



# Functional properties of ovotransferrin from chicken egg white and its derived peptides: a review

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**Abstract** With emerging trends in the food and pharmaceutical industries, potential applications of egg-derived bioactive compounds were recognized. Ovotransferrin is a major egg white functional protein responsible for multiple bioactivities. The objectives of this review are to provide scientific evidence of the functional properties of chicken ovotransferrin and its derived peptides and to identify future research approaches and applications. Various easy, economical, and non-toxic methods have been reported to produce ovotransferrin with high yield and purity, and chemical and enzymatic approaches have been employed to release bioactive peptides. The native ovotransferrin is known to have antimicrobial, antioxidant, anticancer, and immunomodulatory activities. The peptides produced from ovotransferrin also are reported to have antioxidant, antimicrobial, antihypertensive, and anticancer properties. However, little or no application of these compounds in the food and pharmaceutical areas is available yet. Therefore, the practical application of OTF in nutraceutical and pharmaceutical areas are among the emerging areas of research.

**Keywords** Ovotransferrin · Egg white protein · Bioactive peptide · Nutraceutical · Pharmaceutical

## Introduction

Eggs play a significant role in the human diet as an affordable nutrient-rich food that sustains human life, health, and growth. With the increase of human health concerns, natural bioactive proteins and peptides received enormous attention from both the consumers as well as the food industries (Wu, 2014) because scientific evidence proved the existence of bioactive peptides derived from food might have beneficial effects on improving human health and preventing diseases (Möller et al., 2008). Egg contains highly digestible proteins (12%), lipids (12%), carbohydrates (1%), and traces of vitamins and minerals (1%) (Kovacs-Nolan et al., 2005). In addition to their use in food processing, egg white proteins such as ovalbumin (54%), ovotransferrin (12%), ovomucoid (12%), lysozyme (3.4%), and ovomucin (3.5%) and their derived peptides are highly recognized for their biological activities (Abeyrathne et al., 2013a).

Ovotransferrin from chicken egg white and its derived peptides display a wide range of functional properties. Many methods, including chemical precipitation and chromatography, have been employed to separate ovotransferrin from chicken egg white (Abeyrathne et al., 2014a; 2014b; Ko and Ahn, 2008; Wu and Acero-Lopez, 2012). The native ovotransferrin can carry two irons and exhibits multiple bioactivities, including antimicrobial activity for a wide spectrum of bacteria, fungi, yeasts, and parasites (Ibrahim et al., 1998; Ko et al., 2008a; 2008b; Moon et al., 2012; Valenti et al., 1982), antioxidant activity (Ibrahim et al., 2007; Kim et al., 2012), anticancer activity (Ibrahim and Kiyono, 2009; Moon et al., 2013) and immunomodulatory activity (Xie et al., 2002).

The peptides produced from ovotransferrin using dilute acid hydrolysis (Ibrahim et al., 2000; Kim et al., 2012;

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Moon et al., 2013) or enzymatic hydrolysis (Huang et al., 2010; Keung et al., 1982; Kim et al., 2012; Lee et al., 2006; Lei et al., 2011; Ma et al., 2020; Moon et al., 2017; Shen et al., 2010; Wang et al., 2017) also have been reported to have antioxidant, antimicrobial, antihypertensive and anticancer properties. Hence, egg white ovotransferrin and its derived peptides may have great potentials to be used as antimicrobial and antioxidative agents in the food industry and anticancer, antiviral, antihypertensive, and metal-supplementing agents in the medical and pharmaceutical industries. This review is aimed to investigate the research progress of the functional properties of ovotransferrin from egg white and its derived peptides, and to identify their emerging research and application opportunities in the future.

### Structure and chemical properties of ovotransferrin

Ovotransferrin is a monomeric glycoprotein consists of 686 amino acids with 15 disulfide bonds and no free sulfhydryl groups (Wu and Acero-Lopez, 2012). The molecular mass of ovotransferrin is 78–80 kDa and the isoelectric point is 6.0 (Ibrahim, 2000). Ovotransferrin comprises two globular lobes called N-terminal lobe (residues 1–332) and C-terminal lobe (residues 342–686) that are linked by an alpha helix of 333–341 amino acid residues and interact through noncovalent and hydrophobic interactions (Charter and Largade, 2004). Nine of the 15 disulfide bonds are located in the C-terminal lobe and 6 in the N-terminal lobe (Williams et al., 1982). The two lobes of the ovotransferrin molecule are superposed by a rotation of  $167.6^\circ$  with a translation of 24.7 Å. Each lobe of ovotransferrin is further divided into two subdomains (named as N1, N2, and C1, C2) of 160 residues each, and those subdomains are linked through two antiparallel  $\beta$ -strands (Kurokawa et al., 1995). The N- and C-terminal lobes have homologous amino acid sequences with about 40% identical residues (Williams et al., 1982). Both of the lobes have the capabilities to reversibly bind one  $\text{Fe}^{3+}$  ion with a bicarbonate anion but differ in binding capacities (iron-binding constant of C-terminal lobe:  $1.5 \times 10^8$ ; N-terminal lobe:  $1.5 \times 10^{14}$ ) (Lin et al., 1994). Also, ovotransferrin can transfer 1 atom of ions such as  $\text{Cu}^{2+}$  and  $\text{Al}^{3+}$  (Ko and Ahn, 2008). The overall structure of chicken ovotransferrin is identical to those of the human transferrin and rabbit serum transferrin (Kurokawa et al., 1995), and each lobe has an iron-binding site within the interdomain cleft of the lobe (Ibrahim et al., 2000).

The native ovotransferrin is found in two main forms: metal-free (apo) and metal-bound (holo). However, the physicochemical properties of these two forms differ

significantly. According to Ko and Ahn (2008), apo-ovotransferrin is colorless and can be easily denatured by physical and chemical treatments, whereas holo-ovotransferrin has a salmon-pink color and shows resistance against thermal denaturation and proteolytic hydrolysis. The isoelectric point of apo-ovotransferrin is 6.73, whereas that of holo-ovotransferrin is 5.78 (Wenn and Williams, 1968). Furthermore, ovotransferrin has three isoforms; a-ferric (no iron atoms), mono-ferric (carrying one iron atom), and di-ferric (carrying two iron atoms) (Desert et al., 2001). The ovotransferrin has two putative glycosylation sites in the C-terminal lobe (473–476; Asn-Arg-Thr-Gly and 618–621; Asn-Gly-Ser-Glu) and attached with 4 residues of mannose and 8 residues of N-acetylglucosamine (Williams, 1968). However, Acero-Lopez et al. (2012) reported that high-pressure treatment resulted in structural changes of ovotransferrin. At pH 8 and pressure higher than 200 MPa, total sulfhydryl groups decreased whereas surface hydrophobicity increased along with partial aggregation. Also, the conformational structure of ovotransferrin was changed from helices, sheets turn, and aggregated strands to intermolecular  $\beta$ -sheets. However, the structure returned to its original structure at a pressure greater than 500 MPa.

### Separation and purification of ovotransferrin

The isolation and purification of egg white proteins have been emerged due to the presence of a broad range of functional properties. Separation techniques such as liquid chromatography that includes gel permeation, ion exchange, and immobilized ligand affinity chromatography were employed most prominently to separate egg white proteins (Chang et al., 2018). Ovotransferrin was first purified using ammonium sulfate at pH 3 (Fraenkel-Conrat and Feeney, 1950). However, the procedure was not easy to scale up because the product purity was low and the amount of ammonium sulfate used was very high. Warner and Weber (1951) fractionated ovotransferrin in crystalline form (both as the iron complex and iron-free protein) using 50% ethanol in 0.02 M sodium chloride at pH ranging from 6 to 9. However, the purity of the product was low. Later, many chromatographic approaches were employed to improve product purity and yield and to minimize the structural alterations during purification (Wu and Acero-Lopez, 2012). Carboxymethyl cellulose (CMC) chromatography was employed to separate ovotransferrin with 0.1 M ammonium acetate (Rhodes et al., 1958) and 0.3 M ammonium sulfate at pH 8.5 (Azari and Baugh, 1967). Further, the use of CMC along with 0.1 M ammonium acetate had advantages such as direct isolation of ovotransferrin without resorting to the preliminary crystallization of other proteins or conventional  $(\text{NH}_4)_2\text{SO}_4$

fractionation steps, which enabled the purification of large quantities of ovotransferrin (Azari and Baugh, 1967). Introduction of immobilized metal affinity chromatography (Al-Mashikhi and Nakai, 1987) and DEAE-affinity gel blue chromatography (Chung et al., 1991) significantly improved the purity of the ovotransferrin (94–98%) as indicated in SDS-PAGE. The development of two-step chromatographic methods such as gel permeation on a Superose 6 Prep Grade column and anion-exchange chromatography on Q Sepharose Fast-Flow column (Awade et al., 1994); frontal chromatography (also called displacement chromatography) on a Q Sepharose Fast Flow column followed by purification by preparative chromatography on the same column) (Croguennec et al., 2001); anion exchange chromatography on Q-Sepharose Fast-Flow column followed by CM-Toyopearl 650 M cation exchange chromatography (Tankrathok et al., 2009), and anion exchange chromatography on Q-Sepharose Fast-Flow column followed by cation exchange chromatography on SP-Sepharose Fast-Flow column (Omana et al., 2010) further improved the purity of ovotransferrin (75–80%). Vachier et al. (1995) developed a single anion-exchange chromatography technique using the Q-Sepharose Fast-Flow column to isolate ovotransferrin with 97.5% product purity. Non-denaturing two-dimensional electrophoresis (2DE) was employed to separate lysozyme-binding ovotransferrin along with ovalbumin. After separating lysozyme-binding protein, ovotransferrin was extracted using isoelectric focusing (IEF) (Shimazaki and Takahashi, 2018). However, they were good for only laboratory use with no scale-up capability.

A simple, economic protocol and scalable production of apo-ovotransferrin with over 80% purity and around 99% yield were proposed by Ko and Ahn (2008). They used 43% ethanol at pH 9.0 to separate holo-ovotransferrin and ion exchange resin to convert holo-ovotransferrin into the apo-form. Ko and Ahn (2008) further explained that the recovery and stability of ovotransferrin were very high. Therefore, ovotransferrin is more suitable for food applications. The separation methods of multiple proteins from egg white, including ovotransferrin and other egg white proteins such as ovalbumin, ovomucin, and lysozyme using countercurrent chromatography with the polymer-phase system at pH 8.0, were developed by Shibusawa et al. (1998) and Shibusawa et al. (2001). As a part of the sequential separation method of egg white proteins, Abeyrathne et al. (2013b, 2014a; 2014b) developed a scalable ovotransferrin separation method without using organic solvents (using ammonium sulfate and citric acid), and the method was simpler and more cost-effective than previous methods and had over 85% purity and over 83% yield. Recently, Ji et al. (2020) also developed a sequential separation method for 5 major egg white proteins,

including ovotransferrin, and reported that the method was easy, rapid, and scalable, and the purity and yield of all 5 proteins were higher than 90% and 77%, respectively. The development of simple, efficient, and scalable ovotransferrin separation methods is a very important step for the practical application of ovotransferrin and its derived peptides in the nutraceutical and pharmaceutical industries.

## Functional properties of ovotransferrin

### Antibacterial activity

The bacteriostatic activity of ovotransferrin is directly derived from its ability to sequester  $\text{Fe}^{3+}$  necessary for microbial growth (Giansanti et al., 2015). The antimicrobial activity of ovotransferrin was first identified by Schade and Caroline (1944). The antimicrobial domain of ovotransferrin was found in the N-lobe (residues 1–332) of ovotransferrin (Ibrahim et al., 1998) and showed antimicrobial activity against a broad spectrum of bacteria, fungi, yeast, and parasites. Ovotransferrin inhibits common foodborne pathogens such as *Staphylococcus aureus* KCCM 32,395, *Bacillus cereus* KCCM 40,935, *Listeria monocytogenes* ATCC 15,313, *Escherichia coli* 0157:H7 ATCC 43,895, and *Helicobacter pylori* HpKCTC 26,695 (Moon et al., 2012), and the antibacterial activity of ovotransferrin was dose-dependent. Gram-positive bacteria were more sensitive to ovotransferrin than gram-negative bacteria. Baron et al. (2014) postulated another antibacterial mechanism that is related to the damage of bacterial membrane by ovotransferrin. They reported that ovotransferrin lysed bacterial cell membranes and showed strong antibacterial activity against *B. cereus*. Bacterial species, including *Pseudomonas* spp., *E. coli*, and *Staphylococcus mutans*, were the most sensitive, whereas *Staphylococcus aureus*, *Proteus* spp. and *Klebsiella* were the most resistant to ovotransferrin (Valenti et al., 1983). Ovotransferrin along with lysozyme and ethylenediaminetetraacetate (EDTA) exhibited strong antibacterial activity against *L. monocytogenes* (Ko et al., 2008a). The presence of bicarbonate ions increased the bacteriostatic activity of ovotransferrin (Ko et al., 2008b) and the antagonizing effect was reported after adding citrate in the presence of bicarbonate (Valenti et al., 1983). Ibrahim et al. (2000) and Valenti et al. (1987) reported that  $\text{Zn}^{2+}$ -saturated ovotransferrin exhibited stronger antimicrobial activity than apo-ovotransferrin or other metal-complexes of ovotransferrin. However, Valenti et al. (1987) noted that the direct contact of  $\text{Zn}^{2+}$ -saturated ovotransferrin with the bacterial surface is required to express desired antibacterial activity. Valenti et al. (1982) reported that ovotransferrin immobilized on Sepharose 4B also had the iron-binding

capacity and showed a bacteriostatic effect on *E. coli*. Shimazaki and Takahashi (2018) investigated the antibacterial activity of lysozyme-binding ovotransferrin separated with 2DE and IEF and found that it had bacteriolytic activity against *B. subtilis* and *E. coli*. Cooper et al. (2019) reported that the overexpression of ovotransferrin in chicken embryonic cells improved their antimicrobial activity against *Campylobacter jejuni*. Also, the ovotransferrin significantly reduced the total aerobic and coliform bacteria in chicken muscle samples stored at 7 °C for 5 days. Zhu et al. (2019) reported that ovotransferrin enhanced gut health by reducing pathogenic microorganisms such as *Helicobacter* and *Desulfovibrio* in guts.

### Antioxidant activity

The ability of ovotransferrin to retard lipid oxidation is derived from its free-radical scavenging activity. Moon et al. (2012) reported that ovotransferrin maintained approximately 60% of its 2,2-diphenyl-1-picryl hydroxyl (DPPH) radical scavenging capacity for 48 h, but Kim et al. (2012) indicated that approximately 78.2% of 2,2-diphenyl-2-picrylhydrazyl radical scavenging activity was remaining after 32 h of incubation. Ovotransferrin exhibited a distinct thiol-linked self-cleavage in a redox-dependent process and the superoxide dismutase-like superoxide-scavenging activity of ovotransferrin was promoted by binding copper and manganese over ascorbate or serum albumin (Ibrahim et al., 2007). Hence, the metal-bound (holo) ovotransferrin shows greater dismutation than the metal-free (apo) ovotransferrin. You et al. (2014) reported that when the lysine (residues 327) and glutamic acid (residues 186) in ovotransferrin were conjugates with catechin from green tea, the oxygen radical scavenging capacity of the conjugated ovotransferrin was 4–5 times higher than that of the native one. These results indicated that a slight modification of ovotransferrin is a useful strategy to improve the functional properties of ovotransferrin, but further studies are needed to confirm their effects.

### Antifungal and antiviral activity

Ovotransferrin possessed inhibitory activity against genus *Candida*. Valenti et al. (1985) tested the antifungal effect of ovotransferrin against over 100 strains of *Candida* and found that all the *Candida* strains but *C. krusei* showed sensitivity to ovotransferrin. They also found that iron saturated-ovotransferrin possessed antifungal activity. Although the addition of bicarbonate ions increased the antibacterial activity, it did not inhibit the *Candida* spp. Valenti et al. (1986) reported that both lactoferrin and ovotransferrin exhibited antifungal activity against *C.*

*Albicans* through a transferrin adsorption mechanism of *C. Albicans*, but not in *C. krusei*. The antiviral activity of ovotransferrin against the infection of chicken embryo fibroblast by Marek's disease virus (MDV) and avian herpesvirus was demonstrated by Giansanti et al. (2002) who reported that ovotransferrin was more effective than human transferrin and bovine lactoferrins in inhibiting MDV infection. However, there was no correlation between the antiviral efficacy and the iron saturation of ovotransferrin. Zhou et al. (2018) reported that ovotransferrin suppressed the replication of vesicular stomatitis virus and increased the production of type I interferon in the ovotransferrin-pretreated mouse peritoneal macrophages.

### Cytotoxic activity

Proteins in egg whites play important roles in preventing infections during the development of chicken embryos. Ibrahim and Kiyono (2009) investigated the effect of ovotransferrin on the proliferation of MCF-7 (breast cancer) and HCT-116 (colon cancer) cells and reported that thiol-linked auto-cleaved ovotransferrin exhibited cytotoxic effects against the breast and colon cancer cells. Moon et al. (2013) also tested the cytotoxicity of promod 278P hydrolysate of ovotransferrin against various cancer cell lines, including the larynx (Hep-2), stomach (AGS), lung (SK-MES-1), liver (HepG2), breast (MCF-7), cervix (HeLa) and colon (HT-29) cancer, and found that they had little growth inhibitory effects to those cancer cell lines.

### Immunomodulatory activity

Ovotransferrin is an acute-phase protein, the levels of which increase significantly during inflammation and infections. Xie et al. (2002) reported that ovotransferrin stimulated the production of interleukin-6 (IL-6) and matrix metalloproteinase (MMP) by HD11 cells and augmented phorbol ester-induced respiratory burst activity and degranulation in chickens, and suggested that ovotransferrin modulated macrophage and heterophil functions. Lee et al. (2018) reported that ovotransferrin stimulated the production of nitric oxide in RAW 264.7 macrophages, expression of inducible nitric oxide synthase, and pro-inflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$ , and IL-6 and increase the phosphorylation level of mitogen-activated protein kinase (MAPK) signaling pathways that are associated with macrophage activation. Thus, ovotransferrin can be used as a natural immunomodulator to enhance immune responses in innate and adaptive immune systems to prevent diseases. Chiu et al. (2017) reported that ovotransferrin from hyperimmune chicken eggs possessed immunological

characteristics similar to immunoglobulin Y that prevented resistance to antibiotics. Also, ovotransferrin increased major histocompatibility complex class II (MHC-II) and a cluster of differentiation 83 (CD83) levels to enhance maturation of intestinal dendritic cells (DCs). Also, ovotransferrin promoted the expression of cytokines and the genes of TNF- $\alpha$  interferon- $\gamma$  (IFN- $\gamma$ ), interleukin-4 (IL-4), and interleukin-10 (IL-10). Ovotransferrin enhanced the secretion of immunoglobulin A (IgA) and secretory immunoglobulin A (slgA) to promote intestinal humoral immune response and ameliorated the immune dysfunction caused by cyclophosphamide (CP) (Zhu et al., 2018). Zhu et al. (2019) reported that the thymus and spleen index of ovotransferrin-treated mice significantly higher than that of the cyclophosphamide (CP)-treated mice. Also, the secretion of TNF- $\alpha$ , IL-10, and IgA in the CP-treated mice returned to normal levels after treating them with ovotransferrin. Shang (2019) reported that egg white ovotransferrin is responsible for enhancing the expression of type I collagen and alkaline phosphate, inhibition of the production of receptor activator of nuclear factor kappa-B (RANKL). (Shang et al., 2020) reported that the supplementation of ovotransferrin regulated the expression of genes that are involved in lipid metabolism, signal transduction, and the endocrine system, and showed that lipoprotein receptor-related protein 1 worked as a scavenger receptor and regulated the ability of ovotransferrin to promote bone formation. Ovotransferrin increased the beneficial microbiota population such as *Akkermansia* and affected mucosal morphology by increasing crypt depth. The expression of tight junction protein was also increased by supplementation of ovotransferrin in young and adult mice (Zhang et al., 2020).

### Production of ovotransferrin-derived functional peptides

Various approaches to hydrolyze ovotransferrin, including chemical and enzymatic methods, have been reported (Ibrahim et al., 2000; Kim et al., 2012; Lee et al., 2010; Moon et al., 2013). Among the reported methods of hydrolyzing ovotransferrin, enzymatic hydrolysis was more widely used than other methods. Especially, commercially available plant, animal, and fungi or bacterial enzymes have been utilized with the enzyme-to-substrate ratio at 1:25 (Kim et al., 2012) and 1:50 (Moon et al., 2017). Single enzyme hydrolysis was the most commonly used method, but the use of two or more enzyme combinations also have been tested. However, little works were done to produce functional peptides from ovotransferrin using food-grade enzymes such as promod 278P (Table 1).

### Functional properties of ovotransferrin-derived peptides

#### Antimicrobial activity

The presence of an antimicrobial domain is a unique characteristic of the transferrin family (Wu and Acero-Lopez, 2012). A 92 amino acid-residue peptide of ovotransferrin (OTAP-92; Leu109-Asp200) kills *E. coli* through a membrane damage mechanism and it is capable of destructing Gram-negative bacteria by crossing the outer membrane by a self-promoted uptake that causes damage to the biological function of the cytoplasmic membrane (Ibrahim et al., 2000). Also, bioactive peptides obtained by the hydrolysis of ovotransferrin with trypsin and ficin exhibited antimicrobial effects against *S. aureus* (G<sup>+</sup>) and *Salmonella typhimurium* (G<sup>-</sup>) (Zohreh et al., 2014). A novel antimicrobial peptide of ovotransferrin (Ala-Gly-Leu-Ala-Pro-Tyr-Lys-Leu-Lys-Pro-Ile-Ala) was reported to have excellent antimicrobial activities against both Gram-positive (*S. aureus* ATCC 25,923, *L. monocytogenes* ATCC 19,111, *B. subtilis* ATC 23,631) and Gram-negative (*E. coli* ATCC 25,922, *P. aeruginosa* ATCC 27,553, *S. Typhimurium* ATCC 50,013) bacteria (Ma et al., 2020). Ovotransferrin contains antiviral peptides that are related to the natural immunity of chickens. Giansanti et al. (2005) reported that hen egg ovotransferrin consisted of two fragments Asp-Gln-Lys-Asp-Glu-Tyr-Glu-Leu-Leu and Lys-Asp-Leu-Leu-Phe-Lys, which contribute to the antiviral activity against Marek's disease virus that infects chicken embryo fibroblasts. Therefore, these protein fragments have a potential application as an antiviral agent.

#### Antioxidant activity

Ovotransferrin hydrolysates have been reported to have strong antioxidant activities. Kim et al. (2012) produced ovotransferrin hydrolysates using 10 enzymes and reported that all the hydrolysates had approximately 3.2 to 13.5 times higher superoxide anion scavenging and oxygen radical absorbance capacities than the natural ovotransferrin. Shen et al. (2010) hydrolyzed ovotransferrin with thermolysin and found two tetrapeptides (Trp-Asn-Ile-Pro and Gly-Trp-Asn-Ile) with very high antioxidant activity and postulated that the Trp-Asn-Ile portion could be the core motif responsible for the high antioxidant activity. Also, Huang et al. (2010) obtained a peptide (Ile-Arg-Trp) from ovotransferrin using thermolysin and pepsin and found that this peptide possessed significantly higher oxygen radical absorbance capacity than the whole ovotransferrin. They also suggested that ovotransferrin hydrolysates could be used as a natural antioxidant in

**Table 1** Methods for the hydrolysis of ovotransferrin and its derived activities

Method of hydrolysis	Used enzymes or chemicals	Activity	References
Enzymatic hydrolysis	Subtilisin	Iron binding	Keung et al. (1982)
Acid hydrolysis	0.03 N HCl	Antimicrobial	Ibrahim et al. (2000)
Enzymatic hydrolysis	Chymotrypsin	ACE-inhibitory, Antihypertensive	Lee et al. (2006)
Enzymatic hydrolysis	Thermolysin, pepsin	Oxygen radical absorbance capacity	Huang et al. (2010)
Enzymatic hydrolysis	Protamex, alcalase, trypsin, pepsin, neutrase, flavorzyme	ACE-inhibitory	Lee et al. (2010)
Acid hydrolysis	0.03 N HCl	ACE-inhibitory	Lee et al. (2010)
Enzymatic hydrolysis	thermolysin, pepsin	Antioxidant	Shen et al. (2010)
Enzymatic hydrolysis	Thermolysin	ACE-inhibitory	Lei et al. (2011)
Acid hydrolysis	0.03 N HCl	Antioxidant	Kim et al. (2012)
Enzymatic hydrolysis	protamex, alkalase, trypsin, neutrase, flavorzyme, maxazyme, collupulin, protex, promod 278, $\alpha$ -chymotrypsin	Antioxidant	Kim et al. (2012)
Acid hydrolysis	0.03 N HCl	Cytotoxic	Moon et al. (2013)
Enzymatic hydrolysis	Neutrase, alcalase, protamex, protex 6L, flavorzyme, $\alpha$ -chymotrypsin, trypsin, collupulin	Cytotoxic	Moon et al. (2013)
Enzymatic hydrolysis	Trypsin, ficin	Antimicrobial	Zohreh et al. (2014)
Auto-cleave hydrolysis	0.2 mmol – 1 tris(2-carboxyethyl) phosphine in citrate–phosphate buffer	Antioxidant, Mineral chelating	Moon et al. (2014)
Enzymatic hydrolysis	Promod 278P	Cytotoxic, Antioxidant	Moon et al. (2017)
Enzyme hydrolysis	Promod 278P, thermolysin, a combination of promod 278P-thermolysin	Antioxidant, Anticancer	Lee et al. (2017)
Enzyme hydrolysis	Pepsin	Immunomodulatory	Liu et al. (2017)
Enzymatic hydrolysis	Pepsin, trypsin	Anti-inflammatory	Wang et al. (2017)
In Silico hydrolysis	19 enzymes and 1 enzyme combination	Potent ACE-inhibitory	Yu et al. (2019)
Enzymatic hydrolysis	Pepsin	Antimicrobial	Ma et al. (2020)

functional foods, aiming to enhance the antioxidant properties of foods to prevent oxidation reactions during food processing, which would benefit human nutrition and health. Lee et al. (2017) reported that ovotransferrin hydrolysates obtained from promod 278P (OTH-P), thermolysin (OTH-T), and the combination of promod 278P-thermolysin (OTH-PT) had strong antioxidant properties. The DPPH radical scavenging activities of the hydrolysates were OTH-P 35.04%, OTH-T 41.49%, OTH-PT 43.53%, and the ABTS-scavenging activities were OTH-P 47.23%, OTH-T 62.36%, OTH-PT 60.60% at 2 mg/mL concentration.

### ACE-inhibitory activity

The ACE-inhibitory activity of the native ovotransferrin has not been reported yet. However, peptides derived from ovotransferrin were reported to have strong ACE-inhibitory activity. Moon et al. (2017) investigated the ACE-inhibitory activity of promod 278P hydrolysates of ovotransferrin and reported that the hydrolyzed products exhibited a strong ACE-inhibitory activity. Lee et al. (2010) reported that ovotransferrin hydrolysates produced with trypsin, acid, and pepsin reduced the activity of ACE by 60.2%, 55.8%, and 42.6%, respectively. Also, the ACE-inhibitory peptide (Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr) of ovotransferrin significantly reduced the blood pressure in spontaneously hypertensive mice (Lee et al., 2006). They suggested that Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr acted as

a pro-drug of Lys-Val-Arg-Glu-Gly-Thr to inhibit ACE activity in vivo. Majumder et al. (2015) reported that the ovotransferrin-derived peptide Ile-Arg-Trp increased the ACE-inhibitory activity but decreased the expression of proinflammatory gene in mesenteric arteries of spontaneously hypertensive rats. Recently, Yu et al. (2019) reported that Glu-Trp-Leu obtained from in silico hydrolysis of ovotransferrin using 19 enzymes and one enzyme combination showed potent ACE-inhibitory activity. Moon et al. (2017) reported that the promod 278P hydrolysate of ovotransferrin had  $76.82 \pm 1.28\%$  ACE-inhibitory activity at 10 mg/mL concentration.

### Cytotoxic activity

The hydrolysates of ovotransferrin showed a cytotoxic effect on various cancer cells. Ovotransferrin hydrolyzed with promod 278P expressed over 90% of cytotoxicity against human breast adenocarcinoma (MCF-7), human colon adenocarcinoma (HT-29), human colon adenocarcinoma (LoVo), human cervix adenocarcinoma (HeLa), and human liver hepatoblastoma (HepG2) cancer cell lines (Moon et al., 2017). Majumder et al. (2013) reported that peptides (Ile-Arg-Trp and Ile-Gln-Trp) derived from ovotransferrin attenuated tumor necrosis factor, inflammatory responses, and oxidative stress in endothelial cells. Moon et al. (2013) reported that the hydrolysates of ovotransferrin with trypsin and neutrase showed over 90% cytotoxicity against cervical cancer cell lines and those with trypsin, protamex, collupulin MG and protex 6L caused 95, 96, 87, and 86% growth inhibition, respectively, in stomach cancer cell lines. The hydrolysis of ovotransferrin by promod 278P (OTH-P), thermolysin (OTH-T), and its combination (OTH-PT) increased the cytotoxic activity against various cancer cells and the hydrolysate of OTH-PT exhibited the strongest cytotoxic activities against human stomach adenocarcinoma (AGS), LoVo, HT-29 and HeLa with IC50 values 0.79, 0.78, 0.92 and 0.78 mg/mL, respectively (Lee et al., 2017).

### Immunomodulatory and anti-inflammatory activity

The peptides derived from ovotransferrin also exhibited immunomodulatory activities. The Ile-Arg-Trp peptide of ovotransferrin exhibited a modulatory effect on vascular smooth muscle cells against Angiotensin II stimulation, superoxide production, and inflammation (Liao et al., 2016). Liu et al. (2017) reported that pepsin hydrolysates of ovotransferrin inhibited the maturation of lipopolysaccharide-induced bone marrow-derived dendritic cells (BMDCs) generated from KM mice by reducing the expression of MHC-II, CD83, and CD86, and the production of TNF- $\alpha$ , lipopolysaccharide (IL-12p70), and

RANTES. Also, the pepsin hydrolysates of ovotransferrin impaired the ability of lipopolysaccharide-stimulated BMDCs to induce allogeneic T lymphocyte proliferation and decreased the production of IFN- $\gamma$  by activating T cells. Yi et al. (2017) reported that Ile-Arg-Trp inhibited the H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity and production of reactive oxygen species (ROS). Also, Ile-Arg-Trp normalized mitochondrial membrane potential and inhibited the activation of caspase-9 and caspase-3, and exhibited a protective effect against oxidative stress. Ma et al. (2019) showed that Ile-Arg-Trp and Ile-Gln-Trp derived from ovotransferrin improved intestinal health by regulating microbial composition in the colon, colonic inflammation caused by *Citrobacter rodentium*, and serum metabolism of mouse models. Wang et al. (2017) reported that ovotransferrin-derived peptides also exerted anti-inflammatory effects. Dipeptides (Cys-Arg, Phe-Ile, His-Cys, Leu-Leu, and Met-Lys) of ovotransferrin decreased IL-8 secretion, inhibited gene expression of 5 pro-inflammatory cytokines (TNF- $\alpha$ , IL-8, IL-6, IL-1 $\beta$ , IL-12). In contrast, the gene expression of anti-inflammatory cytokines IL-10 in Caco-2 cells was promoted by those 5 di-peptides. Cys-Arg and His-Cys were also reported to inhibit the phosphorylation of p-JNK and p-p-38 (Wang et al., 2017). In an in vivo study, the Ile-Arg-Trp from ovotransferrin inhibited lipopolysaccharide (LPS)-induced peritonitis in rat models by inhibiting the synthesis of serum TNF and IL-8 and myeloperoxidase. Also, Ile-Arg-Trp treatment increased the Shannon index and decreased the Simpson index and suggested that modification of gut microbial diversity due to the potential ability of Ile-Arg-Trp to enhance the barrier properties of the intestine (Jiao et al., 2019) (Table 2).

### Applications of ovotransferrin and its derived peptides

The presence of a wide spectrum of functional properties in the ovotransferrin and its derived peptides suggests their potential applications in food, as well as medical and pharmaceutical industries, are very high.

### Food and nutraceutical applications

As mentioned earlier, due to antimicrobial and antioxidant activities, ovotransferrin and its derived peptides can be used as functional food ingredients to enhance human health. However, few practical applications have been reported. Seol et al. (2009) developed a  $\kappa$ -carrageenan-based film containing ovotransferrin combined with EDTA and used it as a packaging material to extend the shelf-life of the fresh chicken breast by inhibiting *E. coli*. You et al. (2014) reported that ovotransferrin-catechin conjugate

**Table 2** Ovotransferrin-derived peptides and their activities

Ovotransferrin peptide	Activity	References
OTAP-92 (Leu109-Asp200)	Antimicrobial	Ibrahim et al. (2000)
Asp-Gln-Lys-Asp-Glu-Tyr-Glu-Leu-Leu	Antiviral	Giansanti et al. (2005)
Lys-Asp-Leu-Leu-Phe-Lys		
Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr	ACE-inhibitory	Lee et al. (2006)
Trp-Asn-Ile-Pro	Antioxidant	Shen et al. (2010)
Gly-Trp-Asn-Ile		
Ile-Arg-Trp	Oxygen radical absorbance capacity	Huang et al. (2010)
Ile-Arg-Trp	Anticancer	Majumder et al. (2013)
Ile-Gln-Trp		
Ile-Arg-Trp	ACE-inhibitory	Majumder et al. (2015)
Ile-Arg-Trp	Modulatory, ACE-inhibitory	Jiao et al. (2019), Liao et al. (2016), Ma et al. (2019)
Ile-Gln-Trp	Immunomodulatory	Ma et al. (2019)
Cys-Arg	Anti-inflammatory	Wang et al. (2017)
Phe-Ile		
His-Cys		
Leu-Leu		
Met-Lys		

could be used as an antioxidant in functional foods. Recently, ovotransferrin was used to fabricate gum arabic particles by electrostatic interactions, hydrophobic interactions, and hydrogen bonding to employ as a surfactant-free food-grade Pickering emulsion with applications of protein-polysaccharide nanocomplexes (Wei and Huang, 2019a). Ovotransferrin-glucose conjugate (OGC) and ovotransferrin-lactose conjugate (OLC) obtained from Maillard reaction through glucosylation and galactosylation respectively, were tested for their functional properties. However, the covalently bound saccharides weakened the emulsifying ability of ovotransferrin fibrils and had larger emulsion droplet sizes of OGC fibrils-stabilized emulsions than OLC fibrils-stabilized emulsions and OGC exerted the lowest viscosity and storage modulus. Hence, the rheological properties of oil emulsions stabilized by ovotransferrin fibrils were dependent on the nature of the covalently bound saccharides (Wei and Huang, 2019b).

### Medical and pharmaceutical applications

As described previously, ovotransferrin and its derived peptides have great potential to be applied in medical and pharmaceutical industries as antimicrobial, antiviral, anticancer, and antihypertensive agents. Ovotransferrin and the peptides from ovotransferrin, Ile-Arg-Trp, Ile-Gln-Trp, and Lys-Val-Arg-Glu-Gly-Thr, showed ACE-inhibitory, antihypertensive, anti-inflammatory, and antioxidant properties, and thus could be used as a clinical treatment for cardiovascular diseases (Chen et al., 2017). Also, ovotransferrin was suggested to be used as an antibiotic

substitute to prevent chlamydiosis (Van Droogenbroeck et al., 2008). Van Droogenbroeck et al. (2011) clinically used ovotransferrin as an antimicrobial agent to treat turkey and successfully reduced the amount of *Chlamydia psittaci*, *Ornithobacterium rhinotracheale*, and avian metapneumovirus. Abdallah and Chahine (1999) suggested using ovotransferrin as a metal supplementing agent for humans due to its iron-binding and transportation capacity. Moreover, ovotransferrin has been reported to have an osteogenic activity that promotes bone formation while preventing bone resorption. Ovotransferrin can also act as a therapeutic and anabolic agent to prevent osteoporosis and promote bone repair (Shang and Wu, 2018). Son et al. (2018) investigated angiotensin II-stimulated insulin of tri-ACE-inhibitory peptides of ovotransferrin (Ile-Arg-Trp, Ile-Gln-Trp, Leu-Lys-Pro) and reported that only Ile-Arg-Trp decreased insulin-stimulated glucose uptake in rat-derived skeletal muscle cells. Also, egg white ovotransferrin suppressed the Receptor Activator of Nuclear Factor  $\kappa$ B ligand (RANKL) and inhibited osteoclast differentiation, which promoted bone health (Shang and Wu, 2019). Wei et al. (2019) reported that the formation of a hetero protein complex between ovotransferrin and lysozyme stabilized Pickering emulsions, which was an effective delivery tool for hydrophobic nutraceuticals. Ovotransferrin is also tested as a therapeutic agent to treat retained placenta, reproductive tract infection, and inflammation such as metritis to improve the health of the reproductive tract and fertility of cows (Talukder et al., 2019).



## Egg allergenicity and safety aspects

Eggs are considered as one of the main allergenic foods that account for 38.3% of all food allergies (Sakai et al., 2015) and its estimated prevalence is 0.2–7.0% (Rona et al., 2007). As described by Ebbehøj et al. (1995) egg white proteins, including ovomucoid, ovalbumin, ovotransferrin, and lysozyme, are the major allergenic proteins in egg white. As eggs are consumed as a functional food, the avoidance of allergenicity is very important. Scientific evidence revealed that various processing procedures and safety measures can be applied to reduce/eliminate allergenicity of eggs. Hoffman (1983) reported that allergenicity can be simply decreased by boiling eggs. Also, the combination of heat and enzyme treatments reduced the allergenicity of pasteurized eggs without significantly changing their texture and flavor. Furthermore, the enzymatic hydrolysis of proteins into peptides and amino acid monomers is an effective method to eliminate the egg allergenicity. Also, the allergenicity of egg proteins can be decreased by processing egg with other food ingredients: heating of eggs with wheat flour made ovomucoid insoluble by forming intermolecular disulfide protein-polysaccharide polymers and became less extractable and minimized allergenicity (Kato et al., 2001).

## Summary and future research approaches

This study reviewed the structure, separation, and functional properties of ovotransferrin and the production of functional peptides from ovotransferrin. Ovotransferrin and its derived peptides have been recognized for their excellent functional properties. Among the functional properties, antimicrobial, antioxidant, and ACE-inhibitory activities were highly recognized and investigated. Although the *in vitro* experiments for the functional properties of ovotransferrin and its derived peptides were established well, further researches are needed to investigate the bioactivities of ovotransferrin products to apply in biosystems via food, animal, and clinical trials. The antimicrobial activity of ovotransferrin and its derived peptides can be used as a natural preservative to prevent microbial spoilage in foods while enhancing nutritional value. Also, due to the presence of antioxidant properties, ovotransferrin and its bioactive peptides have future potential applications in food processing to prevent undesirable oxidative reactions caused by substances such as metal ions. Also, ovotransferrin and its functional hydrolysates can be used as a pharmaceutical agent that can regulate high blood pressure through its ACE-inhibitory activity. The industrial purification and application of ovotransferrin and its peptides are

not implemented yet, but simple, economical, and scalable separation methods for ovotransferrin are already available, but the identification of particular bioactive peptides of ovotransferrin with their sequences and their application needs further studies. Because various processing methods, such as heating, can influence the physiological and functional properties of the protein, further research is needed to analyze the activities of ovotransferrin and its derivatives *in vivo*. The determination of novel functional properties creates new research approaches for the ovotransferrin and its derived peptides even though most of the approaches used single enzyme hydrolysis of ovotransferrin and few pieces of research were carried out with enzyme combinations. The separation of ovotransferrin and the identification of its derived peptides as a food additive creates a novel trend in the food industry and commercial production opportunities with economical value as well. Although it is well known that ovotransferrin is an iron carrier protein in the animal body, little work has been done to use it as an iron supplementing agent for humans, which needs further attention. The ovotransferrin nanofibrils and their activities were identified as novel applications of ovotransferrin with advantages in the food and pharmaceutical industries, this area needs further studies.

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