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Review

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Egg yolk proteins and peptides with biological activity

Biologiczna aktywność białek i peptydów pochodzących z żółtka jaja

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Summary

Many proteins of food reveal biological activity. In the sequence of these proteins also numerous biologically active peptides are encrypted. These peptides are released during proteolysis naturally occurring in the gastrointestinal tract, food fermentation or during designed enzymatic hydrolysis *in vitro*. Biopeptides may exert multiple activities, affecting the cardiovascular, endocrine, nervous and immune systems.
An especially rich source of bioactive proteins and biopeptides is egg. Bioactive peptides released from egg white proteins have been well described, whereas egg yolk proteins as precursors of biopeptides are less well characterized. This manuscript describes biologically active proteins and peptides originating from egg yolk and presents their potential therapeutic role.

Key words: **egg yolk proteins • phosvitin • biopeptides • antioxidant • ACE-inhibitory activity**

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Composition of egg yolk

Egg yolk contains numerous vital nutrients and preservative substances, due to its original role as an embryonic chamber. The major constituents of yolk are proteins (16.0%) and lipids (32.0%), present mainly in the form of lipoproteins. It also contains carbohydrates (1.0%), mostly oligosaccharides, which are bound to proteins and minerals (1.0%) [34].

Yolk is a complex system composed of particles suspended in yellow fluid named plasma, which contains proteins. Granules are the main type of particles [23]. The granules contain α- and β-lipovitellins, high-density lipoproteins (HDLs), phosvitin, and low-density lipoproteins (LDLs) (15%). In plasma low-density lipoproteins (85%) dominate. It also contains livetins, which are lipid-free globular proteins. Among them γ-livetin, also referred to as immunoglobulin Y, occurs [23,25].

The precursor of the main egg yolk proteins is vitellogenin. Its production takes place in hen's liver and increases with sexual maturation due to estrogen secretion. During egg formulation, vitellogenin is enzymatically cleaved into fragments termed lipovitellin I (120 kDa), phosvitin (44 kDa), and lipovitellin II (32 kDa), located in yolk granules, and a 40 kDa glycoprotein, YGP40, located in yolk plasma and transferred to the oocyte [9,36].

Phosvitin and other proteins from granule fractions as precursors of biologically active peptides

Chicken eggs have been recognized as an excellent source of proteins and peptides with well-documented biological activity [1,35,38]. One of the most important proteins of egg yolk is phosvitin. It represents 11% of yolk proteins. Egg yolk phosvitin is a highly phosphorylated protein, which contains 10% phosphorus. Phosvitin is a mixture of two polypeptides: α-phosvitin (160 kDa) and β-phosvitin (190 kDa) [13]. It has a unique amino acid composition in which more than 55% of the amino acids are serine residues and most of them are monoesterified with phosphoric acid [19,35]. Only the C-terminal region, composed of approximately 15 amino acids, has hydrophobic characteristics [2]. The primary structure makes it one of the strongest metal-binding agents. It shows strong binding ability for positively charged metal ions: Fe (II), Fe(III), Co(II), Mn(II), Ca(II) and Mg(II). Owing to this chelating property, phosvitin shows strong antioxidant activity. It is also resistant to proteolytic enzymes [19,25]. The negative charge of the phosphate groups surrounds the phosvitin molecule and prevent access of enzymes to peptide bonds. However, phosvitin is an attractive substrate to produce functional phosphopeptides for various nutraceutical applications. Proteolytic cleavage of phosvitin using trypsin causes release of phosphopeptides. They have been found to inhibit the formation of insoluble calcium phosphates

Table 1. Protein composition of hen egg yolk (authoring based on Trziszka [34])

Protein	MW [kDa]	pI	Characterization
Livetins	80 45 150	4.3- 5.7 5.7-7.6	α-albumin, allergen, β – α-2 –glycoprotein, highly stable to heat, γ- γ-globulin, protein referred to as IgY, exert immunostimulatory activity
Phosvitin	160 (α) 190 (β)	4.0	mixture of α- and β-polypeptides, highly phosphorylated protein consists of about 50% serine, exerts metal chelating, antioxidant and antimicrobial activities.
Lipoproteins	10.3x103	6.3– 7.5	
LDL	3x103 130 15		LDL1, LDL2, VLDL, HDL possess emulsifying properties,
Main apoproteins of LDL fraction	55 -80 9.4-180		main constituents of yolk (66%),
Apovitellins of HDL fraction			constituents of VLDL fraction,
Lipovitellenins	9-13		
Polypeptides of HDL fraction (apolipoproteins)	420 (dimers)		highly stable to heat,
	28;44;74;109		sensitive to heat

or iron phosphates, which helps the absorption of calcium and iron in the gut [4]. These peptides are characterized by a high content of amino acids such as histidine, methionine and tyrosine [35].

It was shown that phosphopeptides having molecular masses of 1-3 kDa exert strong abilities to inhibit the oxidation of linoleic acid, scavenge DPPH free radicals and chelate iron ions (II) [35]. Moreover, phosvitin and its enzymatic digests protect DNA against oxidative damage induced by Fe(II) and peroxide [12]. The effective protection of these phosphopeptides against oxidative stress was also confirmed by Katayama et al., [17] in an assay *in vitro* using human intestinal epithelial cells.

Therefore antioxidative peptides can be useful for the prevention of iron-mediated oxidative stress related diseases, such as colorectal cancer [12,21]. The consumption of egg yolk protein hydrolysates has been demonstrated to inhibit tumor cell proliferation in the colon [11]. Studies have shown that this effect results primarily from better antioxidant protective systems in the mucosa of the colon. This can be explained by the fact that phosphooligopeptides released from phosvitin have the ability to modulate the secretion of antioxidant enzymes such as catalase and glutathione reductase [11]. Moreover, it was demonstrated that these phosphooligopeptides have the ability to increase activity of intracellular GSH and to regulate the expression of γ-glutamylcysteine in intestinal epithelial cells which catalyze the synthesis of GSH [16].

In addition, this multivalent metal binding capacity of egg yolk phosvitin indicates other important attributes, including antimicrobial activity. Phosvitin and its peptides exhibit antibacterial activity against *Escherichia coli*, under thermal stress, causing disruption of cells and DNA leakage, which may be a result of the synergistic effect of its high metal chelating ability and high surface activity [5].

Peptides derived from phosvitin are also promising agents in prevention of osteoporosis. Phosphopeptides released during tryptic hydrolysis of phosvitin and partially dephosphorylated phosvitin increase the binding of calcium in the bones and prevent the formation of insoluble calcium phosphate [8].

Apart from egg yolk phosvitin and phosvitin-derived peptides of protein, hydrolysates of lecithin-free egg yolk are recognized as antioxidant agents [31,32,39]. It has been reported that egg yolk hydrolysates composed of peptides with molecular weight lower than 1000 Da, obtained with the use of proteinase from *Bacillus* ssp., exhibit antioxidant capacities investigated by using several methods. Superoxide-scavenging activity and suppression of discoloration by β-carotene have been observed [31]. Egg yolk protein hydrolysates also showed strong DPPH radical and hydroxyl radical-scavenging activities. Furthermore, in food modeling systems, these peptides effectively inhibited lipid oxidation processes in beef and tuna muscle homogenates [31,32]. Two peptides obtained from lecithin-free egg yolk hydrolyzed with Alcalase also showed antioxidant activity in a linoleic acid model system. These peptides, composed of 10 and 15 amino acid residues, both contained a leucine residue at their N-thermal positions [26].

Antioxidative properties of lecithin-free egg yolk protein hydrolysates have also been described by other authors [27,39]. Trypsin hydrolysate obtained after a 4-hour reaction (DH 13.0%) demonstrated strong DPPH free radical scavenging activity (0.85 μmol $Trolox_{eq}$/mg) as well as hydrolysates obtained after treatment of the protein with a neurtase protease [27,39]. The scavenging capacity, ferric reducing power, and chelating capacity in the last hydrolysate were observed at the following levels: 0.44 μmol $Trolox_{eq}$/mg, 177.35 μg Fe^{2+}/mg and 549.87 μg Fe^{2+}/mg, respectively [27].

Chay Pak Ting et al. [3] have developed laboratory-scale ultrafiltration processes to separate antioxidative peptides from tryptic hydrolysates of delipidated egg yolk protein. They obtained a peptide fraction enriched in peptides of molecular weight lower than 5 kDa, which was characterized by antioxidant activity, as determined by the oxygen radical absorbance capacity (ORAC) assay.

Numerous proteins as precursors of antihypertensive peptides were found in egg albumin [24,25,28]. On the other hand, there is limited information about antihypertensive peptides derived from yolk proteins.

Heart disease and strokes will surpass infectious diseases as the leading cause of death and disability worldwide in the near future [10]. Hypertension is one of the major risk factors for cardiovascular disease and stroke [24]. Consequently, effective antihypertensive agents including peptides are receiving special attention. The most common strategy for the identification of antihypertensive food-derived peptides is based on the measurement *in vitro* of ACE inhibitory activity. Then the *in vivo* effects are tested in spontaneously hypertensive rats (SHR), which constitute an accepted model for human essential hypertension [22].

Angiotensin-converting enzyme or ACE (EC 3.4.15.1) is an exopeptidase that removes the dipeptide from the C-terminus of various peptide substrates. It is a zinc metallopeptidase with broad substrate specificity *in vitro* [6]. Renin (angiotensinase, EC 3.4.23.15) affects angiotensinogen (plasma protein), an inert precursor, thereby releasing a decapeptide – angiotensin I. Angiotensin I is a substrate for ACE, which produces angiotensin II by removing the C-terminal dipeptide (HL) from angiotensin I. Then angiotensin II, by causing strong muscle contraction in the small blood vessels, significantly raises blood pressure and increases the heart rate. ACE also removes the C-terminal dipeptide of bradykinin (a vasodilator), resulting in the creation of an inactive peptide fragment [6,24].

These peptides displaying inhibitory activity against the dipeptidyl-carboxypeptidase ACE play an important role

in controlling the development of hypertension by regulating the renin-angiotensin system. Whole egg yolk in native form as a potential source of ACE-inhibitory peptides was tested by You and Wu [38]. The level of this activity for hydrolysates prepared using gastrointestinal (pepsin, pancreatin) and microbial (thermolysin, Alcalase) proteases ranged from 133.4 μg/ml to 210.2 μg/ml [38]. The hydrolysate of egg yolk treated with a crude enzyme from *Rhizopus* also exhibited ACE inhibitory activity *in vitro* [37]. The peptide fraction of this hydrolysate with molecular mass lower than 1 kDa inhibited the development of hypertension in SHR rats after oral administration [37]. Effects of proteolytic modification of an egg yolk protein preparation, a byproduct of egg yolk phospholipid extraction, on its biological activity were described by Pokora et al. [27]. Treatment by neutral protease of this byproduct caused release of peptides of molecular weight (MW) range from 4.94 to 41.23 kDa, which show an ACE inhibitory effect *in vitro* (IC_{50}= 59.2 μg).

Lipovitellenin fraction (low-density lipoproteins), lipovitellin fraction (high-density lipoproteins) and their peptides

The lipovitellenin fraction is synthesized in the liver of the laying hen. These lipoproteins are composed of 11-17% protein and 83-89% lipid, the latter comprising 74% neutral lipid and 26% phospholipid. There are six major apoproteins with MW in the range 130-15 kDa [13]. The native lipovitellin fraction forms a complex with phosvitin. The second lipoprotein fraction comprises HDLs present in the form of a dimer of 2 monomers of about 200 kDa each. Each monomer is composed of about 5 main apoproteins, with MW ranging from 35 to 110 kDa. Apoproteins of HDL are glycosylated: mannose, galactose, glucosamine, and sialic acid [13].

Egg yolk lipoproteins are important for lipid-mediated antimicrobial activity. It was documented that these lipoproteins inhibited the growth of *Streptococcus mutans in vitro* [25]. Furthermore, high-density lipoproteins (HDL) and their peptides are associated with antiadhesive activities of egg yolk [14,15]. A recent study revealed that egg yolk supplementation inhibited colonization of some bacteria such as *Salmonella typhimurium*, *Campylobacter jejuni* and *E. coli O157:H7* in internal organs. It was suggested that these effects were due to the presence of antiadhesive factors of egg yolk mentioned above. Egg yolk lipoproteins are protein precursors of peptides with other biological activities. Digestion of vitellenin, which is one of the apoproteins of egg yolk lipovitellenin, with pronase gives two glycopeptides named A and B. Glycopeptide A is characterized by high content of sialic acid (N-acetylneuraminic acid). Sialic acid is a naturally occurring carbohydrate with numerous biological functions, including blood protein half-life regulation, toxin neutralization, regulation of cellular adhesion and inhibition of cell cytolysis [1]. The report suggests that lipovitellenin-derived peptides can serve as carriers of sialic acid, improving its absorption/bioavailability.

Plasma proteins and peptides

Besides low-density lipoproteins, plasma also contains a livetin fraction, which is a heterogeneous fraction composed of lipid-free globular proteins (α-, β- and γ-livetin). Recent studies provide information about livetin fraction proteins as precursors of bioactive peptides. This fraction hydrolyzed with different enzymes has bone growth promotion activity *in vitro* and *in vivo* [18]. These hydrolysates increased preosteoblastic MC3T3-E1 cell proliferation and alkaline phosphatase activity in a dose-dependent manner. Furthermore, livetin hydrolysates potently suppressed osteoclastogenesis from bone marrow-derived precursor cells. The osteoprotective effect of them was confirmed in an assay conducted *in vivo* on ovariectomized (OVX) rats. The study showed that it promotes the elongation of rat tibia bone [1,18].

Among proteins of the livetin fraction, the most important is γ-livetin, also referred to as immunoglobulin Y [23,25]. The structure of the γ-livetin molecule consists of two light and two heavy chains. The heavy chains are indicated by the Greek letter Y. Each light chain consists of one variable and one constant domain (MW 18,660 Da); each heavy chain has a MW of 65,105 Da. pH of IgY is in the range 5.7-7.6 [33]. This protein exerts the same immunomodulatory activity as immunoglobulin G. IgY is produced in plasmatic cells named B lymphocytes during 5 or 6 days after the presence of the antigen in the organism. Serum IgY is selectively transferred to the yolk via a receptor on the surface of the oocyte membrane that is specific for IgY translocation [33]. IgY can be produced on a large scale from eggs laid by hens immunized with selected antigens. Immunoglobulin Y against a number of bacteria and viruses has been demonstrated, and it has been shown to bind gastrointestinal pathogens and inhibit infection [20]. Furthermore, this protein was shown to reduce the incidence of diarrhea caused by rotavirus or *E. coli*. IgY also conferred protection against dental caries formed by *S. mutans* [25].

Hen egg yolk immunoglobulin Y occurs as a complex with peptides exhibiting immunostimulating properties, which were first characterized by Polanowski et al. [29,30]. It has been found that these peptides named yolkin possess significant immunological activity and are strong inducers of cytokine IL-1 β and IL-6 release. The mechanism of immunomodulatory action of biopeptides may rely on direct interaction with pathogens as well as suppression or stimulation of certain immune responses [20]. Cytokines play a significant role in regulating such immune responses [25]. Yolkin is an acidic mixture of peptides rich in Asp/Asn and Glu/Gln but poor in methionine and proline. Yolkin is composed of several peptides of MW ranging from over 1 to about 35 kDa. N-terminal amino acid sequences of eight of the electrophoretically purified yolkin constituents were homological to the C--terminal domain of vitellogenin (Vtg) II. The fractions of MW of about 4 and 12 kDa were free of carbohydrates and their N-terminal amino acid sequences start at posi-

tion 1732 in the Vtg II amino acid sequence, whereas the remaining fractions (MW about 16, 19, 23, 29, 32 and 35 kDa) appeared to be glycoproteins corresponding to the amino acid sequence of Vtg II fragments starting at position 1572 [29]. Probably, yolkin is released from the protein precursor vitellogenin, by limited proteolysis inside the oocyte by cathepsin D produced in the hen's liver [36].

Due to the biological activity of IgY, it seems to be an attractive substrate to produce biopeptides for various nutraceutical and pharmaceutical applications. However, not much information is available about this protein as a precursor of biologically active peptides. Pure IgY obtained as a second product during yolkin isolation from egg yolk was hydrolyzed with various microbial proteases by Eckert et al. [7]. Enzymatic hydrolysates in the DH range from 3.0% to 26.6% possessed significant antioxidant activity expressed as the ability to reduce the oxidation state of metal ions, scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals and chelate iron ions. IgY hydrolysate obtained using *Bacillus thermoproteolyticus Rokko* protease was able to reduce the oxidation state of iron ions at the level 409.7 μg Fe^{2+}/mg. Also, the 24-hour hydrolysate of IgY obtained after degradation with the proteinase from *Aspergillus melleus* possessed free radical scavenging activity equal to 1.46 μM Trolox/mg [7].

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