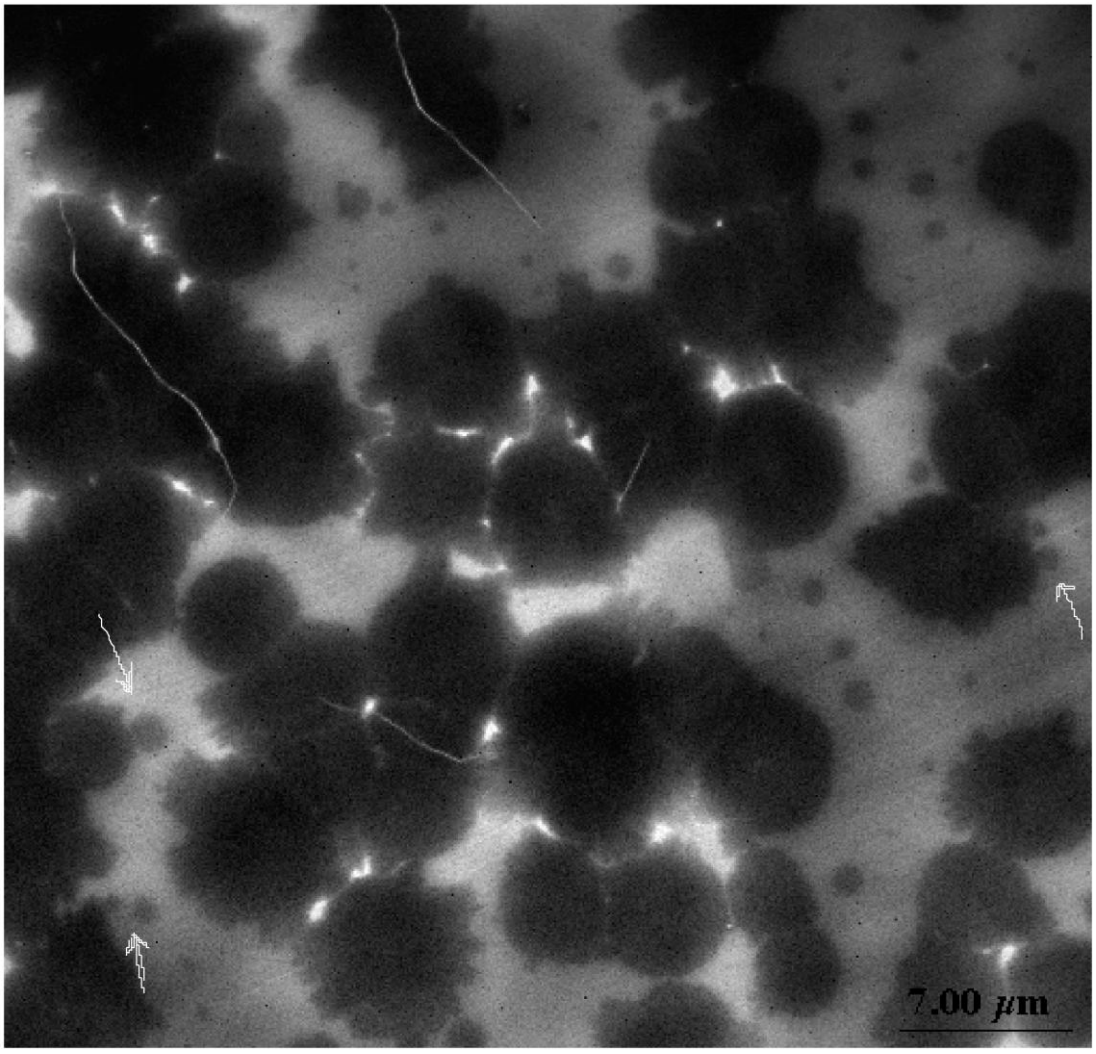


Figure 16. TEM photographs of chitobiose in the presence of the peptide. It was taken after 2 hours incubation at room temperature.

The sizes of ball-like shapes seen in Figure 16 are different. It seems the bigger ones are made by joining the small ones, which is shown by arrows. It may be an indication toward that the structures went through a self-assembly process.

Chitohexaose was another derivative of chitin that was examined. After two hours incubation at room temperature, TEM photographs were taken Figure 17. It seems the peptide caused the chitohexamer units to get closer to each other. It may be due to hydrogen interactions between hexamers' units and the peptides residues. However, it needs to be

investigated with advance equipment. Then, the same samples were used for next step. The samples were incubated at 42 ˚C for 15 hours, and then were used to take photographs using SEM (Figure 18).

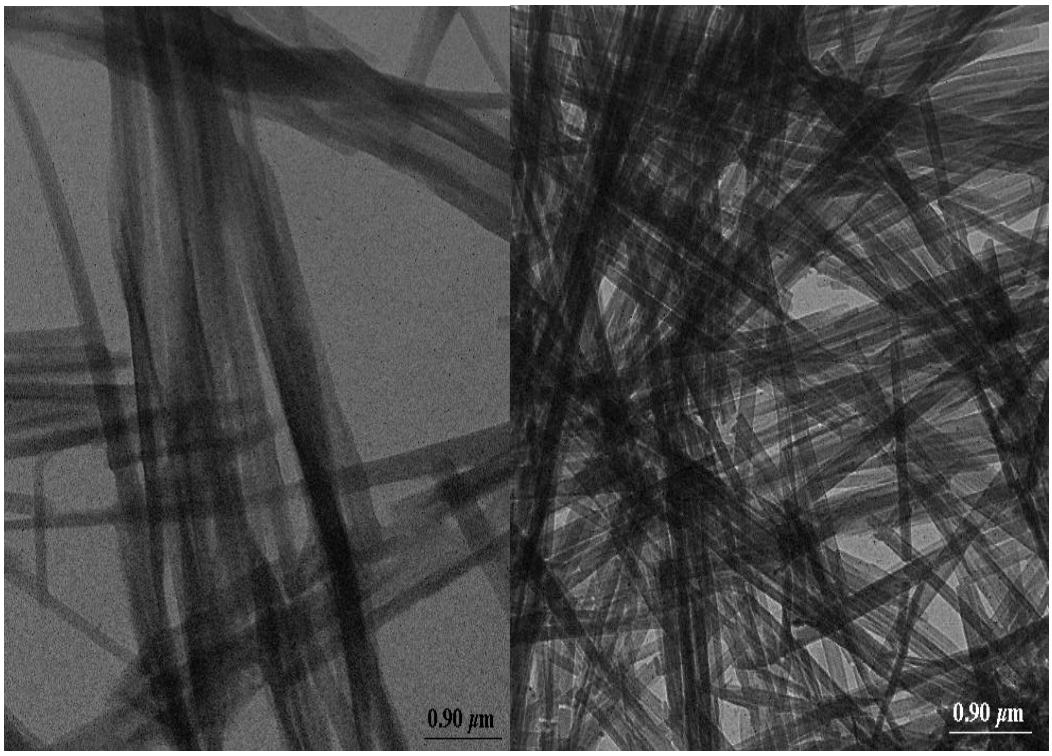


Figure 17. TEM photographs of peptide-chitohexaose vs control, after 2 hours of incubation at room temperature. Left: Control (contains only chitohexamer), right: peptide-chitohexaose.

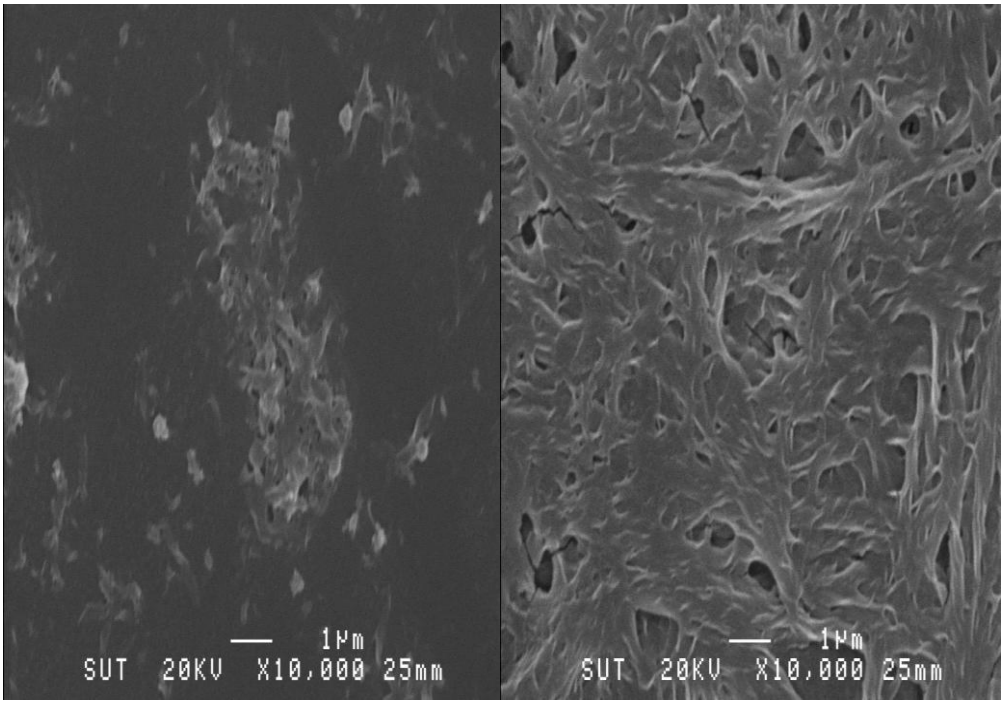


Figure 18. SEM photographs after 15 hours incubation at 42^{o}C. Control contains only chitohexaose (left) vs. Chitohexaose with the peptide(right).

Obviously hexamer of chitin was induced to form a porous structure with nano- and micro-size holes. These results suggested that the short charged peptide was able to bind to chitohexaose and change its behavior toward a well-order shape. It is worthwhile to be mentioned that this structure was disappeared after leaving it at 4˚C overnight, and re-appeared by increasing the temperature to 42˚C. However, the structure was stable at room temperature. This phenomenon will be more discussed later.

Based on the information obtained from chitohexaose experiment, another experiment was done in which, as well as chitohexaose and the peptide, $CaCO_3$ was also added into the reaction. The hypothesis was that, if the structure formed by chitohexaose and the peptide in the last experiment was real so it must form again while entrapping the

$CaCO_3$ crystals. Results showed, Figure 19, that $CaCO_3$ crystals were trapped in structure formed by chitohexaose and the peptide.

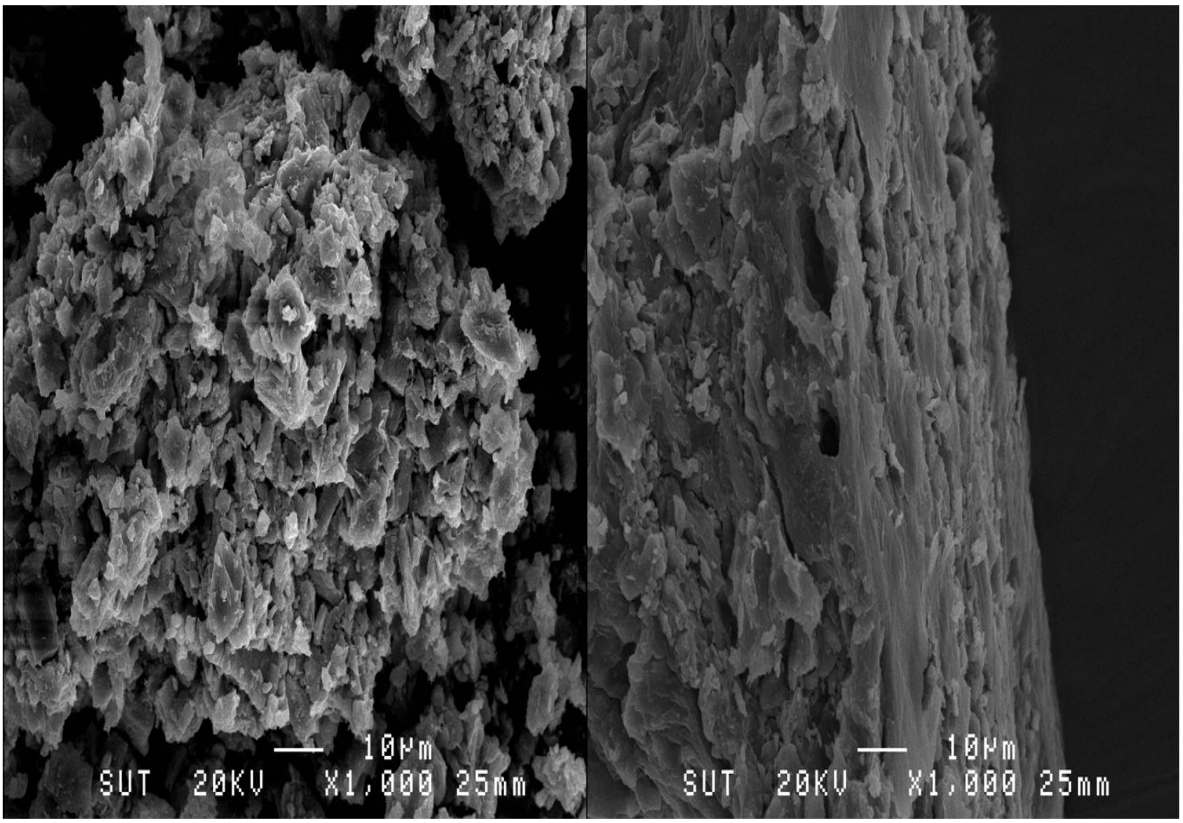


Figure 19. SEM photograph of the structure formed by chitohexaose and $CaCO_3$ with and without the addition of the peptide. Left: $CaCO_3$ and Hexamer of chitin, right: $CaCO_3$-Hexamer-peptide, while in the presence of chitin alone $CaCO_3$ formed rosette-like aggregate; it seems it lost this form in the presence of the peptide.

To find out more details about the structure formed by chitohexaose and the peptide in the presence of $CaCO_3$, another photograph was taken with much higher magnification. Figure 20 shows that the structure is similar to the one formed by chitohexaose in the presence of the peptide.

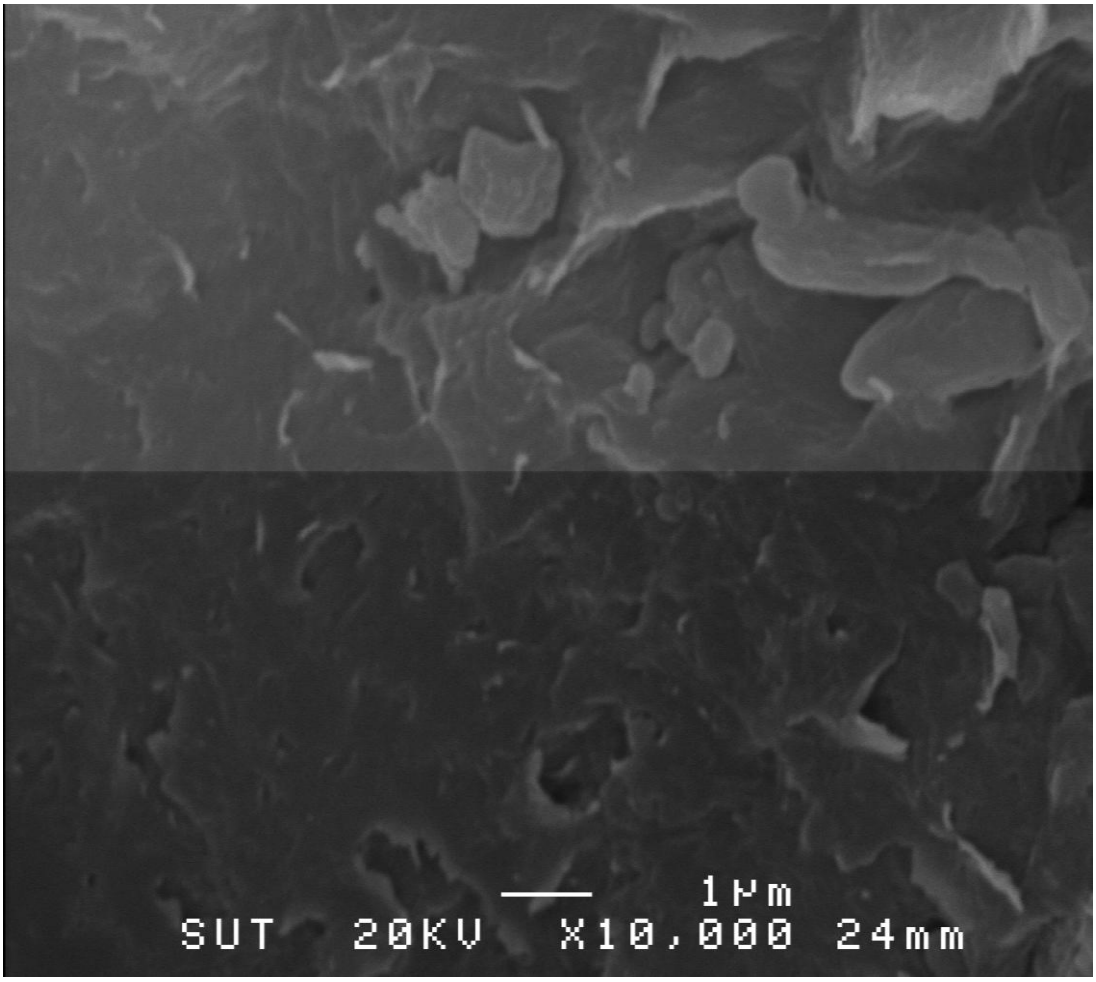
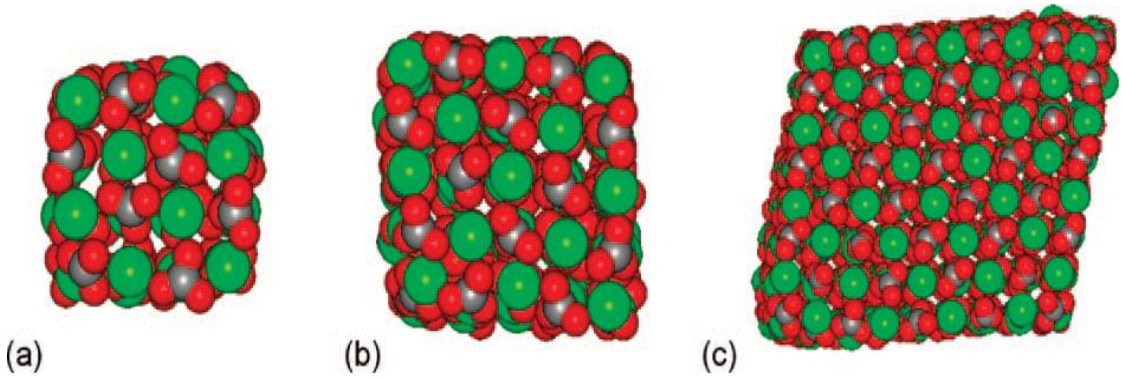


Figure 20. The Structure formed by $CaCO_3$-Hexamer-peptide with higher magnification.

Next photo (Figure 21) showing that in later case $CaCO_3$ forms typical rhombohedral crystals, which may indicates that peptide interrupt interaction between chitin and $CaCO_3$ crystals allowing them to grow differently.



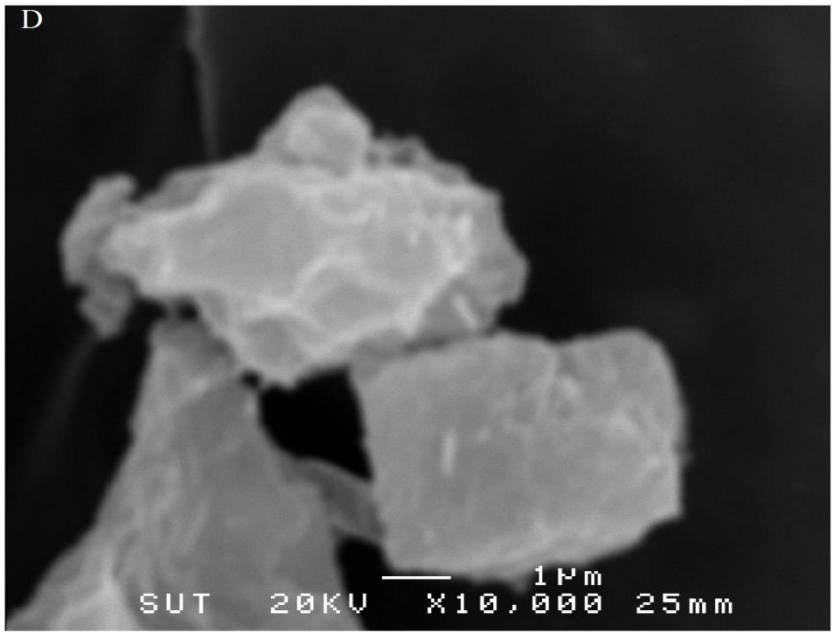


Figure 21. Snapshot of structures of (a) 32 $CaCO_3$, (b) 75 $CaCO_3$, and (c) 324 $CaCO_3$ nanoparticles with an initial calcite structure after 1 ns molecular dynamics simulation in vacuo at 300 K, where green: calcium, red: oxygen, and gray: carbon. Cooke, D. J.; Elliott, J. A. *J. Chem. Phys.* 2007, *127, 104706. Copyright 2007, American Institute of Physics.* (D) Crystal of $CaCO_3$ entrapped into structure formed by chitohexaose and the peptide.

As for this experiment, more research need to be done to answer the following questions; how mineralization is harnessed in nano- and/or micro-level, how to, basically, direct the spatiotemporal deposition of macromolecules in solution to produce structures with different functions for different applications such as development of bioinspired instruments, or complexes.

After all mentioned experiments a question was arisen, which was whether or not the peptide can be used to harness the behavior of colloidal chitin. So, another experiment was set and carried out. Colloidal chitin was used to coat the surface of a tube. As shown in Figure 22, by aid of the peptide it coated the surface of the tube.

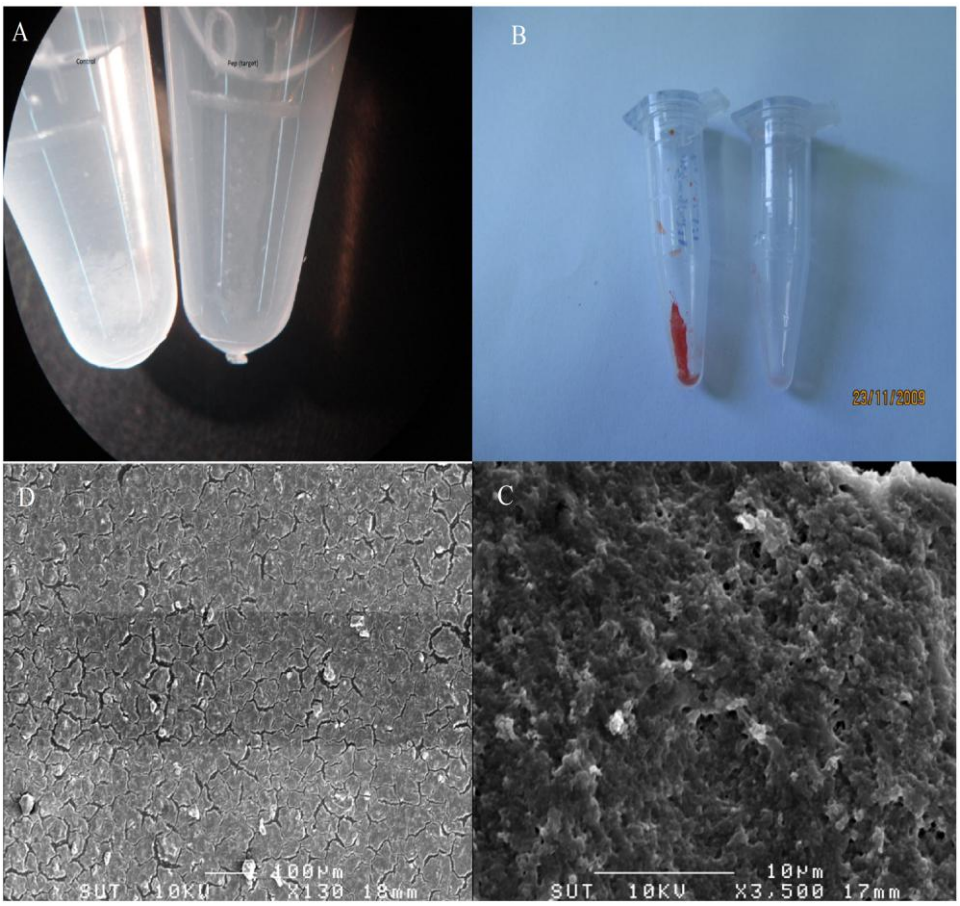


Figure 22. Colloidal chitin coated the surface by aid of peptide. A, left is tube contains chitin and right tube contains chitin and the peptide both in water. It shows in the presence of peptide, chitin stretched out over the inner surface of the tube; B, the same tube as part A, after washing and dying with Congo red, red spot is structure formed by chitin in the presence of the peptide, C and D are SEM photographs of the structure of part A and B.

Temperature was increased to 42 ˚C for 15 hours and then on lowering the temperature to room temperature the coating phenomenon was seen to have taken place. No coating structure developed if it was just left at room temperature without heating beforehand. This may indicate that the peptide perhaps belong to type II self-assembling peptides. These type of peptides show different structures in different temperatures.

Immediately after dissolving in water they show a α-helical structure, when stored at 4 ˚C they form β-sheet structure and by increasing temperature to around 50 ˚C the β-sheet structure is converted to α-helical structure. And at around room temperature, e.g. 23 ˚C, they regain the β-sheet structure (Zhang and Altman, 1999).

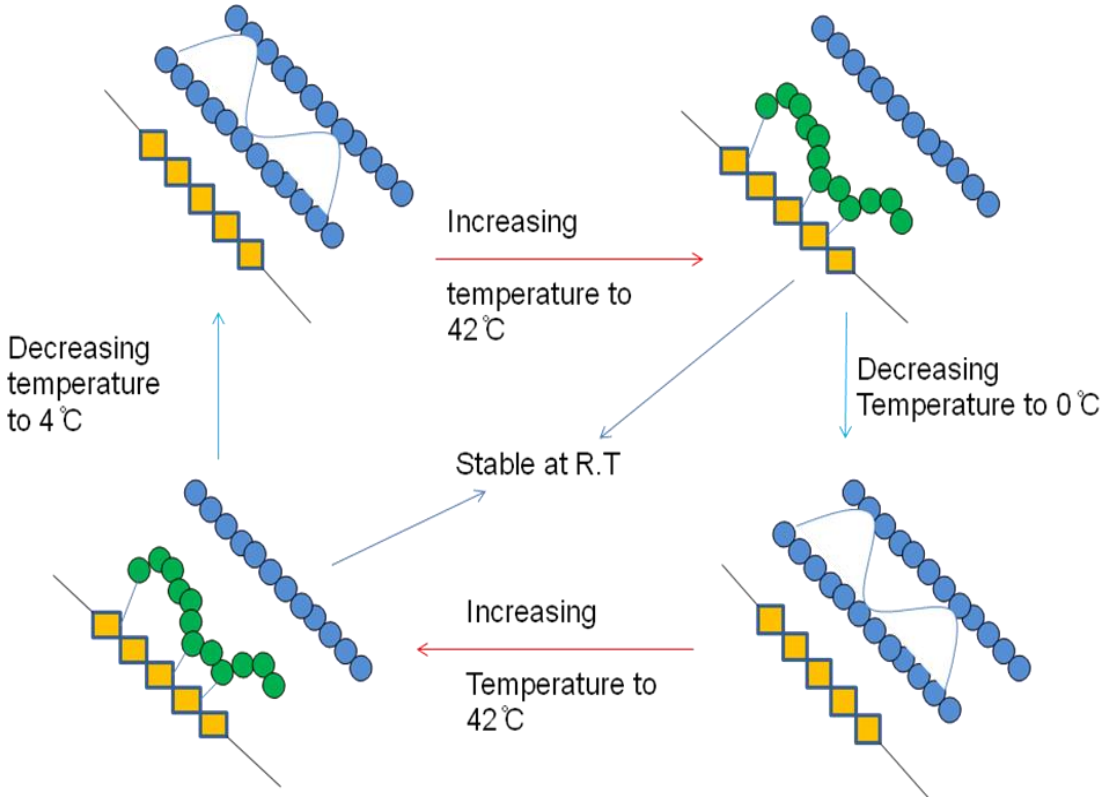


Figure 23. Speculative events at molecular level. Blue and green balls: peptide residues, blue line: interaction behavior, brick-like shapes: chitin and/or derivatives.

Our system also showed different behaviors at different temperatures. For instance, our system formed structures (surface covering) at 42 ˚C while no structure was seen at room temperature without initially increasing the temperature. In another experiments (data not shown) the structure formed by the system lost its integrity at 4 ˚C and re-gained it again at 42 ˚C. We speculate (Figure 24) that at 4 ˚C and room temperature the peptide forms β-sheet structure and in this form it has no or weak interaction with chitin, thus, no

structure is formed or if there is already a structure such as film or membrane it will dissociate. On the other hand, when it is in high temperature with α-helical structure probably has better and stronger interaction with chitin, which may be due to helical dipole moment of α-helix. All α-helices have this helical dipole moments which stabilize them (Sali et al., 1988). It should be noted that this is within intra and inter helices interaction.

Regarding the fact that there is no full electrostatic interaction between peptide and neutral saccharides, we anticipate that the main interactions between peptide and chitin are hydrophobic and Hydrogen bonding. (Fukusaki et al., 2004).

The aforementioned experiment is a simple and straightforward coating method. Another advantage of this system is that only water was used as solution. As can be noticed, the structure remained porous, which is another advantage of the system if it is going to be used for tissue engineering, drug delivery carrier, or stem cell research. This porosity is crucial, especially for cell growth (Drury and Mooney, 2003b).

CHAPTER V

PROPOSED MECHANISM OF SURFACE-CHITIN-PEPTIDE INTERACTION

There are several mechanisms that can be proposed to explain how chitin-peptide complex interacts with the surface (Figure 24). Figure 24.A indicates that the binding may be due to interaction between one of the two ends of the peptide that contain charge residues. Whilst Figure 24.B shows that the binding may comes from the hydrophobic interaction between chitin and the hydrophobic residues of the peptide. In this case electrostatic interaction between charges residues of the peptide and water molecules stabilize the complex. Part C of figure 24 proposed a mechanism which is a mixture of the parts A and B.

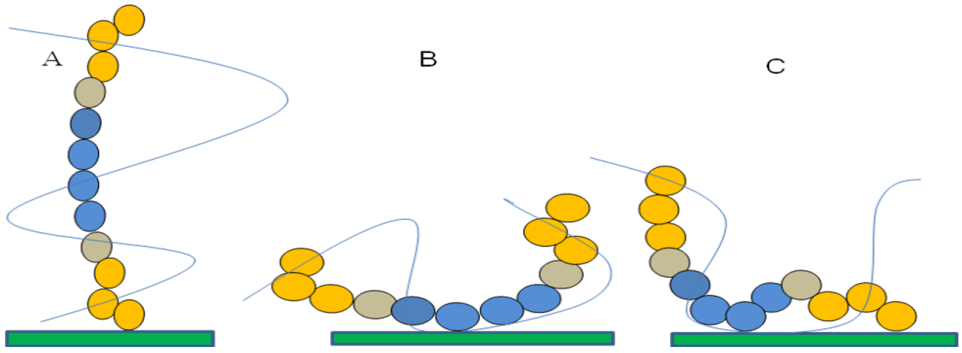


Figure 24. Proposed mechanisms of surface-chitin-peptide interaction. Green: surface, orange: charge residues, blue: hydrophobic part of peptide, gray: flexible residues such as Gly , and blue: a chitin chain.

CHAPTER VI

CONCLUSION AND FUTURE PERSPECTIVE

These results have proved the suitability and usefulness of phage display technology for biomaterial research and specifically chitin research. Chitin was used without chemical modification, a form with the highest mechanical strength. It is speculated that the ChiBP peptides can be another variants of R.R consensus sequence. In other words, they could be squeezed forms of R.R consensus sequence, which means they could be used to build chitin-based nano- and micro-structures. The coating process is simple and straightforward. Only water was utilized which has many advantages in comparison with other chemicals, which are harmful for organisms and the environment. The results of this research have potential applications in several fields of bio-nanotechnology; surface coatings for implantable devices, scaffold for tissue engineering and stem cell research, drug delivery, etc.

The next step would be evaluation of the effects of the peptide on cell growth or cell adhesion. Further studies needs to be done to optimize this system for *in vivo* experiment. More research must be conducted to determine the mechanism of formation of the formed structure, which may be helpful to understand peptide/protein-carbohydrate interaction in nature. As well, affinity of the peptide(s) toward chitin needs to be measured, using which machine?. Applying supercomputer running what? may give more information as to understanding the behaviour of the peptide(s) while interacting with chitinous materials. Regarding the fact that chitin is one of main component of fungi cell wall, antifungal activity of the peptide(s) is an attractive project. Finally, optimizing the biomineralization process would be helpful to design and construct nano- and micro-structures.

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APPENDIX

สำเนา

คำขอรับสิทธิบัตร/อนุสิทธิบัตร

☑ การประดิษฐ์
☐ การออกแบบผลิตภัณฑ์
☐ อนุสิทธิบัตร ยื่นผ่านพาณิชย์จังหวัด

ข้าพเจ้าผู้ลงลายมือชื่อในคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ ขอรับสิทธิบัตร/อนุสิทธิบัตร ตามพระราชบัญญัติสิทธิบัตร พ.ศ 2522 แก้ไขเพิ่มเติมโดยพระราชบัญญัติสิทธิบัตร (ฉบับที่ 2) พ.ศ 2535 และ พระราชบัญญัติสิทธิบัตร (ฉบับที่ 3) พ.ศ 2542

สำหรับเจ้าหน้าที่

วันรับคำขอ 28 ธ.ค. 2552	เลขที่คำขอ 0901005873
วันยื่นคำขอ 14 ธ.ค. 52	

สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ

ใช้กับแบบผลิตภัณฑ์
ประเภทผลิตภัณฑ์

วันประกาศโฆษณา	เลขที่ประกาศโฆษณา

วันออกสิทธิบัตร/อนุสิทธิบัตร	เลขที่สิทธิบัตร/อนุสิทธิบัตร

ลายมือชื่อเจ้าหน้าที่

1.ชื่อที่แสดงถึงการประดิษฐ์/การออกแบบผลิตภัณฑ์

กรรมวิธีการตรึงไคตินและอนุพันธ์ของไคตินสำหรับใช้กับเทคโนโลยีเฟจและเปปไทด์ ไคตินที่ได้จากกรรมวิธีนี้

2.คำขอรับสิทธิบัตรการออกแบบผลิตภัณฑ์นี้เป็นคำขอสำหรับแบบผลิตภัณฑ์อย่างเดียวกันและเป็นคำขอลำดับที่
ในจำนวน คำขอ ที่ยื่นในคราวเดียวกัน

3.ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร และที่อยู่ (เลขที่ ถนน ประเทศ)

มหาวิทยาลัยเทคโนโลยีสุรนารี
111 ถนนมหาวิทยาลัย ตำบลสุรนารี อำเภอเมือง จังหวัดนครราชสีมา 30000 ประเทศไทย

3.1 สัญชาติ	ไทย
3.2 โทรศัพท์	(044) 224818
3.3 โทรสาร	(044) 224818
3.4 อีเมล์	-

4.สิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร

☐ ผู้ประดิษฐ์/ผู้ออกแบบ ☑ ผู้รับโอน ☐ ผู้ขอรับสิทธิโดยเหตุอื่น

5.ตัวแทน(ถ้ามี)/ที่อยู่ (เลขที่ ถนน จังหวัด รหัสไปรษณีย์)

5.1 ตัวแทนเลขที่	---
5.2 โทรศัพท์	---
5.3 โทรสาร	---
5.4 อีเมล์	---

6.ผู้ประดิษฐ์/ผู้ออกแบบผลิตภัณฑ์ และที่อยู่ (เลขที่ ถนน ประเทศ)

รองศาสตราจารย์ ดร.มณฑารพ ยมาภัย 292 ซอยริคลองมอญ แขวงบ้านช่างหล่อ เขตบางกอกน้อย กรุงเทพมหานคร
นายพีอิลซอล โคชอบ 111 ถนนมหาวิทยาลัย ตำบลสุรนารี อำเภอเมือง จังหวัดนครราชสีมา

7. คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิม

ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้ถือว่าได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ ในวันเดียวกับคำขอรับสิทธิบัตร
เลขที่ วันยื่น เพราะคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิมเพราะ

☐ คำขอเดิมมีการประดิษฐ์หลายอย่าง ☐ ถูกคัดค้านเนื่องจากผู้ขอไม่มีสิทธิ ☐ ขอเปลี่ยนแปลงประเภทของสิทธิ

หมายเหตุ ในกรณีที่ไม่อาจระบุรายละเอียดได้ครบถ้วน ให้จัดทำเป็นเอกสารแนบท้ายแบบพิมพ์นี้โดยระบุหมายเลขกำกับข้อและหัวข้อที่แสดงรายละเอียดเพิ่มเติมดังกล่าวด้วย

8.การยื่นคำขอนอกราชอาณาจักร

วันยื่นคำขอ	เลขที่คำขอ	ประเทศ	สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ	สถานะคำขอ
8.1				
8.2				
8.3				

8.4 ☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอสิทธิให้ถือว่าได้ยื่นคำขอนี้ในวันที่ได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรในต่างประเทศเป็นครั้งแรก โดย

☐ ได้ยื่นเอกสารหลักฐานพร้อมคำขอนี้ ☐ ขอยื่นเอกสารหลักฐานหลังจากวันยื่นคำขอนี้

9.การแสดงการประดิษฐ์ หรือการออกแบบผลิตภัณฑ์ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรได้แสดงการประดิษฐ์ที่หน่วยงานของรัฐเป็นผู้จัด

วันแสดง วันเปิดงานแสดง ผู้จัด

10.การประดิษฐ์เกี่ยวกับจุลชีพ

10.1 เลขทะเบียนฝากเก็บ	10.2 วันที่ฝากเก็บ	10.3 สถาบันฝากเก็บ/ประเทศ

11.ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอยื่นเอกสารภาษาต่างประเทศก่อนในวันยื่นคำขอนี้ และจะจัดยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ที่จัดทำเป็นภาษาไทยภายใน 90 วัน นับจากวันยื่นคำขอนี้ โดยขอยื่นเป็นภาษา

☐ อังกฤษ ☐ ฝรั่งเศส ☐ เยอรมัน ☐ ญี่ปุ่น ☐ อื่นๆ

12.ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้อธิบดีประกาศโฆษณาคำขอรับสิทธิบัตร หรือรับจดทะเบียน และประกาศโฆษณาอนุสิทธิบัตรนี้ หลังจากวันที่ เดือน พ.ศ

☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอให้ใช้รูปเขียนหมายเลข ในการประกาศโฆษณา

13.คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ประกอบด้วย

ก. แบบพิมพ์คำขอ 2 หน้า

ข. รายละเอียดการประดิษฐ์ หรือคำพรรณนาแบบผลิตภัณฑ์ 3 หน้า

ค. ข้อถือสิทธิ 1 หน้า

ง. รูปเขียน 1 รูป 1 หน้า

จ. ภาพแสดงแบบผลิตภัณฑ์

☐ รูปเขียน รูป หน้า

☐ ภาพถ่าย รูป หน้า

ฉ. บทสรุปการประดิษฐ์ 1 หน้า

14.เอกสารประกอบคำขอ

☑ เอกสารแสดงสิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร

☐ หนังสือรับรองการแสดงการประดิษฐ์/การออกแบบผลิตภัณฑ์

☐ หนังสือมอบอำนาจ

☐ เอกสารรายละเอียดเกี่ยวกับจุลชีพ

☐ เอกสารการขอนับวันยื่นคำขอในต่างประเทศเป็นวันยื่นคำขอในประเทศไทย

☐ เอกสารขอเปลี่ยนแปลงประเภทของสิทธิ

☑ เอกสารอื่น ๆ

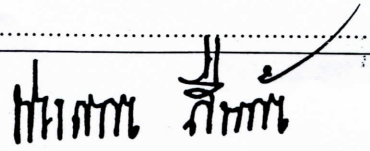
- ประกาศแต่งตั้งอธิการบดี

15. ข้าพเจ้าขอรับรองว่า

☑ การประดิษฐ์นี้ไม่เคยยื่นขอรับสิทธิบัตร/ อนุสิทธิบัตรมาก่อน

☐ การประดิษฐ์นี้ได้พัฒนาปรับปรุงมาจาก.....

16.ลายมือชื่อ (☑ ผู้ขอรับสิทธิบัตร / อนุสิทธิบัตร; ☐ ตัวแทน)



(ศาสตราจารย์ ดร.ประสาท สืบค้า)

หมายเหตุ บุคคลใดยื่นขอรับสิทธิบัตรการประดิษฐ์หรือการออกแบบผลิตภัณฑ์ หรืออนุสิทธิบัตร โดยการแสดงข้อความอันเป็นเท็จแก่พนักงานเจ้าหน้าที่ เพื่อให้ได้ไปซึ่งสิทธิบัตรหรืออนุสิทธิบัตร ต้องระวางโทษจำคุกไม่เกินหกเดือน หรือปรับไม่เกินห้าพันบาท หรือทั้งจำทั้งปรับ