Immunomodulatory activity of egg yolk protein hydrolysates prepared by novel two-step hydrolysis: A study of mechanism and stability after in vitro digestion model

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ABSTRACT The aim of this study was to determine the immunomodulatory activity of 2-step egg yolk protein hydrolysates. A two-step hydrolysate of egg yolk protein was prepared using 2 enzymes sequentially, pancreatin and neutrase (**EYPH-PN**). Our results illustrated that EYPH-PN increased the expression of inducible nitric oxide synthase (**iNOS**) mRNA in macrophages, resulting in increased nitric oxide (**NO**) production. EYPH-PN could also enhance the production of tumor necrosis factor (**TNF**)-α and interleukin (**IL**)-6 at both the mRNA and protein levels in macrophages. In addition, treatment with EYPH-PN increased the phagocytic activity of macrophages. According to the evaluation with specific inhibitors, both p38 and JNK cell signaling pathways were involved in the activation of macrophages induced by EYPH-PN. As the TLR-2 receptor of macrophages was blocked, the NO production induced by EYPH-PN was decreased. These results suggest that EYPH-PN activates RAW 264.7 macrophages via the TLR-2/p38/ JNK pathway to increase the production of NO, TNF-α, and IL-6, and increases phagocytic activity. Furthermore, the immunomodulatory activity of EYPH-PN was maintained even after applying the in vitro digestion model. Taken together, EYPH-PN could be used as a functional food ingredient with excellent immunomodulatory activity in the food industry. Therefore, this study suggests a new alternative method to effectively utilize egg yolk protein, a by-product of the poultry industry.

Key words: egg yolk protein, two-step hydrolysis, immunomodulatory activity, TLR/MAPK signaling pathway, in vitro digestion model

INTRODUCTION

Eggs have been widely used in the food industry worldwide as an economical protein source since ancient times (Lesnierowski and Stangierski, 2018). Current studies have suggested that eggs not only serve as a nutritious food, but can also play a role as a functional substance that can treat and prevent a variety of diseases. Various studies have reported that egg proteins, hydrolysates, and peptides have antioxidant, antibacterial, antihypertensive, anticancer, and immunomodulatory activities (Abeyrathne et al., 2013; Lee and Paik, 2019; Moreno-Fernández et al., 2020). Egg protein exists in both the egg white and yolk, and ovalbumin, ovotransferrin, ovomucin, and phosvitin are known as representative egg proteins (Lee and Paik, 2019). In contrast to egg whites, which have the highest proportion of protein in their composition, egg yolk has a higher proportion of lipids than protein (Kovacs-Nolan et al., 2005). Therefore, egg yolk has long been used in the food and cosmetics industry to produce lecithin, a mixture of phospholipids (Peñaranda-López et al., 2020). Egg yolk protein is obtained as a by-product after lecithin extraction in industry (Lesnierowski and Stangierski, 2018). The representative proteins of egg yolk protein, phosvitin, and IgY, are known to have various biological activities such as antioxidant, anticancer, antimicrobial, and immunomodulatory activity (Cook and Trott, 2010; Moon et al., 2014; Lee and Paik, 2019). However, these proteins are denatured by the solvent used to extract lecithin; thus, their functionality and value are reduced (Choi et al., 2022). Therefore, enzymatic hydrolysis is widely used to increase the utility of egg yolk proteins (Eckert et al., 2014).

Enzymatic hydrolysis of proteins is a common method for increasing the functional activity of proteins.

Generally, peptides with functional activity do not exhibit functional activity when present in the sequence of a protein molecule. However, they are known to be active when the peptide is released through hydrolysis (Chalamaiah et al., 2014). Additionally, the low molecular weight of the peptides facilitates digestion and absorption (Chalamaiah et al., 2018). The main reason for using enzymatic hydrolysis to produce peptides is that each enzyme has a specific cleavage site. Since each enzyme has a different cleavage site, various enzymes have been used to make functional hydrolysates and peptides from proteins (e.g., pepsin, trypsin, alcalase, neutrase, and thermolysin). Furthermore, studies have reported the sequential use of hydrolysates composed of peptides of various patterns using 2 different enzymes (Lee et al., 2017a; Huang et al., 2019; Bu et al., 2020; Zhao et al., 2021). Compared with single enzymatic hydrolysis, 2-step enzymatic hydrolysis has the advantage that secondary enzymes can hydrolyze new cleavage sites exposed due to primary enzymatic hydrolysis, thereby increasing the diversity and hydrolysis efficiency of the produced peptides (Huang et al., 2019). In addition, an increase in functionality has also been reported (Lee et al., 2017a).

Macrophages are well known to play an important role in the immune system. They have essential abilities such as regulating cytokine production, engulfing invading pathogens, and presenting antigens (Lee et al., 2017b; Lim et al., 2021). Moreover, they exist in all tissues, and because of their iniquitousness, are one of the first cell populations to encounter invading pathogens (Dukhinova et al., 2021). Macrophages also play a role in effectively regulating the inflammatory response by promoting inflammation or suppressing overexpressed inflammatory reactions (Koh and DiPietro, 2011). Various functions of macrophages are mediated by toll-like receptors (**TLR**s) (Iwasaki and Medzhitov, 2004). Activated TLRs initiate downstream signaling pathways, such as nuclear factor (**NF**)-κB and mitogen-activated protein kinases (**MAPK**s), followed by the production of pro-inflammatory mediators such as nitric oxide (**NO**), prostaglandin E2 (**PGE2**), and pro-inflammatory cytokines (Lim et al., 2021). Therefore, substances that can boost the immune system by activating macrophages through TLRs are attracting attention as immune-related functional materials in the food and pharmaceutical industries.

In this study, we produced four types of hydrolysates using single enzymatic hydrolysis and 2-step enzymatic hydrolysis, and investigated the immunomodulatory activity of the hydrolysates. The immunomodulatory activity of the hydrolysates was determined using RAW 264.7 cells. In more detail, the effects on the production of pro-inflammatory mediators such as NO, tumor necrosis factor (**TNF**)-α, and interleukin (**IL**)-6 of macrophages, and their effects on the phagocytic activity of macrophages were evaluated. In addition, we confirmed the immunomodulatory mechanism using TLR/MAPK pathway inhibitors and the stability of the hydrolysates using an in vitro digestion model.

MATERIALS AND METHODS

Materials and Reagents

Dulbecco's modified Eagle medium (**DMEM**), phosphate-buffered saline (**PBS**), fetal bovine serum (**FBS**), and penicillin-streptomycin were purchased from Hyclone Inc. (Logan, MI, USA). Neutrase, pancreatin, pepsin, trypsin, α-chymotrypsin, thiazolyl blue tetrazolium bromide (**MTT**), lipopolysaccharide (**LPS**), Griess reagent, and neutral red were obtained from Sigma-Aldrich (St. Louis, MO, USA). SB 202190, PD 98059, SP 600125, anti-TLR-2 antibody, and anti-TLR-4 antibody were purchased from Abcam (Cambridge, UK). Mouse TNF-α and IL-6 ELISA kits were obtained from AB Frontier (Seoul, Korea). All other chemical reagents used in this study were of analytical grade.

Preparation of Egg Yolk Protein Hydrolysate

The by-product egg yolk protein produced after extracting the yolk lecithin used in this study was obtained from Join Co. (Yongin, Korea). Briefly, egg yolk was separated from egg whites and dried using a vacuum dryer. The dried egg yolk powder was extracted with 95% ethanol at 50°C for 1 h. Finally, the filtered solid obtained through a 1 μm filter was dried in a dry oven at 70°C and used in the experiment.

The enzymatic hydrolysis was prepared using the following method: (1) One-step hydrolysis was conducted by adding the enzyme with 2% E/S ratio (enzyme/substrate ratio) and reacting at pH 7.0, 50°C, for 4 h. (2) Two-step hydrolysis was conducted by adding another enzyme with 2% E/S ratio after one-step hydrolysis and reaction at pH 7.0, 50°C, for an additional 4 h. After hydrolysis, the enzyme was inactivated at 100°C for 10 min, centrifuged at 3,000 × g for 20 min, and the supernatant was lyophilized and used as a hydrolysate of egg yolk extract protein (**EYPH**). Hydrolysates were classified as EYPH-N (one-step hydrolysates using neutrase), EYPH-P (one-step hydrolysates using pancreatin), EYPH-NP (2-step hydrolysates using neutrase and pancreatin in sequence), and EYPH-PN (2-step hydrolysates using pancreatin and neutrase in sequence), respectively.

Cell Culture

The RAW 264.7 cell line was purchased from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured in DMEM with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were kept in a humidified incubator (MCO-18AIC incubator, Sanyo, Japan) containing 5% CO$_2$ at 37°C.

Measurement of Cell Viability

Cell viability was determined using the MTT assay, as described by Lee et al. (2017a). RAW 264.7 cells were added to a 96-well plate at a concentration of 2 × 10^5 cells/well and incubated for 2 h. The EYPHs were added

to each well at various concentrations (25, 50, 100, and 200 μg/mL) and then incubated for 24 h. MTT solution (2.5 mg/mL in PBS) was added and incubated for an additional 4 h. After removing the supernatant, the formazan crystals were dissolved by adding 100 μL of DMSO. The absorbance of the cell plate was measured using a microplate reader (Bio-Rad, Hercules, CA) at 570 nm. The control group treated with DMEM was considered 100% viable cells. Cell viability was expressed as a percentage of that of the control group.

Measurement of NO Production and iNOS Expression

NO production was measured using the Griess assay (Lee et al., 2017b). RAW 264.7 cells were added to a 96-well plate at a concentration of 2×10^5 cells/well and incubated for 2 h. The EYPHs were added to each well at various concentrations (25, 50, 100, and 200 μg/mL) and then incubated for 24 h. LPS (10 ng/mL) was used as a positive control, and the amount of NO produced was quantified using Griess reagent (1:1 mixture with 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in distilled water and 1% sulfanilamide in 5% phosphoric acid). The absorbance of the cell plate was measured using a microplate reader at a 540 nm wavelength. Sodium nitrate was used as the standard NO curve.

The effect of EYPH on inducible nitric oxide synthase (**iNOS**) mRNA expression was determined using quantitative real-time polymerase chain reaction (**qRT-PCR**). RAW 264.7 cells were added to a 6-well plate at a concentration of 1×10^6 cells/well and incubated for 24 h. After treating the wells with various concentrations of EYPH (25, 50, 100, and 200 μg/mL), the wells were incubated for an additional 24 h. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Milan, Italy) and converted to cDNA using the Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Carlsbad, CA). SYBR green reagent (PhileKorea, Daejeon, Korea) and PikoReal Real-Time PCR System (Thermo Fisher Scientific) were used to measure the expression levels of iNOS mRNA. The delta-delta Ct method was used to analyze the amplified mRNA levels, and β-actin was used as the housekeeping gene. The primer sequences used in this study are listed in Table 1.

Measurement of Pro-Inflammatory Cytokine Production

The effects of EYPH on the expression and production of pro-inflammatory cytokines (TNF-α and IL-6) were determined using qRT-PCR and ELISA. qRT-PCR analysis was performed to confirm the effect of expression of TNF-α and IL-6 at the mRNA level using the same method as described above. ELISA was performed to confirm the effect of the production of TNF-α and IL-6 at the protein level. Briefly, RAW 264.7 cells were added to a 12-well plate at a concentration of 4×10^5 cells/well and incubated for 24 h. After treating the

Table 1. qRT-PCR primer sequence.

Genes	Primer sequence (5' - 3')
iNOS	Forward: CCCTTCCGAAGTTTCTGGCAGCAGC Reverse: GGCTGTCAGAGCCTCGTGGCTTTGG
TNF-α	Forward: TTGACCTCAGCGCTGAGTTG Reverse: CCTGTAGCCCACGTCGTAGC
IL-6	Forward: GTACTCCAGAAGACCAGAGG Reverse: TGCTGGTGACAACCACGGCC
β-actin	Forward: GTGGGCCGCCCTAGGCACCAG Reverse: GGAGGAAGAGGATGCGGCAGT

wells with various concentrations of EYPH (25, 50, 100, and 200 μg/mL), the wells were incubated for an additional 24 h. Levels of pro-inflammatory cytokines in the supernatant were analyzed using an ELISA kit according to the manufacturer's instructions.

Determination of Phagocytic Activity

The effect of EYPH on the phagocytic activity of RAW 264.7 cells was measured using the neutral red uptake method (Tang et al., 2019). RAW 264.7 cells were added to a 24 well plate at a concentration of 2×10^5 cells/well and incubated for 4 h. After treating the wells with various concentrations of EYPH (25, 50, 100, and 200 μg/mL), the wells were incubated for an additional 24 h. Thereafter, the supernatant was removed and a neutral red solution (0.075% in PBS) was added for 1 h. Finally, the wells were washed with PBS three times and then lysis solution (acetic acid: ethanol = 1:1, v/v) was added to the wells to dissolve the neutral red. The absorbance of the cell plate was measured using a microplate reader at 540 nm wavelength.

Investigation of Cell Signaling Pathway

To confirm whether the MAPK signaling pathway is involved in the activation of RAW 264.7 cells by EYPH, the effect of EYPH treatment on NO production in RAW 264.7 cells was confirmed after pretreatment with a MAPK pathway-specific inhibitor (p38, SB 202190; JNK, SP 600125; ERK, PD 98059). RAW 264.7 cells were added to a 12-well plate at a concentration of 4×10^5 cells/well and incubated for 24 h. The cells were then incubated with a specific inhibitor (40 μM of SB 202190, PD 98059, and 20 μM of SP 600125) and 200 μg/mL of EYPH for 8 h. Finally, the cells were replaced with a fresh medium and cultured for an additional 16 h, and the amount of NO production was determined using Griess reagent.

Investigation of Cell Membrane Receptors

To determine the receptor of EYPH-PN, RAW 264.7 cells were treated with TLR-2 and TLR-4 antibody (10 μg/mL) for 1 h to block each receptor. Then, 200 μg/mL of EYPH was added to each antibody-treated cell and non-treated cell (control group), and

incubated for a further 24 h. Finally, the amount of NO production was determined using Griess reagent.

In vitro Intestinal Digestion Model

Subsequent hydrolysis was performed using some enzymes (pepsin, trypsin, and α-chymotrypsin) to perform simulations of the gastric and small intestine digestion processes as described by Vercruysse et al. (2009), with slight modifications. First, for the digestion in the stomach step, EYPH was dissolved in distilled water, and the pH of the solution was lowered to 2 with 4 M HCl. Pepsin was added to the solution at an E/S ratio of 1/250 and incubated for 2 h at 37°C. Second, for the digestion in the small intestine step, trypsin and α-chymotrypsin were added to the solution at an E/S ratio of 1/250 after the solution pH was adjusted to 6.5, with 10 M NaOH, and incubated for an additional 2.5 h at 37°C. Finally, the enzymes were inactivated by heating the solution for 10 min at 100°C, the solution was centrifuged at 3,000 × g for 20 min, and the supernatant was lyophilized.

Statistical Analysis

All results are presented as the mean ± standard deviation (**SD**) from triplicate measurements of the analyses, and the data were analyzed with SPSS statistics (version 20.0; SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA, followed by Duncan's multiple test) and Student's t-test were applied to measure the significance of the difference in mean values between samples.

RESULTS AND DISCUSSION

Effects of EYPHs on the Cell Viability and the Production of NO and iNOS

The effects of hydrolysates on the viability of RAW 264.7 macrophages were determined using the MTT assay following the treatment of cells with each hydrolysate (25, 50, 100, and 200 μg/mL) for 24 h. As shown in Figure 1, none of the four enzymatic hydrolysates showed cytotoxic effects on RAW 264.7 macrophages at any of the tested concentrations. Therefore, to evaluate the immunomodulatory activity, the following experiments were conducted using hydrolysate concentrations of less than 200 μg/mL.

NO is a molecule that acts as a mediator of inflammation and is produced by activated macrophages. Its role is to eliminate tumor cells, invade pathogenic microorganisms, and mediate various biological functions, such as vasodilation, neurogenesis, and wound healing (Li et al., 2015). The effects of EYPHs on NO production in RAW 264.7 macrophages are shown in Figure 2A. Among the 4 enzymatic hydrolysates, only EYPH-PN (hydrolyzed using pancreatin and neutrase in sequence) significantly stimulated NO production in a dose-dependent manner ($P < 0.05$). In contrast, it was confirmed that the one-step hydrolysates (EYPH-N and EYPH-P) and EYPH-NP (hydrolyzed using neutrase and pancreatin in sequence) had no effect on NO production in macrophages. At the 200 μg/mL concentration, EYPH-PN increased the NO amount to 25.10 ± 0.84 μM, which is higher than that of LPS (10 ng/mL) treatment. By treating macrophages with 100, 50, and 25 μg/mL of EYPH-PN, 22.55 ± 0.74, 22.19 ± 1.07, and 21.70 ± 0.77 μM of NO was produced, respectively. Among the various isoforms of NO synthase, NO is synthesized by iNOS (Lee et al., 2017b). Therefore, in this study, the amount of iNOS mRNA expression in macrophages treated with EYPH-PN was measured to confirm the relationship between NO production and iNOS expression. As shown in Figure 2B, iNOS mRNA expression was increased by EYPH-PN treatment. This result suggests that increased NO production in RAW 264.7 macrophages after EYPH-PN treatment may result from increased iNOS expression. Similar results have been reported in studies of other functional substances
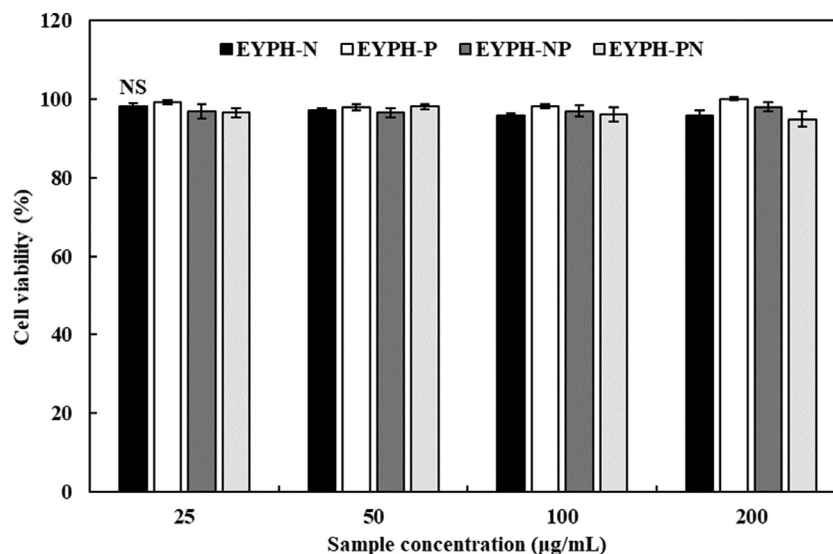

Figure 1. Effects of EYPHs on cell viability of RAW 264.7 macrophages. Values are expressed as the mean ± SD. NS: not significant difference between samples ($P > 0.05$).
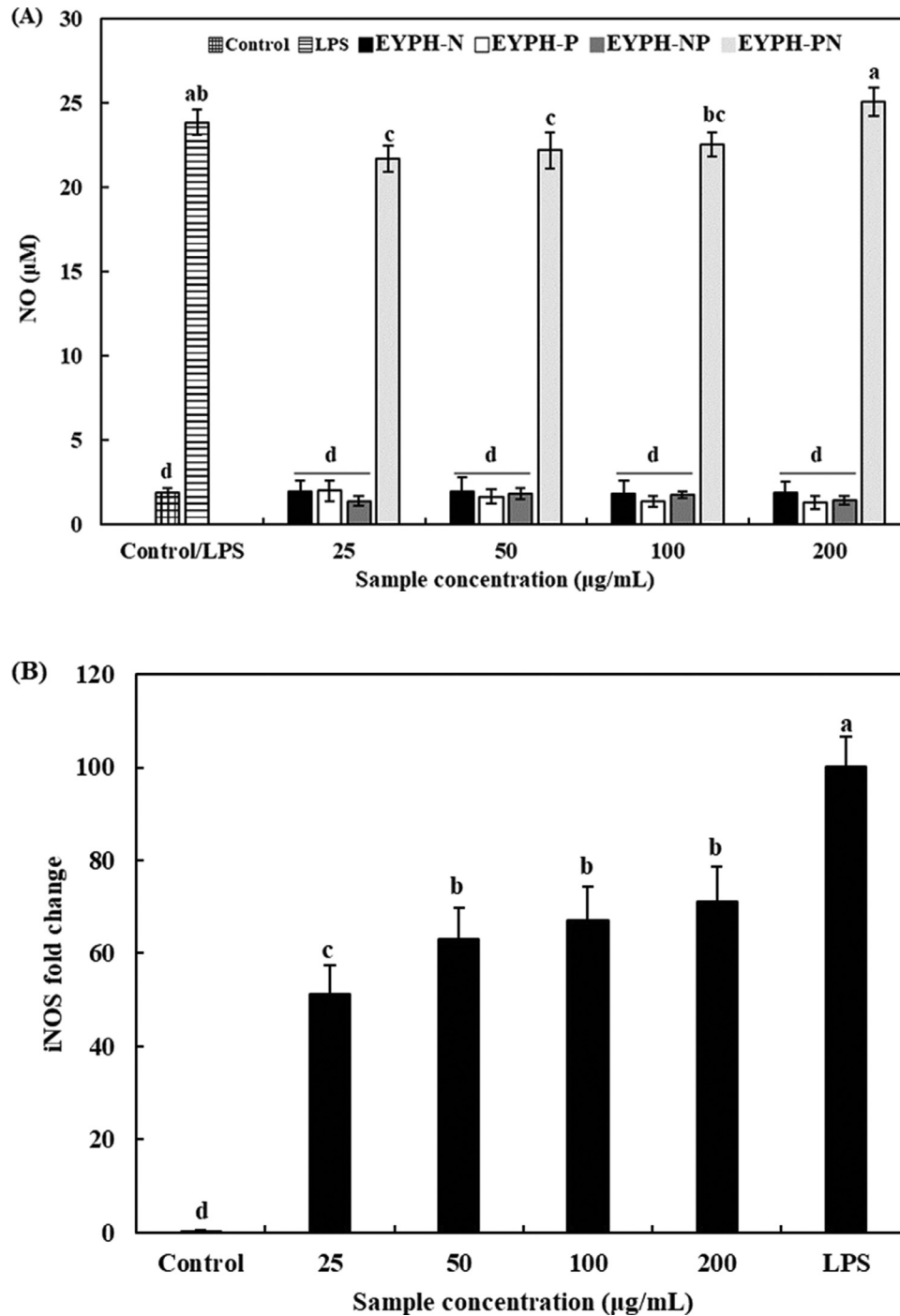
Figure 2. Effects of EYPHs on the production of NO (A) and effects of EYPH-PN on the secretion of iNOS mRNA (B) in RAW 264.7 macrophages. Control: only medium-treated group. Values are expressed as the mean ± SD. Different lowercase alphabet letters were significantly different ($P < 0.05$).

(e.g., polysaccharides, protein hydrolysates, and herbal extracts) showing increased NO production due to increased iNOS expression (Shen et al., 2017; Yang et al., 2019; Geum et al., 2020). In the above studies, various functional substances significantly increased the expression level of iNOS in macrophages, thereby increasing the production of NO. In addition, the authors explained that this increased NO could be of great help in improving immunity.

In this study, of the four enzymatic hydrolysates, only EYPH-PN increased iNOS expression and promoted NO production. This means that when hydrolysis was performed with pancreatin and neutrase alone, there was no production of peptides that promoted NO production. In addition, in the case of EYPH-NP, which was hydrolyzed in the order neutrase and then pancreatin, it was confirmed that there was no production of peptides that promoted NO production, even though it was the same 2-step hydrolysate as EYPH-PN. This means that the enzymes used in the 2-step hydrolysis and the sequence of hydrolysis are important. This is because proteins do not consist of simple amino acids, but have a complex tertiary structure (Sbroggio et al., 2016). This means that, depending on the type of the

primary enzyme, the binding site to which the secondary enzyme can bind may be exposed to the outside or buried in the protein core (Sbroggio et al., 2016). Due to this, if the order of the enzymes is changed, the type of peptide produced may also be changed Huang et al. (2019). reported that 2-step hydrolysis increases the degree of hydrolysis of hydrolysates and imparts a diversity of peptides present in hydrolysates. Moreover, it has been reported that 2-step hydrolysis is a good strategy for producing functional peptides that is more effective than single enzymatic hydrolysis (Asoodeh et al., 2016; Lee et al., 2017a). Based on the experimental results, the following experiments were conducted using only EYPH-PN.

Effects of EYPH-PN on the Expression and Production of Pro-Inflammatory Cytokines

Inflammatory cytokines are known to play an important role in the inflammatory reaction and are known to be involved in regulating the proliferation, differentiation, and function of immune cells (Kubo et al., 2003). Therefore, the cytokine levels of macrophages are being applied as an evaluation method to evaluate the immune-modulating capacity of bioactive compounds (Wu et al., 2018).

The effects of EYPH-PN on the expression and production of pro-inflammatory cytokines in RAW 264.7 macrophages were determined using qRT-PCR and ELISA. As shown in Figure 3A and B, EYPH-PN treatment stimulated the expression of pro-inflammatory cytokines (TNF-α and IL-6) in RAW 264.7 macrophages. It was confirmed that the mRNA expression levels of both TNF-α and IL-6 increased in a dose-dependent manner following EYPH-PN treatment ($P < 0.05$). Compared to LPS used as a positive control, when the concentration of EYPH-PN was 200 μg/mL, the expression levels of TNF-α and IL-6 mRNA increased to 70.58% and 60.77%, respectively. These results suggested that treatment with EYPH-PN upregulated the secretion of pro-inflammatory cytokines in RAW 264.7 macrophages. To confirm the effect of EYPH-PN on the production of TNF-α and IL-6 at the protein level, an ELISA was conducted. As shown in Figure 3C and D, it was confirmed that the production of TNF-α and IL-6 in macrophages increased in a dose-dependent manner following treatment with EYPH-PN. Following treatment with 200 μg/mL EYPH-PN, it was confirmed that TNF-α and IL-6 were produced at levels of 47.11 ng/mL and 16.02 ng/mL, respectively. This was similar to the effect on mRNA expression. Furthermore, it was confirmed that EYPH-PN can exert immune-modulating effects by regulating pro-inflammatory cytokines in macrophages at the mRNA and protein levels.
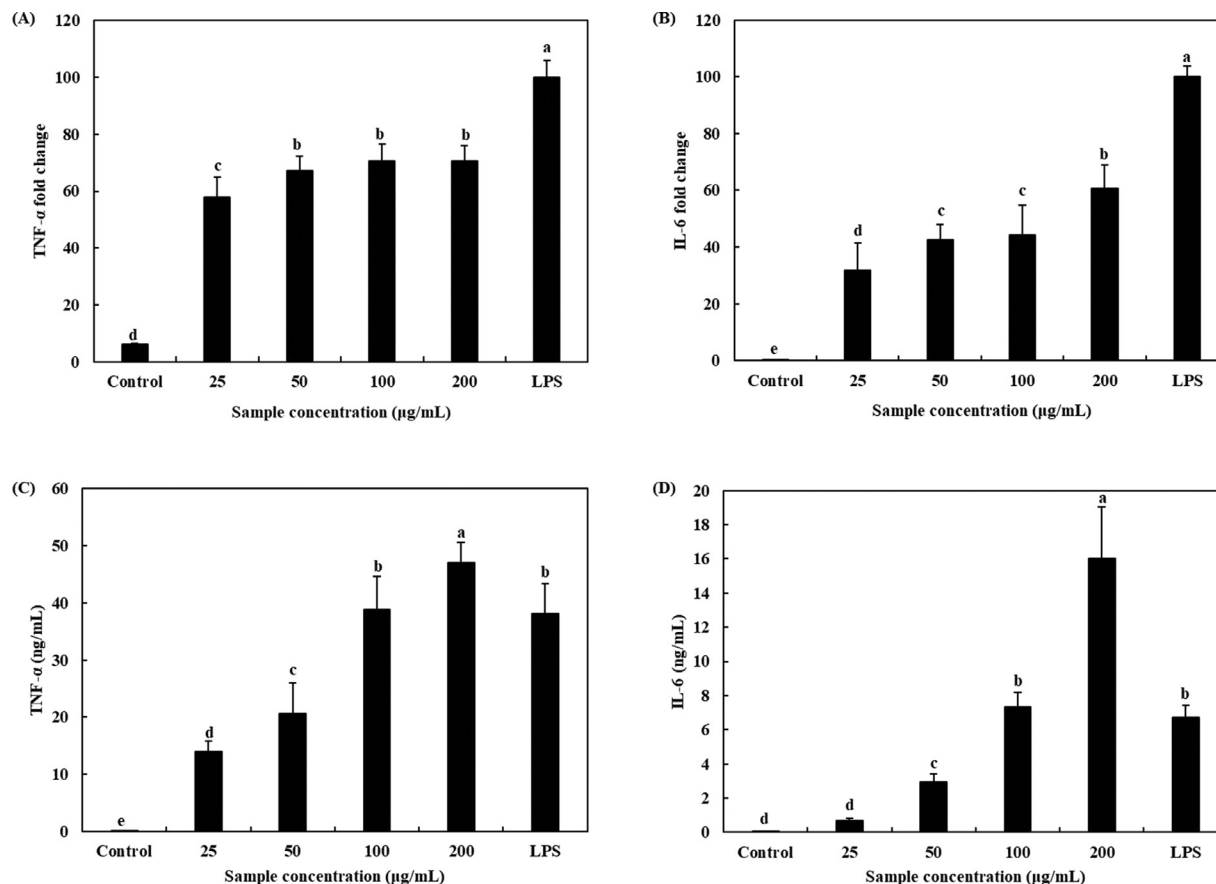

Figure 3. Effects of EYPH-PN on the production of TNF-α and IL-6 at the mRNA level (A,B) and the protein level (C,D) by RAW 264.7 macrophages. Control: only medium-treated group. Values are expressed as the mean ± SD. Different lowercase alphabet letters were significantly different ($P < 0.05$).

Many studies have confirmed the immune-modulating activity of various functional substances by confirming the induced production of pro-inflammatory cytokines (Shen et al., 2017; Wu et al., 2018; Geum et al., 2020). TNF-α is an essential cytokine for innate immune reactions and is one of the first cytokines released when macrophages are activated (Shen et al., 2017). TNF-α can induce the secretion of other inflammatory cytokines and plays an important role in macrophage proliferation and function (Yang et al., 2019). In addition, IL-6 acts as a signaling molecule that links innate and adaptive immune responses (Wu et al., 2018). It is known that IL-6 not only stimulates the production of macrophages and neutrophils in innate immunity, but also plays an important role in the maturation of B cells in adaptive immunity (Naugler and Karin, 2008). Therefore, the production of appropriate pro-inflammatory cytokines by EYPH-PN contributes to immune enhancement.

Effects of EYPH-PN on the Phagocytic Activity

The effects of EYPH-PN on the phagocytic activity of RAW 264.7 macrophages were determined using a neutral red uptake assay. As shown in Figure 4, compared with the non-treated group, EYPH-PN treatment increased the phagocytic activity of RAW 264.7 macrophages in a dose-dependent manner ($P < 0.05$). It was confirmed that the phagocytic activity increased to 146.22% at the EYPH-PN concentration of 200 μg/mL, whereas the LPS-treated group used as a positive control showed a phagocytic activity of 128.31%. The phagocytic activity of macrophages is an important step in the response to pathogens and cellular debris and is one of the distinguishing features of activated macrophages (Li et al., 2015). EYPH-PN significantly promoted the phagocytic activity of macrophages, suggesting that EYPH-PN enhanced the function of

macrophages. Similar to our results, Cheng et al. (2019) reported that polysaccharides isolated from mushrooms exhibit immunomodulatory activity by increasing the phagocytic activity of macrophages.

The Roles of TLR/MAPK Pathways in the Production of NO Induced by EYPH-PN in RAW 264.7 Cells

EYPH-PN produced from egg yolk extract protein using 2-step hydrolysis induced macrophage activation and showed immunomodulatory activities such as NO production, expression and production of pro-inflammatory cytokines, and increased phagocytosis (Figures 2−4).

To confirm whether the MAPK pathways were involved in the activation of macrophages by EYPH-PN, cells were co-treated with MAPK pathway-specific inhibitors (p38, ERK, and JNK). As shown in Figure 5A, co-treatment with p38 and JNK pathway-specific inhibitors significantly reduced NO production in macrophages induced by EYPH-PN treatment ($P < 0.001$). Compared to the EYPH-PN treatment group, the p38 pathway inhibitor (SB 202190) and JNK pathway inhibitor (SP 600125) treatment groups reduced NO production in macrophages by 59.55% and 32.60%, respectively. However, it was confirmed that the ERK pathway inhibitor (PD 98059) treatment group had no effect on NO production following EYPH-PN treatment. The important function of the MAPK pathway is the regulation of the innate immune response and adaptive immunity (Liu et al., 2007). There are also 3 MAPK pathways: p38, ERK, and JNK pathways in mammalian cells (Lim et al., 2021). These pathways are involved in the production of pro-inflammatory mediators, such as NO and pro-inflammatory cytokines. The results of our study showed that the production of NO induced by EYPH-PN treatment was inhibited by treatment with
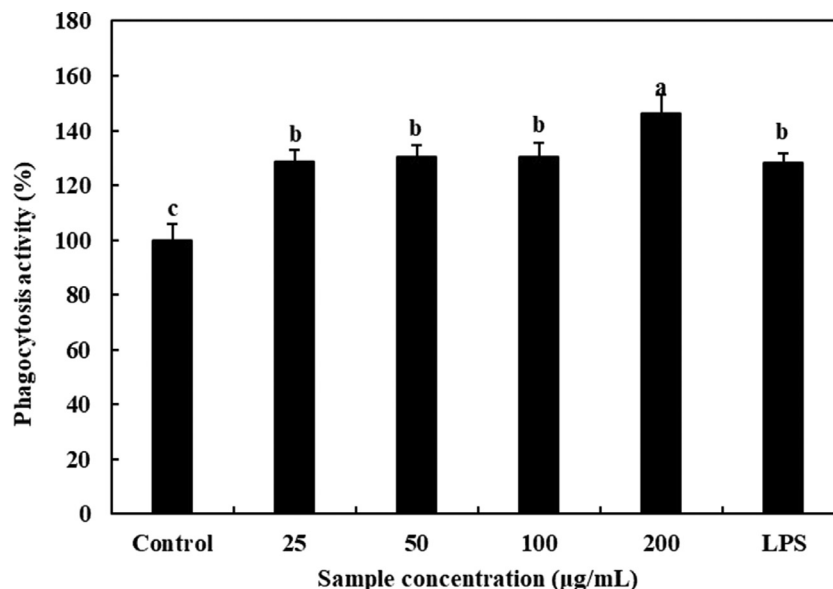

Figure 4. Effects of EYPH-PN on the phagocytic activity of RAW 264.7 macrophages. Control: only medium-treated group. Values are expressed as the mean ± SD. Different lowercase alphabet letters were significantly different ($P < 0.05$).
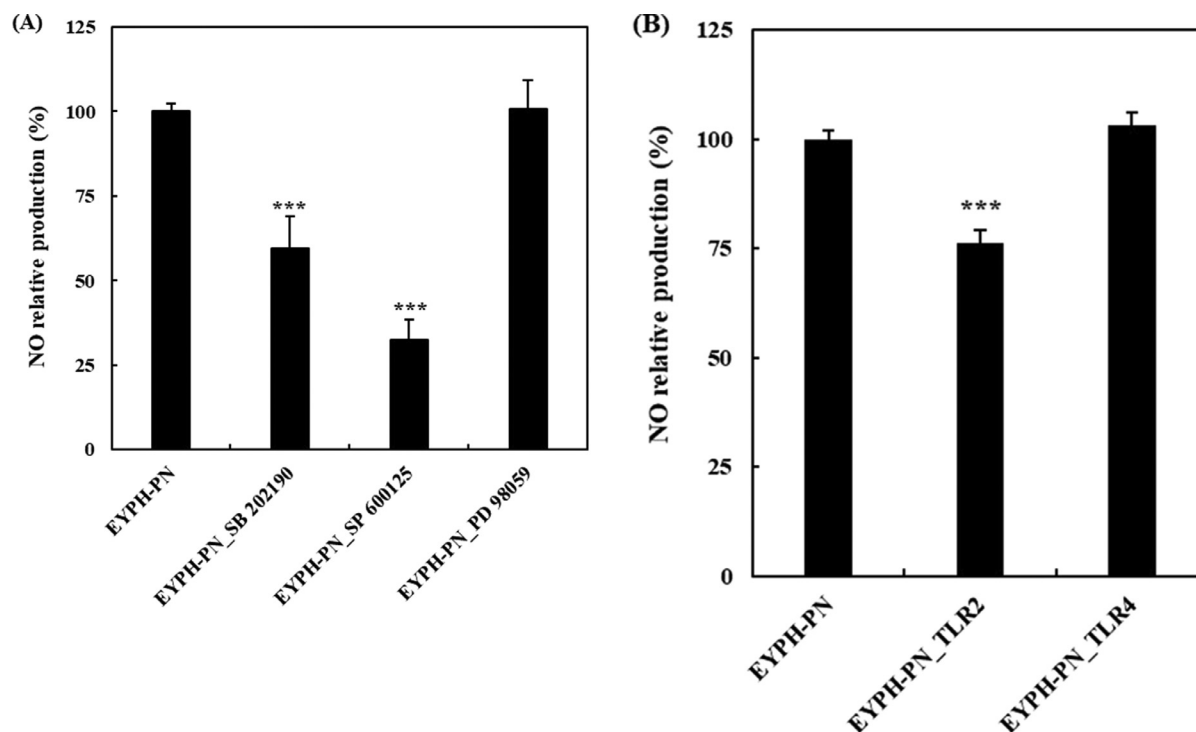
Figure 5. Roles of MAPK pathways (A) and TLR-2, TLR-4 on EYPH-PN (200 μg/mL) in induced NO production by RAW 264.7 macrophages. Values are expressed as the mean $\pm$ SD. *** mean statistical difference for $P < 0.001$ (Student's t-test) compared with EYPH-PN group (without treat inhibitor).

specific inhibitors of these pathways, indicating that EYPH-PN macrophage activation occurs via the p38 and JNK pathways. According to Ha et al. (2013), the activation of macrophages by lysozyme through the JNK pathway was inhibited by a specific inhibitor, resulting in a decrease in the production of pro-inflammatory cytokines. In addition, phosvitin, an egg yolk protein, increased NO production by inducing macrophage activation, but it was confirmed that NO production was decreased when NF-κB, Akt, and MAPK pathway inhibitors were co-treated (Lee et al., 2017b). This finding is similar to our results. In addition, another representative egg yolk protein, IgY, has been reported to have an immunomodulatory activity by effectively reducing overproduced TNF-α caused by *Salmonella typhimurium* infection in a mouse model (Li et al., 2016).

To confirm whether the TLR pathway was involved in the activation of macrophages by EYPH-PN, the cell membrane receptor of macrophages was blocked with anti-TLR2 and anti-TLR4. As shown in Figure 5B, compared with the EYPH-PN treatment group, the group pretreated with anti-TLR2 showed a significant decrease in NO production ($P < 0.001$). Anti-TLR2 pretreatment reduced the NO production level of macrophages by EYPH-PN treatment by 23.67%. However, no significant decrease in NO levels was observed in the groups pretreated with anti-TLR4. The recognition of pattern recognition receptors (**PRR**s), such as TLRs, in cells is important for triggering intracellular signaling pathways that play a variety of roles in immune responses (Liao et al., 2015). When blocking was performed using an antibody of TLR-2, the reduction in NO production by EYPH-PN treatment means that EYPH-PN

stimulated the immune response by mainly activating RAW 264.7 cells through binding to TLR-2 and not TLR-4. Similar to our results, Wu et al. (2018) also reported that the amount of NO produced by CS (chondroitin sulfates; one of the glycosaminoglycans) decreased when the TLR-2 receptor was blocked. It has been reported that this means that the TLR-2 receptor is utilized as the main receptor for CS-induced macrophage activation. It is known that TLR-2 binds to bacterial lipoproteins, and TLR-4 is mainly bound by bacterial lipopolysaccharides (Roshan et al., 2016). Therefore, it is thought that binding to TLR-2 was caused by the lipoprotein present in EYPH-PN. This is because egg yolk contains a large proportion of lipoproteins (Kovacs-Nolan et al., 2005). Therefore, it can be deduced that TLR-2 is a major receptor for EYPH-PN in activating macrophages, and it was confirmed that macrophage activation was induced through the p38 and JNK signaling pathways.

In vitro Intestinal Digestion Model

In general, food-derived proteins and peptides are decomposed by various enzymes after ingestion in the body, resulting in loss of functionality (Wang et al., 2019). Therefore, an in vitro digestion model was used to determine whether the functionality of EYPH-PN was maintained even after treatment with digestive enzymes (Vercruysse et al., 2009).

After applying the digestion model using pepsin, trypsin, and α-chymotrypsin, the amount of NO produced was measured to confirm the change in the macrophage
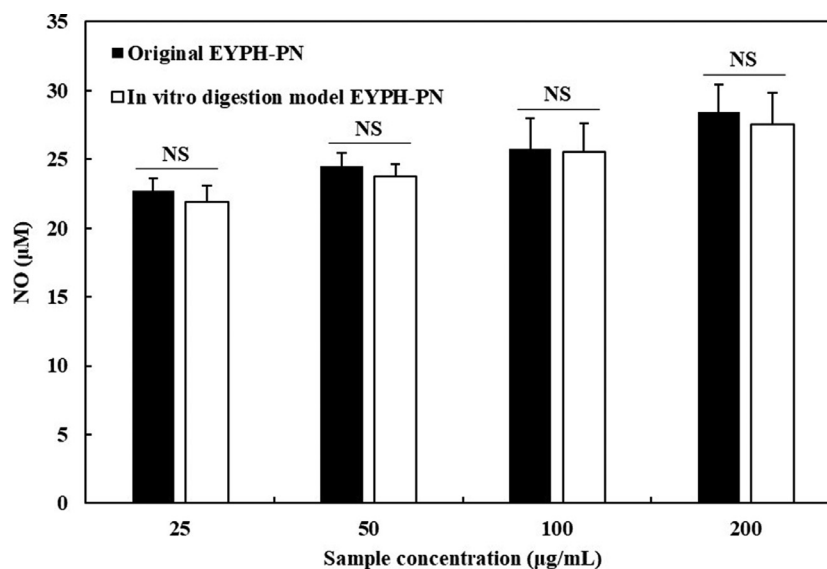
Figure 6. Changes in the effects of EYPH-PN on NO production after applying in vitro digestion model. Values are expressed as the mean ± SD. NS: not significant difference between samples ($P > 0.05$).

activation activity of EYPH-PN. As shown in Figure 6, it was confirmed that there was no difference in the NO production amount of macrophages according to EYPH-PN treatment even after the in vitro digestion model was applied ($P > 0.05$). According to Chen and Li (2012), peptides over 3 kDa can be more easily degraded during gastric digestion than peptides below 3 kDa. They also reported that low-molecular-weight peptides showed excellent structural stability. In the case of EYPH-PN produced by 2-step hydrolysis, it was confirmed that the molecular weight was 3 kDa or less by SDS-PAGE analysis (data not shown), and this was thought to be the reason why the immunomodulatory activity of EYPH-PN was maintained even after the in vitro digestion model was applied.

CONCLUSIONS

In the present study, we found that EYPH-PN produced by 2-step hydrolysis using egg yolk protein exhibited immunomodulatory activity in RAW 264.7 macrophages. EYPH-PN increased the production of NO in macrophages by increasing the secretion of iNOS mRNA. In addition, we confirmed that EYPH-PN affects the production of TNF-α and IL-6 at both the mRNA and protein levels of macrophages. Finally, EYPH-PN increased the phagocytic activity of macrophages. When macrophages were treated with EYPH-PN, it was confirmed that the TLR-2 receptor of macrophages acted as the main receptor, and macrophages were activated through p38 and JNK cell signaling pathways. Furthermore, the immunomodulatory activity of EYPH-PN was maintained even after applying the in vitro digestion model. EYPH-PN showed excellent immunomodulatory activity, but in the case of single enzymatic hydrolysates EYPH-P and EYPH-N, it was confirmed that there was no effect on macrophage activation, confirming that 2-step hydrolysis was excellent for functional peptide production. In addition, when it

was confirmed that EYPH-NP, which had undergone 2-step hydrolysis in the order of neutrase and pancreatin, had no immunomodulatory activity, it was confirmed that the sequence of enzymes is also important in the 2-step hydrolysis. Taken together, these results suggest that EYPH-PN could be used as a functional food ingredient in the food industry. This has not only developed novel functional materials for immune modulation, but also presented a new alternative method to effectively utilize egg yolk protein, a by-product of the poultry industry.

DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that might influence the work reported in this paper.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.101802.

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