Development of IgY Binding Peptide using T7 Phage Display Technology and its Applications for Antibody Purification

(T7 ファージ提示技術による IgY 結合ペプチドの開発と抗体精製への応用)

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Abstract

Chicken egg yolk immunoglobulin (IgY) is a functional substitute for mammalian IgG for antigen detection. Traditional IgY purification methods involve multi-step procedures resulting in low purity and recovery of IgY. In this study, we developed a simple IgY purification system using IgY-specific peptides identified by T7 phage display technology. From disulfide-constrained random peptide libraries constructed on T7 phage, we identified three specific binding clones (Y4-4, Y5-14, and Y5-55) through repeated biopanning. We synthesized two peptides derived from Y4-4, Y5-55 phage clones. In surface plasmon resonance analysis, those peptides showed high binding specificity to IgY-Fc and moderate affinity for IgY-Fc ($K_d = 7.3 \pm 0.2$ µM for Y4-4 and 4.4 ± 0.1 µM for Y5-55). To evaluate the ability to purify IgY, we performed immunoprecipitation and affinity high-performance liquid chromatography using IgY-binding peptides; the result indicated, that these peptides can be used as affinity ligands for IgY purification. To avoid the viscous property of egg yolk in the purification process, we also optimized the egg yolk delipidation technique using distilled water dilution followed by ammonium sulfate precipitation to remove insoluble lipids from the egg yolk. Finally, we then used a peptide-conjugated column to purify IgY from pre-treated egg yolks. Here, we report the construction of a cost-effective, one-step IgY purification system, with high purity (~92%) and recovery.

Schematic overview of this research
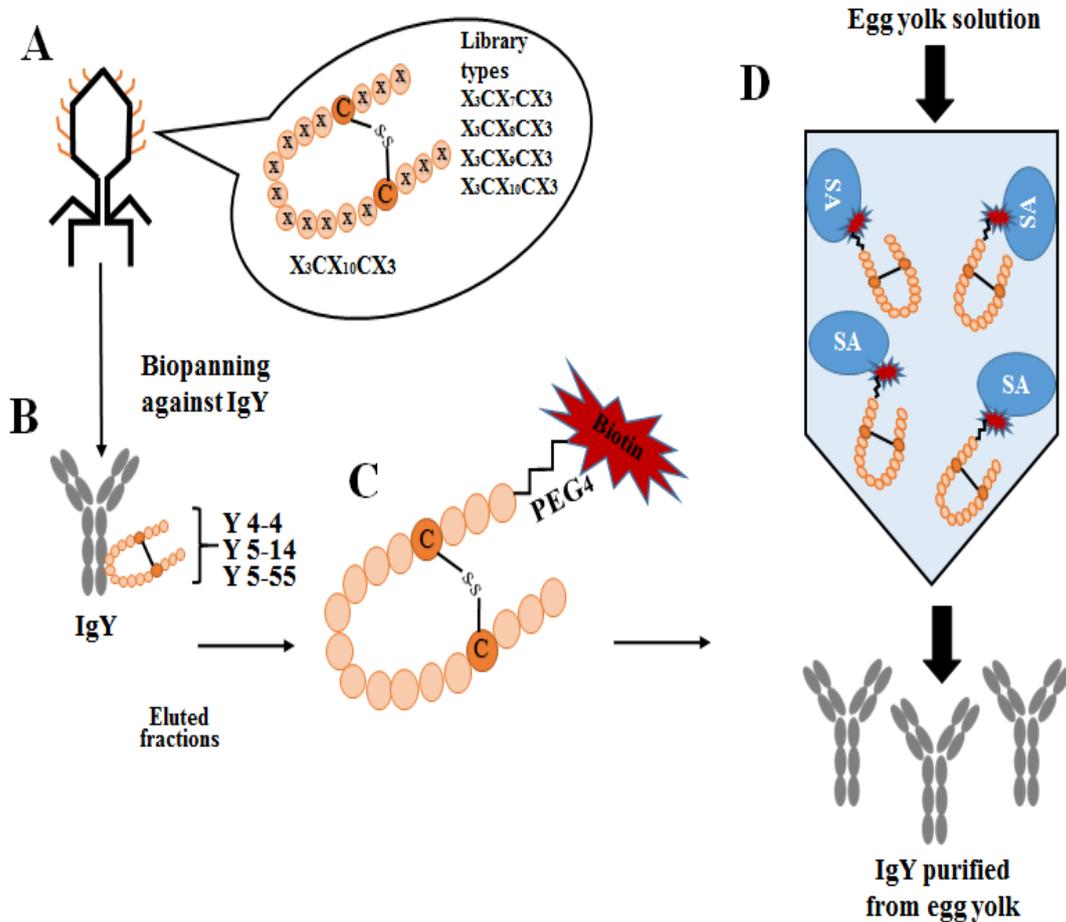
Schematic diagram for the development of IgY binding peptide using T7 phage display technology and its applications for antibody purification in this study.

(**A**) Disulfide-constrained random peptide libraries ($X_3CX_{7-10}CX_3$ random peptides, where X represents the randomized amino acid positions) were constructed on T7 phage using phage display technology (described in **Chapter 2**). (**B**) Three IgY binding phage clones (Y4-4, Y5-14, and Y5-55) were successfully identified thorough repeated biopanning against IgY from random peptide libraries (described in **Chapter 3**). (**C**) Biotinylated IgY-binding peptides derived from Y4-4 and Y5-55 phage clones were synthesized for their characterizations (described in **Chapter 3**). (**D**) Egg yolk were pre-treated using an optimized delipidation technique (described in **Chapter 4**) and used in affinity column conjugated with biotinylated Y4-4 peptide to purify IgY (described in **Chapter 5**).

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Chapter 1

General Introduction

1.1 Background

An antibody is a large Y-shaped protein produced by the immune system of the host body to prevent the pathogen from harming the body (1). There are many types of pathogens like virus, bacteria or chemicals, which are called antigens. When an antigen invades into the host body, the antibodies will be produced by plasma cell to mark the antigen and finally to degrade them. Antibodies can specifically target and neutralize foreign antigens by binding to them (2-3), activate the phagocytosis and complement system of the immune system . Antibodies consist of two heavy (H) and two light (L) chains (Figure 1), where the L- chain can consist of either κ or λ chain. N-terminal domains of the H chain and L chain which being called variable region (VH and VL), represent the most diverse amino acid sequence. The hypervariable region is also known as complementarity determining regions (CDR), the binding specificity of antibody against the antigen is determined by the amino acid sequence of this portion. On the other hand, the portion other than the variable domain are called constant regions ($CH_{1,2,3}$ and CL), has a constant amino acid sequence, this part is mainly involved in effector functions such as the promotion of cytotoxicity by natural killer cells and phagocytosis by activated the phagocytic cells of the complement system.
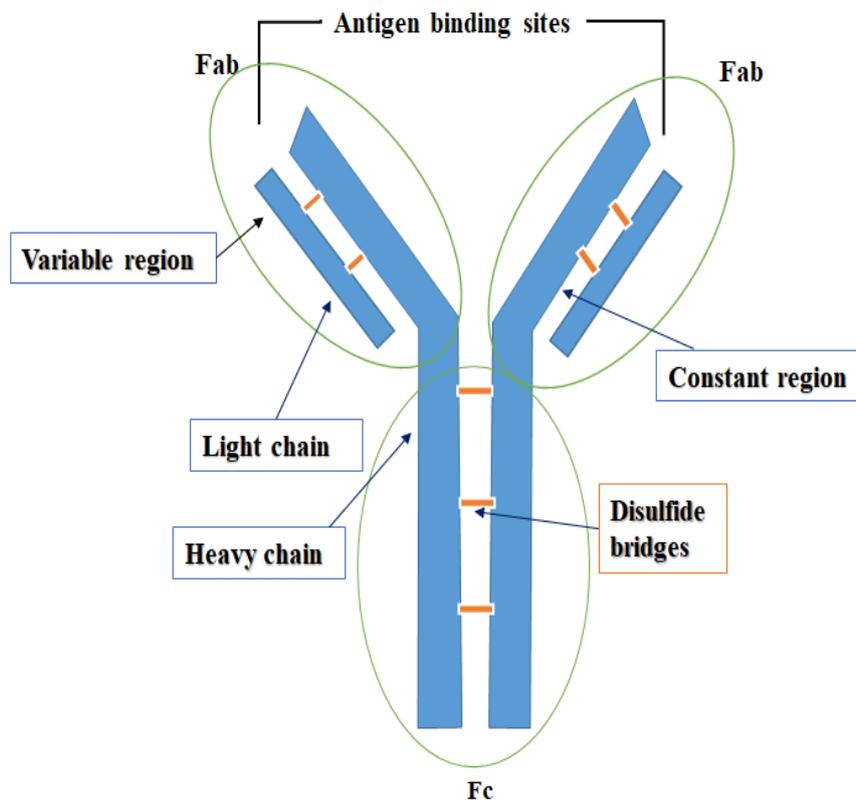
Figure 1 The basic structure of an antibody.

The ability of antibodies to recognize their antigens with exquisite specificity and high affinity makes them an attractive class of molecules to bind extracellular targets and generate a desired pharmacological effect. Antibodies also benefit from their ability to harness an active salvage pathway, mediated by the neonatal Fc receptor (FcRn), thereby enhancing their pharmacokinetic (PK) life span and mitigating the need for frequent dosing. The antibodies and antibody derivatives approved in the United States and the European Union span a wide range of therapeutic areas, including oncology, auto-immunity, ophthalmology, and transplant rejection. They also harness disparate modes of action like a blockade of ligand binding and subsequent signalling, and receptor and

signal activation, which target effector functions, namely antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), and delivery of the cytotoxic payload. Antibodies are generated by the assembly of two heavy chains and two light chains to produce two antigen-binding sites and a single constant domain region. The constant domain sequence in the heavy chain designates the subtype. The light chains can belong to two families (λ and κ), with most of the currently marketed antibodies belonging to the k family. The antigen-binding regions can be derived by proteolytic cleavage of the antibody to generate antigen-binding fragments (Fab) and the constant fragment (Fc, also known as the fragment of crystallization). The Fab comprises the variable regions (variable heavy (VH) (4) and variable light (VL)) and constant regions (CH1 and Cκ/Cλ). Within these variable regions reside loops called complementarity determining regions (CDRs) responsible for direct interaction with the antigen. Because of the significant variability in the number of amino acids in these CDRs, there are multiple numbering schemes for the variable domains (5, 6) but only one widely used numbering scheme for the constant domain (including portions of the CH1, hinge, and the Fc) called the EU numbering system (7).

There are two general methods to generate antibodies in the laboratory. The first utilizes the traditional methodology employing immunization followed by recovery of functional clones either by hybridoma technology or, more recently, by recombinant cloning of variable domains from previously isolated B cells displaying and expressing the desired antigen-binding characteristics. There are several variations of these approaches. The first approach includes the immunization of transgenic animals expressing subsets of the human Ig repertoire (8) and isolation of rare B-cell clones from

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humans exposed to specific antigens of interest (9). The second approach requires selecting from a large *in vitro* displayed repertoire either amplified from natural sources (i.e., human peripheral blood lymphocytes in Ref. (10) or designed synthetically to reflect natural and/or desired properties in the binding sites of antibodies (11, 12). This approach requires the use of a genotype-phenotype linkage strategy, such as phage or yeast display, which allows for the recovery of genes for antibodies displaying appropriate binding characteristics for the antigen.

The antibody has different varieties known as isotypes or classes. In mammals there are five antibody isotypes could be found, which are known as IgA, IgD, IgE, IgG and IgM. Here, each type of the isotypes named with an "Ig" that stands for immunoglobulin and each of the immunoglobulin has differed in their properties, functions and location of the action. IgA is mainly found in the mucosal areas like gut, respiratory tract, urogenital tract, saliva and tears of the mammalians. The function of IgA is to prevent the colonization of the antigens. IgD found in B cell surface and functions as an antigen receptor on B cell and basophils and mast cell to produce antimicrobial factors (13). IgE mainly attaches to the basophils and give protections from parasitic worms, also binds to allergens and triggers histamine release from mast cell for the allergic reaction. Among the antibodies, IgG is the most important and abundant immunoglobulin found in the mammalian cell. IgG has the ability to cross the placenta to give passive immunity to the fetus (14). And finally, IgM expressed on the surface of the B cells gives protection in the early stages of B cell mediated immunity (15). There are other isotypes of antibodies, such as IgY and IgW which are not found in mammals. IgY is major serum antibodies for birds and replies, also found in egg yolk of the birds plays

the equivalent role as IgG in mammalians. IgW is mainly found in sharks and skates, which works like mammalian IgD (16).

Table 1 Comparison of properties between different isotypes of antibody.

Properties	IgA	IgD	IgE	IgG	IgM
Molecular forms	Monomer or dimer	Monomer	Monomer	Monomer	Pentamer
% total Ig in serum	10-20	<1	<1	70-80	10
Where found in body	Found in bodily secretions.	Found on B –cell surface	Attach to basophils and mast cells.	Blood & extracellular fluid	Blood & extracellular fluid
Functions	Protect external openings	Unknown; may be antigen detection	Allergic response and defend infection by the large parasite.	Long-term Ab that protects the body.	Appear earlier in the infection and offer valuable defence during a critical stage of the infection.
Transferrable to offspring's	*Via* colostrum and breast milk.	No	No	*Via* placenta	No

1.2 Introduction of IgY

IgY antibodies refer to a predominant class of immunoglobulin serum found in oviparous animals, plays the equivalent role of mammalian IgG. Recently IgY targeted as an alternative for mammalian antibodies (17). As purposeful homologues of mammalian IgG, IgY antibodies are transferred to egg yolk via the particular receptor placed on the membrane of ovarian follicle to verify passive immunity for the developing offsprings. (18-19). the use an avian antibody as a research tool is not a novel concept. As early as 1893, Klemperer described an experiment in which he showed that the immunization of a chicken resulted in a transfer of specific antibodies into the egg yolk.

In the year of 1990s, for the availability of commercial secondary reagent like IgY-purification kits the use of IgY-antibody increase more widely. The production and use of IgY-antibody are designated internationally as a standard terminology since 1996. In order to minimize painful situations due to invasive antibody sampling, ECVAM (European Centre for the Validation of Alternative Method) recommended the use of IgY instead of mammalian IgG. ECVAM also recommended about the practice of rearing of laying hens, chicken immunization, use of adjuvants, IgY extraction methods, storage of IgY, etc. In the meantime, there was a lot of research work, which were covering all the aspects of IgY-technology perhaps due to the advantages of using IgY antibodies.

The immunization of chickens provides an attractive alternative (20-21) to use mammals as hosts for antibody production. IgY is the major low molecular weight immunoglobulin which considers as distinctive properties that can be exploited in various ways in research, diagnostics and therapy. One important advantage arises from the

phylogenetic distance and genetic background that distinguishes birds from mammals. This improves the likelihood that an immune response will be elicited against antigens or epitopes that may be non-immunogenic in mammals. The deposition of IgY into the egg yolks of the immunized bird then provides an elegant source of polyclonal immunoglobulins. Since polyclonal IgY can be recovered from the eggs of laying hens for prolonged periods, this approach provides a long-term supply of substantial amounts of antibodies. In addition, such antibodies exhibit biochemical and structural features, which can render them superior in virtually all types of immunoassays, especially those designed to detect molecules in specimens like mammalian blood or serum (22-23). Due to the technical difficulties of avian hybridoma techniques, and the problem that existing immortalized B cell lines (such as the ALV-induced bursa-derived lymphoma line DT40) undergo Ig gene conversion during *in vitro* culture (24), the production of chicken antibodies languished somewhat until it became possible to generate monoclonal IgY through the *in vitro* selection from combinatorial antibody libraries by phage display (25). In the chicken, only a single functional V and J segment is present in the light and heavy chain gene loci. As a result, diversification of the avian immune repertoire is introduced into the rearranged V (D) J segments by gene conversion using pseudo V genes as donors. This greatly simplifies the construction of combinatorial recombinant antibody libraries while the selective power of phage display provides a way of accessing unique binders.

1.3 Importance of IgY antibody

For the antibody production, immunization of chicken provides an attractive alternative to the using mammals as host (26-29). IgY has some distinctive properties which can be used for various types of research and therapy. Distinguishable phylogenetic and genetic

properties of IgY from mammalian immunoglobulin can be used accustomed to being elicited highly specific antibody against conserved mammalian proteins. IgY doesn't show any cross-reactivity with class IgG, rheumatoid factor (RF), human anti-mice antibodies (HAMA) and heterogeneity glycoprotein (30), As a result, IgY provides a distinctive feature in various immunology and diagnostic identification methods with reducing the risk of the false-positive result (31). Immunized chickens IgY depositional properties into egg yolks could be a distinguished originator of polyclonal immunoglobulin with several edges over existing class antibodies. Besides those advantages, IgY technology has some moral values like, as isolation from egg yolk does not need animal trauma (31). Chicken Antibodies can be easily sampled by a non-invasive method based on the simple action of egg collection, instead of the stressful bleeding of animals to obtain serum. IgY technology also offers economic advantages because the costs of handing hen are lower than those for rabbits. Furthermore, a large amount of antibody can be produced from one hen, approximately 17–35g of total IgY/chicken/year, of which 1–10% can be expected to be antigen-specific. If chickens and rabbits are immunized with the same mammalian antigen, the chickens respond with an Antibody-specificity that can rarely be achieved in rabbits, as for instance, PIIINP (32), parathyroid hormone-related protein (33) and YKL-40 glycoprotein (34). The cost-effective and availability of huge antibodies explore the new fields of IgY applications, such as immunotherapy applied to several viral and bacterial infections in veterinary and human medicine.
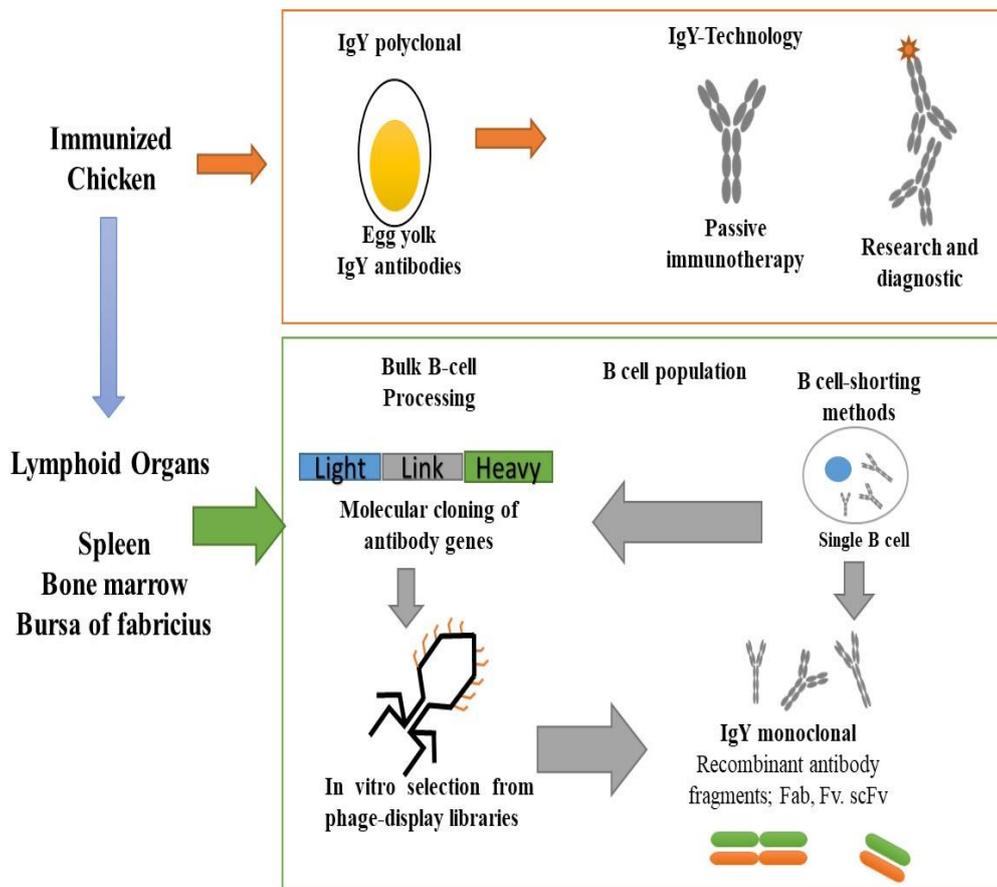
Figure 2 Overall potential use of IgY antibodies (38). Immunized birds naturally deposit protective antibodies in the yolk of their eggs. These antibodies are termed IgY, they can be used as passive immunotherapies in humans, animals for the research and diagnostic immunoassays. Furthermore, from avian lymphoid organs, the recovered B-cell population can be processed as bulk, leading to naïve or immune libraries of recombinant antibody fragments. By using standard phage display methods subsequent screening of antigen-specific monoclonal antibodies can be performed. Alternatively, sophisticated single-cell sorting methods can be applied to the initial B-cell population in order to select B-cells producing an antibody of interest.

Generally, one of the most intriguing and extraordinary characteristics of IgY is the lack of most interactions with mammalian immune components. This makes IgY especially suited to applications in which the use of its mammalian counterparts is prone

to unwanted cross-reactivity. For instance, in proteomics, pretreating of serum samples with IgY to specifically neutralize highly abundant serum components was found to improve downstream analyses (35). In another study, the identification of underrepresented serum proteins and disease marker candidate discovery was simplified when specific IgY was used as a blocking reagent (36-37). This approach was facilitated by the general characteristics of IgY such as the ease of production and the low incidence of cross-reactivity.

1.4 Structural and functional properties of IgY

IgY is a low molecular weight serum immunoglobulin isotype found in amphibians, reptiles, and birds. Among the three avian isotypes (IgY, IgM and IgA), IgY is the most abundant in serum and in the laying hens its concentrations ranging from 5 to 15 mg/ml in (39-40) compared to the lower concentrations of IgM (1-3 mg/ml) and IgA (0.3-0.5 mg/ml). In chicken, the organs which are responsible for antibody production differ significantly from those in mammals. The central lymphoid organs are represented by the thymus and bursa of Fabricius (BF), whereas peripheral lymphoid organs include the spleen, harderian glands, bone marrow, conjunctival-associated lymphoid tissue (CALT), bronchial-associated lymphoid tissue (BALT) and gut-associated lymphoid tissue (GALT). In chickens instead of lymph nodes, the lymphoid nodules associated with the lymphatics (41). The bursa of fabricius is located above the cloaca in the caudal body cavity and plays a crucial role in avian B cell development and antibody diversification (42). From a small number of B cell precursors, cells expressing surface immunoglobulin undergo rapid proliferation such that at about two months of age there are approximately 10^4 follicles in the BF (43). A few weeks after hatching, about 5% of the bursal cells

migrate into the blood and then into the spleen, thymus, and caecal tonsils, where they produce immunoglobulins subsequently. The spleen is the largest secondary lymphoid organ which is important in the production of antibodies and for antigen processing after hatching (44). IgY contains two heavy chains and two light chains, similar to mammalian IgG. But compared to IgG IgY have higher molecular weight due to an extra heavy chain constant domain (Table 2), lacks a well-defined hinge region and IgY has unique oligosaccharide side chains (45). It has been assumed that the extra domain (CH_2) is the evolutionary precursor to the mammalian IgG hinge region (46). In mammals, IgG forms immune complexes by opsonization activate the complement system and facilitate protection for the foetus by transport across the placenta. IgE usually sensitizes effector cells and mediates anaphylactic reactions (47). But IgY appears to combine the function of mammalian IgG- and IgE since it not only provides defence against infections (48) but may also mediate anaphylaxis (49). In compares to mammals, basophils are much more numerous in birds than mast cells (50) and antibody-dependent hypersensitivity and fatal systemic anaphylaxis (51-52) are mainly mediated by these cells (53). Besides its function and interaction with Fc receptors, IgY differs from IgG in a variety of aspects, as mentioned earlier, the phylogenetic distance between the avian immune system and mammalian proteins most likely increases the immune response towards the respective antigens. This means that IgY can often be raised against epitopes on highly conserved proteins while other mammals cannot provide an immunological response (54-55). Compared to mammalian IgG, IgY is lacking the flexible hinge region which makes them more rigid immunoglobulin. This hinge-less structure is also found in mammalian IgE. IgY, therefore, exhibits structural features of both mammalian IgE and IgG, a finding also supported by a structural analysis of the IgY Fc portion (56). Potentially reduced

molecular flexibility might be associated with decreased susceptibility to proteolytic degradation or fragmentation. Nevertheless, IgY can be fragmented by papain or trypsin (57). IgY, like mammalian IgG, is reasonably stable and can be stored for several months under standard conditions (58). In contrast to IgG, the antigen binding activity of IgY decreases significantly under acidic conditions. To identify the IgY structural and functional characteristics a comparison between the mammalian IgG and IgY, IgY-Fc is given in next page (59-64).

Table 2 Functional comparison between IgG, IgY and IgY-Fc.

Functional characteristics	IgG	IgY	IgY-Fc
Species	Mammals	Birds, Reptiles, Amphibians Lungfish	Birds, Amphibians, Lungfish
Molecular weight (kD)	150	180	118
Isoelectric point (pI)	6.4-9.0	5.7-7.6	5.2-7.3
Number of constant domains	4	3	2
Carbohydrate content (%)	2-4	4	0.6
Hinge region	Yes	No	No
Antigen valence	2	2	2
Major serum antibody	Yes	Yes	Yes
Source	Serum	Chicken serum/egg yolk	Chicken serum/egg yolk
Concentration (mg/ml)	10-12	8-10	3-12
Mammalian complement binding	Yes	No	No
Rheumatoid factor binding	Yes	No	No
Fc receptor binding	No	Yes	No
Mediates anaphylaxis	No	Yes	No
Binding to protein A	Yes	No	No
Binding to protein G	Yes	No	No

1.5 Applications of IgY

IgY-based immunoassays are being used to measure the concentration of proteins or peptides via ELISAs, RIAs or other assays in clinical chemistry and basic research. IgY antibodies are used in immunohistochemistry for detection of antigens of viral, bacterial, plant and animal origin, to assess the incidence of intestinal parasites in domestic animals (65) and the contamination of foods with toxins or drugs (66).

1.5.1 Therapeutic use of IgY

Eggs constitute a very common component of our diet and are therefore tolerated by the human immune system. Administration of IgY may, therefore, represent an attractive approach to immunotherapy with a reduced risk of toxic effects. Chicken antibodies are well-established as anti-toxins and/or for passive vaccination. For instance, specific anti-venom IgY can neutralize bacterial toxins (67) and be used to treat snake bites (68-75). Indeed, anti-venom IgY shows a higher bioactivity than antidotes raised in horses (76). In such applications, egg yolks can provide a continuous supply of potentially superior reagents. Today's consumers have become increasingly interested in foods that supposedly promote health and reduce the risk of disease. The different use of IgY is listed below.

1.5.1.1 Treatment of intestinal infection

For most of the viral and bacterial pathogens, the adherence is a major prerequisite for the successful colonisation, especially with respect to the host's respiratory and intestinal mucosae. It has been shown that IgY antibodies specific against *Salmonella* antigens are able to inhibit *in vitro* the adhesion of this bacterium to epithelial cells (77). Carlander *et*

al. (78), and Sarker *et al.* (79) investigated the action of hyper-immune bovine colostrum (HBC) and isolated IgY against human rotavirus from infected children. The oral administration of IgY showed a significant protective effect (79). An anti-human rotavirus (strains Wa, RV5, RV3, ST3) IgY was also effective, although to a lower extent than with HBC.

1.5.1.2 Treatment of *Helicobacter pylori*

Therapeutic use of IgY against *Helicobacter pylori* has also been investigated in animals (80) and humans (81-82). Shin *et al*. (83) identified the immune-dominant proteins of *H. pylori*. Antibodies specific for these proteins were more effective as a prophylactic reagent as compared to antibodies directed against the whole bacterial lysate. Altogether, all studies demonstrated a curative effect of the anti-*H. pylori* Antibody. In most cases, no complete *H. pylori* eradication could be achieved. But in view of the increasing bacterial resistance, the use of specific IgY minimizes the use of antibiotics. Horie *et al.* (84) carried out a study with 42 volunteers to test the protective effect of a drinking yogurt fortified with anti-*H. pylori* urease IgY, obtaining a significant decrease in urea breath values of the treated group (fed with IgY-yogurt).

1.5.1.3 Treatment of colitis and celiac disease

The colitis and celiac disease refers to an immune-mediated disorder, an autoimmune enteropathy, triggered by the ingestion of gluten in the genetically susceptible patient. This disease primarily affects the gastrointestinal tract and is characterized by chronic inflammation of the small bowel mucosa that may lead to atrophy of intestinal villi, malabsorption, and a variety of clinical manifestations (85). Worledge *et al*. (86)

demonstrated significant protective effects of IgY specific against tumour necrosis factor (TNF) after oral application in an experimental rat model of colitis. TNF is implicated in the pathogenesis of inflammatory bowel disease. The oral use of antibodies is considered to have fewer systemic side-effects than the intravenous infusion of a humanized murine anti-TNF monoclonal antibodies (Infliximab, Centocor, Malvern, Pennsylvania, USA). Sunwoo and Sim (87) identified gluten proteins which play a role in the autoimmune disorder of the celiac disease and reported on the use of IgY against gluten protein. The authors immunized chickens with gliadins and low- and high molecular glutenin. The isolated IgY can be used in different forms, such as table eggs, liquid and powdered eggs, and encapsulated nutraceuticals for the treatment of celiac disease.

1.5.1.4 Treatment of cystic fibrosis

Cystic fibrosis (CF), known as the most common fatal genetic disease of the Caucasian population in Europe and the USA. It caused by a mutation in the gene for a chloride channel protein, which results in the secretion of an abnormally thick mucus. This leads to secondary infections in the respiratory tract, caused by several bacterial species, one of which, *Pseudomonas aeruginosa* infects virtually all CF patients. Researchers found the benefits of IgY as a prophylactic tool for CF patients (88). They treated CF patients orally with an aqueous IgY anti-*P. aeruginosa* solution (70 ml, 0.7 mg/ml IgY), as a mouth rinse. Approximately 8 hours after the treatment, a high level of the specific chicken Abs could be demonstrated in the saliva via an ELISA. Later, the IgY concentration gradually declined and was completely undetectable in the saliva 16 hours after the treatment. These oral IgY treatments were successful in reducing chronic *P. aeruginosa* infections in CF patients (89).

1.5.1.5 The use of IgY in veterinary medicine

In veterinary medicine, powdered whole eggs or egg yolks have been used as an inexpensive source of IgY for the treatment of enteric diseases. The IgY has been used in the treatment of calves and piglets with specific Antibodies against *Escherichia coli* (K88, K99, 987P), rotaviruses and coronavirus (90). Several types of research have been reported (91- 94) that IgY has significant prophylactic and therapeutic benefits for the treatment of diarrhea in calves and piglets. Pokorova *et al.* reported that IgY can be used to protect dogs against canine parvovirus by interacting with canine parvovirus surface components (95). Sunwoo *et al.* demonstrated *in vitro* a marked growth inhibiting the effect of specific IgY on *E. coli* 0157: H7, showing that growth inhibition was actually caused by binding of specific IgY to bacterial surface antigens, which caused significant changes in the bacterial surface structure (96). Another effect of IgY binding to bacterial surface antigens is a marked impairment of bacterial attachment to the intestinal mucosa (97, 77). Therefore, therapeutic IgY administration might reduce the clinical use of antibiotics, minimizing the risk of bacteria developing antibiotic resistance.

1.5.2 The use of IgY in immunoassays

One of the most intriguing and extraordinary characteristics of IgY is the lack of most, if not any, interactions with mammalian immune components. This property of IgY is suited to applications in which the use of its mammalian counterparts is prone to unwanted cross-reactivates. In proteomics, pretreating of serum samples with IgY to specifically neutralize highly abundant serum components were found to improve downstream analyses (98). In another study, the use of IgY as blocking reagents simplified the identification of underrepresented serum proteins and disease marker candidate discovery

(99-100). This approach was facilitated by the general characteristics of IgY such as the ease of production and the low incidence of cross-reactivity. In the case of immunoassays, homologous mammalian immunoglobulins may have deleterious activities on the performance of many different types of immunoassays. In particular, approaches using immunoglobulins as bioactive molecules to capture or detect the analyte is often affected by heterophilic antibodies and/or high levels of non-specific binding. It was estimated of the prevalence of assay interference by heterophilic antibodies range from 1 to 80% (101-106). In addition, antigen-independent specific binding via immunoglobulin Fc receptors or lectins and non-immunoglobulin-based interactions, e.g. those mediated by complement factors (107), can result in false-positive and false-negative results (108-109).

To avoid this problem, chicken IgY offers several advantages over their mammalian homologues since they do not interact with rheumatoid factor (RF), human anti-mouse IgG antibodies (HAMA), complement components or mammalian Fc receptors. Nowadays most currently used immuno-tests are based on murine monoclonal antibodies, here monoclonal IgY can be used (110) as a way of avoiding interference by RF and heterophilic antibodies in human serum samples (111). It has shown that monoclonal and polyclonal IgY antibodies bind neither to mammalian Fc receptors CD64 and CD16A (111) nor to the human high-affinity IgE receptor, despite having similarities in the amino acid sequences of human IgE and avian IgY. For some diagnostic applications, the advantages of IgY may be undermined by the prevalence of anti-chicken antibodies in certain individuals. One study (112) demonstrated that 15 in 28 egg-allergic patients exhibited specific IgE binding against one or more egg yolk-derived antiviral chicken immunoglobulins. In contrast, according to another study, the overall allergenic

potential of IgY in animal models appear to be low.

1.6 Summary

Today, there is no doubt that chicken IgY can be produced and used in similar ways to the use of mammalian antibodies. Depending on the circumstances, the use of IgY shows significant advantages over the use of mammalian Antibodies. In summary, the IgY technology is a fast developing field and in this chapter, the IgY technology has been introduced with some of its uses. The ready availability of polyclonal IgY and the rise of recombinant technologies made IgY available for more widely used in research, diagnostics and therapeutics. It is to be expected that in future the studies on the therapeutic use of IgY will be intensified and offer new alternatives and solutions to science, to medicine and to the society as a whole.

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Chapter 2

Construction of random peptide library using T7 phage display technique

2. 1 Introduction to Phage-display technique

Phage display technology has been used as a screening method for the generation/ identification of functional peptides, proteins, or monoclonal antibodies (1), studying protein/DNA–protein interactions, screening cDNA expression, epitope mapping of antibodies, engineering human antibodies (2), optimizing antibody specificities, identifying peptides that home to specific organs or tissues, and generating immunogens for vaccine design (3–6). This technique offers two basic features 1. The linkage of genotype and phenotype, 2. The ability to construct peptide or protein display libraries which could contain up to 10^{10} different variants (7). The physical linkage between the displayed target protein and the encoded gene facilitates characterization of the target protein on phage and target protein-displaying phage could be isolated by selection process with its desired binding property. Phage display was first described by G. Smith in 1985 and used to display short peptide fragments (8), and the then first patent (9) was filed in 1991. Since then, this technique has proven to be a reliable method for the generation of peptides with potential therapeutic or diagnostic use (10). In 1990 phage display of single-chain V-domain antibody fragments (scFv) was reported, and target-displaying phage was recovered on the basis of antigen binding (11). It becomes a major discovery platform for the identification of fully human monoclonal antibodies (12). More recently Fab fragments of antibody displaying-phage library have described, which can be readily reformatted to full-length IgG antibodies without loss of binding function.

E. coli filamentous bacteriophages (f1, fd, M13) are commonly used for phage display technique. Phage proteins pIII (13) and pVIII (14) are used for displaying of antibodies and peptides. The successful expression of gene 8 is responsible for the production of phage major coat protein (pVIII) and it can produce up to 3000 copies, therefore it is used to elevate detection signal when phage-displayed antibody associated with the antigen. Moreover, modifications of pVIII are made to increase the potency of the display onto pVIII coat protein (14). On the other hand, minor coat protein (pIII) consists of 406 amino acid residues and expressed at the phage tip in 3 to 5 copies. As a result, larger peptides and folded proteins are displayed as fusions with pIII protein, whereas pVIII, for preserving its functionality only short peptides (6–7 residues) without cysteine residues could be used for display (15). The major limitation of the phage display technology is the loss of coat protein functionality, however, this problem could overcome by hybrid phages and coat protein modifications (14).In this step, the virion consist of the complete wild-type genome and a copy of fusion gene was inserted into phage genome (16) or as phagemid (17) vector. The phagemid vector contains the origins of replication for phage and its host, gene 3 with appropriate cloning sites and an antibiotic-resistance gene. Moreover, the phagemid encoding polypeptide-PIII fusion requires hybrid with helper phage for packing into the M13 particle. The helper phage contains a slightly defective origin of replication (such as M13KO7 or VCSM13) and supplies all the structural proteins required for generating a complete virion. Thus, both wild pIII protein and target polypeptide pIII fusion protein will be present on the phage surface. The ratio of polypeptide-PIII fusion protein to wild-type pIII may range between 1 to 9 and 1 to 1,000, which depends on the type of phagemid, growth conditions, the nature of the polypeptide fused to pIII and proteolytic cleavage of antibody-PIII fusions (18). This ratio also ensures

that the fusion protein, used as a minor component of the phage coat, does not affect phage viability. However, when hyper-phage was used, achieving this ratio is unnecessary. Hyper-phage has wild-type non-functional pIII gene, as a result, antibody fused pIII is the only source of pIII for phage assembly.
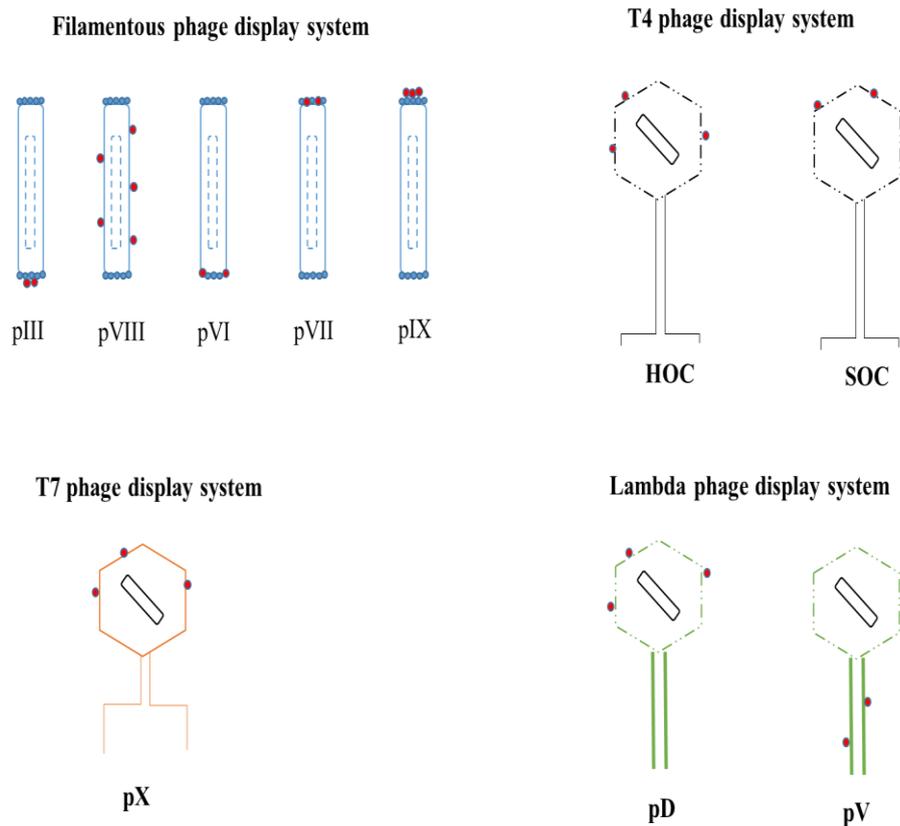

Figure 1 Schematic presentation of phage display systems. The displayed molecules on the various protein are indicated by a red circle (30).

Therefore, it allows the use of increasing the number of scFv for fusion and also 10-fold increases the binding of phage to antigen could be achieved comparing to M13KO7 helper phage. Hybrid phage system enables displaying of larger proteins with all five M13 coat proteins as N-terminal fusions with pIII, pVIII, (19) pVII, pIX (20) and

also as C-terminal fusions with pVI, pIII, and pVIII (21-22). The usage of T7 phage is an alternative for M13 phage display system (23). One of the advantages of T7 phage display system is its extreme robustness and stability in conditions that inactivate other phages (24-25). In this system, small peptides (less than 50 residues) in high copy number (415 per phage), larger peptides or proteins (up to 1200 amino acid) in low (0.1-1 per phage) or mid-copy number (5-15 per phage) could be displayed with stop codon on the C-terminal of pX capsid protein. T7 phage display over M13 display techniques is connected with the fact that the capsid is not involved in the phage to host adsorption and avoid the secretion of displayed peptides through the periplasm and the cell membrane. However, this approach restricts the possibility of posttranslational modification of polypeptides in eukaryotic systems (23). The T4 phage HOC/SOC bipartite display system (26) could be applied to cDNA expression. This system allows larger proteins in high copy number and inserts with stop codon on the C-terminal of SOC (small outer capsid) protein that occurs in 810 copies or N-terminal of HOC (highly antigenic outer capsid) protein that occurs in 155 copies. Phage λ is also capable of displaying complicated, high molecular mass proteins as fusions with N- or C-terminal of PD head protein that occurs in 405 copies or C-terminal of PV tail protein that occurs in 6 copies (27-28). λ Phage-display system does not require translocation through the *Escherichia coli* membrane. In comparison to filamentous phage system, lambda phage display showed a higher immune response in spite of displaying a wide variety of proteins in multiple copies.

2.2 T7 phage display system

T7 phage is lytic viruses with double-stranded linear genome DNA of about 40kbp comprising 60-degree genes (30-31). This phage attached via the Lipopolysaccharide

(LPS) on the surface of *E. coli* and infected by injecting DNA into the cell. Phage assembly takes place inside the *E. coli* cell and mature phage released by cell lysis. As a result, T7 phage display enables various peptides or proteins to be displayed on the surface of lytic T7 phage particles with a reduced bias of amino acids generated by the mixed nucleotides in the display peptides. Unlike the filamentous phage display systems, peptides or proteins displayed on the surface. It takes 2-3 hours at 37 °C for plaques formation and cultures lyse 1–2 hours after infection, decreasing the time allows to perform the multiple rounds of growth usually required for the selection process. The T7 phage particle is extremely robust, stable to harsh conditions like detergents and denaturants including 1% sodium dodecyl sulfate (SDS) and urea (up to 4 M) (32). This property of T7 phage expands the variety of agents that can be used in biopanning selection procedures. T7 phage is an excellent general cloning vector. There are commercial *in vitro* packaging systems available by which purified DNA could be obtained in large amounts (32).
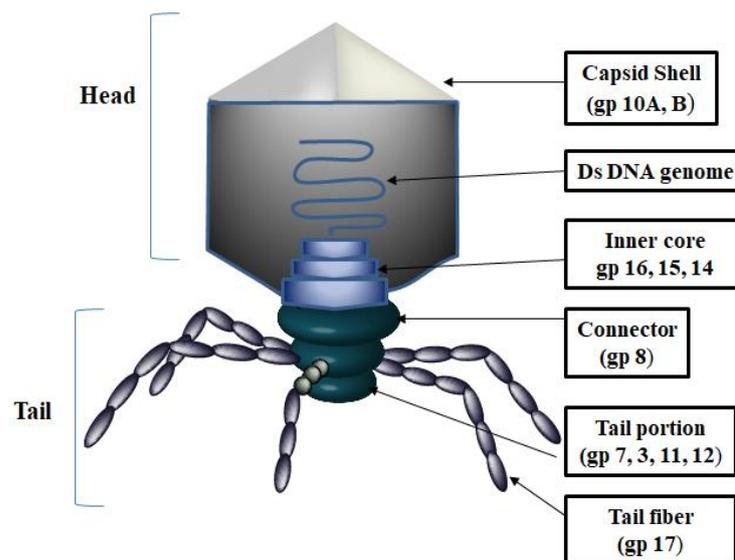

Figure 2 Structure of T7 bacteriophage.

2.2.1 T7 phage structure and assembly

T7 is an icosahedral phage contains a capsid shell composed of 415 copies of the T7 capsid protein (expressed by gene 10) arranged as 60 hexamers on the faces of the shell and 11 pentamers at the vertices (33). Remaining part of the phage is attached by the head-tail connector vortex (gene 8), a short conical tail (genes 11 and 12) and 6 tail fibres (gene 17) (shown in Figure 2). T7 phage assembly process is similar to that of other double-stranded DNA phages (34). DNA is packaged into a scaffolding protein (gene 9) made the procapsid shell, capsid protein, the head-tail connector, and an internal protein structure (genes 13, 14, 15, and 16). The DNA is usually packaged in a linear concatemers forms, and when it enters the procapsid shell the scaffolding protein is released and a conformational change occurs to form the mature particle. Tail and tail fibres attach at the head-tail connector vertex. In the T7 Phage Display System, the T7 capsid protein is mainly used to display peptides or proteins on the surface of the phage. The capsid protein is normally made in two forms, 10A (344 aa) and 10B (397 aa). 10B is produced by a translational frameshift at amino acid (aa) 341 of 10A and makes up about 10% of the capsid protein (35). However, the entire functional capsids can be composed of either 10A or 10B or of various ratios of the proteins. This property provided the initial suggestion that the T7 capsid shell could accommodate variation and the unique 10B region of the capsid protein could be used for displaying target protein or peptide.
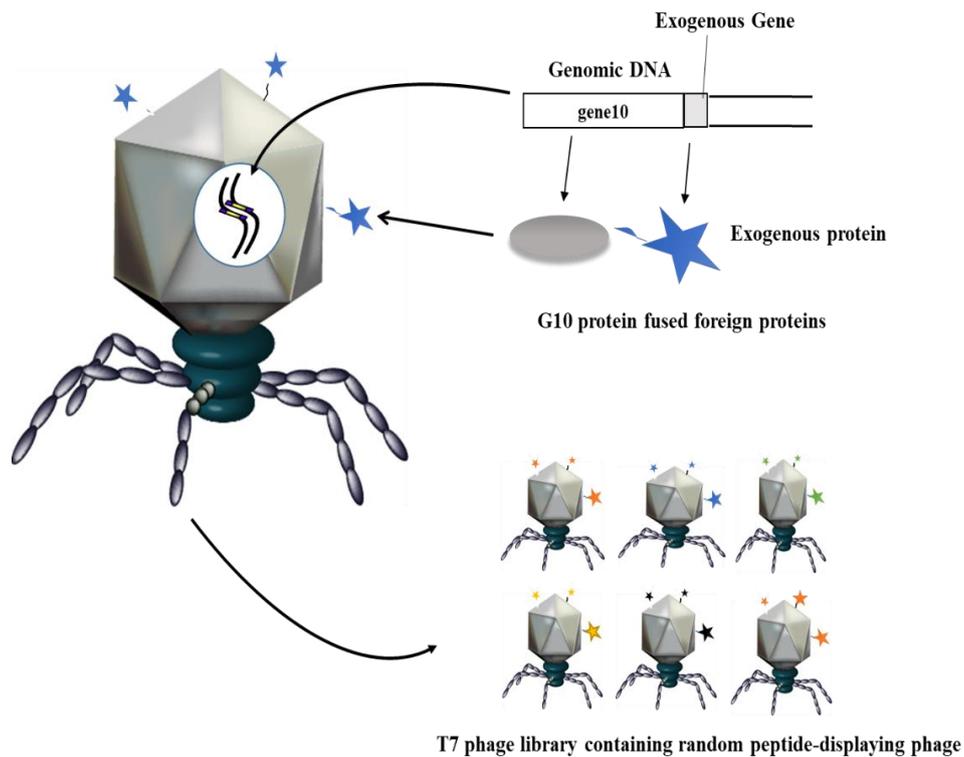
Figure 2 Diagrams for T7phage display technology.

2.2.2 Construction of T7 phage library

Construction of T7 phage library usually performed by using commercially *in vitro* packaging systems. T7Select Cloning Kit vectors from Merck (Kenilworth, NJ, USA) is most commonly used commercial cloning kit used for T7 phage display technology. In this procedures vector arms are prepared and ligated with target inserts, the resulting DNA is incubated with an *in vitro* packaging extract, and the phage products are used for infection of a suitable host. There are multiple cloning sites in the T7 vectors, which are compatible with many existing vectors, including the pET vectors used in the T7 expression system. The target insert DNA usually contain a limited coding region for variant amino acids (Figure 3). The vector arms and T7 packaging extracts provided in

the T7Select System routinely produce $> 10^8$ recombinant plaques per μg of arms. The high-efficiency T7 packaging extracts (2×10^9 plaques per μg intact DNA) are made with a specially designed phage that reduces the non-recombinant cloning background to below 0.1%. (36).
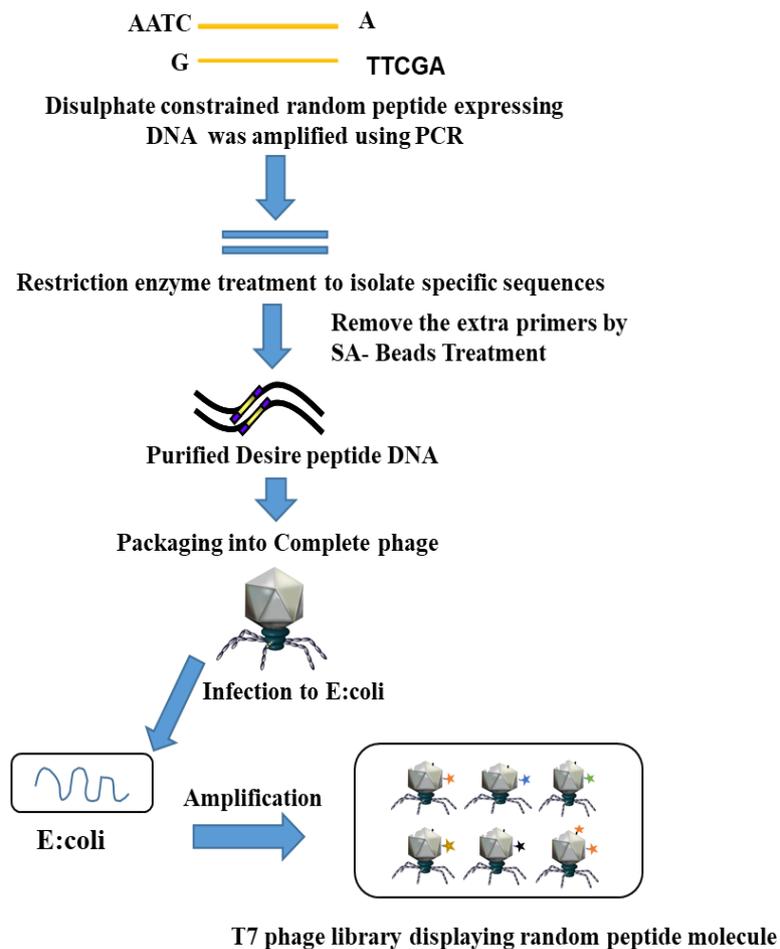

Figure 3 Schematic diagram for the construction of peptide-displaying T7 phage library.

2.3 Experimental procedures

2.3.1 Design of the structure of random peptide-displaying T7 phage library

The design of the insert random peptides to the T7 phage is a cyclic peptide having a disulfide bond with Cysteine and Cysteine reduce, using four kinds of forms of $X_3CX_{7-10}CX_3$. Here X represent the random amino acid sequences.
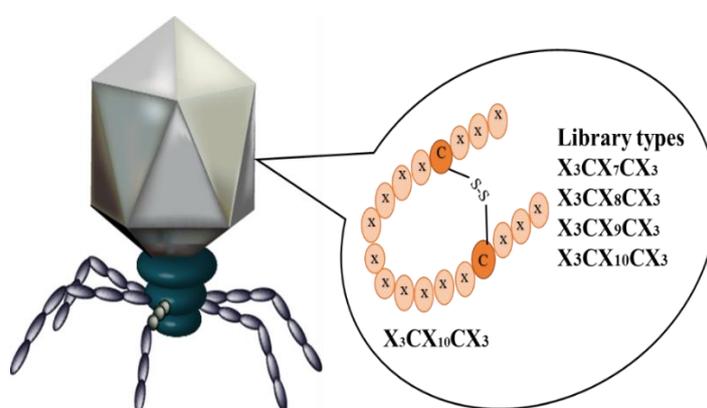
Figure 4 Design of random peptide displaying T7 phage libraries.

2.3.2 Design of insert DNA for the T7 phage library

The DNA fragment encoding the sequence of the random peptides ($X_3CX_{7-10}CX_3$) was amplified using biotinylated primers containing the restriction enzyme sites *Eco* RI and *Hind* III (indicated by underline). The design of the primers was given below.

KEB553 (With *Eco* RI restriction site)	Bio-ATGAATACCAGGATCC<u>GAATTC</u>AGGTGGAGGTTCG
KEB547 (With *Hind* III restriction site)	Bio-ACTATCGCCGGCCGC<u>AAGCTT</u>TTATCC

2.3.3 Amplification of insert DNA by PCR

Random peptide coding inserts DNA was amplified by polymerase chain reaction (PCR)

using the primers. DNA polymerase (Takara Bio, Dalian, China) was used to amplify the gene under following conditions: 95ºC for 5 min, 30 cycles (95ºC 30 s; 60ºC 30 s; 72ºC 60 s), and a final extension of 72ºC for 3 min. The PCR products were analyzed on 2% agarose gel.

2.3.4 Restriction enzyme treatment of insert DNA

After confirming the insert DNA amplification by PCR, The insert DNA was used for the *Hind* III and *Eco* RI restriction enzyme treatment for 2 hours in RT and analyzed on 2% agarose gel.

2.3.5 Streptavidin beads treatment

First, streptavidin beads were taken into the tube and the water portion of the beads was removed by pipetting. The restriction enzyme treated DNA was added and incubated for 1 h with shaking. Finally, DNA sample was recovered from a new tube and analyzed on 2% agarose gel.

2.3.6 Ligation of insert DNA with T7 vector arms

After SA bead purification, the purified DNA was ligated with a T7select10-3b vector using T7select packaging kits (Novagen) manufactures instruction.

2.3.7 Amplification of synthetic phage in *E.coli*

E.coli (BTL5403) cell was grown into 2TY culture media and T7 packaging solution was added to the bacterial culture and the synthetic phage was amplified by 2 hours of incubation. Amplified phage was recovered by centrifugation for 30 min at maximum

speed. After recovering the phage in the culture supernatant, 50% polyethene glycol was added and incubated overnight at 4 °C to perform a precipitation reaction. Finally, the precipitate phages were recovered by centrifugation and dissolved in PBS buffer solution for further experiments.

2.4 Results and Discussion

This experiment was conducted to construct random peptide displaying T7 phage library. Total four types of random peptide-displaying phage library were constructed, where in every library, the insert DNA consists 7, 8, 9 and 10 random peptide expressing amino acid sequences between two cysteine residues respectively (Figure 4). After designing the insert DNA for four kinds of phage library, the synthetic insert DNA was amplified by PCR using *Hind* III and *Eco*RI restriction site containing primers. Figure 5 shows the gel electrophoresis analysis of the amplified DNA by PCR. This result confirms that the (113base pair) DNA was amplified successfully.
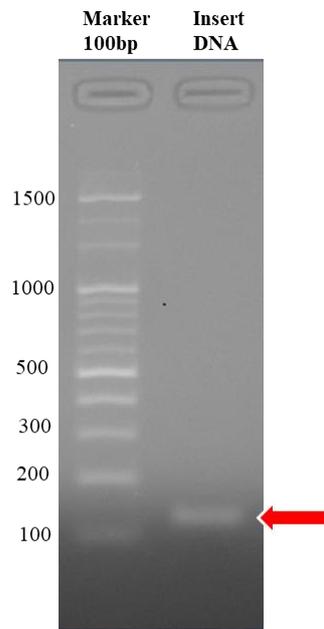
Figure 5 Gel electrophoresis analysis of the amplified insert DNA by PCR. Arrow indicates the target PCR product.

After the amplification of insert DNA with *Hind* III and *Eco* RI restriction site containing primers. The insert DNA was purified by *Hind* III and *Eco* RI restriction enzyme treatment respectively. In figure 6, the gel electrophoresis image shows the insert DNA was sliced successfully by both of the restriction enzymes respectively.
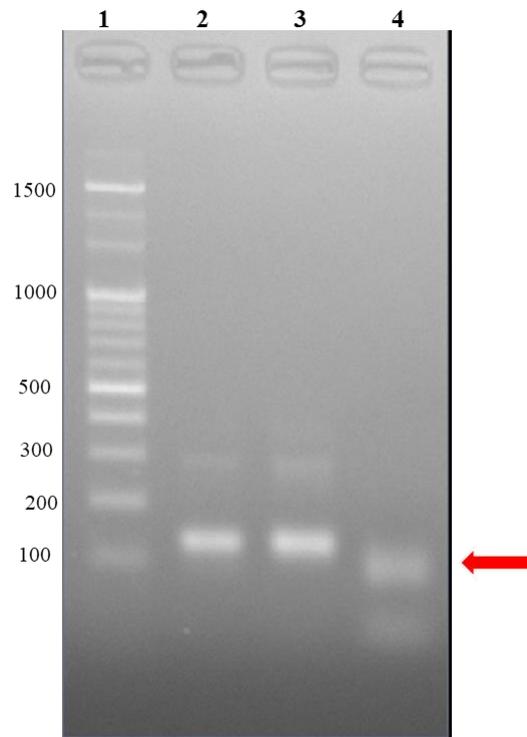
Figure 6 The gel electrophoresis analysis of the insert DNA after *Hind* III and *Eco* RI restriction enzyme treatment. , Lane 1 indicates the marker line, Lane 2: Insert DNA before restriction enzyme treatment, Lane 3: after *Hind* III restriction enzyme treatment. Lane 4: after *Eco* RI restriction enzyme treatment. Arrow indicates the target PCR product.

After the *Hind* III and *Eco* RI restriction enzyme treatment, the insert DNA solution exhibit different types of sliced primers and end product of restriction enzyme treatment. To purify the insert DNA from those unwanted by-product, Streptavidin beads treatment was used. Figure 7 shows that after SA beads treatment the insert DNA was purified successfully.
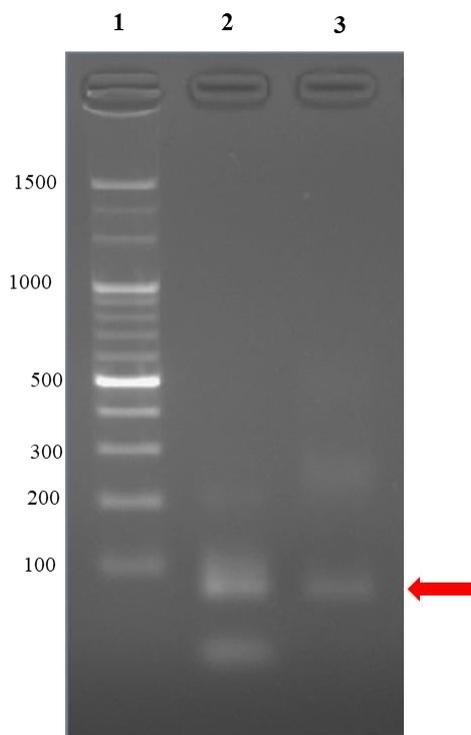
Figure 7 The gel electrophoresis analysis of the insert DNA after Streptavidin beads treatment. Lane 1 indicates the marker line, Lane 2: Insert DNA before Streptavidin beads treatment, Lane 3: after Streptavidin beads treatment. Arrow indicates the target PCR product.

The purified insert DNA of random peptide displaying was incorporated into T7select10-3b vector (Novagen) by using T7select packaging kits manufactures instruction and synthetic phage was replicated using *E.coli* (BTL5403) cell culture (Figure 8). And after successful amplification, single phage clones (Figure 9) were isolated into semi solid top agarose medium (2TY-top agarose). Finally, the amino acid sequences of the random peptide-displaying single phage clones were analyzed (Table 1).

**Figure 8** *E.coli* (BTL5403) cell culture after growing in to 2TY liquid medium.

Figure 9 T7 phage clones grown into the semi-solid top agarose medium.

Table 1 Amino acid sequence analysis for phage library. In this process, 5 phage clones were randomly collected and their amino acid sequences were examined to confirm the condition of phage libraries.

Randomly collected clones	Amino acid sequences
Clone-1	STNCSF*FGEIVHCAFL
Clone-2	SLSCSWYCGHMSCCGGE
Clone-3	STSCSLQLGGLGHCANV
Clone-4	SFRCSLHCGHFSCCVMP
Clone-5	SFDCTCYCGRLIFCVER

After comparing 5 random phage clones displaying amino acid sequence it was observed that all the phage clones displayed disufide constrained random amino acid sequence $X_3CX_9CX_3$ (X= random amino acid, *= stop Codon) with two cysteine amino acid residue in 4th and 10th position except clone-1 shows one mutation (stop Codon).

2.5 Summary

Phage display is a powerful technique for identifying peptides or proteins that have desirable binding properties. In this method, target peptide or protein could be displayed on the surface of a phage as a fusion to a protein. The most common bacteriophages used for phage display are M13, fd filamentous phage, T4, T7, and λ phage. Among these, T7 phage display enables various peptides or proteins to be displayed on the surface of lytic

T7 phage particles with a reduced bias of amino acids generated by the mixed nucleotides in the display peptides. Increased peptide diversity and rapid plaque formation properties (2–3 h) (24) made T7 phage-based screening procedures superior to those of a filamentous phage-based (like M13). In this chapter, the construction of random peptide-displaying T7 libraries was described briefly, including the designing, amplification and purification procedure of insert DNA, construction and amplification of synthetic phage libraries using T7Select Cloning Kit.

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Chapter 3

Isolation and Identification of IgY binding peptides from random peptide libraries

3.1 Introduction

Combinatorial phage-displayed random peptide libraries are very valuable research tools for studying the interaction between peptides and other substances or materials. From all different available molecular display techniques, phage display has become the most popular approach (1). Screening phage-displayed random peptide libraries is an effective technique for identifying peptides that can bind to its target molecules and regulate their function. Phage-displayed peptide libraries can be used for (i) B-cell and T-cell epitope mapping, (ii) selection of bioactive peptides bound to receptors or proteins, disease-specific antigen mimics, peptides bound to non-protein targets, cell-specific peptides, or organ-specific peptides, and (iii) development of peptide-mediated drug delivery systems and other applications. In the past, scientists have been focused on identifying B/T cell epitopes (2-10), disease-specific antigen mimics (11-13), receptor agonists/antagonists (14-15), enzyme inhibitors (16-22) and protein partners (23-30). In recent years, there is an increasing number of researchers who apply this technique to new areas of chemistry (31-33) and materials (34-44) science. Although phage display is a powerful technique, its efficiency still depends on several factors. Choosing suitable phage display libraries (i. e. the number of phage-displayed amino acid residues), using applicable selection condition, ensuring the stability and the quality of phage display libraries and following the appropriate screening protocols are all important factors that could impact the quality and desirability of the ligand peptides generated. Considering those key parameters functional target peptides are more likely to be obtained. Furthermore, a combination of

peptide-displaying phage libraries with bioinformatics resources may improve the quality of peptides. Tian *et al.* (45) and Sandman *et al.* (46) used genetically-encoded non-natural amino acids into phage-displayed libraries for obtaining wider chemical diversities. Woiwode *et al.* (47) also constructed a new phage-displayed hybrid system with synthetic chemistry through one-compound-one-clone principle (each compound was encoded by a unique nucleotide sequence inserted in a non-coding region of the phage genome). These new techniques wider the advance the possible applications and potentials of phage display going forward. Target specific peptides can be identified by affinity selection called biopanning. For biopanning, molecule-displayed phage libraries are incubated with an immobilized target, followed by extensive washing to remove non-binding phages. Specific binders are usually eluted using acid or high salt and are enriched by amplification in the appropriate host cells. The biopanning procedure usually performed three to five times in order to obtain target binder that binds with high affinity (Figure 1).

This chapter described the isolation and identification of IgY-binding peptides from random peptide-displaying phage libraries. The previous chapter described the construction of four types of random peptide libraries. By using biopanning process, IgY-binding phages were isolated from four types of random peptide libraries. The binding properties of IgY-specific phage were evaluated by ELISA, Surface plasmon resonance (SPR) and immunoprecipitation, respectively.
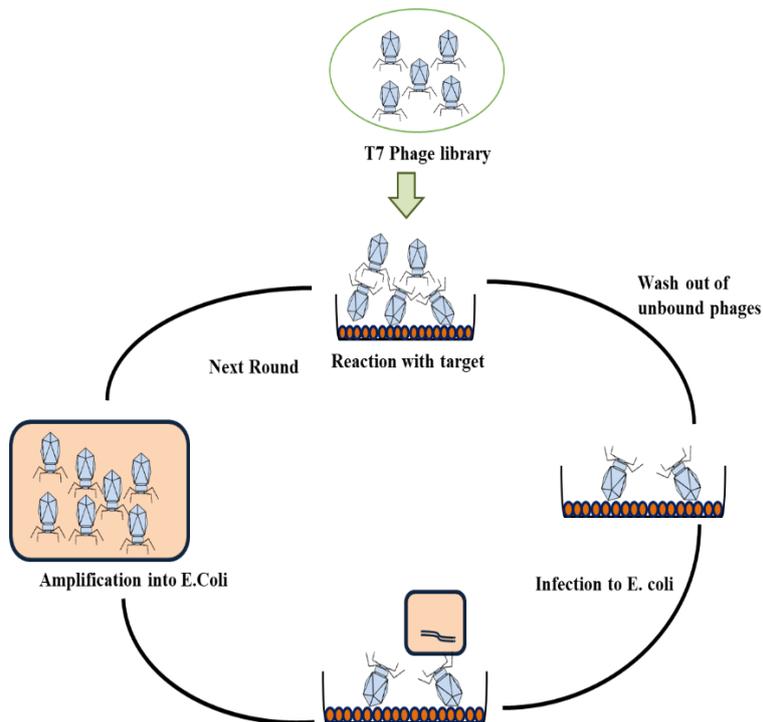
Figure 1 The schematic diagram of biopanning with phage-displayed random peptide libraries. Random peptide-displayed on phage libraries could be used for a number of target candidates, including purified antibodies (B-cell epitopes), receptors (agonists or antagonists), enzymes (enzyme inhibitors), and carbohydrates (antigen-mimetic peptides). After three to five rounds of biopanning, specific individual phage clones are selected and analyzed.

3.2 Experimental procedure

3.2.1 Materials

Polyclonal IgY and IgG were purchased from Acris Antibodies GmbH (Herford, Germany), Athens Research & Technology (Athens, GA), and ICN/Cappel Biomedicals (Aurora, OH), respectively.

3.2.2 Isolation of IgY binding phages from random peptide displaying Phage Library by the biopanning procedure.

The T7 phage libraries displaying typically $X_3CX_{7-10}CX_3$ random peptides, where X represents the randomized amino acid positions generated using mixed oligonucleotides on template DNA, were constructed using the T7Select vector 10-3b from Novagen (Tokyo, Japan), according to methods described previously (Chapter 2). 96-well microplate wells (Nunc Maxisorp) were coated with IgY dissolved into the solution (5.5μg / 200μl / well) and blocked with 0.25% BSA in PBS. The T7 phage libraries (1 × 10^{10} pfu) of $X_3CX_{7-10}CX_3$ were incubated for 1 h in wells coated with BSA to remove non-specific phages and were then added to IgY-coated wells. After 2-1 hour incubation, the plate was washed 5–30 times with PBS containing 0.1% Tween 20 (PBST). *Escherichia coli* BLT5615 cells (300 μL) (Novagen) in log-phase growth (the OD600 0.7-0.8) were added to the wells, infected with phages for 10 min, and propagated in the 2TY medium at 37 °C. After bacteriolysis, 5M NaCl (0.1 times of the bacterial culture solution) was added and phages were recovered from the culture supernatant by centrifugation (15000 rpm for 20 min). Then, 50% polyethylene glycol (0.2 times of the bacterial culture solution) was added for precipitation reaction. After centrifugation (15000 rpm for 20 min), the supernatant was discarded, the remaining phage pellet was dissolved in PBS, and the recovered phage solution was used for the next round of biopanning.

3.2.3 Preparation of Synthetic Peptides

C-terminally amidated synthetic peptides Y4-44 and Y5-55 were synthesized by solid phase synthesis using Fmoc chemistry. The protected peptides were coupled to the resin with NHS-PEG$_4$-biotin (ThermoFisher, Waltham, MA). After removal of the protecting

groups, the peptides were mildly oxidized to form intra-molecular disulfide bonds in DMSO containing 1% pyridine. The generated disulfide-constrained peptides were purified by reversed phase-HPLC. The purity of the peptides was checked on Acquity SQD ultra-performance liquid chromatography system (Waters Corp., Milford, MA) and the disulfide bond formation of the peptides was confirmed by MALDI-TOF mass spectrometry on Voyager System 6366 (Applied Biosystems). The physicochemical properties of these peptides are summarized in Table 1. After lyophilization, the peptides were dissolved in the appropriate buffers and used for assay after centrifugation.

Table 1 Physicochemical characterization of IgY binding peptides used in this study.

Peptide	Purity (%)	Theoretical mass (Daltons)	MALDI-TOF-MS (Daltons)
Biotin-PEG4-Y4-4	96	2442.8	2442.9
Biotin-PEG4-Y5-55	94	2482.8	2482.9

3.2.4 Identification of synthetic peptide binding with IgY by ELISA

The wells of a Microplate (Nunc Maxisorp) were coated with IgY, IgY-Fc, hIgG (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan), mIgG (PharMingen, San Diego, CA, USA), human serum albumin, and BSA (50 ng/50 µL/well) overnight at 4 °C, washed three times with PBST, and blocked for 2 h with 0.5% BSA in PBS. Phage solution was added to each well and incubated for 2 h. After washing the plate, bound phage was detected with biotinylated anti-T7 phage antibody (Novagen,) and horseradish peroxidase (HRP)-conjugated streptavidin (SA) (Vector Laboratories, Burlingame, CA, USA). For IgY-binding 2nd and 3rd phage libraries binding capacity was identified by same ELISA procedure as described here.

For peptide binding, biotinylated peptide (40 nM) was pre-incubated with HRP-conjugated SA (10 nM) to form a tetrameric peptide complex. The mixture was added to IgY-coated wells of a plastic plate. After 1 h incubation, the wells were washed five times with PBST, and binding was detected with tetramethylbenzidine (Wako Pure Chemical, Osaka, Japan) reagent. Finally, 1 M hydrochloric acid (40 μL/well) was added to stop the reaction and binding was measured by the absorbance at 450 nm in a microplate reader (680XR, Bio-Rad, Hercules, CA, USA).

3.2.5 Surface Plasmon Resonance (SPR) Analysis

SPR analysis was performed on a BIAcore T200 (GE Healthcare, Little Chalfont, UK) at 25 °C. All reagents and sensor chips were purchased from GE Healthcare. IgY and IgY-Fc were immobilized on a CM5 sensor chip according to the manufacturer's instructions. The amount of the immobilized IgY was adjusted to within 1300–4800 response units. The association reaction was monitored by injecting the peptides into the sensor chip at a flow rate of 50 μL/min for 180 s. The dissociation reaction was performed in HBS-EP buffer (10 mM HEPES; pH 7.4 containing 150 mM NaCl, 3 mM EDTA, and 0.005% Tween 20).Binding kinetic parameters were calculated using BIA evaluation Version 3.2 Software (GE HealthCare).

3.2.6 Immunoprecipitation on Streptavidin beads

Immunoprecipitation was performed on SA beads by washing with PBS. Biotinylated IgY-binding peptides (100 mM) were added and shaken for 30 min. Next, IgY and human IgG (30 μg/100 μL) were added, shaken for 1 h, and centrifuged. The supernatant was removed and dispersed precipitated beads were mixed with buffer and centrifuged.

Finally, 30 μL of precipitated beads was used for SDS-PAGE analysis. For SDS-PAGE analysis of the obtained fractions, the sample was mixed with SDS sample buffer and subjected to SDS-PAGE on a 4–20% gradient gel (Mini-PROTEIN TGX; Bio-Rad).After electrophoresis, the gel was stained with Coomassie Brilliant blue R-250 stain solution (Bio-Rad).

3.3 Results and Discussion

3.3.1 Isolation of IgY- specific phage clones from random peptide libraries

IgY-binding T7 phage clones were enriched by five rounds of biopanning against IgY from two random libraries, $X_3CX_{7-8}CX$ and $X_3CX_{9-10}CX_3$, where X represents random amino acid positions. The binding activities of the phage libraries after biopanning were examined by ELISA (**Figure 2**). Phage binding increased after repeated biopanning in both libraries, but in the $X_3CX_{7-8}CX$ library, binding to human serum albumin was observed indicating non-specific binding. In contrast, the $X_3CX_{9-10}CX_3$, the library showed increased binding to IgY. The phage was randomly cloned after 4 and 5 rounds of biopanning of the $X_3CX_{9-10}CX_3$ library and subjected to binding screening by ELISA.
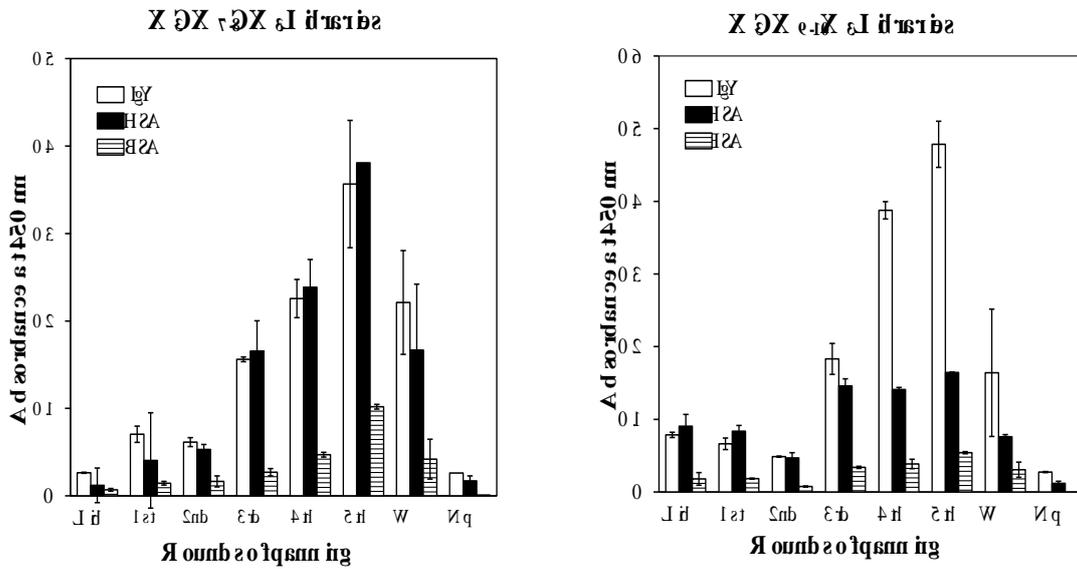
Figure 2 Isolation of IgY-specific phage clones from T7 phage-displayed random peptide libraries. Isolation of IgY-specific phage from the initial libraries ($X_3CX_{7-8}CX$ or $X_3CX_{9-10}CX_3$). After five rounds of biopanning, specific phage enrichment against IgY was examined by ELISA. Lib, phage libraries before biopanning; 1st–5th, phage after 1–5 rounds of biopanning; W, wild- phage; Np, measurement without phage.

Among the 30 clones, 25 clones showed high binding to IgY (Figure 3). Sequence analysis of 16 clones displaying peptides revealed three individual motifs (Y4-4, Y5-14, and Y5-15), as shown in Table 2. These binding clones bound specifically to IgY but not to other immunoglobulins or proteins (Figure 4).
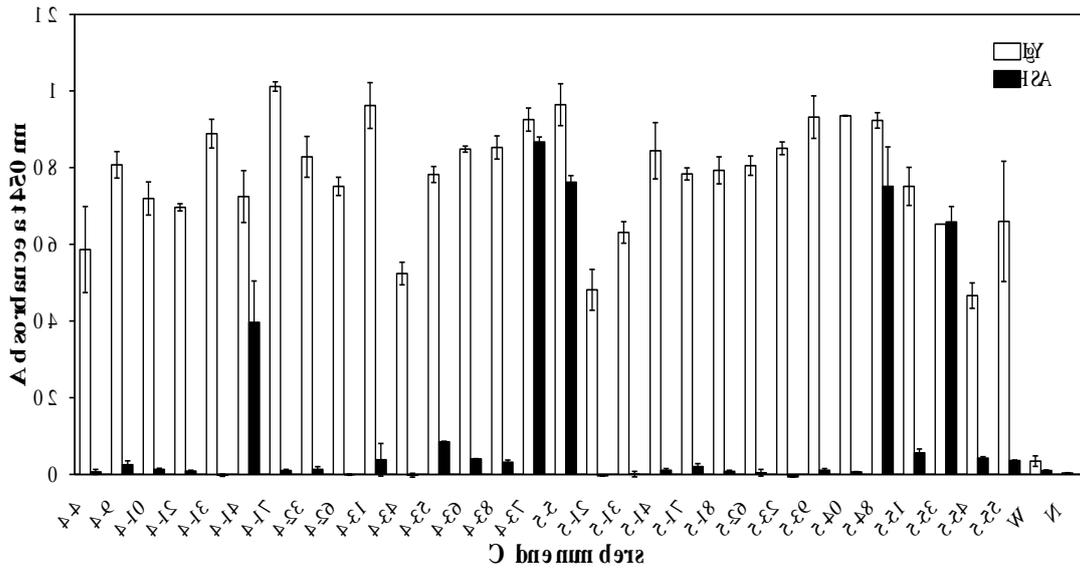
Figure 3 Identification of IgY-binding phage clones isolated from 4th and 5th phage pool by ELISA. 4-4 - 4-37, phage clones isolated after 4 rounds of biopanning. 5-5 – 5 55, Phage clones isolated after 5 rounds of biopanning. W, wild- phage; Np, measurement without phage.

Table 2 Comparison of amino acid sequences of IgY binding peptides. Amino acid positions are numbered based on $X_3CX_9CX_3$ sequence.

Clone	Sequence				Frequency	Library source
	1	5	10	15		
4-4	GVKCTWSSIVDWVCVDM				11/16	$X_3CX_9CX_3$
5-14	GTRCDW̄SAAYGW̄LCYDY				4/16	$X_3CX_9CX_3$
5-55	RSVCVW̄TAVTGW̄DCRND				1/16	$X_3CX_9CX_3$

62
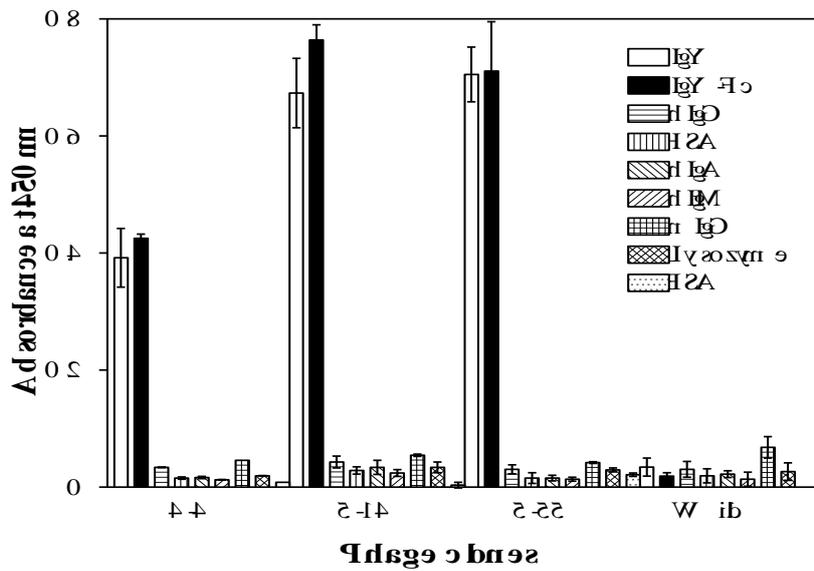
Figure 4 Identification of binding specificity of IgY binding specific phage clones by ELISA. Wild indicates the wild-type phages binding properties.

3.3.2 Functional evaluation identification of IgY binding synthetic peptides

The peptide sequences displayed on the 16 phage clones shared common peptide sequence pattern $X_3CXWX_5WXCX_3$ (Table 2) on three types of peptide-displaying phage clones. We have synthesized most abundant peptide sequence derived from Y4-4 phage clone (Table 2) and Y5-55 phage clones displayed peptide sequences and analyzed for its functionality using ELISA, SPR (Surface Plasmon Resonance) and immunoprecipitation. The binding specificity of the synthetic peptide Y4-4 and Y5-55 were compared with various antibodies and control proteins by ELISA (Figure **5**). Both peptides showed higher binding against IgY and IgY-Fc region indicating their specificity.
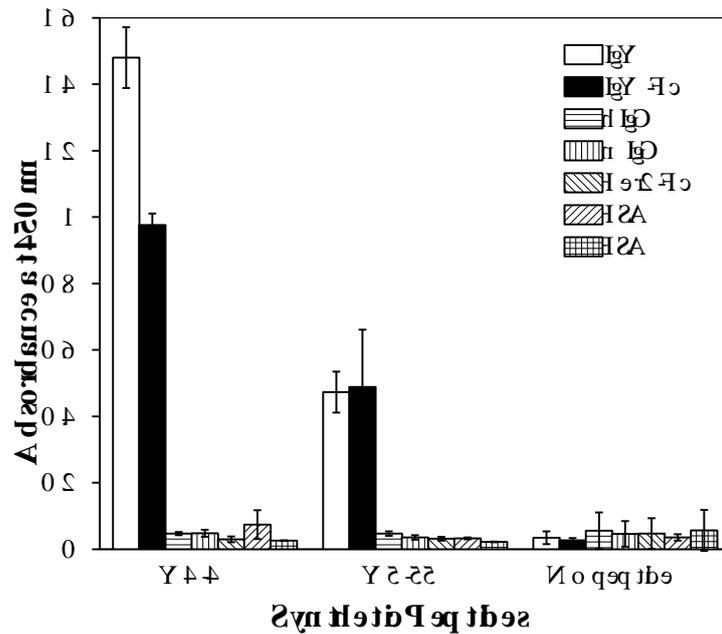
Figure 5 IgY binding synthetic peptides binding specificity identification by ELISA. No peptide indicates measurement without peptides.

Synthetic peptides binding affinity for the IgY were analyzed by SPR. The equilibrium constant for the dissociation (K_d) between IgY immobilized on a CM5 sensor chip and Y4-4, Y5-55 peptides were estimated 6.9μM and 4.2μM respectively using pH 7 running buffer, which is not sufficient for an affinity ligand. (Figure 6)**.** The SPR analysis repeated again using acetate running buffers (pH 4 and pH 5) to verify the binding force between IgY and peptides under acidic conditions but the binding affinity was estimated is nearly equal to pH7.
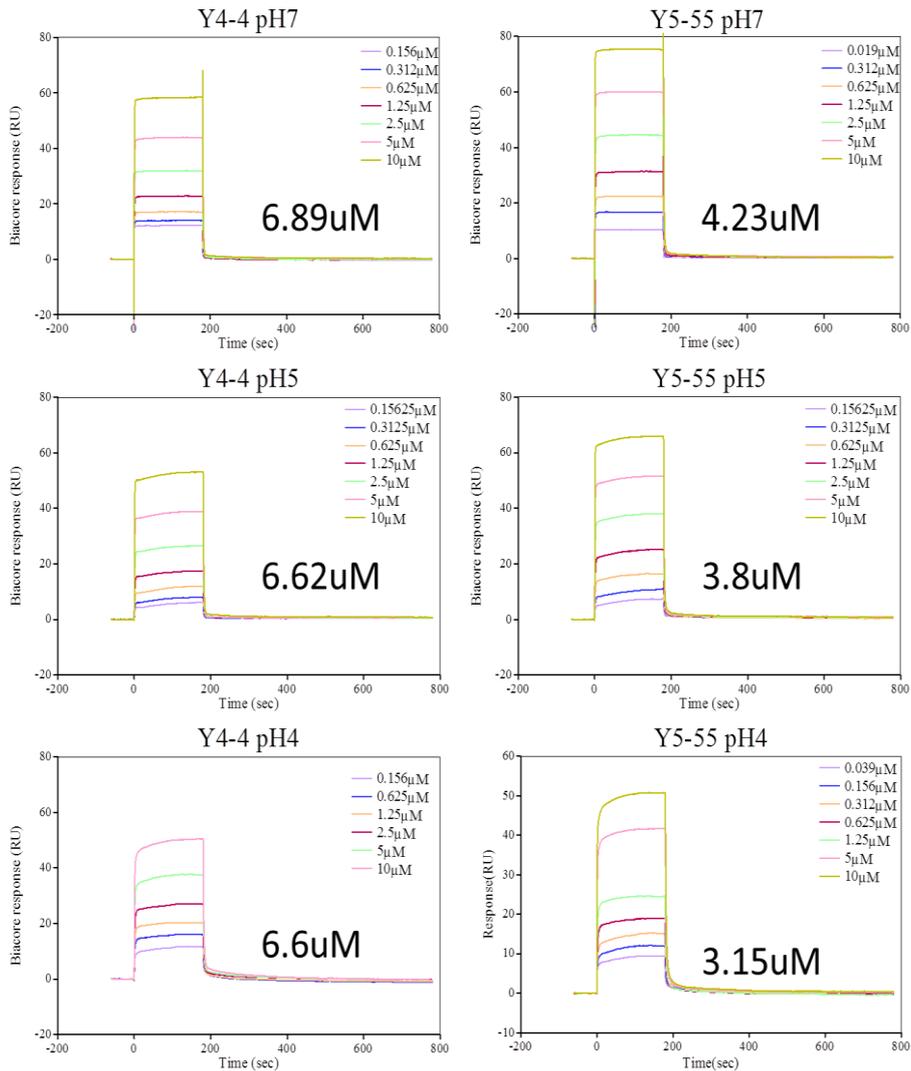
Figure 6 Affinity measurement of IgY-binding synthetic peptide using the SPR.

Finally, to confirm the peptides have the potential binding ability for recovering IgY from the solution in the case of immobilized peptide column carrier, IgY binding peptides (Y4-4, Y5-55) were evaluated by immunoprecipitation. In this experiment, IgY binding biotinylated peptides were immobilized to SA-agarose beads and IgY and human IgG (used as a control) were immune-precipitated, and analyzed by SDS-PAGE (Figure 7).The result shows IgY is specifically collected by both the peptides.
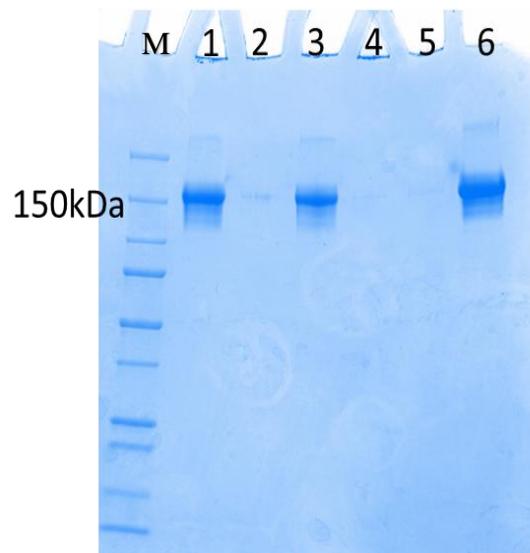
Figure 7 Immunoprecipitation analysis of Y4-4 and Y 5-55 synthetic peptides. Lane M, molecular weight marker (Precision Plus Protein™ All Blue Standards, Bio-Rad). Lane 1, Y4-4 peptide beads mixed with IgY. Lane 2, Y4-4 peptide beads mixed with IgG. Lane 3, Y5-55 peptide beads mixed with IgY. Lane 4, Y5-55 peptide beads mixed with IgG. Lane 5, beads only. Lane 6, standard IgY.

3.4 Summary

This chapter described the importance of combinational phage library and its application in research and diagnosis. This chapter also described the experiment for the isolation of IgY-binding peptide from a random peptide-displaying phage library. In this experiment, we have confined two IgY-binding peptides (Y4-4 & Y5-55) from T7 phage-displayed random peptide libraries. We have distinct logics to adopt disulfide-constrained T7 phage display tactics from two most prevalent (M13 and T7) phage display system, such as T7 phage display library show less bias in displayed amino acids which acquiesce greater coverage of a mixture of peptide sequences in the library (48)**,** disulfiide-constrained

cyclic peptide has higher affinity compared to linear form because of the reduction of the conformational chain entropy(49).

Binding properties of IgY-specific peptides were confirmed by ELISA but in the SPR analysis, they have shown an average binding affinity (K_d = 4-7 μM) with IgY. We again endorsed their binding specificity towards IgY by SA-beads immunoprecipitation. From the immunoprecipitation analysis, both of our peptides showed specific binding against IgY and IgY-Fc respectively.

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Chapter 4

Development of a simple, cost-effective and efficient method for isolating chicken egg yolk immunoglobulin using de-lipidation solution and ammonium sulfate

4.1 Introduction

In Chicken egg, the yolk is usually a shade of yellow color, which is separated from the surrounding egg white by the three-layered vitelline membrane. Chicken egg yolk provides the maternal antibodies for the first immune defence for the embryo. Furthermore, lipids, proteins, vitamins, and minerals are also provided by the egg-yolk in the developing embryo. Yolk consists of approximately 48% water, 33% lipid, and 17% protein (1). The component of the yolk is synthesized in the liver, transported with the blood to the ovary, and incorporated into the egg cell by receptor-mediated endocytosis (1–4). Apart from its function in avian reproduction, egg yolk is widely used in protein source as food. In industry, they are used as a binding agent, emulsifier, and natural antioxidant. Several research has established the therapeutic applications of egg yolk (5–8) in life science. Some experiments suggested egg proteins as an important raw material for the production of bioactive peptides (7, 8). The major egg yolk proteins and lipoproteins are distributed among two yolk compartments as plasma portion and a granular fraction. The plasma contains the water-soluble fraction, which mainly consists of a-livetin (serum albumin), b-livetin (a2-glycoprotein), and g-livetin (IgY). The granular (or globular) fraction contains the lipovitellins as components of high-density lipoprotein (HDL), phosvitin, and low-density lipoprotein (LDL) apoproteins (apo). Lipovitellins, the highly phosphorylated phosvitins, and the yolk plasma glycoproteins YGP40 and YGP42 are synthesized in the liver and transported to the oocyte as 210–260

kDa precursors, the vitellogenins (VTGs), which are constituents of blood HDL particles (9, 10). These are cleaved in the yolk to yield the mature proteins. Yolk LDL apoproteins are also synthesized in the liver and transported in the blood to the ovary as constituents of very-low-density lipoprotein (VLDL) particles. Yolk LDL has been shown to yield nine major protein bands on PAGE in a recent study (11). Two of these bands were identified as monomer and dimer of the,9 kDa apovitellenin I, while all other bands contained fragments of apolipoprotein B, which was already suggested earlier to give rise to all yolk LDL apoproteins with the exception of apovitellenin I (1, 12). In addition to these major proteins, it was reported that the yolk contains many enzymes, which were often characterized by their activity only (1, 13). Cathepsin D is important exceptions among the enzymes, which was suggested to be a key enzyme in yolk protein and aminopeptidase Ey (14) maturation.

Chicken egg yolk has been considered an ideal source of immunoglobulin and IgY is referring to be the predominant antibody in chicken egg yolk (15). It has many significant advantages of IgY over mammalian IgG have described in chapter 1. IgY has also drawn considerable attention as a means of preventing and controlling such diseases as bovine mastitis, diarrhoea in piglets, campylobacteriosis, shrimp white spot syndrome virus (16), immunological supplement in infant formula and other food (17). Due to the unnecessary complexity and time-consuming purification steps, the practical use of IgY in research and diagnostics becomes limited. IgY from egg yolk could be isolated to remove the water-insoluble components such as lipids and lipoproteins to get water-soluble protein fraction (WSF). Several methods are used to isolate IgY, including water dilution (18), salt precipitation (19), high polymer precipitation (20), organic solvent

extraction (21), ultrafiltration (22), and chromatography (23). However, most of these methods have drawbacks, such as low IgY yield, the complexity of procedures, or compatibility with human uses. Chang *et al*. (24) suggested that anionic polysaccharides could interact with lipoproteins to isolate IgY, but they just focused on the interactions between polysaccharides and lipoproteins without considering the purification of IgY. Souza *et al*., again pointed out that polysaccharides and lipoproteins could form complex coacervates, and the changes of pH value in egg yolk solution influenced the extent of the precipitation of polysaccharide lipoprotein complexes. Tan *et al*. (25) reported a rapid way to isolate IgY using a combination solution followed by ammonium sulfate. Because of some factors (e.g., pH, types and concentrations of polysaccharides, and diluted rate), the yield and purity of IgY isolated in this way were not very satisfying (26). Sock Hwee Tan *et al.* (25) reported IgY extraction method with a de-lipid solution (mix of κ-Carrageenan, pectin and $CaCl_2$) followed by precipitation using various concentrations ammonium sulfate ((NH_4)$_2SO_4$), but in that experiment, it was not possible to recover IgY using 20%-30% ammonium sulfate precipitation. In addition, after adding the de-lipid solution, a considerable viscosity was observed in the samples subjected to the ammonium sulfate precipitation which was considered to be due to the gelatin ability of κ-Carrageenan.

To investigate the properties of IgY extraction by de-lipid solutions, in this study, we adopted a simple, safe, and effective extraction method using distilled water as delipidation solution to remove insoluble lipids and lipoproteins in the first step, followed by ammonium sulfate precipitation. Using distilled water as delipidation solution confirms no use of undesirable reagents which was considered as a better IgY extraction

method without affecting the future experiments. So the isolated IgY can be applied to large-scale production in the commercial industries and medical field.

4.2 Experimental procedures

4.2.1 IgY Extraction by de-lipid extraction solution

Egg yolk was separated from egg white, and after washing the egg with distilled water, 5 mL of egg yolk was mixed with five volumes of (25 mL) of delipidation solution containing 0.072% κ- Carrageenan (Sigma, St. Louis, USA), 0.12% low-methoxyl pectin (Cp Kelco APS, Atlanta, GA, USA), and 12 μM $CaCl_2$ (Sigma). The solution was then incubated at 4 °C for 2 h with shaking and then centrifuged for 20 min at 12 000 ×g at 4 °C. After centrifugation, soluble IgY in the delipidated supernatant was collected and precipitated by $(NH_4)_2 SO_4$, which accounted for up to 35–40% of the total solution, followed by 1 h incubation with shaking at 4 °C and centrifugation for 20 min at 12,000 ×g. Finally, the supernatant was removed, and the pellet was suspended in 10 or 50 mM phosphate buffer.

4.2.2 IgY Extraction by distilled Water

First, 5 mL of egg yolk separated from egg white was mixed with 10 volumes of distilled water and incubated at 4 °C with shaking overnight followed by centrifugation at 10,000 ×g for 25 min. After centrifugation, the supernatant was collected and precipitated using four different concentrations (30%, 35%, 40%, and 50% w/w) of $(NH_4)_2 SO_4$. Finally, all the samples were incubated with shaking at 4 °C for 1 h, followed by centrifugation for 20 min at 12 000 ×g, and the pellet was suspended in 10 mM phosphate buffer (pH 7.0) after removing the supernatant.
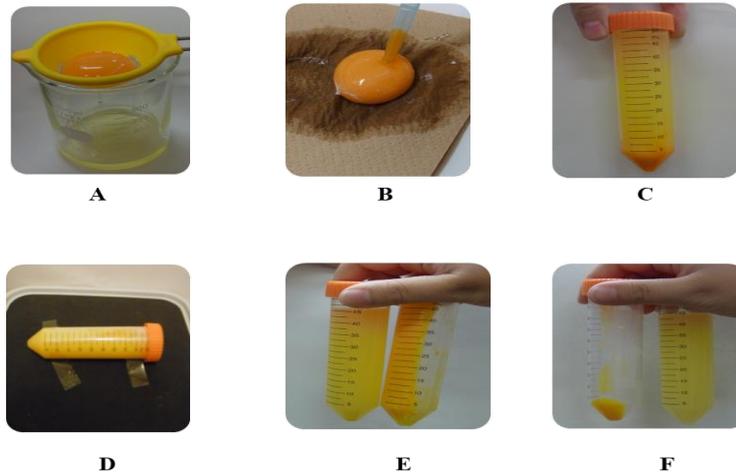
Figure 1 Schematic diagram of IgY extraction from egg-yolk by distilled water. (A) Egg yolk separated from the egg white portion. (B) By using micro-pipette 5ml of yolk separated. (C) Egg yolk diluted with 10 times distilled water. (D) Diluted egg yolk incubated with shaking for overnight at 4ºC. (E) Egg diluted solution centrifuged at high speed for 20 minutes, in the recovered supernatant 35% of total volume of the solution ammonium sulfate added and centrifuged again at high speed. (F) Supernatant removed and pellet suspended into 5ml of phosphate buffer.

4.3 Results and Discussion

Several IgY extraction methods have been reported before (24-29), here we examined IgY extraction by de-lipid solution (containing κ-Carrageenan) and distilled water respectively (31). We have prepared egg samples by adding de-lipid solution (diluted 2-fold by PB and incubated with and without shaking) followed by 35% and 40% ammonium sulfate precipitation respectively. After the precipitations, to identify the influence of the PB concentration, samples were suspended in 10mM and 50mM respectively and IgY was measured by SDS-PAGE analysis (Figure 2). In figure 2, it was

observed that IgY could be recovered efficiently by 35% ammonium sulfate precipitation. Accordingly, we have also tried to extract IgY from the egg by adding 10 volumes of distilled waters with egg yolk with shaking followed by different concentrations of ammonium sulfate precipitation. In the SDS-PAGE analysis (Figure 3), it was confirmed that IgY could be efficiently recovered without contamination by distilled water extraction method with 35% ammonium precipitation (Figure 3, lane 3). Moreover, to identify the best IgY extraction condition, IgY recovered with the de-lipid extraction method (Figure 2, lane 9) was also used in SDS-PAGE (Figure 3, lane 6). As compared between the two IgY extraction methods, in IgY extraction by distilled water method there is no use of undesirable reagents which was considered as a better IgY extraction method without affecting the future experiments.
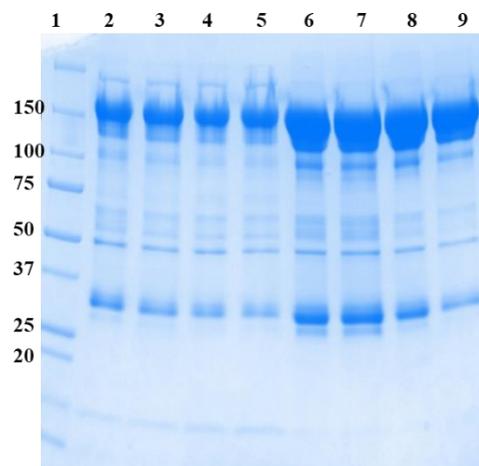
Figure 2 SDS-PAGE analysis of IgY extraction with κ-Carrageenan. Lane 1, IgY (standard control). lanes 2 and 3; egg yolk suspended in 10 mM and 50 mM PB buffer with shaking, respectively and precipitated by 40 % (w/w) $(NH_4)_2SO_4$. lanes 4 and 5; egg yolk suspended in 10 mM and 50 mM PB buffer with shaking, respectively and precipitated by 35 % (w/w) (NH_4) lanes 6 and 7; egg yolk suspended in 10 mM and 50 mM PB buffer without shaking, respectively and precipitated by 40 % (w/w) $(NH_4)_2 SO_4$. lanes 8 and 9; egg yolk suspended in 10 mM and 50 mM PB buffer without shaking, respectively and precipitated by 35 % (w/w) $(NH_4)_2SO_4$.
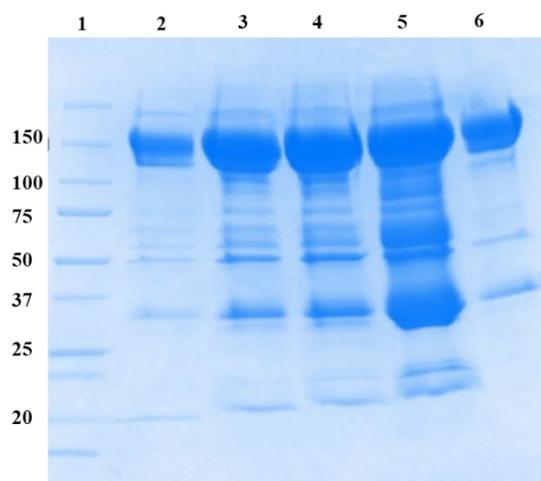
Figure 3 Optimization for extraction condition of IgY from delipidated egg yolk solution. Lane 1, IgY (standard control). Proteins (lanes 2–5) sample diluted in 10times distilled water and precipitated by 30, 35, 40, and 45% (w/w) $(NH_4)_2SO_4$, respectively. Lane 6, protein extracted by κ-Carrageenan, suspended in 50 mM PB buffer without shaking, and precipitated by 35 % (w/w) $(NH_4)_2SO_4$.

4.4 Summary

The high lipid content of egg yolk interferes with affinity-based IgY purification. To avoid this, a delipidation step is required to remove insoluble lipids and lipoproteins (22). We first investigated an IgY de-lipid extraction procedure using vegetable gum (κ-Carrageenan) followed by ammonium sulfate precipitation, as reported by Tan *et al*. (25). However, the κ-Carrageenan de-lipid solution based IgY extraction procedure cause the extract solution to become very viscous, which interrupted column chromatography. Therefore, we employed a simple dilution of egg yolk with distilled water followed by ammonium sulfate precipitation. This water-based IgY extraction offers a clear and non-viscous IgY extraction solution, removing lipids as a floating bubble layer. We confirmed

that IgY extraction by distilled water and 35% (w/w) ammonium sulfate precipitation to remove insoluble lipids from the egg yolk was effective for extracting IgY.

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Chapter 5

Applications of IgY binding peptides for IgY purification

5.1 Introduction

Chicken egg yolk immunoglobulin or IgY is the functional equivalent of mammalian IgG (1).It is found in the serum of the chicken and provides passive immunity to the embryo via the egg yolk, as a result the high concentration of IgY is available in the egg yolk (2). Comparing to the mammalian IgG, chicken IgY has many advantages (2) such as 1) no sacrificing or bleeding of the animal, 2) high protein yield comparing to another source (100–150 mg IgY/each yolk), and 3) less cross-reactivity with mammalian proteins. Traditional methods for IgY purification involve multi-step, complex, and time-consuming procedures such as ammonium sulfate precipitation (3), polyethene glycol precipitation (4), water dilution (5), ultrafiltration (6), gel filtration (7), thiophilic gel chromatography (8), and ion exchange chromatography (9). But the recovery of IgY via these methods are very low and the abundant lipoprotein in egg yolk is usually denatured with chloroform and removed by centrifugation.

Recently, Verdoliva *et al.* (10) reported a synthetic ligand consisting of a tetrameric tripeptide (ArgThr-(Tyr) 4-K2-K-G) for IgY affinity purification. However, this ligand detaches from the matrix and its binding capacity is reduced by 30% after exposure to 0.1 M NaOH for 1 h. For industrial use, the ligand column needs to be cleaned and used repeatedly in production, this deficiency greatly limits this ligand's industrial-scale application for IgY purification. Recently affinity chromatography is one of the most widely used methods for antibody purification (9) for the rapidity and high

selectivity. Immunoglobulin binding proteins (IBPs), such as protein A and protein G, have been broadly used for affinity purification of mammalian immunoglobulin (11). Protein A and G ligands bind to Fc region of most IgG and IgA subclasses, meanwhile, protein A also binds to the Fab region at the framework of VH (Kd ~ 20 nM) while protein G binds to the Fab at the CH1 (Kd ~ low M) (12). However, it is important to address that those commonly used IBPs have no affinity with IgY due to the different specific amino acid sequence of IgY-Fc to IgG-Fc. There is no suitable affinity chromatography for IgY purification except small-scale antigen-antibody affinity chromatography. Recently, protein M, a transmembrane protein from human mycoplasma, has been reported to bind to all types of mammalian IgG, IgA, IgD and IgM with high affinity (11). Protein M mainly blocks antibody-antigen union depends on ten conserved hydrogen bonds and one salt bridge made from Protein M to each Fab VL, almost all of the bonds are highly conserved among human antibodies with both and light chains (11). It could be rational to propose that protein M might also bind to IgY according to the variable region amino acid sequence alignment of human IgG and chicken IgY, which could lead to methodology innovation on IgY purification. W.Maciej *et al., 2015* also presented the method for generation of peptide-specific IgY antibodies directed to *Staphylococcus aureus* extracellular fibrinogen binding protein epitope (12-13). However, bacterial protein based antibody purification always require extra attention to prevent bacterial endotoxin or bacterial protein contamination in the pharmaceutical industries (14). Human Mycoplasma and *Staphylococcus aureus* both have highly toxic and antigenic nature which may have led to high risk of contamination for large scale pharmaceutical use (15).

In this chapter, we have described the establishment of the IgY purification system using IgY binding peptides. The preparation of affinity column immobilized with IgY-binding peptide and the determination of IgY antibody purification condition was performed to establish the purification process of IgY antibody from egg yolk in a single step.

5.2 Experimental procedure

5.2.1 HPLC analysis

Y4-4 and Y5-55 peptides were biotinylated via a PEG_4 linker to the N-terminus, and then 385 and 324 nmol/column were immobilized, respectively, on a HiTrap™ Streptavidin HP column (1 mL; GE Healthcare) according to the manufacturer's instructions. IgY, IgY-Fc, and human IgG were used as standard proteins to identify binding against peptides. After injecting the standard proteins, the column was washed with PBS and peptide-absorbed proteins were eluted with elution buffer (0.1 M glycine-HCl, pH 2.5 and pH 3).

5.2.2 Chicken immunization and egg collection

Two Boris Brown Chickens were injected intramuscularly with JWH-KLH (JWH conjugated to Keyhole Limpet Hemocyanin) (ARC Resources, Calgary, Canada) and the first immunization was carried out in complete adjuvant. Four booster injections with incomplete adjuvant were given at 14, 28, 42, and 63 days after the first immunization. The eggs were collected each day starting on the second day after immunization and stored at 4 °C.

5.2.3 Purification of chicken egg yolk IgY by affinity peptide column

Approximately 385 nmol of the Y4-4 peptide was immobilized on a HiTrap™ Streptavidin HP column (1 mL; GE Healthcare), according to the manufacturer's instructions. Egg yolk IgY was extracted from a commercial egg by injecting distilled water directly into the peptide-immobilized affinity column (1 mL), which was connected to a Profinia purification system (Bio-Rad). Washing buffer (PBS) was used to remove unbound materials. Binding IgY was eluted with 0.1 M glycine-HCl/0.25 M NaCl (pH 2.5 and pH 3) and the eluate was immediately neutralized with neutralization buffer (1 M Tris-HCl, pH 8.5) and stored at 4 °C until use. The obtained fractions were evaluated for immunoreactivity by enzyme-linked immunosorbent assay (ELISA) and purity by SDS-PAGE analysis. From the SDS-PAGE data, the purity of the obtained fractions was estimated using GelAnalyzer2010a software. Protein concentrations were estimated from the absorbance at 280 nm using an absorbance coefficient of 1.55 mL/mg for IgY and other proteins.

5.2.4 ELISA

For anti-KLH antibody measurements, KLH (50 ng/50 μL/well) and 0.5% BSA were coated overnight at 4 °C, washed three times with PBST, blocked with 0.5% BSA for 2 h, and washed again with PBST. Egg samples (before and after peptide-conjugated column purification) were diluted by 1000-fold using distilled water. Next, 50 μL/well of the solution was added. Finally, the binding was detected with tetramethyl-benzidine (Wako Pure Chemical, Osaka, Japan) reagent after incubating anti-IgY-conjugated with HRP (diluted by 2000-fold) for 1hour with shaking. 1 M hydrochloric acid (40 μL/well) was added to stop the reaction, and binding was measured by the absorbance at 450 nm in a

microplate reader (680XR; Bio-Rad, Hercules, CA, USA).

5.2.5 SDS-PAGE analysis

For SDS-PAGE analysis of the obtained fractions after column purifications, the sample was mixed with SDS sample buffer and subjected to SDS-PAGE on a 4–20% gradient gel (Mini-PROTEIN TGX; Bio-Rad).After electrophoresis, the gel was stained with Coomassie Brilliant blue R-250 stain solution (Bio-Rad).

5.3 Results and Discussion

To investigate the absorption ability of Y4-4 and Y5-55 peptide-conjugated affinity columns, an N-terminal biotinylated peptide was immobilized on a HiTrap Streptavidin HP column. IgY, IgY-Fc, or human IgG were injected into the column connected to HPLC. To elute the adsorbed protein fraction from the Y4-4 and Y5-55 peptide columns, two acidic elution buffers (0.1 M glycine-HCl) were used with pH 2.5 and pH 3.0, respectively. Although human IgG passed through both peptide-immobilized columns, IgY and IgY-Fc proteins were absorbed onto both columns (Figure 1). Furthermore, IgY and IgY-Fc proteins were eluted from the column at pH 2.5 and 3.0, although the elution peaks at pH 2.5 were sharper than those at pH 3.0 for both peptides.

A peptide-conjugated column prepared by immobilization of the biotinylated Y4-4 peptide (385 nmol) in a HiTrap Streptavidin HP column was used for one-step purification chromatography on Profinia (Bio-Rad) to purify IgY from egg yolk extract

precipitated with 35% (w/w) ammonium sulfate. After applying the egg yolk solution to the peptide affinity column, the column was washed with 10 column volumes of PBS and eluted with 0.1 M glycine-HCl with pH 2.5 and 3.0, respectively (Figure 2).
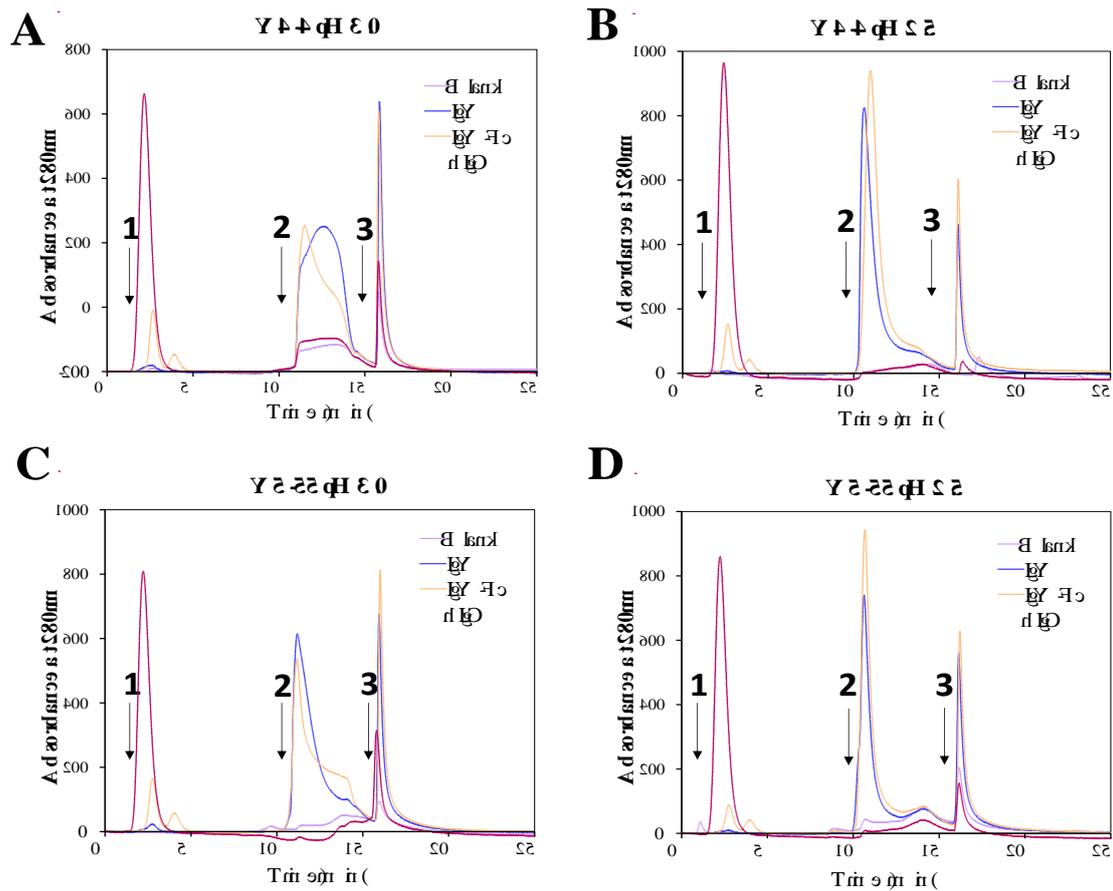

Figure 1 IgY absorption/elution profiles on IgY binding peptide immobilized column by HPLC. Biotinylated Y4-4 and Y5-55 peptides were immobilized into SA-HiTrap columns. After equilibration with PBS, IgY, IgY-Fc or IgG were injected. Elution was performed by 0.1 M glycine-HCl buffers at pH 3.0 (A, C) or 2.5 (B, D) to monitor the absorption/elution properties. Arrows 1, 2 and 3 indicate the starting point for sample injection, elution and regeneration with PBS, respectively.
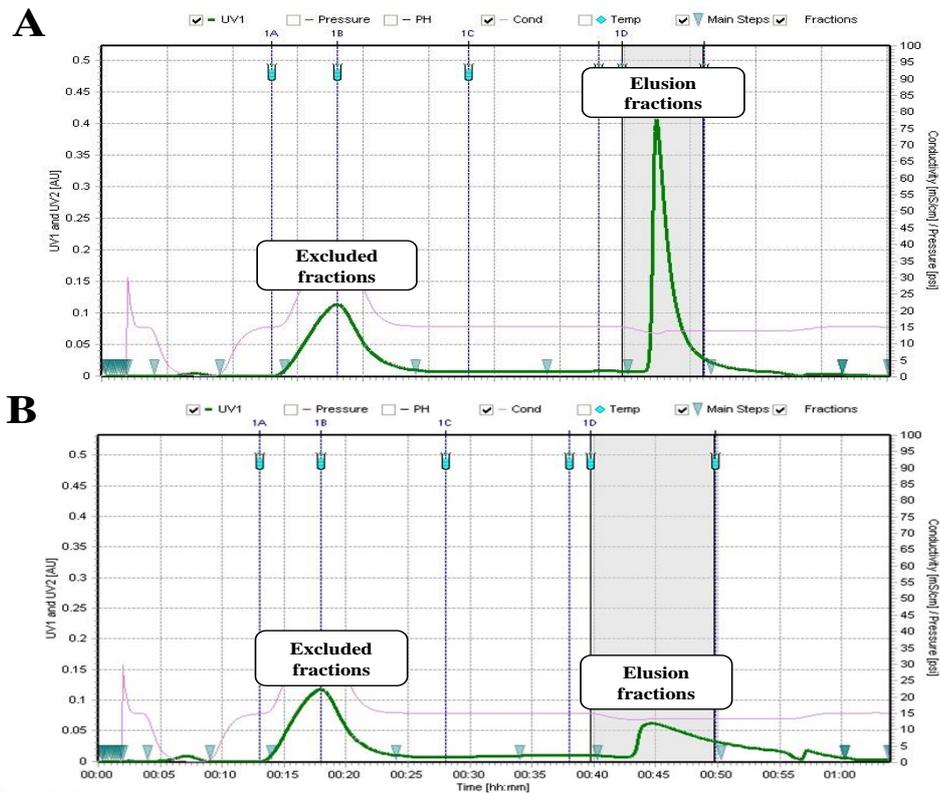
Figure 2 IgY purification from egg yolk (non-immunized) extract by peptide column at pH 2.5 (A) and 3.0 (B).

We attempted to purify functional IgY from eggs collected from chickens 30–37 days after immunization with KLH antigen four times every week after initial immunization. IgY was purified from these eggs as described in Figure 3. After applying the egg yolk solution to the peptide affinity column, the column was washed with 10 column volumes of PBS and eluted with 0.1 M glycine-HCl with pH 2.5.
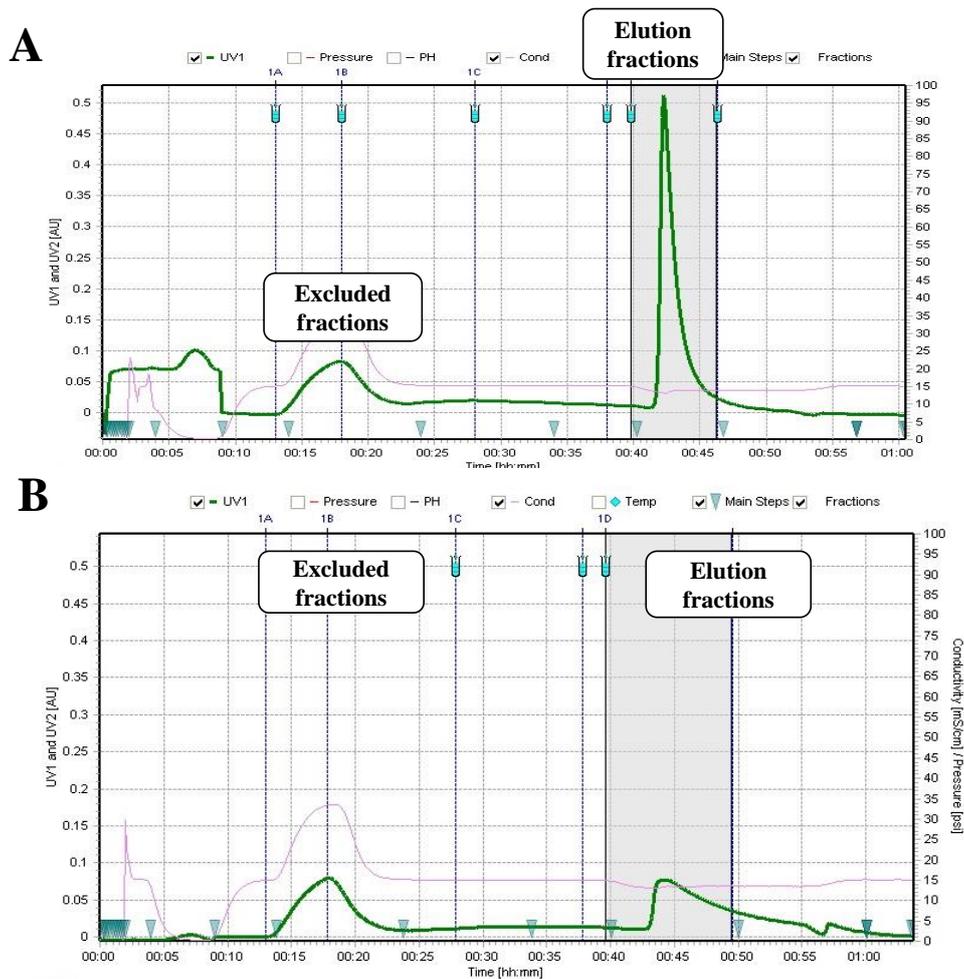
Figure 3 IgY purification from KLH-immunized egg yolk extract by peptide column at pH 2.5 (A) and 3.0 (B).

After purification, the non-immunized and KLH-immunize chicken egg yolk, the excluded and eluted fractions by both elution condition (pH 2.5 and 3.0) were subjected to SDS-PAGE to evaluate the purity of IgY in each fraction (Figure 4). IgY (150 kDa) was not detected in the flow-through fractions but appeared as a single band in the immunized and non-immunized egg eluted fractions at pH 3.0 (Figure 4, lane 7 and 12), indicating the successful isolation of IgY from both egg yolk with high purity (93%). In contrast, proteins with higher molecular mass (>250 kDa) were observed by SDS-PAGE

following elution at pH 2.5 (lane 5 and 10), resulting in low purity (63%) of IgY. The recovery and purity of IgY after purification are summarized in Table 1.
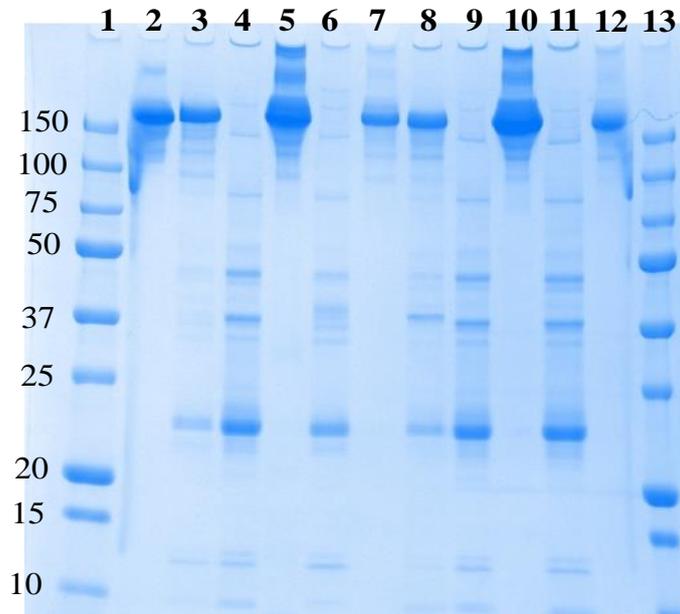
Figure 4 SDS-PAGE of purified KLH-immunized and non-immunized egg yolk fractions. Lane 1, molecular weight marker (Precision Plus Protein™ All Blue Standards, Bio-Rad). Lane 2, standard product IgY 5µg. Lane 3, KLH-Immunize egg delipidated solution before purification. Lane 4, exclusive fraction. Lane 5, elusion fraction at pH 2.5. Lane 5, exclusive fraction. Lane 6, elusion fraction at pH 3.0. Lane 7, Non-immunize egg delipidated solution before purification. Lane 8, exclusive fraction. Lane 9, elusion fraction at pH 2.5. Lane 10, exclusive fraction. Lane 11, elusion fraction at pH 3.0. Lane 12, molecular weight marker. Arrow indicates the position of IgY.

Table 1 Recovery and purity of IgY (non-immunized egg) in peptide affinity column purification. The protein amount (starting from 1 mL delipidation solution corresponding to 1 mL egg yolk) was indicated at each step of purification. The purity of IgY in each fraction was estimated from CBB-gel image on SDS-PAGE using GelAnalyzer2010a software.

pH of elution buffer	Protein in egg yolk delipidation solution		Flow-through fractions		Elution fractions	
	Amount (mg)	Purity (%)	Amount (mg)	Purity (%)	Amount (mg)	Purity (%)
2.5	1.47	70	0.68	0	0.74	63
3.0			0.65	0	0.43	93

Finally, the antigen binding ability of the purified IgY was tested by ELISA. As shown in Figure 4, purified IgY from eggs of immunized chickens showed KLH-specific binding ability, which was also observed in the egg yolk extraction before column chromatography. This suggests that our method can be used for to purify functional IgY from the eggs of immunized chickens.
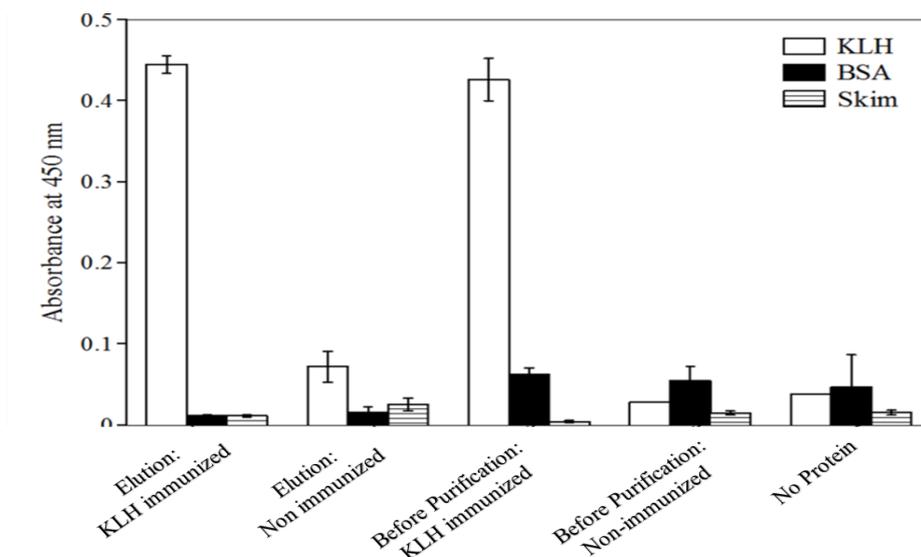
Figure 5 The function of purified IgY. The binding specificity of IgY from the KLH-immunized chicken egg was analyzed by ELISA. Extract from KLH-immunized egg; delipidated solution from KLH-immunized chicken egg yolk. IgY from KLH-immunized egg; IgY purified by Y4-4 peptide conjugated column from the KLH-immunized chicken egg. Extract from the non-immunized egg: delipidated solution from non-immunized chicken egg yolk. IgY from the non-immunized egg; IgY purified by Y4-4 peptide conjugated column from the non-immunized chicken egg. No protein; measurement without protein.

5.4 Summary

Using the peptide-conjugated column, we purified IgY from egg yolks of non-immunized and KLH-immunized chickens and successfully isolated highly pure IgY (>90%) in both cases with a high recovery yield (approximately 70%). However, the purified IgY contained several bands with high molecular weights larger than IgY (Figure 4). Because these bands were not observed before chromatography, they were generated by the

chromatographic procedures, likely by the formation of oligomers of IgY induced by acid treatment (pH 2.5) during elution. To avoid this, a mildly acidic condition of pH 3.0 was used for elution (lane 7 and 12 of Fig. 4), although the recovery yield from the column decreased from 0.74 to 0.43 mg (Table 1).

In summary, we successfully refined a highly specific and functional IgY binding peptide from the T7 phage library. This was achieved by identifying a specific IgY binding peptide-displaying phage by biopanning from a random peptide-displaying library. Our IgY-binding novel peptide is compact and highly functional as an affinity ligand and may be an excellent reagent for low-cost purification of IgY from chicken egg yolks with high yield and purity.

5.5 References

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Chapter 6

Summary

6.1 Summary and conclusion

In this thesis, we present a study about the identification of IgY binding peptides with unique properties and their application for the purification of IgY from egg yolk. Through biopanning from the random peptide-displaying phage libraries constructed by T7 phage display technology, IgY-specific binding phages were isolated and their synthetic peptides were characterized by several analytical techniques. The obtained data provided detail properties about the IgY-binding peptides and indicated the potentials in use for purifying IgY antibody from the egg. Therefore, the egg yolk dilapidation technique was validated by comparing the different types of lipid extraction followed by ammonium sulfate precipitation methods. Finally, IgY purification system was established by combining the dilapidation technique and the chromatographic technique using the column conjugated with the IgY-binding peptide.

Chapter 1, described the general introduction of the IgY antibody's structure and functions as an attractive tool for biological research, diagnostic use and preparation of immunotherapy. Especially, this chapter also analysed the advanced features of IgY antibodies, such as its higher specificity, their low cross-reactivity to mammalian proteins due to the maximum phylogenetic distance between them, improved pH/temperature stability and cost-effective preparations over mammalian IgG.

Chapter 2, summarised the introduction and the preparation of random peptide-displaying T7 phage library used here. To construct T7 phage library harbouring the disulfiide constrained random peptide library, the design of DNA template expressing random peptides, PCR amplification of template, the ligation of amplified DNA to T7 phage vector and the phage propagation using *E. coli* were described. Finally, the quality of peptide-displaying T7 phages libraries constructed here was also validated.

Chapter 3, described the isolation of IgY-binding peptide from random peptide displaying T7 phage libraries and the characterizations of the obtained peptides. The biopanning procedure was applied to identify IgY-binding phage and the specific phages were isolated. The synthetic peptides derived from the sequences of the phages were prepared and their binding properties including their specificity and affinity were analyzed.

Chapter 4, reports the pretreatment of chicken egg including delipidation of egg yolk for the use of IgY purification. Two previously developed egg delipidation techniques were compared and validated for the pretreatment of egg yolk. Finally, a novel and potential egg delipidation technique which can serve lipid extraction solution for better IgY purification were identified.

Chapter 5, described the establishment of the IgY purification system using IgY binding peptides. The preparation of affinity column immobilized with IgY-binding peptide and the determination of IgY antibody purification condition was performed to establish the purification process of IgY antibody from egg yolk in a single step.

In Chapter 6, the results of this study were summarized and the usefulness of our method was discussed from viewpoint of industrial applications, comparing with other methods.

6.2 Acknowledgements