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ARTICLE

Antioxidant, Antimicrobial, and Cytotoxic Activities of Ovotransferrin from Egg White

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Abstract

The antioxidant, antimicrobial, and cytotoxic activities of ovotransferrin were investigated *in vitro*. The antioxidant capacity of ovotransferrin was evaluated using the 2,2-Diphenyl-1-picryl hydrazyl (DPPH) radical scavenging method, antimicrobial effects using the agar well diffusion method, and cytotoxicity using the 3-(4,5-dimethylthizol-2-yl)-2,5-diphenylatetetrazolium bromide (MTT) assay. The DPPH radical-scavenging capacity of ovotransferrin at 1 mg/mL level reached approximately 60% after 48 h of reaction. The antimicrobial effects of ovotransferrin against common food-borne pathogens, *Staphylococcus aureus* KCCM 32395, *Bacillus cereus* KCCM 40935, *Listeria monocytogenes* ATCC 15313, *Escherichia coli* O157:H7 ATCC 43895, and *Helicobacter pylori* HpKCTC 26695 were dose dependant. Gram-positive bacteria was more sensitive to ovotransferrin than gram-negative bacteria. Ovotransferrin showed stronger antimicrobial effect against *L. monocytogenes* than other gram-positive bacteria tested. The cytotoxicity of ovotransferrin was evaluated in human cancer cell lines, various tissue origins, including the larynx (Hep-2), stomach (AGS), lung (SK-MES-1), liver (HepG2), breast (MCF-7), cervix (HeLa), and colon (HT-29). Ovotransferrin displayed relatively high cytotoxicity (≤60% inhibition effects) at 40 mg/mL. At lower concentrations (≤10 mg/mL), however, ovotransferrin cytotoxic effects were not significant in all cancer cell lines tested. These results indicated that ovotransferrin has potential to be used as an antioxidant or antimicrobial agent in foods or a pharmaceutical agent against cancers.

Key words: ovotransferrin, antioxidant activity, antimicrobial activity, cytotoxic activity

Introduction

Chicken egg has been used as an important source for protein, energy, vitamins, and minerals for human (Yi *et al.*, 2003), and is an excellent source for biologically active substances (Mine, 2007). Ovotransferrin from egg white was first characterized by Schade and Caroline (1944) who called it conalbumin, subsequently renamed as ovotransferrin after being confirmed as an iron-binding protein, and became a member of the transferrin family (Williams, 1968; Yamamoto, 1997).

Ovotransferrin accounts for approximately 12% of egg white protein and is best known for its ability to bind iron. It is comprised of 686 amino acids with 77.9 kDa molecular weight and an isoelectric point of 6.38. It contains 15 disulfide cross-links and has no free sulfhydryl groups (Wu *et al.*, 2011). Ovotransferrin is reported to have antimicrobial, antioxidative, antiviral, and immunomodulatory activities (Giansanti *et al*., 2005; Rath *et al.,* 2009; Schade and Caroline, 1944; Valenti *et al.*, 1985; Xie *et al.,*2002). The antimicrobial activity of ovotransferrin was recognized soon after it was first purified (Schade and Caroline, 1944). Since then, the antibacterial properties of ovotransferrin against a variety of pathogens including *Escherichia coli*, *Pseudomonas* spp., *Streptococcus mutans*, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella* Enteritidis, and *Candida* spp. have been reported (Ko *et al.*, 2008a; Ko *et al*., 2008b). The iron-binding capacity of ovotransferrin was initially believed to be responsible for its antibacterial activity, but antimicrobial mechanisms of ovotransferrin are not fully defined yet (Ibrahim, 1997; Ibrahim *et al*., 2000).

It has previously been reported that the antioxidant activ-

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ity of ovotransferrin exhibits superoxide dismutase-like activity, which is promoted by its metal binding activity (Ibrahim *et al*., 2007; Shen *et al*., 2010), but the presence of free radical scavenging activity in ovotransferrin has not been reported. Ibrahim and Kiyono (2009) demonstrated that ovotransferrin autocleaved under reducing conditions (reduction with 0.2 mM tris (2-carboxyethyl) phosphine in 20 mM citrate-phosphate, pH 4.0, for 6 h at 37°C) exhibited stronger SOD-like activity, and anticancer effects against human colon and breast cancer cells than the natural ovotransferrin.

The objective of this study was to determine the antioxidant and antimicrobial capacities of ovotransferrin, and its cytotoxic effects against human cancer cells.

Materials and Methods

Materials

The apo-ovotransferrin (iron-free) used in this study was separated from chicken eggs using the method by Ko and Ahn (2008). 2,2-Diphenyl-1-picryl hydrazyl (DPPH), L-Ascorbic acid, and 3-[4,5-dimethythiazol-2-yl]-2,5-diphenyl tetrazolium bromide were purchased from Sigma Chemical Co. (St. Louis, USA). RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM) medium, minimum essential medium, fetal bovine serum (FBS), horse serum, and penicillin-streptomycin were from Gibco-BRL (Grand Island, USA) and tryptic soy broth (TSB), bacto peptone, yeast extract, and brucella agar were from Difco Laboratories (Sparks, USA).

DPPH radical-scavenging activity

The DPPH radical-scavenging activity was determined by the method of Xu *et al*. (2007), with some modifications. Briefly, 1 mL of 0.5 mM DPPH ethanol solution was mixed with 1.5 mL of ethanol and added to 2.5 mL of sample solution at different concentrations. This mixture was maintained at 37°C during 72 h, and absorbance at 517 nm was measured at every 12 h. L-Ascorbic acid (0.5 mg/mL) was used as a reference compound. The control was the absorbance of 2.5 mL of sample solution mixed with 2.5 mL of ethanol, and the blank was the value for 2.5 mL of water mixed with 1.5 mL of ethanol and 1 mL of 0.5 mM DPPH ethanol solution. The residual radicals were calculated as follows:

$$\text{Residual radicals (\%)} = 1-\left(\frac{Absorbance\ of\ sample - Absorbance\ of\ control}{Absorbance\ of\ the\ blank}\right)\times 100$$

Bacterial strains and culture conditions

Bacterial strains *Staphylococcus aureus* KCCM 32395, *Bacillus cereus* KCCM 40935, *Listeria monocytogenes* ATCC 15313, and *Escherichia coli* O157:H7 ATCC 43895 were purchased from the Korean Culture Center of Microorganisms (KCCM, Korea). *Helicobacter pylori* HpKCTC 26695 was obtained from the *H. pylori* Korean Type Culture Collection (HpKTCC) (Jinju, Korea). Each strain was cultured through 2 consecutive 24 h growth cycles in tryptic soy broth supplemented with 0.6% yeast extract (TSB-YE) at 37°C and used for further studies. *H. pylori* was activated in brucella agar plates supplemented with 5% (v/v) horse serum and was cultured under micro-aerophilic conditions (10% CO_2 atmosphere) for 3 d. For these studies, the strains were then inoculated in brucella broth supplemented with 5% horse serum and cultured for 1 d at 37°C before use.

Agar well diffusion assay

Inoculum of each strain was prepared in TSB-YE and incubated overnight at 37°C. All bacteria were suspended in 0.1% sterile peptone water and diluted to 10^5 CFU/mL. One milliliter of each bacterial suspension was added to 99 mL of 0.75% soft TSB-YE agar or brucella agar, after which 20 mL of inoculated agar was poured into a petridish. Once the agar plates solidified, 6-mm diameter wells were aseptically cut with a sterilized cork borer. The freeze-dried ovotransferrin samples were dissolved in distilled water to a final concentration of 80 mg/mL. All solutions prepared were sterilized using 0.22 μM syringe filters before performing this experiment. Hundred-microliter aliquots of the ovotransferrin solutions were subsequently delivered onto the dried TSB-YE agar or brucella agar plates. The TSB-YE agar plates were then incubated at 37°C for 24 h, and brucella agar plates were cultured under micro-aerophilic conditions (10% CO_2 atmosphere) for 2-3 d. The antimicrobial activity of ovotransferrin against each bacterial strain was estimated via the observation of clear zones (Taye *et al.*, 2011; Voidarou *et al*., 2011). Three replicates were prepared per sample.

Cell lines and culture conditions

Human cancer cell lines were purchased from the Korean Cell Line Bank (KCLB; Seoul National University, Seoul, Korea). The HT-29 (human colon adenocarcinoma), AGS (human stomach adenocarcinoma), and MCF-7 (human breast adenocarcinoma) cell lines were maintained in RPMI 1640 (Gibco Laboratories, Grand Island, USA) containing 10% heat-inactivated FBS (HyClone, USA), pen-

icillin (100 U/mL), and streptomycin (100 μg/mL). HeLa (human uterine adenocarcinoma) and HepG-2 (human liver hepatoblastoma) cells were grown in minimum essential medium containing 10% heat-inactivated FBS, penicillin, and streptomycin. SK-MES-1 (human lung carcinoma) and Hep-2 (human larynx carcinoma) cells were grown in DMEM supplemented with 10% FBS, penicillin, and streptomycin. All cell lines were cultured at 37°C in a humidified incubator containing 5% CO_2. For the testing of cytotoxic activity, cells were seeded in new dishes and grown to 80% confluency.

***In vitro* cytotoxicity assay (MTT assay)**

The *in vitro* cytotoxic effects of ovotransferrin were determined by 3-(4,5-dimethylthizol-2-yl)-2,5-diphenylate-tetrazolium bromide (MTT) assay as described by Park *et al.* (1999). Briefly, 100 μL aliquots of cell suspension were transferred to 96-well micro plates and incubated for 24 h. One hundred microliter of ovotransferrin and its hydrolysates were added to each cell suspension, followed by incubation at 37°C for 44 h. At the end of the incubation, MTT solution (2.5 mg MTT/mL of phosphate-buffered saline [PBS]) was added, and the plate was further incubated for 4 h. The supernatant was then removed from each cell suspension, and 100 μL of dimethyl sulfoxide was added to dissolve the colored formazan crystals produced from reaction with MTT. The optical density values of each solution were then measured with a microplate reader at 540 nm (Carmichael *et al*., 1987; Mosmann, 1983).

Statistical analysis

All results are presented as mean±SE, and statistical analysis was performed using the SPSS (Chicago, IL, USA) package for Windows (Ver. 18.0). The mean values were compared using one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. *p*-values of less than 0.05 were considered significant. Correlations were determined using Pearson's correlation coefficient.

Results and Discussion

DPPH radical scavenging effect of ovotransferrin

The DPPH radical scavenging assay has been widely used for the screening of antioxidant capacity, most likely due the simplicity and short detection time of the procedure. DPPH is a stable free radical compound that accepts an electron to become a stable molecule. DPPH is deep violet in its free radical form, but assumes a light yellow-ish color when it reacts with the hydrogen contained in the antioxidant (Lee *et al*., 2011).

L-Ascorbic acid is a strong free radical scavenger. The DPPH radical residual percentage of L-ascorbic acid reached under 10% after 1 h of incubation. Ovotransferrin (1 mg/mL) showed radical scavenging activity lower than that of L-ascorbic acid (0.5 mg/mL), but it continued to possess radical-scavenging capability even after 24 h (Fig. 1). Ovotransferrin was relatively good antioxidants with radical-scavenging capability that can last for a long time.

Kechaou *et al.* (2009) demonstrated that this results may have been caused by differences in protein composition of the materials. Huang *et al.* (2010) reported that whole protein of ovotransferrin only exhibited weak oxygen radical-scavenging effect, giving an 0.21 μmol trolox equivalent (TE)/mg. Another study showed that ovotransferrin is an SOD mimic protein with potent superoxide anion scavenging activity under the natural xanthine oxidase coupling system (Ibrahim *et al*., 2007). Davalos *et al.* (2004) reported that the antioxidant activity of peptides produced by enzymatic hydrolysis of crude egg white with pepsin has strong antioxidant activities. Ovalbumin also exhibited a high free radical activity through the naturally occurring Maillard reactions (Nakamura and Kato, 2000).

Formation of clear zones by agar well diffusion assay

Ovotransferrin at 80 mg/mL showed a slight clear zone against *E. coli* O157:H7 ATCC 43895 and *H. pylori* HpKCTC, but less than 60 mg/mL did not yield any clear zones (Table 1). Egg white has a number of proteins with

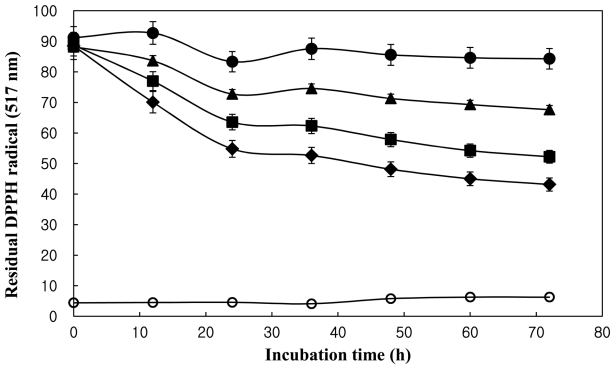

Fig. 1. DPPH free radical scavenging activity of ovotransferrin. Ovotransferrin concentrations were adjusted to 0.125 mg/mL (●), 0.25 mg/mL (▲), 0.5 mg/mL (■), and 1 mg/mL (◆), the positive control (○) had L-ascorbic acid (0.5 mg/mL) instead of ovotransferrin solution. Values are mean with standard error.

Table 1. Zone of inhibition by ovotransferrin against five foodborne pathogens

Strains	Ovotransferrin (mg)			
	20	40	60	80
Staphylococcus aureus KCCM 32395	-	-	+	++[1)]
Bacillus cereus KCCM 40935	-	-	+	++
Listeria monocytogenes ATCC 15313	-	+	++	+++
Eschrerichia coli O157: H7 ATCC 43895	-	-	-	+
Helicobacter pylori HpKCTC 26695	-	-	+	++

[1)]-, No inhibition (≤6.0 mm); +, slight inhibition (6.1-7.9 mm); ++, moderate inhibition (8.0-9.9 mm); +++, strong inhibition (≥10 mm)

demonstrated antimicrobial activities, which act as part of the natural defense system of the egg (Li-Chan *et al*., 1989). As ovotransferrin is a member of an iron-binding protein family, this property is thought to be due to its ability to sequester iron, an activity that would deplete the iron necessary for the growth of microorganisms (Bullen *et al.*, 1978; Jang *et al.*, 2005; Wu *et al*., 2011).

The outer membrane of gram-negative bacteria is reported to be relatively resistant to ovotransferrin (Davis *et al*., 2002). Gram-positive bacteria such as *Staphy. aureus* KCCM 32395*, B. cereus* KCCM 40935, and *L. monocytogenes* ATCC 15313 were more sensitive to ovotransferrin than gram-negative bacteria. Ovotransferrin showed stronger antimicrobial effect against *L. monocytogenes* than other gram-positive bacteria tested. Ibrahim *et al*. (1997) reported that holo- and apo-ovotransferrin, and other metal-ovotransferrin complexes have antibacterial activities against *Staphy. aureus*, *B. cereus,* and *E. coli*. Zn^{2+}-saturated ovotransferrin was more active against bacteria than apo-ovotransferrin and other metal-ovotransferrin complexes. The combinations of ovotransferrin with sodium bicarbonate and zinc also improved antibacterial effects of ovotransferrin against *E. coli* and *L. monocytogenes* (Ko *et al*., 2008A; Ko *et al*., 2008B).

In vitro cytotoxicity assay of ovotransferrin

MTT is a tetrazolium salt, which is reduced to formazan by living cells via the "succinate-tetrazolium reductase" system. The formazan produced by cellular suspensions is directly correlated with the number of metabolically active cells, and thus, the colorimetric MTT assay is used to determine cell proliferation (Jee *et al.*, 2008). To determine the cytotoxic effects of ovotransferrin on cancer cells, Hep-2, AGS, SK-MES-1, HepG-2, MCF-7, HeLa,

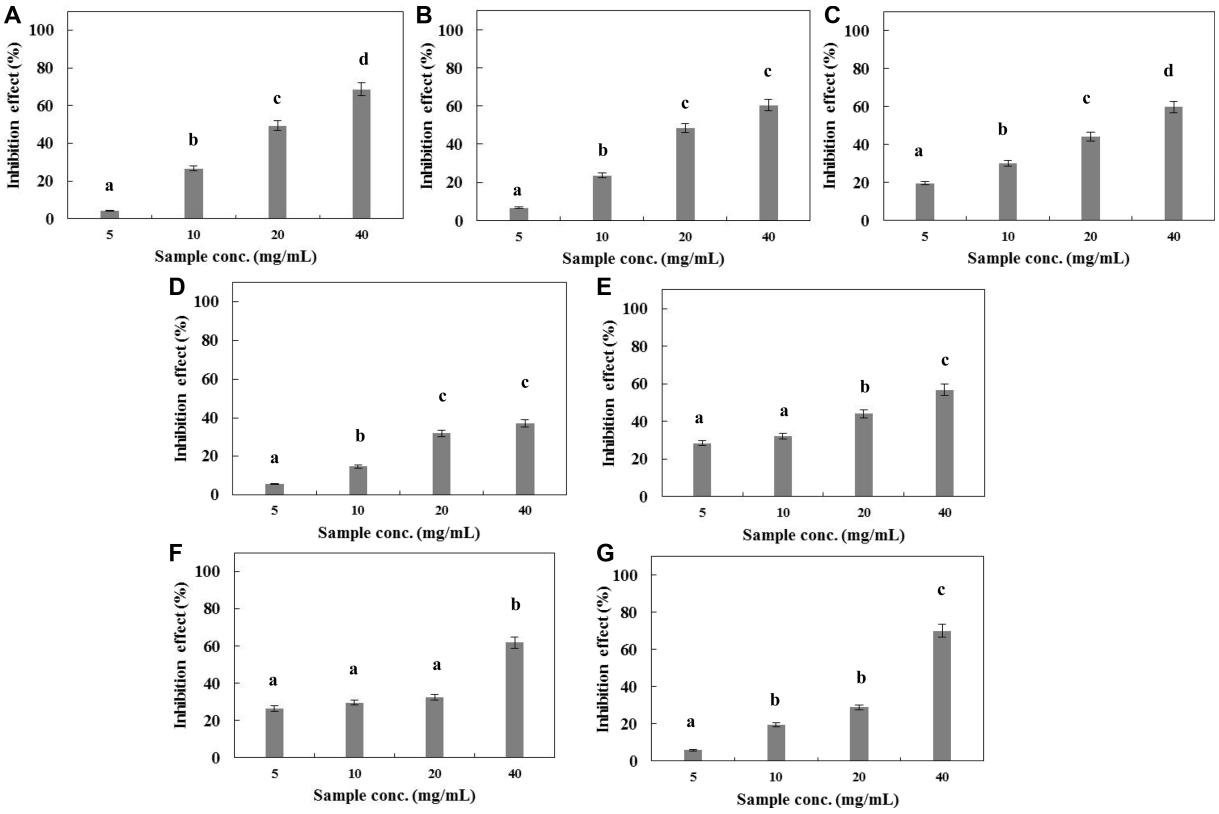

Fig. 2. The cytotoxic effects of ovotransferrin. (A) Hep-2 cell, (B) AGS cell, (C) SK-MES-1 cell, (D) HepG-2 cell, (E) MCF-7 cell, (F) HeLa cell, and (G) HT-29 cell. Values are mean with standard error. Values not sharing the same letter are significantly different from one another (p<0.05) by Duncan's multiple range test.

and HT-29 cells were treated with a range of concentrations (5-40 mg/mL) of ovotransferrin for 48 h (Fig. 2). A significant suppression of cell growth was detected in the presence of ovotransferrin, which occurred in a dose-dependent manner. Ovotransferrin showed relatively high cytotoxicity to most of the cell types at a high concentration (40 mg/mL). At lower concentrations (<10 mg/mL), however, the cytotoxic effects were not significant. In particular, more than 70% of cytotoxicity was observed in HT-29 cell line by ovotransferrin at 40 mg/mL concentration. Among the egg white proteins, glycopeptides of ovomucin demonstrated antitumor effects and inhibited tumor growth in a double-grafted tumor system in mice (Oguro *et al*., 2001; Watanabe *et al*., 1998).

In conclusion, ovotransferrin showed an antioxidant activity in the DPPH radical assay system, and the activity increased as the concentrations increased and lasted for over 72 h.

Ovotransferrin at 80 mg/mL showed antimicrobial activities against *Staphy. aureus* KCCM 32395, *B. cereus* KCCM 40935, *L. monocytogenes* ATCC 15313, *E. coli* O157:H7 ATCC 43895, and *H. pylori* HpKCTC 26695. Ovotransferrin also had cytotoxic activity against several human cancer cell lines. These data suggested that ovotransferrin exhibited multifunctional properties *in vitro*, but further studies are needed to elucidate the same activities *in vivo* systems.

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