Chapter 5

Functional Proteins and Peptides of Hen's Egg Origin

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1. Introduction

Hen's egg has long history as a food. It contains a great variety of nutrients to sustain both life and growth. Egg provides an excellent, inexpensive and low calorie source of high-quality proteins. Moreover, Eggs are a good source of several important nutrients including protein, total fat, monounsaturated fatty acids, polyunsaturated fatty acids, cholesterol, choline, folate, iron, calcium, phosphorus, selenium, zinc and vitamins A, B_2, B_6, B_{12}, D, E and K [1]. Eggs are also a good source of the antioxidant carotenoids, lutein and zeaxanthin [2]. The high nutritional properties of eggs make them ideal for many people with special dietary requirements.

Egg proteins are nutritionally complete with a good balance of essential amino acids which are needed for building and repairing the cells in muscles and other body tissues [3]. Egg proteins are distributed in all parts of the egg, but most of them are present in the egg white and egg yolk amounting to 50% and 40%, respectively. The remaining amount of protein is in the egg shell and egg shell membranes.

In addition to excellent nutritional value, egg proteins have unique biological activities. Hyperimmunized hens could provide a convenient and economic source of specific immunoglobulin in their yolks (IgY) that have been found to be effective in preventing many bacteria and viruses infections [4]. Proteins in the egg white as lysozyme, ovotransferrin, and avidin have proven to exert numerous biological activities. Moreover, a specific protein in eggshell matrix shows unique activity; enhancement of calcium transportation in the human intestinal epithelial cells.

It is well-known that egg proteins are a source of biologically active peptides. Many researches are aiming to unlock the hidden biological functions of peptides hidden in egg proteins. These peptides are inactive within the sequence of parent proteins and can be released during gastrointestinal digestion or food processing and exerting biological

activities. Once bioactive peptides are liberated, they may act as regulatory compounds, and exhibit various activities such as anti-hypertensive, bone growth promoting, anticancer or exaggerated antimicrobial activities.

For development of bioactive peptides from parent proteins, following techniques have been conventionally used; the establishment of an assay system of biological activities, hydrolysis of proteins by digestive enzymes, isolation of peptides, determination of structures and synthesis of peptides. Recently, bioengineer technique; synthesis of peptides within egg proteins based on sequence similarities of peptides having known biological activity, has been used. The functional characteristics of either natural or modified egg proteins and the use of eggs components as "functional ingredients" are relatively new applications. A truly impressive volume of researches is now available for the egg industry to apply these new applications. Herein, some aspects concerning biologically functional egg proteins or peptides, biochemical and physiological properties as well as possible applications of egg proteins or peptides are discussed.

2. Egg yolk bio proteins

The major portion of egg yolk exists as lipoproteins, which can be separated by centrifugation into a plasma fraction (which remains soluble) and a granular fraction (which precipitates). Lipovitellenin, lipovitellin, phosvitin, livetin, yolk immunoglobulins (IgY), and some minor components have been isolated and identified in egg yolk.

2.1. Lipoproteins

Low density lipoprotein (LDL), which contains between 80 and 90% lipids, characterized by its emulsifying capacity. LDL is the major protein in egg yolk, accounting for 70% of yolk proteins. When LDL is treated with ether, residual fraction is referred to lipovitellenin containing 40% lipid. [5]. Shinohara et al. (1993) studied the effect of some constituents of egg yolk lipoprotein on the growth and IgM production of human-human hybridoma cells and other human-derived cells [6]. LDL-rich fractions were found to enhance the growth and IgM secretion of HB4C5 cells. The promoting activity was found in the commercial LDL.

High density lipoprotein (HDL) or lipovitellin comprise about one sixth of egg yolk solids in the granular yolk proteins. It has a molecular weight of $4X10^5$ and composed of 80% protein and 20% lipid. HDL exists as a complex with a phosphoprotein referred to phosvitin [5]. The addition of one or two eggs a day to a healthy person's diet does not adversely affect lipoprotein levels, and can actually increase plasma HDL levels [1].

2.2. Phosvitin

The name phosvitin comes from both its high phosphorus content (10%) and its source in the egg yolk. The emulsification properties of phosvitin, particularly emulsion-stabilizing activity, were found to be higher than those of other food proteins. Phosvitin is water

insoluble but under low ionic strength and acidic conditions, it becomes soluble and can become complex with various metal ions (*e.g.* Ca^{++}, Mg^{++}, Mn^{++}, Co^{++}, Fe^{++} and Fe^{+++}) [5]. It could be used as a potent natural antioxidant on the basis of its potential to inhibit metal-catalyzed lipid oxidation [7]. The conjugation of egg yolk phosvitin with galactomannan produces a novel macromolecular antioxidant with significantly improved emulsifying activity and emulsion-stabilizing activity [8]. The antibacterial activity of phosvitin has been investigated against *Escherichia coli* and suggested that a significant part of the bactericidal activity of phosvitin could be attributed to the synergistic effects of the high metal-chelating ability and the high surface activity under the influence of thermal stress [9]. Phosvitin and the phosvitin-galactomannan conjugate may represent safe anti-bacterial agents for foods.

2.3. Vitamin-binding protein

A riboflavin-binding protein exists in the egg yolk. It is a hydrophilic phosphoglycoprotein with a molecular weight of 3.6×10^6 Da, and it can conjugate one mole of riboflavin per mole of apoprotein. Also, biotin and cobalamin-binding proteins could be found in egg yolk.

2.4. Livetin

Livetin is a water-soluble, non-lipid, globular glycoprotein, which is immunologically analogous to the plasma proteins of mammals. α-Livetin is analogous to serum albumin, β-livetin to 2-glycoprotein and γ-livetin to γ-globulin [10]. Most of the research effort has been focused on the immune proteins found in the egg yolk (IgY). Recent advances in IgY technology will be discussed.

2.5. Egg yolk immunoglobulin (IgY)

Immunoglobulin from yolk (IgY) is the major antibody found in hen eggs. In 1893, Klemperer first described the acquisition of passive immunity in birds, by demonstrating the transfer of immunity against tetanus toxin from the hen to the chick [11]. Three immunoglobulin classes analogues to the mammalian immunoglobulin classes; IgA, IgM, and IgG, have been shown to exist in chicken. In the egg, IgA and IgM are present in the egg white, while IgG is present in the egg yolk [12]. IgG in egg yolk has been referred to as IgY to distinguish it from its mammalian counterpart [13].

The concentration of IgY in the yolk is essentially constant (10-20 mg/mL) through the oocyte maturation. Approximately 100-400 mg IgY is packed in an egg. The concentration of IgY in the yolk is 1.23 times to the serum concentration [14]. A delay of 3 to 4 days is observed for the appearance of specific IgY in yolk after first appearance of specific IgG in the serum of a hen.

2.5.1. Structure and Characteristics of Avian IgY Versus Mammalian IgG

Composition differences. General structure of IgY molecule is the same as mammalian IgG with 2 heavy (Hv) chains with a molecular mass of 67–70 kDa each and two light (L) chains

with the molecular mass of 25 kDa each (Figure 1). The major difference is the number of constant regions (C) in H chains: IgG has 3 C regions (Cy1–Cy3), while IgY has 4 C regions (Cv1–Cv4). Due to occurrence of one additional C region with two corresponding carbohydrate chains, molecular mass of IgY (180 kDa) is larger than *mammalian* IgG (150 kDa). IgY is less flexible than mammalian IgG due to the absence of the hinge between Cy1 and Cy2. There are some regions in IgY (near the boundaries of Cv1–Cv2 and Cv2–Cv3) containing proline and glycine residues enabling only limited flexibility. IgY has isoelectric point 5.7–7.6 and is more hydrophobic than IgG [13, 15].

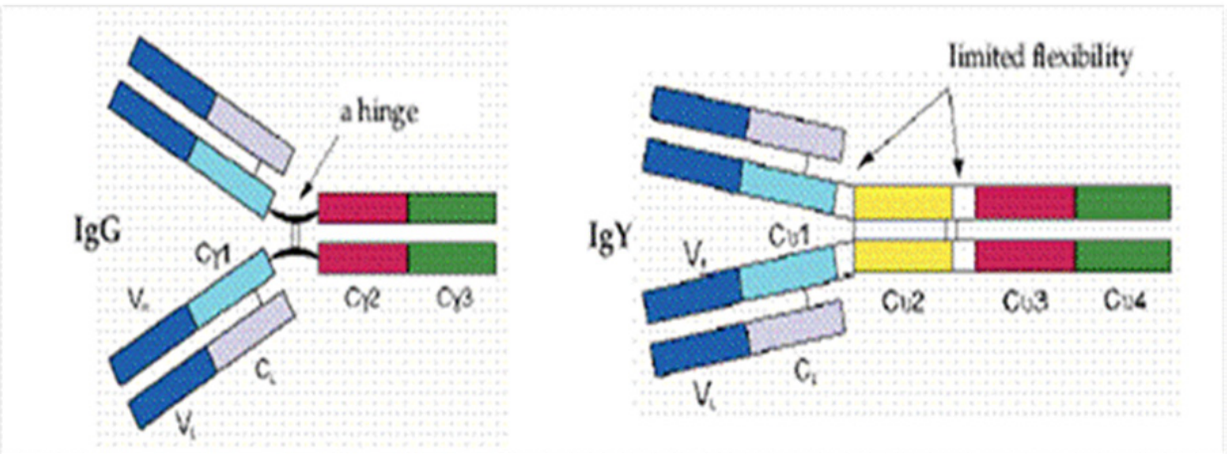


Figure 1. Structure of IgG and IgY.

Advantage of IgY. Most biological effectors' functions of immunoglobulin are activated by the Fc region, where the major structural difference between IgG and IgY is located. Therefore, Fc-dependent functions of IgY are essentially different from those of mammalian IgG. First, IgY does not activate the complement system[16], second, IgY does not bind to protein-A and G [17], third, IgY is not recognized by mammalian antibodies [18] *i.e.* rheumatoid factors (RF, an autoantibody reacting with the Fc portion of IgG) or HAMA (human anti-murine antibodies), and fourth, it does not bind to cell surface Fc receptor [19]. These differences in molecular interactions bring great advantages to the application of IgY antibodies. Then that were IgY has been successfully applied into a variety of methods in different areas of research, diagnostics, and medical areas. For these applications, IgY can successfully compete with antibodies (IgG) isolated from the blood of mammals [20]. The advantage of usage of the immunized hen is that it produces a large number of eggs. Approximately 40g of IgY could be collected from egg of one hen each year, compared with about 1.3g from blood of one rabbit [21]. An industrial scale production of IgY is possible because of the availability of large number of chicken farms and automation of egg breaking and processing.

2.5.2. Applications and uses of IgY

Oral administration of antibodies specific to host pathogens is an attractive approach to establish protective immunity, especially against gastrointestinal pathogens both in human

and animals. Eggs are normal dietary components and there is practically no risk of toxic side effects of IgY given orally. As mentioned above, IgY does not activate mammalian complement system nor interact with mammalian Fc-receptors that could mediate inflammatory response in the gastrointestinal tract.

On the basis of these facts, IgY has been used for suppression of growth of food-bone pathogens [11]. Whole egg yolks and water soluble fractions were prepared from the egg of the hens that had been immunized with pathogens such as *E. coli* O157:H7, *Salmonella enteritidis*, *Salmonella typhimurium*, *Campylobacter jejuni*, *Staphylococcus aureus*, and *Listeria monocytogenes*. It has been demonstrated that pathogen-specific IgY is bound to the surface of bacteria, resulting in structural alterations of cell wall and consequently kills bacteria. Sarker et al. (2001) performed a study for children with proven rotavirus diarrhea. The patients were treated with IgY from the eggs of chickens immunized with human rotavirus strains [22]. The treatment moderated diarrhea, which was characterized by an earlier clearance of rotavirus from the stools. Recently, IgY has been applied to cancer therapy. Hens were immunized with an antigen purified from human stomach cancer cells. The purified IgY recognized gastrointestinal cancer cells. Conjugation of antibodies and drugs may be an important agent for cancer treatment [23].

These IgY treatments have been shown to provide a safer, more efficient and less expensive method than those using conventional mammalian antibiotics for managing disease-causing pathogens. Recently, successful progresses in industrialization of IgY has been achieved in Japan, where IgY as a bioactive ingredient in food, nutraceuticals, cosmetics and other sectors is applied . The followings are the most recent applications.

2.5.2.1. Anti-Helicobacter pylori IgY

Helicobacter pylori, a spiral gram-negative microaerophilic pathogen, has been shown to be a common inhabitant of the gastric and duodenal mucosa. The microorganism is recognized as one of the most prevalent human pathogens. It infects over 50% of the population worldwide [24], and is recognized as the etiologic agent of gastritis, peptic ulcer, and has been linked to the development of gastric adenocarcinoma and mucosa associated lymphoid tissue lymphoma [25, 26]. The eradication of *H. pylori* by administration of oral antimicrobials is not always successful and may be associated with adverse effects [27]. For colonization in gastric mucosa, *H. pylori* abundantly produces urease enzyme, which degrades urea into ammonia. *Helicobacter pylori* organism uses the ammonia to neutralize microenvironment in gastric mucosa. Accordingly, a novel approach in prevention and reduction of *H. pylori* infection using urease-specific IgY has been developed. It has been reported that oral administration of anti-*H. pylori* urease IgY (IgY-urease) could suppress the bacterial colonization.

Preparation of IgY-Urease Yogurt. Today, consumers prefer foods that promote good health and could reduce risk of diseases. Dairy products are excellent media to generate an array of products that fit into the current consumer demand for functional foods [28]. Scientific and clinical evidence is mounting to corroborate the consumer perception of health from yogurt. Designing a yogurt fortified with IgY-urease could supply passive immunization with a

natural and highly specific attempt to decrease the *H. pylori* infection. In order to suppress *H. pylori* infection, a yogurt fortified with IgY-urease has been designed and developed. Three clinical studies were done to examine the efficacy of a specially designed functional yogurt containing IgY-urease on the suppression of *H. pylori* in humans. IgY-urease containing yogurt (plain and drinking) have been prepared and in markets in Japan, Korea, and Taiwan (Figure 2). IgY-urease was pasteurized and then added to yogurt mix at specific dose after all heat-treatment steps. Yogurts were cooled and stored at 4° C for up to 3 weeks. IgY-urease activity remained in the product throughout the 3 weeks of storage.

Figure 2. Different IgY-urease yogurt products in Japan, Korea, and Taiwan.

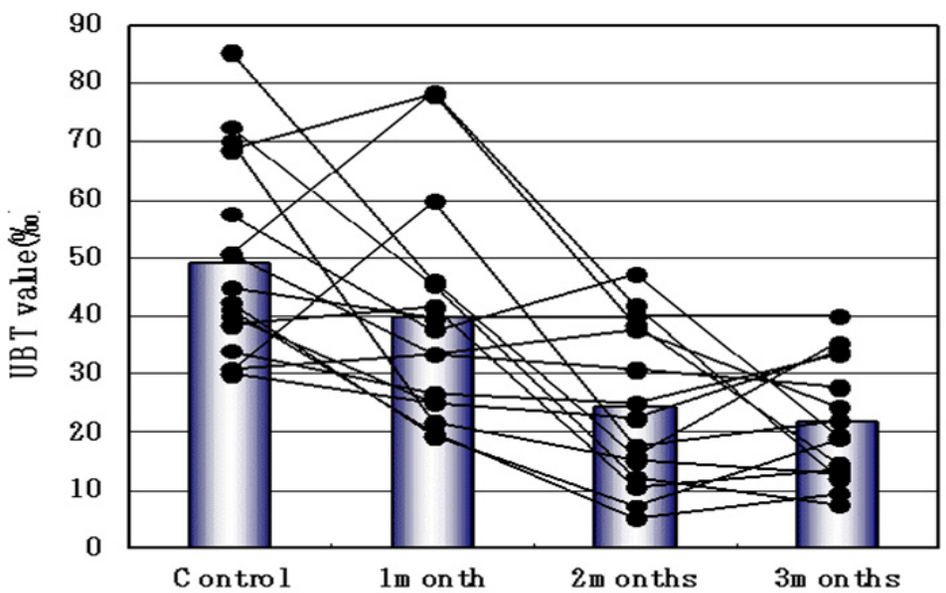


Figure 3. UBT Value Change of volunteers（clinical study in Japan).

Clinical studies. Plain yogurt containing 2g IgY-urease egg yolk was produced commercially in Japan. A clinical study was conducted to determine the effect of IgY-urease yogurt to decrease *H. pylori* in humans [29]. To assess presence of *H. pylori* in stomach, UBT method has been extensively used. This method based on the invasive detection of exhaled ^{13}C-labeled carbon dioxide resulting from *H. pylori* urease activity [30]. One hundred seventy-four volunteers were screened using a ^{13}C-urea breath test (UBT). Heavily infected volunteers (with UBT values over 30‰) were selected (16 subjects) and recruited. Each volunteer consumed 1 cups of yogurt twice daily (4 g/d egg yolk containing 40 mg IgY-urease) for 12 wk. Volunteers were tested after 4, 8 and 12 weeks. The UBT values obtained at week 8 and 12 were significantly different from those obtained at week 0 ($P < 0.001$), showing a 55.1% and 57.2% reduction in UBT values after 8 and 12 weeks, respectively (Figure 4). Other clinical studies using IgY-urease containing drinking yogurt were carried out in Taiwan [31] and Korea [32] showed nearly similar results.

The three different studies demonstrated that administration of a specially designed yogurt with highly specific antibodies from egg yolk could effectively decreases number of *H. pylori* in humans. During the study period of the three clinical studies, the ingestion regimen was well-tolerated and no adverse effects or any complications were observed.

The use of probiotics for the suppression of *H. pylori* in humans has been studied by some investigators [33, 34]. However, none of these studies were able to show a significant suppression of *H. pylori* in humans, and others showed a slight but no significant trend toward a suppressive effect of drinking yogurt containing specific lactic acid bacteria. Anti-*H. pylori* effect of yogurt containing specific lactic acid bacteria has been examined. However, no significant reduction of *H. pylori* in human stomach has been observed, although trend for decrease was observed [35, 36].

The use of IgY against a pathogenic factor of *H. pylori* would be a prudent way to suppress the infection. It was demonstrated that IgY-urease was highly specific and had a significant effectiveness against *H. pylori* because of its ability to inhibit *H. pylori* from adhering to the gastric mucosa [4, 37, 38]. Because IgY-urease binds urease only, the functional efficacy observed was presumably via capture of bacterium-associated urease within the gastric mucus layer, which resulting in bacterial aggregation and clearance via the constant washing action of the gut. By such a mechanism, consumption of IgY-urease yogurt may play a dual role in suppression and prophylaxis against *H. pylori* in humans (Figure 4).

These findings opened new gate of applications of IgY-urease against *H. pylori* in the food industry to prevent *H. pylori*. Recently, a specially designed egg containing IgY-urease was produced in Japan. This egg has been on the Japanese market under a trade name of "stomach friendly egg". Moreover, IgY-urease was incorporated in neutraceutical formulations that launched recently in the Japanese market aiming to prevent and reduce *H. pylori* infection.

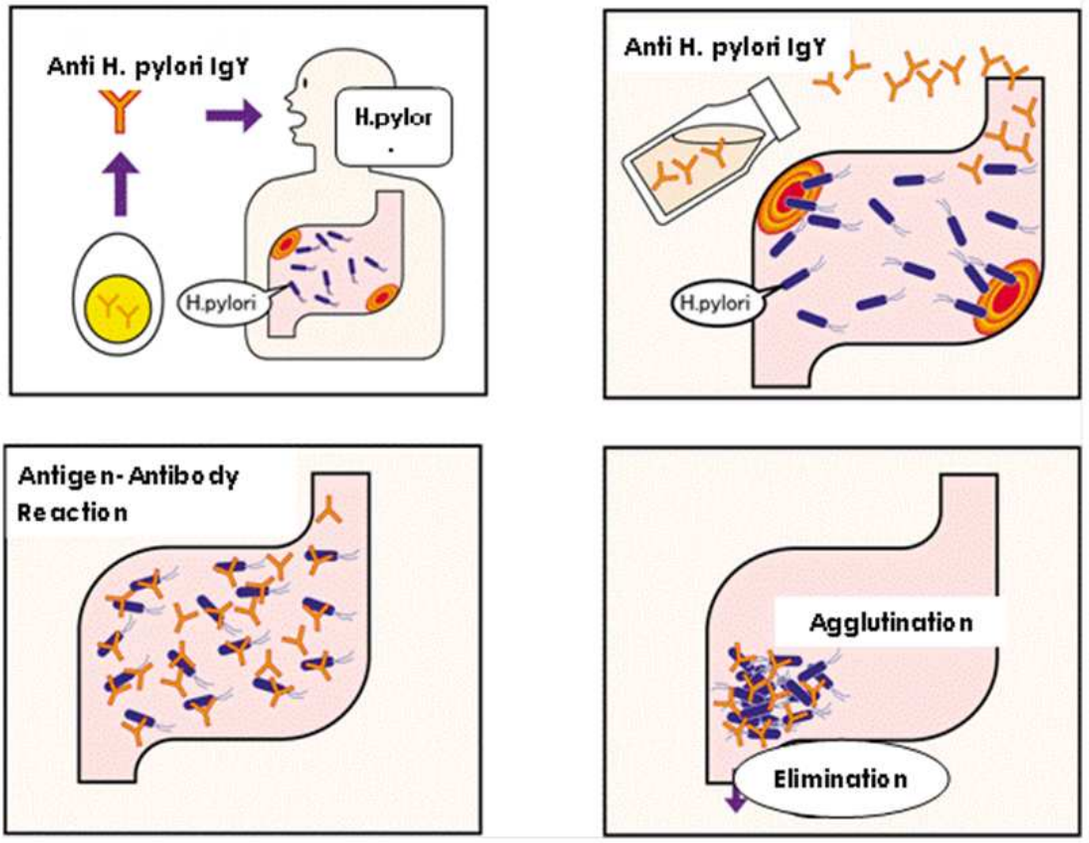


Figure 4. Suppressive mechanism of anti-*H. pylori* urease IgY.

2.5.2.2. Anti-Streptococcus mutans IgY

Dental caries is still one of the most widespread diseases of mankind. Human are frequently infected with cariogenic microorganisms in early life. The cariogenic microorganisms survive in dental biofilm and can emerge under favorable environmental condition and consequently cause dental disease [39]. *Streptococcus mutans* is the main etiologic agent of dental caries and that infection is transmissible [40]. Abilities of mutants' streptococci to adhere tooth surface in the presence of sucrose and release acids by fermention play a significant role in development of dental caries [41]. Initial attachment of *S. mutans* to the saliva-coated enamel surface occurs through the surface protein of *S. mutans*. For the colonization of *S. mutans*, synthesis of water-insoluble and adherent glucan from sucrose by the glucosyl transferases (GTases) is essential. *Streptococcus mutans* produces both cell-associated (CA) and cell-free (CF) forms of GTase; the former primarily synthesizes water-insoluble glucan, while the latter produces water soluble glucan. The combined action of these two GTases on the cell surface of *S. mutans* during its growth in the presence of sucrose is critically important in allowing firm adherence. The GTase system of *S. mutans* has therefore been considered an important virulence factor promoting caries development. Administration of IgY against *S. mutans* CA-GTase specifically inhibited insoluble glucan-synthesizing CA-GTase, resulting in a significant reduction in the development of dental caries. Otake et al. (1991) reported that anti-*S. mutans* CA-GTase IgY suppressed development of dental carries in rat model [42]. Hatta et al. (1997) reported that the

effectiveness of IgY with specificity to *S. mutans* prevented the colonization of mutans streptococci in the oral cavity of humans [43]. Recently, food products such as candies, chocolates and gums containing fourth- or anti-*S. mutans* IgY have been launched in the Japanese market for oral care [37].

2.5.2.3. Anti-Influenza virus IgY (Anti-influenza biofilter)

Influenza caused by a virus is called the influenza virus. Influenza or "flu" is an infection of the respiratory tract that can affect millions of people every year. It is highly contagious and occurs mainly in the late fall, winter, or early spring. Influenza is spread from person-to-person through mists or sprays of infectious respiratory secretions caused by coughing and sneezing. Influenza affects all age groups and causes severe illness, loss of school and work, and complications such as pneumonia, hospitalization, and death.

Recently, specific anti-influenza IgY was successfully produced from hens immunized with inactivated influenza virus strain. This specific IgY significantly reacts virus in vitro [44]. Subsequently, an anti-influenza IgY biofilter which trap the influenza virus has been developed by Daikin Environment Laboratories, Japan, a research arm of Daikin Industries, in cooperation with five Japanese research institutions. It was found that 99.99% of the influenza virus sprayed over the biofilter was captured within 10 minutes. An air cleaner with anti-influenza filter is recently launched in Japan (Figure 5 and 6). Moreover, a facemask with anti-influenza IgY was developed and will be available in the Japanese market.

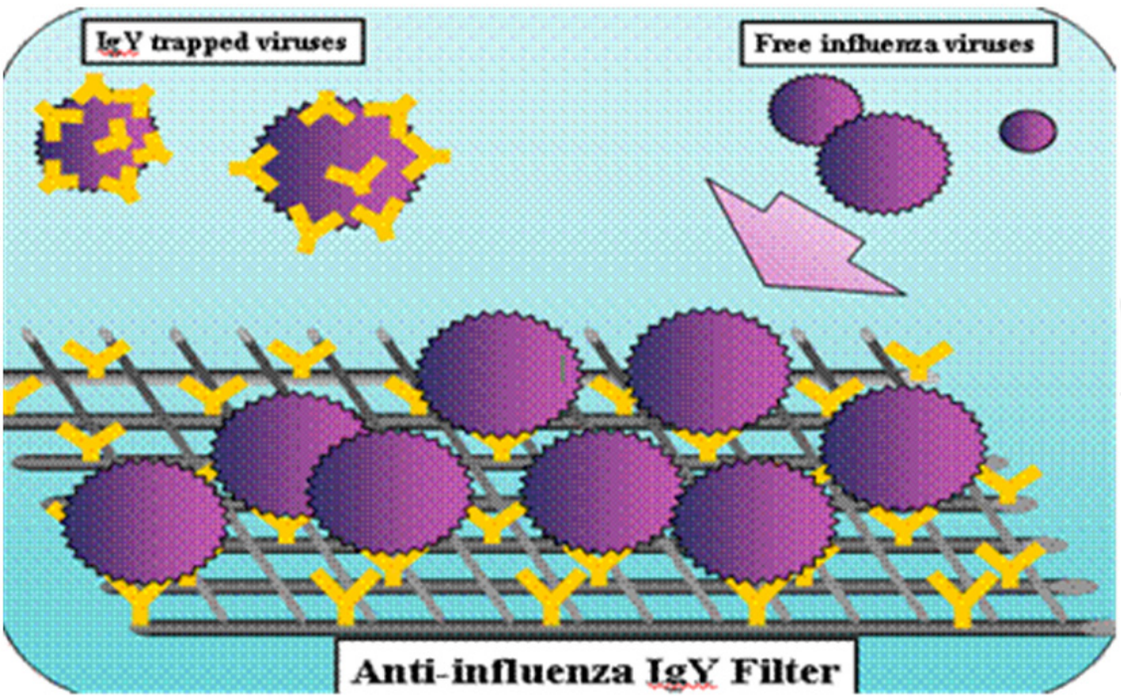


Figure 5. Diagram for the Anti-influenza IgY biofilter.

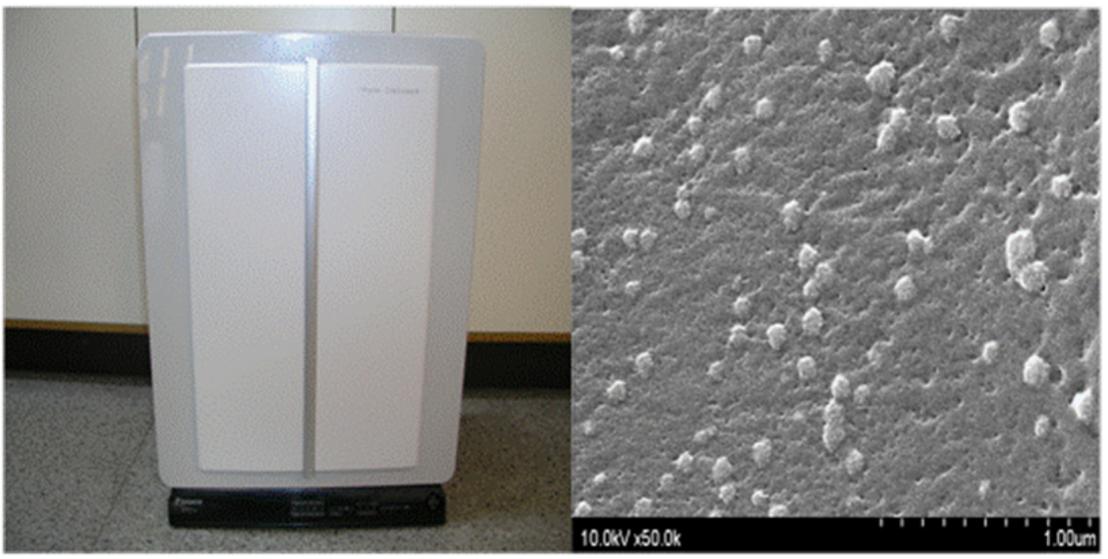


Figure 6. An air cleaner with anti-Influenza virus biofilter available in Japanese market.

2.5.2.4. Future prospects of IgY applications

Many research activities and proposals are going on in order to provide new applications for IgY technology. An anti-*Bacteriodes gengivalis* is under development for improving the oral health. For cosmetics sector, IgY against *Propionibacterium acnes* and its lipases has been developed to prevent and treat acne that is the most common skin disease [45]. For the medical sector, many researchers are working on using transgenic chicken to produce human antibodies in the transgenic hen's eggs in the form of IgY to help for treating human diseases.

3. Egg yolk bio peptides

Recently, it has become clear that proteins are a source for biologically active peptides. These peptides are inactive within the sequence of parent protein and can be released during gastrointestinal digestion or food processing. Egg yolk proteins could be an important source of bioactive peptides. The resultant peptides could show biologically new function with improved stability and/or solubility. In this section, some biologically functional egg yolk derived peptides are introduced and their underlying mechanisms are discussed.

3.1. Lipovitellenin peptide

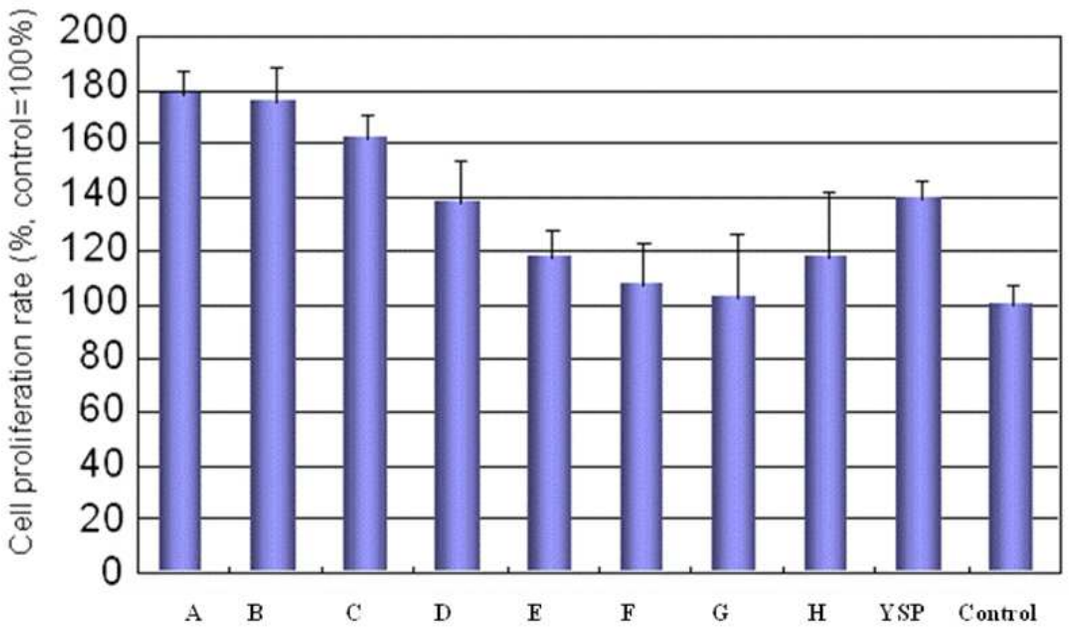
Vitellenin is the apoprotein of lipovitellenin. Digestion of vitellenin with pronase gives two glycopeptides. Glycopeptide A has high content of sialic acid and glycopeptide B contains most of the carbohydrates of vitellenin but devoid of sialic acid (N-Acetylneuraminic acid). Sialic acid is naturally occurring carbohydrate with numerous biological functions, including blood protein half-life regulation, variety of toxin neutralization, regulation of cellular adhesion and glycoprotein lytic protection.

3.2. Phosvitin peptide

Phosvitin phosphopeptides are new functional bioactive peptides derived from egg yolk with molecular masses of 1-3 kDa prepared from tryptic hydrolysate by partial dephosphorylation [46]. The phosvitin phosphopeptides were shown to be effective for enhancing the calcium binding capacity and inhibiting the formation of insoluble calcium phosphate. The results suggest a potential application of phosvitin peptides as novel functional peptides for the prevention of osteoporosis.

3.3. Egg yolk bone peptides (Bonepep®)

Hen egg turns into a full skeleton chick within 3 weeks; based on this fact, some investigations were carried out to explore the biologically active substances in hen egg that would initiate and enhance bone growth. It has been found that a specific yolk water-soluble protein (YSP) has a bone growth promotion activity *in vitro* [47] and *in vivo* [48]. These findings encouraged to search the functional yolk peptides that would promote bone growth. Different enzymes were used to hydrolyze YSP and the effect of the peptide preparations on osteoblast MC3T3-E1 cell proliferation was investigated. A novel peptides preparation (Bonepep®) with bone growth promotion activity was obtained. An *in vitro* study showed that Bonepep enhances the osteoblast MC3T3-E1 cell proliferation (Figure 7). Furthermore, an *in vivo* study showed that it promotes the elongation of rat tibia bone [49]. Compared with control and YSP fed rats; rats fed on Bonepep® showed marked and significant increase of chondrocytes proliferation and the bone formation in the tibia and consequently significantly increased elongation of their bone.



(A: Bonepep, B~H: peptides by other enzymes, YSP: Yolk Soluble Protein).

Figure 7. Promotion of osteoblast MC3T3-E1 cell proliferation by Bonepep®.

4. Egg white bio proteins

Well-known biological functions of egg white proteins are the prevention of microorganisms' penetration into the yolk and supply of nutrients to the embryo during the late stages of development. Most of the egg white proteins appear to possess antimicrobial properties or certain physiological functions to interfere with the growth and spread of invading microorganisms.

Most of egg white proteins are soluble and can easily be isolated. Egg white contains approximately 40 different proteins. Egg white proteins possess unique functional properties, such as antimicrobial, enzymatic and anti-enzymatic, cell growth stimulatory, metal binding, vitamin binding, and immunological activities [50].

4.1. Ovalbumin

Ovalbumin is a predominant protein contributing to the functional properties of egg white [51]. Ovalbumin is a monomeric phosphoglycoprotein with a molecular weight of 44.5 kDa and an isoelectric point of 4.5. Ovalbumin is a key reference protein in biochemistry. As a carrier, stabilizer, blocking agent or standard, highly purified ovalbumin has served the fundamentalists as well as the food industry. It has long been the subject of physical and chemical studies as a convenient protein model.

It is believed that ovalbumin, especially its unphosphorylated form, serves as a source of amino acids for the developing embryo. Despite the intensive investigations undertaken on ovalbumin, its function remains largely unknown. Ovalbumin is the only egg white protein which contains free sulfhydryl groups. The complete amino acid sequence of hen ovalbumin (which comprises 385 residues) and its crystal structure have been reported [52]. The unexpected finding that this protein belongs to the serpin superfamily has stimulated new interest in the structure and function of ovalbumin. The serpins are a family of more than 300 homologous proteins with diverse functions found in animals, plants, insects and viruses, but not in prokaryotes [53]. They include the major serine protease inhibitors of human plasma that control enzymes of the coagulation, fibrinolytic, complement and kinin cascades, as well as proteins without any known inhibitory properties such as hormone binding globulins, angiotensinogen and ovalbumin [54].

4.2. Ovotransferrin

Ovotransferrin (also known as conalbumin) has been identified as the iron-binding protein from avian egg white. Ovotransferrin, which constitutes 12% of the egg white protein, has a molecular weight of 77.7 kDa and a pI of about 6.1. It contains 686 amino acid residues and has 15 disulfide bridges [55]. It is glycosylated and contains a single glycan chain (composed of mannose and N-acetylglucosamine residues) in the C-terminal domain. Ovotransferrin is a neutral glycoprotein synthesized in the hen oviduct and deposited in the albumen fraction of eggs. Furthermore, it has two similar domains in N and C terminal regions, each one binding one atom of transition metal (Fe^{+++}, Cu^{++}, Al^{+++}) very tightly and specifically [55]. It is

implicated in the transport of iron in a soluble form to the target cells. The recognition of transferrin molecules by the target cells is mediated by membrane-bound transferrin receptors [56]. The significant structural similarities between lactoferrin and ovotransferrin justify the similarity of their biological roles. Ovotransferrin can be used as a nutritional ingredient in iron fortified products such as iron supplements, iron-fortified mixes for instant drinks, sport bars, protein supplements and iron-fortified beverages.

There is also extensive evidence of an antibacterial effect of ovotransferrin based on iron deprivation, iron being an essential growth factor for most microorganisms. The high affinity of transferrins for iron means that, in the presence of unsaturated transferrin (apotransferrin), iron will be sequestered and rendered unavailable for the growth of microorganisms. *In vivo*, ovotransferrin has been shown to have therapeutic properties against acute enteritis in infants [57].

4.3. Lysozyme

The name lysozyme was originally used to describe an enzyme which had lytic action against bacterial cells. Lysozyme is one of the oldest egg components to be utilized commercially after it was discovered by Alexander Fleming in 1922. It is a bacteriolytic enzyme commonly found in nature and is present in almost all secreted body fluids and tissues of humans and animals. It has also been isolated from some plants, bacteria and bacteriophages. Avian egg white is a rich and easily available source of lysozyme.

The lysozyme content of a laying hen's blood is 10-fold higher than in mammals because it is being transferred to the egg white. Lysozyme constitutes approximately 3.5% of hen egg white [50]. Egg white lysozyme consists of 129 amino acid residues with a molecular weight of 14.4 kDa. Because of its basic character, lysozyme binds to ovomucin, transferrin or ovalbumin in egg white [58]. In nature, lysozyme is found mainly as a monomer but it has been reported to also exist as a reversible dimer, which can be evoked by pH, concentration and/or temperature-dependent phase transition of the molecule.

It has long been believed that lysozyme's antimicrobial action could only be attributed to its catalytic effect on certain Gram-positive bacteria, by splitting the bond between N-acetylmuramic acid and N-acetyl-glucosamine of peptidoglycan in the bacterial cell wall [59]. Beside this well-known inactivation mechanism, a non enzymatic antibacterial mode of action of lysozyme was achieved by denatured form of lysozyme without enzymatic action.

Lysozyme demonstrates antimicrobial activity against a limited spectrum of bacteria and fungi [60]. However, the antimicrobial activity of lysozyme is greater for certain Gram-positive bacteria. On the other hand, Gram-negative bacteria are less susceptible to the bacteriolytic action of the enzyme [61]. The cell walls of different bacteria show varying degrees of susceptibility to digestion with hen egg white lysozyme. The walls of *Micrococcus lysodeikticus* were the most sensitive and the walls of *Staphylococci* were the less sensitive to the bacteriolytic action of lysozyme. Among Gram-negative bacteria, the walls of *Salmonella* and *Shigella* were the most sensitive whereas those of *E.coli*, *Vibrio* and *Proteus* were much

less sensitive [59]. The susceptibility differences are believed to be due to the complex envelope structure of Gram-negative bacteria such as *E.coli* or *Salmonella typhimurium*. The outer membrane serves to reduce the access of lysozyme to its site of action (peptidoglycan layer).

4.3.1. Molecular modification for functional improvement

Lipophilization. A number of chemical modifications of lysozyme have been undertaken to increase its efficacy as an antimicrobial agent. The effect of lipophilization with long chain fatty acids (palmitic or stearic acid) and shorter chain saturated fatty acids (caproic, capric or myristic acid) on the bactericidal action of lysozyme was investigated [62]. Lipophilization broadened the bactericidal action of lysozyme to Gram-negative bacteria with little loss of enzymatic activity [63].

Glycosylation. It is one of the most promising techniques, involves the attachment of carbohydrate chains to lysozyme. Glycosylation produces more stable proteins with improved conformational stability, protease resistance, modulated charge effects and water-binding capacity [64]. Conjugation of lysozyme with dextran by Maillard reaction increases antimicrobial activity. In addition, emulsifying activity of the conjugate was approximately 30 times that of native lysozyme [65]. Extending the function of lysozyme by conjugation with food compounds gives a novel and potentially useful bi-functional food additive. Similarly, hen egg lysozyme conjugated with xyloglucan hydrolysates; totally conserved enzymatic activity of lysozyme and increased the emulsifying properties 5 times higher than that of the native protein [66]. An antibacterial emulsifier was prepared by conjugating a fatty acylated saccharide with lysozyme through the Maillard reaction; the conjugate exhibited considerable resistance to proteolysis and much enhanced emulsifying activity and emulsion stability. The conjugate maintained approximately 70% of the bactericidal activity of native hen egg lysozyme without significant conformational changes of the protein [67].

Combination of lipophilization and glycosylation. An egg white lysozyme, which had been modified using the Maillard-type glycosylation method prior to lipophilization with palmitic acid, was prepared [68]. The yield of lipophilized lysozyme was increased significantly by pre-glycosylation of the protein and showed strong antimicrobial activity against *Escherichia coli*. Lipophilization of lysozyme combined with glycosylation is a promising method for potential industrial applications of lysozyme due to its enhanced antimicrobial activity towards Gram-negative bacteria and improved yield.

4.3.2. Applications

The bacteriostatic and bactericidal properties of lysozyme have been used to preserve various food items, as well as in pharmacy, medicine and veterinary medicine.

Natural food preservative. Lysozyme has been used as an antimicrobial agent in various foods. In 1992, the Joint FAO/WHO Expert Committee on Food Additives declared that lysozyme

was safe to be used in food [69]. The enzyme shows a number of properties important for food application. It is a heat stable protein, active in a broad range of temperatures (from 1°C to nearly 100°C), withstands boiling for 1-2 min, and stable in freeze-drying and thermal drying. Moreover, lysozyme is not inactivated by solvents and it maintains its activity when re-dissolved in water. It has optimum activity at pH 5.3 to 6.4 (*i.e.* typical for low-acidic food).

In cheese making, lysozyme has been used to prevent growth of *Clostridium tyrobutyricum,* which causes off-flavors and late blowing in some cheeses [70]. Another application of lysozyme may be the possible acceleration of cheese ripening, because lysis of starter bacteria would cause release of cytoplasmic enzymes which play a key role in proteolysis during cheese ripening [71]. Moreover, egg white lysozyme was used as an antimicrobial agent to control lactic acid bacteria in some fermented beverages [72].

Pharmaceuticals. In the pharmaceutical industry, avian egg white lysozyme can protect the body against bacterial, viral or inflammatory diseases [73]. It has been used in aerosols for the treatment of broncho pulmonary diseases, prophylactically for dental caries, for nasal tissue protection and is incorporated into various therapeutic creams for the protection and topical preparation of certain dystrophic and inflammatory lesions of the skin and soft tissues (*e.g.* burns and viral diseases).

Regardless of the direct bacteriolytic action, many other biological functions of lysozyme have recently been reported. These include anti-viral action by forming an insoluble complex with acidic viruses, enhanced antibiotic effects, anti-inflammatory and anti-histaminic actions, direct activation of immune cells and anti-tumor action [74-77].

4.4. Ovomucoid

Ovomucoid is a glycoprotein with heat stable trypsin inhibitor activity. Ovomucoid, which constitutes about 11% of the egg white protein, has a molecular weight of approximately 28 kDa and a pI of 4.1. It has nine disulfides and no free sulfydryl groups. The molecule consists of three tandem domains, each of which is homologous to pancreatic secretory trypsin inhibitor (Kazal-type). It has a putative reactive site for the inhibition of serine proteases. A large proportion of the carbohydrate present in this glycoprotein (about 25%) is joined to the polypeptide chain through asparginyl residues [78]. Ovomucoid can be heated at 100° C under acidic conditions for long periods without any apparent changes in its physical or chemical properties. Ovomucoid may play a more important role in the pathogenesis of allergic reactions to egg white than other egg white proteins [79].

4.5. Ovomucin

Ovomucin comprises 1.5-3.5% of the total egg white solids. It is a highly viscous glycoprotein with an extremely large molecular weight (8300-23000 kDa). The specific jellying property of egg white is attributed to ovomucin. It consists of two subunits; α-subunit and a β-subunit which are bound by disulfide bonds. The biological function of

ovomucin is shown to inhibition of haemagglutination by viruses. Its affinity with viruses such as bovine rotavirus, hen Newcastle disease virus and human influenza virus was already proved [80]. Moreover, the β-subunit from ovomucin was shown to have a cytotoxic effect on the cultured tumor cells [81].

4.6. Avidin

Avidin is a strongly basic glycoprotein synthesized in the hen oviduct and deposited in the albumen fraction of eggs. Avidin is a tetrameric protein, composed of subunits of identical amino acid composition and sequence (15.6 kDa and 128 amino acids each). Avidin is a trace component (0.05%) of egg white, but it has been well studied because of its ability to tightly and specifically bind biotin, one of Vitamin B group. Each subunit of avidin binds to a molecule of biotin. The high affinity of avidin for biotin has been widely used as a biochemical tool in molecular biology, affinity chromatography, molecular recognition and labeling, Enzyme Linked Immuno Sorbent Assay (ELISA), histochemistry and cytochemistry [82].

4.7. Ovoglobulin

In early studies, six globulin fractions were thought to be present in egg white. They are macroglobulin, ovoglobulins G1, G2 and G3 and two other globulins. However, the two globulins were later classified as ovoinhibitors and ovoglobulin G1 was identified as lysozyme. Currently, the name ovoglobulin is given only to ovoglobulins G2 and G3, which have molecular weights of 36 and 45 kDa, respectively. The biological function of these proteins has not been clearly elucidated, but they appear to be important in the foaming capacity of egg white [5].

4.8. Ovomacroglobulin

Ovomacroglobulin is the second largest egg glycoprotein after ovomucin and its molecular weight is 760-900 kDa. Ovomacroglobulin, like ovomucin, has the ability to inhibit hemagglutination [5].

4.9. Ovoflavoprotein

Ovoflavoprotein is acidic protein with a molecular weight of 32-36 kDa, and contains a carbohydrate moiety (14%) made up of mannose, galactose and glucosamines, 7-8 phosphate groups and 8 disulfide bonds. After being transported from the blood to the egg white, most of the riboflavin (Vitamin B2) is stored in the egg white bound to an apoprotein called flavoprotein. One mole of apoprotein binds one mole of riboflavin, but this binding ability is lost when the protein is exposed to a pH below its isoelectric pH 4.2 [5]. It has antimicrobial properties due to depriving the microorganisms from its riboflavin content [50].

4.10. Ovoinhibitor

This trypsin inhibitor was discovered by Matsushima in 1958. While it is a Kazal-type inhibitor (like ovomucoid), ovoinhibitor functions as a multi-headed inhibitor and inhibits bacterial serine proteinase, fungal serine proteinase and mammalian chymotrypsin [5].

4.11. Cystatin

It is the third proteinase inhibitor in egg white (also called ficin-papain inhibitor). In contrast to ovomucin, cystatin is a small molecule (12.7 kDa) and it has no carbohydrates and a high thermal stability. The potential of their broad application in medical treatments has been reported in the literature, which includes antimicrobial and antiviral activities [83], the prevention of cerebral hemorrhage [84] and control of cancer cell metastasis [85].

4.12. Ovoglycoprotein

Ovoglycoprotein is an acidic glycoprotein with a molecular weight of 24.4 kDa. This protein contains hexoses 13.6%, glucosamine 13.8%, and N-acetylneuraminic acid 3%. The biological functions of ovoglycoprotein are still unclear [5].

5. Egg white bio peptides

5.1. Ovoalbumin peptide (Ovokinin)

Ovokinin, a vasorelaxing octapeptide derived from pepsin digest of ovalbumin, has been shown to significantly lower the systolic blood pressure of spontaneously hypertensive rats [86]. Oral vailability of ovokinin is improved after emulsification. When we eat whole egg, ovalbumin peptides will be released by the action of pepsin in the stomach, and the peptide will be instantly emulsified with egg yolk and effectively absorbed from the intestines.

A vasorelaxing peptide – ovokinin (2-7) – was isolated from chymotryptic digest of ovalbumin [87]. However, the mechanisms for the relaxation were different from ovokinin. More anti-hypertensive peptide was obtained by modifying the amino acid residues of ovokinin (2-7). The minimum effective dose of [Pro2, Phe3]-ovokinin (2-7) was about one-thirtieth of that of ovokinin (2-7). [Pro2, Phe3]-ovokinin (2-7) proved to be a potent anti-hypertensive peptide with little effect on normal blood pressure when administered orally [88].

5.2. Ovotransferrin peptides

The ovotransferrin antimicrobial peptide (OTAP-92) is a 92 amino acid cationic fragment of hen ovotransferrin located (109-299) in the N-lobe of ovotransferrin. The peptide OTAP-92 showed strong bactericidal activity against both Gram-positive *S.aureus* and Gram-negative *E.coli* strains [89]. OTAP-92 has also been shown to possess a unique structural motif similar to the insect defensins. Furthermore, this cationic antimicrobial peptide is capable of killing

Gram-negative bacteria by crossing the outer membrane by a self-promoted uptake pathway and damaging the cytoplasmic membrane by channel formation. Knowledge of the structure-function relationship may allow combinations of antimicrobial agents with different mechanisms to be designed for pharmaceutical applications. OTAP-92 may represent a novel antimicrobial agent for the food and pharmaceutical industries [55].

5.3. Ovomucoid and ovomucin peptides

A pepsin-digest of egg white ovomucoid was prepared to enhance digestibility and lower allergenicity [90]. A highly glycosylated peptide fragments (220 and 120 kDa) was separated from pronase digest of avian egg white ovomucin; It was derived from the β-subunit. Both fragments inhibited the growth of tumors [91].

5.4. Egg white peptides (Runpep®)

Egg white hydrolysate (Runpep®) has been produced by standardized technology to provide a highly nutritive source of peptides. Runpep® contains all essential amino acids with amino acid score of 100 [5, 50]. It is rich in branched chain amino acids (BCAA); these are the essential amino acids leucine, isoleucine, and valine. BCAA's are of special importance for athletes because they are metabolized in the muscle, rather than in the liver. Moreover, it is a rich source of sulpher containing amino acids as methionine and cysteine. It is in the form of small peptides (less than 3000 Da) and contains more than 91% protein providing easily absorbable amino acids source (Figure 8). An *in vitro* study showed that Runpep® has anticoagulant activity (reduce the formation of coagulum in blood vessels, reduce the risk of embolism) and helps in lowering the blood platelets aggregations [92, 93].

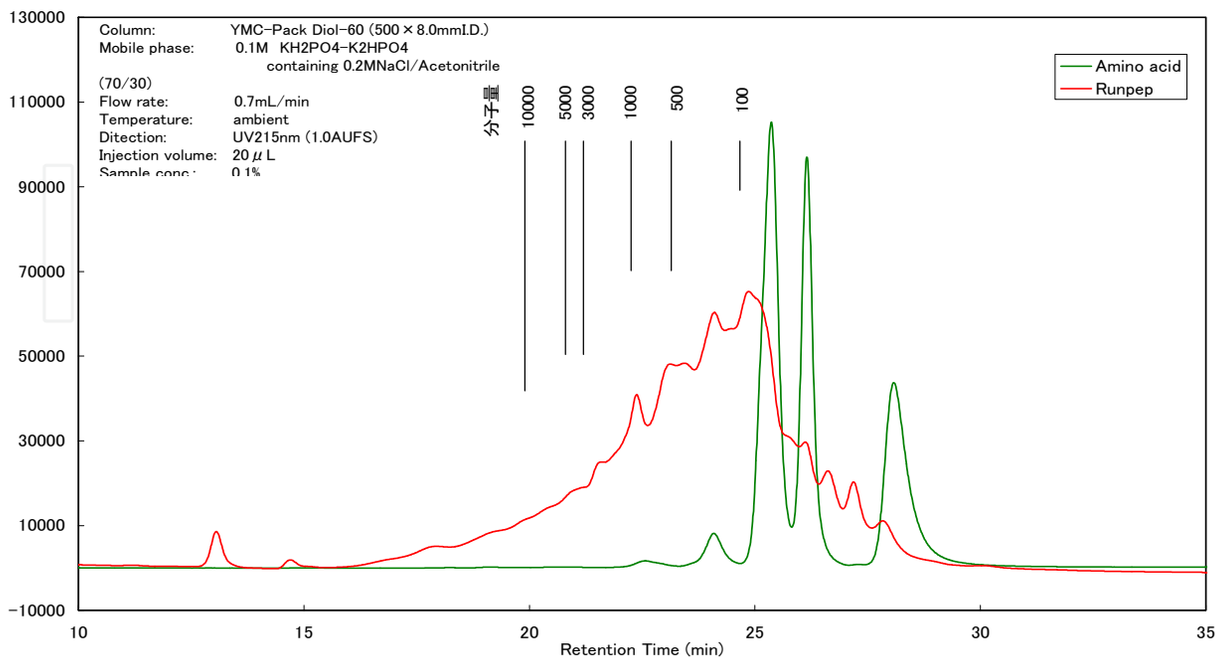


Figure 8. Runpep® profile by GPC.

In vitro, it showed dose-dependent vasorelaxant activity of both aortic and mesenteric vascular smooth muscle. *In vivo*, Runpep® exhibited dose-dependent hypotensive effect on both systolic and diastolic blood pressures with more pronounced effect on the systolic one by ingestion. Runpep® has been proved to have anti-hypertensive effect and has been shown to significantly lower the systolic blood pressure (Figure 9) of spontaneously hypertensive rats [94].

The hydrolysate of egg white with pepsin was found to exhibit a strong angiotensin I–converting enzyme (ACE) inhibitory activity in vitro [95]. Other work reports the antioxidant activity of peptides produced by pepsin hydrolysis of egg white; four peptides included in the protein sequence of ovalbumin possessed radical scavenging activity higher than that of Trolox. The combined antioxidant and ACE inhibition properties make it a very useful multifunctional preparation for the control of cardiovascular diseases, particularly hypertension. No correlation was found between antioxidant and ACE inhibitory activities of pepsin digest of egg white [96].

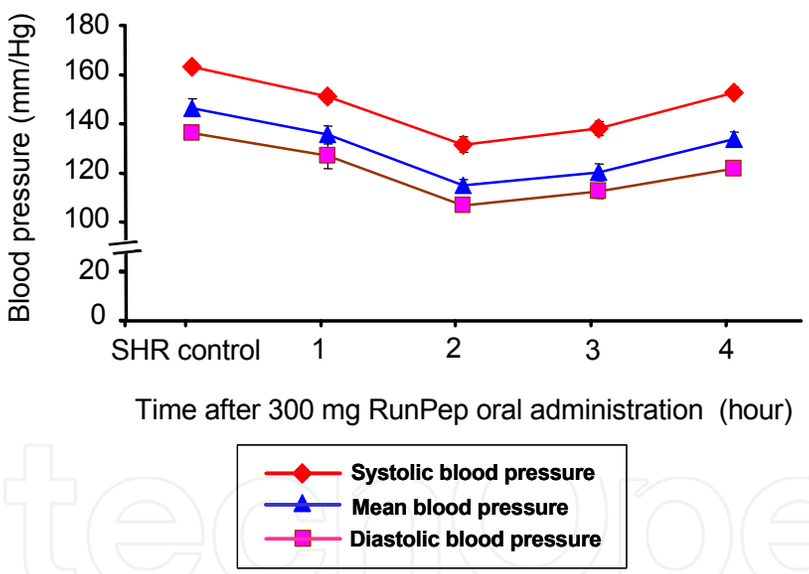


Figure 9. Antihypertensive activity of Runpep® after oral administration to SHR rats using cuff method (Sphygmomanometer).

5.5. Lysozyme peptides

Lysozyme is known to have a variety of folding topologies around the active site cleft [97]. Enzymatic hydrolysis of lysozyme is a novel technology that uses proteolytic enzymes for

hydrolyzing native lysozyme to produce potent antimicrobial peptides that hidden within its folds. Lysozyme was digested by different proteolytic enzymes, such as clostripain [58, 60], pepsin, and trypsin [97, 98, 99]. All these researchers proved that the resulting peptides lost the enzymatic activities of lysozyme, but exhibited strong bactericidal activities against both Gram-negative (*E.coli*, *Salmonella*, *Pseudomonas*, and *Aeromonas*) and Gram-positive bacteria (*Listeria monocytogenes*, *Staph aureus, Bacillus* spp., and *Leuconostic*s spp.) as well as yeasts (*Saccharomyces*). Scanning electron microscopy clearly demonstrated that cell membrane of both Gram-negative and –positive bacteria was damaged by these peptides. Thus these peptides probably have a different mechanism of action than native lysozyme [58, 99]. Mine et al (2004) could isolation, purification, and characterization of novel antimicrobial peptides from chicken egg white lysozyme hydrolysate, obtained by peptic digestion and subsequent tryptic digestion. The hydrolysate was composed of over 20 small peptides of less than 1000 Da, and had no enzymatic activity. The water-soluble peptide mixture showed bacteriostatic activity against Gram-positive bacteria (Staphylococcus aureus 23-394) and Gram-negative bacteria (*E. coli* K-12). Two bacteriostatic peptides were purified and sequenced. One peptide, with the sequence Ile-Val-Ser-Asp-Gly-Asp-Gly-Met-Asn-Ala-Trp, inhibited Gram-negative bacteria *E. coli* K-12 and corresponded to amino acid residues 98-108, which are located in the middle part of the helix-loop-helix. Another novel antimicrobial peptide inhibited *S. aureus* and was identified as His-Gly-Leu-Asp-Asn-Tyr-Arg, corresponding to amino acid residues 15-21 of lysozyme. These peptides have broadened the antimicrobial activity of lysozyme to Gram-negative bacteria. The results obtained in this study indicate that lysozyme possesses nonenzymatic bacteriostatic domains in its primary sequence and they are released by proteolytic hydrolysis [99].

Consumers are increasingly demanding food that is free from pathogens, but with less preservatives and additives. As a response to these conflicting demands, current trends in the food industry include the investigation of alternative natural preservative in foods. Six Gram-negative bacteria (*Escherichia coli*, *Salmonella enteritidis* NBRC 3313, *Salmonella typhimurium*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila*) were checked for sensitivity to native hen egg white lysozyme and hydrolysate preparation of lysozyme derived peptides. Generally, lysozyme peptides preparation acts on the tested organisms and on different strains of *Bacillus* spp. with much more potency comparing to native lysozyme [100, 101]. The resulting peptides lost the enzymatic activities of lysozyme, but exhibited strong bactericidal activities against both Gram-negative and Gram-positive bacteria [97, 102]. Being natural antimicrobial, lysozyme peptides preparation will find its way as a safe shelf-life extender in the food industry.

6. Egg shell membrane bio proteins

The egg shell membrane has been thought to be beneficial in the treatment of some injuries. For example, in Japan, when Sumo wrestlers get flesh abrasions, they will often peel the egg membrane from the egg shell and cover their injuries. They believe that it facilitates their recovery. Peptides product which are stable in water have been prepared from hydrolyzed

egg membrane. The effects of this egg shell membrane protein on cell growth have been studied. The growth of normal human skin fibroblasts on egg membrane protein-coated tissue cultures increased in relation to increasing egg membrane protein concentration.

The egg shell membranes contain several bacteriolytic enzymes (*e.g.* lysozyme and N-acetylglucosaminidase) and other membrane components which may alter the thermal resistance of Gram-positive and Gram-negative bacterial pathogens (*Salmonella Enteritidis, Escherichia coli* 0157:H7, *Listeria monocytogenes* and *Staphylococcus aureus*).

The presence of hydroxyproline in hydrolysates of the egg shell membrane layers has suggested that the membrane layers contain collagen [103]. This has been confirmed using biochemical and immunological tests. It has been established that about 10% of the total proteinaceous content of the membrane structure of an egg shell is collagen. The outer shell membrane contains predominately Type I collagen and the inner shell membrane contains Types I and V collagen. In addition, Type X collagen has been found in both the inner and outer shell membranes using immunohistochemical analysis. It is important to recognize the presence of collagen in egg shell membranes because of its potential value.

7. Egg shell bio proteins

Different Non-collagenous proteins have been identified in the organic matrix of the hen's egg shell. Ovocleidin-17 is a soluble matrix protein component and distributed in palisade and mammillary layers [104].

Ovocalyxin-32 has been identified as a novel 32-kDa protein. It is expressed at high levels in the uterine and isthmus regions of the oviduct, and concentrated in the eggshell. In the eggshell, ovocalyxin-32 localizes to the outer palisade layer, the vertical crystal layer, and the cuticle of the eggshell, in agreement with its demonstration by Western blotting at high levels in the uterine fluid during the termination phase of eggshell formation. Ovocalyxin-32 is therefore identified as a novel protein synthesized in the distal oviduct where hen eggshell formation occurs [105].

Osteopontin, a phosphorylated bone glycoprotein involved in formation and remodeling of the mineralised tissue, has also been demonstrated in the hen egg shell. Gene expression for this protein has been shown to be higher during the period of calcification [106].

Ovalbumin, lysozyme and ovotransferrin, as egg white proteins, have been identified in hen's egg shell. The organic matrix also contains several proteoglycan molecules [107].

Chicken eggshell powder has been proposed as an attractive source of calcium for human health to increase bone mineral density in an elderly population with osteoporosis. However, factors affecting calcium transport of eggshell calcium have not yet been evaluated. Chicken eggshell contains about 1.0% (w/w) matrix proteins in addition to a major form of calcium carbonate (95%, w/w). It was found that soluble eggshell matrix proteins remarkably enhance calcium transport using in vitro Caco-2 cell monolayers grown on a permeable support. The total calcium transport across Caco-2 monolayers showed an

increase of 64% in the presence of 100 microg/well soluble eggshell matrix proteins. The active enhancer with a molecular mass of 21 kDa was isolated by reversed phase high-performance liquid chromatography and did not match to any previously identified protein. The N-terminal sequence was determined to be Met-Ala-Val-Pro-Gln-Thr-Met-Val-Gln. The possible mechanisms of eggshell matrix protein-mediated increase in calcium transport and the potential significance of eggshell calcium as a nutraceutical are discussed [108].

Experimental and clinical studies performed to date have shown a number of positive properties of eggshell powder, such as antirachitic effects in rats and humans. A positive effect was observed on bone density in animal models of postmenopausal osteoporosis in ovariectomized female rats. In vitro eggshell powder stimulates chondrocyte differentiation and cartilage growth. Clinical studies in postmenopausal women and women with senile osteoporosis showed that eggshell powder reduces pain and osteoresorption and increases mobility and bone density or arrests its loss. The bioavailability of calcium from this source, as tested in piglets, was similar or better than that of food grade purified calcium carbonate. Clinical and experimental studies showed that eggshell powder has positive effects on bone and cartilage and that it is suitable in the prevention and treatment of osteoporosis [109].

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