1 **TITLE PAGE**
2 **- Korean Journal for Food Science of Animal Resources -**
3 **Upload this completed form to website with submission**
4

ARTICLE INFORMATION	Fill in information in each box below
Article Title	Ovalbumin hydrolysates inhibit nitric oxide production in LPS-induced RAW 264.7 macrophages
Running Title (within 10 words)	NO inhibitory activity of ovalbumin hydrolysates
Author	Hyun Suk Kim1, Jae Hoon Lee1, Sun Hee Moon2, Dong Uk Ahn3, Hyun-Dong Paik1
Affiliation	1 Department of Food Science and Biotechnology of Animal Resources, Konkuk University, Seoul 05029, Korea 2 Department of Environmental and Occupational Health, University of Arkansas for Medical Science, Little Rock, Arkansas 72205, USA 3 Department of Animal Science, Iowa State University, Ames, Iowa 50011, USA
Special remarks – if authors have additional information to inform the editorial office	
ORCID (All authors must have ORCID) https://orcid.org	Hyun Suk Kim (https://orcid.org/0000-0002-1261-5008) Jae Hoon Lee (https://orcid.org/0000-0002-7440-6842) Sun Hee Moon (https://orcid.org/0000-0002-4787-3912) Dong Uk Ahn (https://orcid.org/0000-0001-9523-7630) Hyun-Dong Paik (https://orcid.org/0000-0001-9891-7703)
Conflicts of interest List any present or potential conflict s of interest for all authors. (This field may be published.)	The authors declare no potential conflict of interest.
Acknowledgements State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available. (This field may be published.)	This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture and Forestry (IPET) through High Value-added Food Technology Development Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA)(118037-3).
Author contributions (This field may be published.)	Conceptualization: Paik HD, Ahn DU. Data curation: Kim HS, Lee JH, Moon SH. Formal analysis: Kim HS, Lee JH. Methodology: Kim HS, Lee JH, Moon SH. Validation: Lee JH, Moon SH. Writing - original draft: Kim HS, Lee JH. Writing - review & editing: Paik HD, Ahn DU, Lee JH, Kim HS, Moon SH.
Ethics approval (IRB/IACUC) (This field may be published.)	This manuscript does not require IRB/IACUC approval because there are no human and animal participants.

5
6 **CORRESPONDING AUTHOR CONTACT INFORMATION**

For the corresponding author (responsible for correspondence, proofreading, and reprints)	**Fill in information in each box below**
First name, middle initial, last name	Hyun-Dong Paik
Email address – this is where your proofs will be sent	hdpaik@konkuk.ac.kr
Secondary Email address	
Postal address	Department of Food Science and Biotechnology of Animal Resources, Konkuk University, Seoul 05029, Korea
Cell phone number	82-10-4586-6189
Office phone number	82-2-2049-6011
Fax number	82-2-455-3082

7
8

ACCEPTED

Abstract

In this study, ovalbumin (OVA) hydrolysates were prepared using various proteolytic enzymes and the anti-inflammatory activities of the hydrolysates were determined. Also, the potential application of OVA as a functional food material was discussed. The effect of OVA hydrolysates on the inhibition of nitric oxide (NO) production was evaluated via the Griess reaction, and their effects on the expression of inducible nitric oxide synthase (iNOS) were assessed using the quantitative real-time PCR and Western blotting. To determine the mechanism by which OVA hydrolysates activate macrophages, pathways associated with the MAPK signaling were evaluated. When the OVA hydrolysates were added to RAW 264.7 cells without lipopolysaccharide (LPS) stimulation, they did not affect the production of NO. However, both the OVA-Protex 6L hydrolysate (OHPT) and OVA-trypsin hydrolysate (OHT) inhibited NO production dose-dependently in LPS- stimulated RAW 264.7 cells. Especially, OHT showed a strong NO-inhibitory activity (62.35% at 2 mg/mL) and suppressed iNOS production and the mRNA expression for iNOS ($p<0.05$). Also, OHT treatment decreased the phosphorylation levels of JNK and ERK in the MAPK signaling pathway. These findings suggested that OVA hydrolysates could be used as an anti-inflammatory agent that prevent the overproduction of NO.

Keywords: Ovalbumin, egg white protein, nitric oxide, anti-inflammatory activity, MAPK pathway

ACCEPTED

Introduction

Inflammation is an immune response that can be activated by various conditions and stimuli which involve the injury of tissue or infection (Medzhitov, 2008). Immune cells and inflammatory mediators are known to be involved in the inflammatory process. Nitric oxide (NO) is one of the inflammatory mediators that play a variety of roles in physiological and pathological immune responses (Chang et al., 2019). NO is synthesized by the nitric oxide synthase (NOS) family, which consisting neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). Under normal conditions, nNOS and eNOS produce the required amount of NO that regulates cell proliferation and survival, while iNOS produces large amounts of NO to enhance immunity (Lundberg et al., 1997). However, the overproduction of NO has been associated with the progression of many diseases, including chronic inflammation, Alzheimer's disease, diabetes mellitus, and cancer (Dail-Youcef et al., 2013; Sharma et al., 2007). Therefore, new compounds that inhibit the overproduction of NO are considered candidates for the production of novel anti-inflammatory agents.

Macrophages play a vital role in immune reactions, and the functions of macrophages that include phagocytosis, antigen presentation, and NO and inflammatory cytokine production (Lee et al., 2017b). Macrophages produce pro-inflammatory factors via several cell signaling pathways, including nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) pathways when they are activated by lipopolysaccharides (LPS), which are one of the main sources of stimulation for macrophages (Ham et al., 2015; Li et al., 2017). The MAPK pathways have three different main families, which are Jun amino-terminal kinases (JNK) pathway, extracellular signal-regulated kinases (ERK) pathway, and p38 pathway (Ham et al., 2015). This has made the MAPK pathways the primary targets for the development of novel anti-inflammatory agents and has placed them at the center of this field of research (Seo et al., 2015).

Many peptides produced from various protein sources, such as soy (de Mejia and Dia, 2009), fish (Ahn et al., 2015) and lupine (Millán-Linares et al., 2014), are known to have anti-inflammatory properties. These peptides inhibit the production of pro-inflammatory cytokines and NO in immune cells after stimulation. de Mejia and Dia (2009) reported that peptides derived from soy proteins suppressed the NF-

ACCEPTED

κB pathway and inhibited the production of inflammatory cytokines in LPS stimulated RAW 264.7 macrophages. Some egg proteins were also used to prepare anti-inflammatory peptides: Ovotransferrin peptides (IRW) inhibited the production of several cell adhesion molecules associated with the onset and progression of inflammation in human endothelial cells (Huang et al., 2010). Sun et al. (2016) reported that ovomucin peptides have anti-inflammatory activities by suppressing the NF-κB pathway. Ovalbumin (OVA) has important functional properties that make it useful for gelling, foaming, and emulsifying of food products. Many studies have shown that the hydrolysates or peptides from OVA possess various bioactivities, including antioxidants (Abeyrathne et al., 2014), antihypertensive (Matoba et al., 1999), and antimutagenic effects (Vis et al., 1998). However, few studies have assessed the anti-inflammatory activity of OVA hydrolysates in relation to their inhibitory effects on the production of NO in immune cells.

The objectives of present study were to determine the effect of OVA hydrolysates on the production of NO and the expression of iNOS mRNA, and elucidate the anti-inflammatory mechanism of OVA hydrolysates in LPS-stimulated RAW 264.7 cells.

Materials and Methods

Materials and reagents

Ovalbumin was isolated from egg white using the method of Abeyrathne et al. (2013). Briefly, egg white was diluted with an equal volume of distilled water, homogenized for 1 min using a hand-blender (Kitchen-Aid) at high speed (set at 9) and used. Lysozyme, ovomucin, and ovotransferrin were sequentially removed from the diluted egg white using the method developed by Abeyrathne et al. (2014). Lysozyme was removed first from the egg white solution: Amberlite FPC 3500 resin (5 g/100 mL diluted egg white) was added to the diluted egg white solution and stirred overnight in a 4 °C walk-in cooler using an overhead stirrer set at 250 rpm (RW20 digital, IKA Works Inc., Wilmington, NC, USA) to trap lysozyme. After filtering out the resins, the pH of the lysozyme-free egg white solution was adjusted to 4.75 using 3 N HCl to precipitate ovomucin. The precipitated ovomucin was removed by centrifugation at 3,500 × g for 30 min at 4°C (Sorvall Evolution RC superspeed centrifuge, Thermo

ACCEPTED

79 Scientific, Waltham, MA, USA), and then the lysozyme- and ovomucin-free egg white was added with
80 ammonium sulfate (5.0%, wt/vol) and citric acid (2.5%, wt/vol) and held for 12 h at 4 °C to precipitate
81 ovotransferrin, which was removed by centrifugation at 3,500 × g for 30 min. The resulting supernatant,
82 which was mainly composed of ovalbumin and ovomucoid, was added with 100% ethanol to the final
83 concentration of 35%, and then centrifuged. After removing the residual ovomucoid using 4 volumes
84 of 35% of ethanol, the resulting precipitant was washed with 4 volumes of distilled water twice, the pH
85 adjusted to pH 12.0 to dissolve ovalbumin, and then brought the pH down to 10.0 using 1N HCl. Finally,
86 the dissolved ovalbumin was heated at 70 ºC for 15 min to precipitate impurities, centrifuged, and then
87 lyophilized using a freeze-dryer (Labconco, Kansas City, MO, USA). The purity and yield of the isolated
88 ovalbumin was 97% and 97.7%.

89 Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, and
90 phosphate-buffered saline (PBS) were purchased from Hyclone Laboratories, Inc. (Logan, MI, USA). N-
91 (1-naphthyl)-ethylenediamine, sulfanilamide, thiazolyl blue tetrazolium bromide (MTT),
92 lipopolysaccharides (LPS), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St.
93 Louis, MO, USA). For PCR analysis, RNeasy Mini Kit for total RNA isolation was purchased from Qiagen
94 (Milan, Italy), RevertAid™ First Strand cDNA Synthesis Kit was purchased from Thermo Fisher Scientific
95 (Carlsbad, CA, USA), and SYBR reagent was purchased from PhileKorea (Daejeon, Korea). For Western
96 blotting analysis, cell lysis buffer, inhibitor cocktail containing protease and phosphatase inhibitor, and a
97 Protein Assay Kit were purchased from Thermo Fisher Scientific. Primary antibodies for total or
98 phosphorous from MAPKs (p38, JNK, and ERK), iNOS, and β-actin were obtained from Santa Cruz
99 Biotechnology (Dallas, TX, USA). A secondary antibody (Goat anti-mouse IgG-HRP) and other reagents
100 for western blotting were purchased from Bio-Rad (Hercules, CA, USA). All other chemicals used were
101 of analytical reagent grade.

102

103

ACCEPTED

104 **Preparation of OVA hydrolysates**

105 Because the functional activity of peptides varies depending on their amino acid sequence, length, and
106 structural characteristics (Hartmann and Meisel, 2007), four different proteolytic enzymes were used in
107 this study. OVA (10 mg/mL) were hydrolyzed using one of the following proteases: Trypsin (from bovine
108 pancreas, EC 3.4.21.5. Sigma-Aldrich), Promod 278P (endopeptidase, EC 3.4.24.28, BISION, Sungnam,
109 Korea), Multifect PR 14L (thermophilic-bacterial protease from *Geobacillus stearothermophilus*, EC
110 3.4.24.27, BISION), and Protex 6L (alkaline protease from *Bacillus licheniformis*, EC 3.4.21.65, BISION).
111 Enzymes were added at 1:50 (enzyme: substrate) ratio, and then the mixture was incubated for 4 h under
112 optimal conditions: Trypsin (pH 8.0, 40°C), Promod 278P (pH 6.5, 45°C), Multifect PR 14L (pH 8.0, 70°C),
113 and Portex 6L (pH 7.0, 60°C). To inactivate the enzymes, the mixture was heated for 10 min at 100°C, and
114 then centrifuged at 2,000 × *g* for 20 min. The supernatant was lyophilized using a freeze-dryer. The OVA
115 hydrolysates produced by Trypsin, Promod 278P, Multifect PR 14L, and Protex 6L were named as OHT,
116 OHPM, OHMF, OHP, respectively, and the size of OVA hydrolysates was determined using SDS-PAGE
117 (15% gel).

118

119 **Cell lines and culture conditions**

120 The RAW 264.7 cell line, which is a murine macrophage, was purchased from the KCLB (Korean Cell
121 Line Bank, Seoul, Korea). The medium used for the growth of RAW 264.7 cells was DMEM that contained
122 1% penicillin-streptomycin and 10% FBS (heat-inactivated). RAW 264.7 cells were grown at 37°C in a
123 humidified 5% CO_2 incubator (MCO-18AIC, Sanyo, Osaka, Japan) and were sub-cultured at 70-80%
124 confluence.

125

126 **Cell viability assay**

127 The MTT assay (Lee et al., 2017b) was used for determining the effects of OVA and OVA hydrolysates
128 on cell viability of RAW 264.7 cells. After measuring NO concentration in the medium, an aliquot of the
129 MTT solution (2.5 mg/mL) was added to each well and incubated for an additional 4 h. Supernatants were

ACCEPTED

130 removed, and dimethyl sulfoxide was added to the wells to dissolve the formazan crystals. The absorbance
131 of each well was then measured using a microplate reader (Emax, Molecular Devices, Sunnyvale, CA,
132 USA) at 570 nm. Cell viability was calculated using the following equation:

133 $$\text{Cell viability (\%)} = \left(\frac{\text{Absorbance of sample}}{\text{absorbance of control}}\right) \times 100.$$

134

135 **Measurement of NO production**

136 RAW 264.7 cells (2×10^5 cells/well) were plated into a 96-well plate for 2 h, then 2 mg/mL of OVA and
137 OVA hydrolysates were treated and incubated for 24 h. Nitric oxide concentration was measured using the
138 Griess reagent (Kim et al., 2018). One hundred μL of supernatant mixed with 100 μL of the Griess reagent
139 and the mixture was incubated for 15 min at room temperature. The absorbance at 540 nm was recorded
140 for each well, using a microplate reader. Nitric oxide concentration was calculated using a standard curve
141 made of sodium nitrate.

142

143 **Inhibition of NO production**

144 RAW 264.7 cells (2×10^5 cells/well) were plated into a 96-well plate and various concentrations (0.5, 1,
145 or 2 mg/mL) of OVA hydrolysates were treated for 2 h. After that, the plate was further incubated with or
146 without LPS (100 ng/mL) for 24 h. Nitric oxide concentration was then measured and calculated using the
147 Griess reagent as mentioned above.

148

149 **PCR analysis (RT-PCR and quantitative real-time PCR)**

150 RAW 264.7 cells (5×10^5 cells/well) were plated into a 6-well plate and various concentrations (0.5, 1, or
151 2 mg/mL) of OVA hydrolysates were added and incubated for 24 h. At the end of incubation, the samples
152 in the plate were treated with LPS (0 or 100 ng/mL) and then incubated for 24 h. Total RNA was isolated
153 using the RNA isolation kit according to the manufacturers' instructions and synthesized to cDNA using a
154 synthesis kit. Synthesized cDNA was used in PCR analysis as template DNA. The primer sequences were
155 shown as follows: iNOS (forward 5´-CCCTTCCGAAGTTTCTGGCAGCAGC-3´, reverse 5´-

ACCEPTED

156 GGCTGTCAGAGCCTCGTGGCTTTGG-3′), and β-actin (forward 5′-

157 GTGGGCCGCCCTAGGCACCAG-3′, reverse 5′-GGAGGAAGAGGATGCGGCAGT-3′). The RT-PCR

158 reaction was conducted under the following reaction conditions: 95°C for 5 min (initiation), and followed

159 by 25 cycles of 95°C for 30 s (denaturation), specified temperature of each primer for 30 s (annealing),

160 72°C for 45 s (extension), and followed by a final extension at 72°C for 10 min. The final products were

161 then evaluated by 1.2% agarose gel electrophoresis with DNA SafeStain (Thermo Fisher Scientific). For

162 quantitative real-time PCR analysis, SYBR reagent was used and the reaction was conducted under the

163 following reaction conditions: 95°C for 2 min (initiation) followed by 40 cycles of 95°C for 5 s

164 (denaturation), 60°C for 30 s (annealing/extension). The amplified results were analyzed using the delta-

165 delta Ct method and normalized to β-actin. The purity of PCR products was assessed by analyzing the

166 melting curve.

167

168 **Western blot analysis**

169 RAW 264.7 cells (2×10^6 cells/well) were plated into a 6-well plate and OVA hydrolysates were treated

170 and incubated for 24 h. After that, LPS was added to each well and further incubated for 45 min. RAW

171 264.7 cells were washed with PBS and lysed using the Radioimmunoprecipitation assay (RIPA) lysis and

172 extraction buffer (Thermo Fisher Scientific) with protease/phosphatase inhibitor. Total protein

173 concentration was measured by Lowry's method. And the same concentration (25 μg) of proteins were

174 separated by 12% SDS-PAGE gel and transferred to PVDF membranes. After blocking with 5% skim

175 milk or 5% BSA dissolved in TBST (Tris-buffered saline with 1% Tween-20) for 1 h, membranes

176 incubated with primary antibody iNOS, β-actin, total or phosphorous from of MAPKs (p38, JNK, and

177 ERK) in 5% skim milk or 5% BSA overnight at 4˚C. After washing 3 times with TBST, the membranes

178 were incubated with secondary antibody (Goat anti-mouse IgG-HRP) for 2 h at room temperature. After

179 washing, the protein bands were detected using a chemiluminescent ECL kit (Thermo Fisher Scientific).

180

181

ACCEPTED

Statistical analysis

SPSS 18.0 software (Chicago, IL, USA) was used for statistical analysis. Data were expressed as mean ± standard deviation (SD). All data were taken from three independent experiments. For analysis between two samples, the Student's *t*-test was used. One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was used for the analysis between multiple samples. The significance of differences was reported at the level of $p < 0.05$.

Results and Discussion

Hydrolysis of OVA

The OVA standard (lane 1) showed two thick bands near 45 kDa, and our OVA (lane 2) showed a similar band pattern (Fig. 1). Lanes 3–6 showed the results for the OVA hydrolysates prepared using Multifect 14L hydrolysate (OHMF), Promod 278P hydrolysate (OHPM), Protex 6L hydrolysate (OHPT), and trypsin (OHT), respectively. Although the same amounts of samples were loaded on lanes 3–6 (1 mg/mL), the density of the band observed around 45 kDa in Lane 4 was lighter than that of other lanes, indicating that Promod 278P hydrolyzed OVA better than other enzymes used. Also, lanes 3–6 showed different band patterns: lanes 5 and 6 showed more peptide bands than lanes 3 and 4 in the areas where the molecular weights were < 25 kDa. This means that the four proteolytic enzymes produced different kinds of peptides from the OVA. Also, this is why each OVA hydrolysate exhibited different activities. Lee and Paik (2019) reported that the biological activity of protein hydrolysates and peptides were closely related to their amino acid sequence, length, and composition. Some researchers purified and identified some of the main peptides to determine their functional activity, but many others examined the functional activity of protein hydrolysates or mixtures of various peptides (Bhaskar et al., 2019; Lee et al., 2017a; Millán-Linares et al., 2014). In this study, we have investigated the anti-inflammatory activity of OVA hydrolysates, instead of individual peptides in the hydrolysates, in LPS-stimulated RAW 264.7 cells.

ACCEPTED

208 **Cell viability and NO production by OVA and OVA hydrolysates**

209 The effects of OVA and OVA hydrolysates on cell viability were evaluated using MTT assay to confirm
210 that the changes of NO production by the LPS-stimulated RAW 264.7 cells were not the result of
211 cytotoxicity of the hydrolysates. As shown in Figure 2, the viability of the RAW 264.7 cells in all groups
212 was above 90%, indicating that all concentrations (0.5–2 mg/mL) of OVA and OVA hydrolysates were not
213 toxic to the RAW 264.7 cells. Thus, we could confirm that the changes of NO production in RAW 264.7
214 cells by the OVA and OVA hydrolysate treatments were not the result of cytotoxicity.

215 Ovalbumin has been widely used as an antigen in experimental animal models (including as an inhalant)
216 and dietary allergy study (Mine and Yang, 2008). Some studies reported that OVA increased iNOS and
217 COX-2 expression by activating NF-κB pathways in RAW 264.7 cells (Lee et al., 2011). However, this
218 allergenicity could be decreased by heat, pH, and enzymatic hydrolysis because these parameters could
219 alter protein structure (Ballmer-Weber et al., 2016; Duan et al., 2014).

220 Thus, to confirm the inflammatory response to OVA (allergenicity) and its hydrolysates, LPS-stimulated
221 RAW 264.7 cells were treated with OVA and OVA hydrolysates and the amount of NO produced was
222 determined. As shown in Figure 3, LPS (100 ng/mL) treatment increased the NO production level to
223 27.61±1.96 μM and addition of OVA to the LPS-stimulated RAW 264.7 cells increased the NO production
224 level to 19.48±1.40 μM. However, the addition of OVA hydrolysate to the LPS-stimulated RAW 264.7
225 cells produced less than 1 μM of NO (OHMF, OHPM, OHPT, and OHT showed 0.20±0.08, 0.17±0.08,
226 0.22±0.07, and 0.13±0.13 μM, respectively), which were similar to the amount produced in the cells added
227 with distilled water (negative control; 0.55±0.26 μM), indicating that OVA hydrolysates did not affect NO
228 production.

229

230 **Inhibitory effects of OVA hydrolysates on NO production**

231 As shown in Figure 4, the positive control (LPS, 100 ng/mL) showed high production of NO (28.39 ±1.63
232 μM). Ovalbumin hydrolysates showed significant inhibitory effects on NO production. At a concentration
233 of 2 mg/mL, OHMF inhibited NO production by 24.24±1.35 μM. The OHPT and OHT treatments showed

ACCEPTED

234 dose-dependent inhibitions of NO production at 0.5, 1, or 2 mg/mL (OHPT: 25.17±1.46, 21.15±1.35, and
235 17.01±0.84 μM, OHT: 23.28±1.35, 19.46±1.07, and 13.99±2.22 μM, respectively).
236 Under normal conditions, NO performs an important function in the inflammatory response (Kumar et
237 al., 2017). When inflammation is induced, an appreciable level of NO is secreted by immune-activated
238 macrophages at the site of inflammation. However, the overproduction of NO causes various complications
239 as a result of chronic inflammation (Cho et al., 2011). Therefore, the inhibitory effect of ovalbumin
240 hydrolysates on NO production in LPS-stimulated RAW 264.7 cells is a relevant measure to releave the
241 inflammatory activity (Kim and Kim, 2019). Several studies have suggested that the peptides in the
242 hydrolysates prepared using proteolytic enzymes exerted anti-inflammatory activity by inhibiting NO
243 production in the LPS-stimulated RAW 264.7 cells (Kim et al., 2013; Lai et al., 2011). As shown in the
244 results, OHT showed the highest inhibitory effect on NO production in the LPS-stimulated RAW 264.7
245 cells. Therefore, OHT was selected for further analysis.
246

247 **Effect of OHT on gene and protein expression of iNOS**

248 To understand the inhibition mechanism of NO production by OTH, the amount of iNOS and the
249 expression of iNOS mRNA were evaluated using Western blotting and PCR analysis, respectively.
250 The inducible nitric oxide synthase is strongly linked to the regulation of NO production during the
251 inflammatory responses. Hence, many researchers have tried to find new substances that can inhibit its
252 enzyme production (Shanura Fernando et al., 2018). As shown in Figure 5 (A), the production of iNOS
253 was significantly decreased dose-dependently by OHT. The expression of iNOS mRNA (Figure 5 (B)) also
254 significantly decreased when treated with OHT ($p<0.05$). These decreases, 23.34±3.56, 43.79±2.59, and
255 63.87±1.47% at 0.1, 0.5, and 2 mg/mL of OHT, respectively, also showed dose-dependence. The
256 ezpression of iNOS was not affected when the RAW 264.7 cells were treated with OHT only.
257 After stimulation, macrophages produced increased amount of iNOS, indicating that the pro-
258 inflammatory response is directly related to the production of NO. The overexpression of iNOS enzyme
259 produces excessive amount of NO and leads to the pathogenesis of various inflammatory diseases, septic

ACCEPTED

260 shock, and cancer (Lai et al., 2011). Many studies reported that natural compounds including carbohydrates,
261 protein, and flavonoids have anti-inflammatory activity by suppressing iNOS expression, resulting in the
262 inhibition of NO production (Ham et al., 2015; Shanura Fernando et al., 2018; Sun et al., 2016). Thus, our
263 results suggested that OHT inhibited NO production by inhibiting iNOS expression.
264

265 **Effect of OHT on phosphorylation of the MAPK pathway**

266 The Western blotting was performed to confirm whether the MAPK pathway is involved in the anti-
267 inflammatory activity of OHT in LPS-stimulated RAW 264.7 cells. The JNK, ERK, and p38 pathway are
268 part of the MAPK pathway whose phosphorylation increases their inflammatory activity. As shown in
269 Figure 6, the phosphorylation of JNK, ERK, and p38 in RAW 264.7 cells increased after the cells were
270 treated with LPS (45 min). However, when RAW 264.7 cells were co-treated with LPS and OHT (2 mg/mL),
271 OHT inhibited the phosphorylation of JNK and ERK but did not affect the phosphorylation of p38.
272 The MAPK pathway is known to regulate several cellular responses (e.g., cell survival, proliferation, and
273 apoptosis) following exposure to various stimuli such as stress, shock, and pro-inflammatory cytokines
274 (Pearson et al., 2001). Also, many studies indicated that the phosphorylation of MAPK was associated with
275 the pro-inflammatory signaling, and up-regulate the production of IL-6, iNOS, and COX-2 in LPS-
276 stimulated macrophages (Gao et al., 2012; Ham et al., 2015). Therefore, effective anti-inflammatory
277 materials should show inhibitory activity on the phosphorylation of MAPK genes. Crebanine, isolated from
278 *Stephania venosa,* inhibits the production of NO, iNOS, COX-2, and PGE_2, and also inhibits pro-
279 inflammatory cytokines including TNF-α and IL-6 by suppressing the MAPK signaling pathways
280 (Intayoung et al., 2016). A study with sophocarpine, which is an alkaloid from Sophora *alopecuroides* L.,
281 indicated that sophocarpine attenuated the phosphorylation of p38 and JNK, resulted in the inhibition of
282 NO production (Gao et al., 2012), which was similar to our results.
283
284

ACCEPTED

285 **Conclusion**

286 This study demonstrated that OVA hydrolysate treated with trypsin (OHT) showed significant anti-
287 inflammatory activity. The OHT inhibited NO production in the LPS-stimulated RAW 264.7 cells by
288 suppressing the production of iNOS and the expression of iNOS mRNA. Also, it was confirmed that the
289 anti-inflammatory activity of OHT was through the inhibition of JNK and ERK pathways. Therefore, the
290 tryptic hydrolysates of OVA could be used as an anti-inflammatory agent in food products.

291

ACCEPTED

References

Abeyrathne EDNS, Lee HY, Ahn, D. U. 2013. Sequential separation of lysozyme and ovalbumin from the chicken egg white. Korean J Food Sci Anim Resour 33:501-507.

Abeyrathne EDNS, Lee HY, Jo C, Nam KC, Ahn, D. U. 2014. Enzymatic hydrolysis of ovalbumin and the functional properties of the hydrolysates. Poult Sci 93:2678-2686.

Ahn CB, Cho YS, Je JY. 2015. Purification and anti-inflammatory action of tripeptide from salmon pectoral fin byproduct protein hydrolysate. Food Chem 168:151-156.

Ballmer-Weber BK, Brockow K, Fiocchi A, Theler B, Vogel L, Ring J, Szepfalusi Z, Mazzina O, Schaller R, Fritsche R, Vissers YM, Nutten S. 2016. Hydrolyzed egg displays a strong decrease in allergenicity and is well tolerated by egg-allergic patients. Allergy 71:728-732.

Bhaskar B, Ananthanarayan L, Jamdar SN. 2019. Effect of enzymatic hydrolysis on the functional, antioxidant, and angiotensin I-converting enzyme (ACE) inhibitory properties of whole horse gram flour. Food Sci Biotechnol 28:43-52.

Chang SH, Lin YY, Wu GJ, Huang CH, Tsai GJ. 2019. Effect of chitosan molecular weight on anti-inflammatory activity in the RAW 264.7 macrophage model. Int J Biol Macromol 131:167-175.

Cho YS, Lee SH, Kim SK, Ahn CB, Je JY. 2011. Aminoethyl-chitosan inhibits LPS-induced inflammatory mediators, iNOS and COX-2 expression in RAW 264.7 mouse macrophages. Process Biochem 46:465-470.

Dail-Youcef N, Mecili M, Ricci R, Andres E. 2013. Metabolic inflammation: connecting obesity and insulin resistance. Ann Med 45:242-53.

de Mejia EG, Dia VP. 2009. Lunasin and lunasin-like peptides inhibit inflammation through suppression of the NF-κB pathway in the macrophage. Peptides 30:2388-2398.

Duan C, Yang L, Li A, Zhao R, Huo G. 2014. Effects of enzymatic hydrolysis on the allergenicity of whey protein concentrate. Iran J Allergy Asthma Immunol 13:231-239.

ACCEPTED

316 Gao Y, Jiang W, Dong C, Li C, Fu X, Min L, Tian J, Jin H, Shen J. 2012. Anti-inflammatory effects of
317 sophocarpine in LPS-induced RAW 264.7 cells via NF-κB and MAPKs signaling pathways. Toxicol
318 Vitro 26:1-6.

319 Ham YM, Ko YJ, Song SM, Kim J, Kim KN, Yun JH, Cho JH, Ahn G, Yoon WJ. 2015. Anti-
320 inflammatory effect of litsenolide B2 isolated from *Litsea japonica* fruit via suppressing NF-κB and
321 MAPK pathways in LPS-induced RAW264.7 cells. J Funct Food 13:80-88.

322 Hartmann R, Meisel H. 2007. Food-derived peptides with biological activity: from research to food
323 applications. Curr Opin Biotechnol 18:163-169.

324 Huang W, Chakrabarti S, Majumder K, Jiang Y, Davidge ST, Wu J. 2010. Egg-derived peptide IRW
325 inhibits TNF-α-induced inflammatory response and oxidative stress in endothelial cells. J Agric Food
326 Chem 58:10840-10846.

327 Intayoung P, Limtrakul P, Yodkeeree S. 2016. Antiinflammatory activities of crebanine by inhibition of
328 NF-κB and AP-1 activation through suppressing MAPKs and Akt Signaling in LPS-Induced RAW
329 264.7 macrophages. Biol Pharm Bull 39:54-61.

330 Kim EK, Kim YS, Hwang JW, Kang SH, Choi, DK, Lee KH, Lee JS, Moon SH, Jeon BT, Park PJ. 2013.
331 Purification of a novel nitric oxide inhibitory peptide derived from enzymatic hydrolysates of *Mytilus*
332 *coruscus*. Fish Shellfish Immunol 34:1416-1420.

333 Kim HS, Yu HS, Lee JH, Lee GW, Choi SJ, Chang PS, Paik HD. 2018. Application of stabilizer
334 improves the stability of nanosuspended branched-chain amino acids and anti-inflammatory effect in
335 LPS-induced RAW 264.7 cells. Food Sci Biotechnol 27:451-459.

336 Kim L, Kim JY. 2019. Chondroprotective effect of curcumin and lecithin complex in human
337 chondrocytes stimulated by IL-1β via an anti-inflammatory mechanism. Food Sci Biotechnol 28:547-
338 553.

339 Kumar S, Singh RK, Bhardwaj TR. 2017. Therapeutic role of nitric oxide as an emerging molecule.
340 Biomed Pharmacother 85:182-201.

ACCEPTED

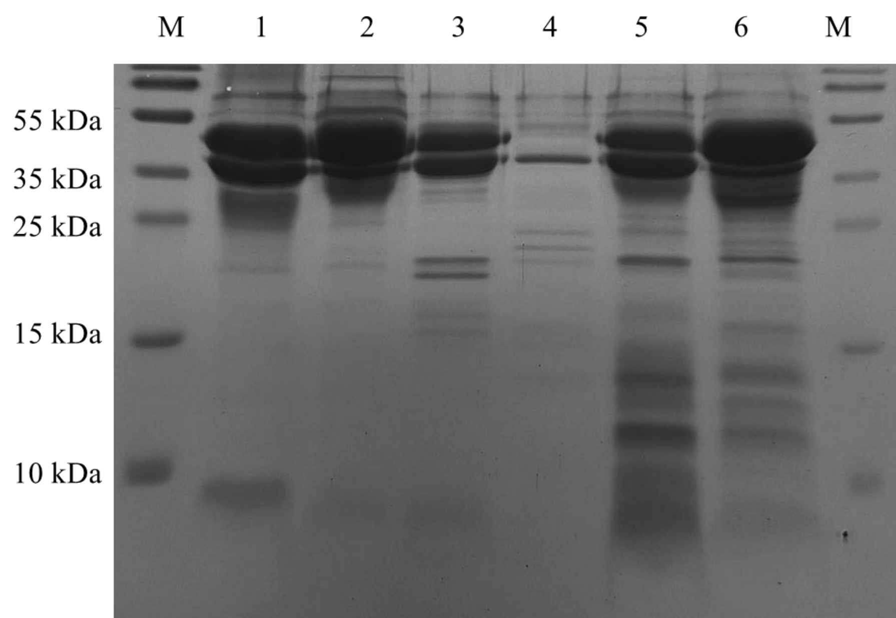
341 Lai CS, Lai YS, Kuo DH, Wu CH, Ho CT, Pan MH. 2011. Magnolol potently suppressed
342 lipopolysaccharide-induced iNOS and COX-2 expression via downregulating MAPK and NF-κB
343 signaling pathways. J Funct Food 3:198-206.
344 Lee AN, Park SJ, Jeong AR, Lee JR, Park HJ, Kim SJ, Min IS, Youn HS. 2011. Ovalbumin induces
345 cyclooxygenase-2 and inducible nitric oxide synthase expression. Korean J Food Sci Technol 43:110-
346 113.
347 Lee JH, Moon SH, Kim HS, Park E, Ahn DU, Paik HD. 2017a. Antioxidant and anticancer effects of
348 functional peptides from ovotransferrin hydrolysates. J Sci Food Agric 97:4857-4864.
349 Lee JH, Moon SH, Kim HS, Park E, Ahn DU, Paik HD. 2017b. The immune-enhancing activity of
350 phosvitin by stimulating the production of pro-inflammatory mediators. Poult Sci 96:3872-3878.
351 Lee JH, Paik HD. 2019. Anticancer and immunomodulatory activity of egg proteins and peptides: a
352 review. Poult. Sci. 98:6505-6516.
353 Li Y, Meng T, Hao N, Tao H, Zou S, Li M, Ming P, Ding H, Dong J, Feng S, Li J, Wang X, Wu J. 2017.
354 Immune regulation mechanism of Astragaloside IV on RAW 264.7 cells through activating the NF-
355 κB/MAPK signaling pathway. Int Immunopharmacol 49:38-49.
356 Lundberg JON, Lundberg JM, Alving K, Weitzberg E. 1997. Nitric oxide and inflammation: The answer
357 is blowing in the wind. Nat Med 3:30-31.
358 Matoba N, Usui H, Fujita H, Yoshikawa M. 1999. A novel anti-hypertensive peptide derived from
359 ovalbumin induces nitric oxide-mediated vasorelaxation in an isolated SHR mesenteric artery. FEBS
360 Lett 452:181-184.
361 Medzhitov R. 2008. Origin and physiological roles of inflammation. Nature 454:428-435.
362 Millán-Linares MC, Bermúdez B, Yust MM, Millán F, Pedroche J. 2014. Anti-inflammatory activity of
363 lupine (*Lupinus angustifolius* L.) protein hydrolysates in THP-1-derived macrophages. J Funct Food
364 8:224-233.
365 Mine Y, Yang M. 2008. Recent advances in the understanding of egg allergens: Basic, industrial, and
366 clinical perspectives. J Agric Food Chem 56:4874-4900.

ACCEPTED

367 Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH. 2001. Mitogen-
368 activated protein (MAP) kinase pathways: Regulation and physiological functions. Endocr Rev 22:153-
369 183.
370 Seo JY, Lee CW, Choi DJ, Lee J, Lee JY, Park YI. 2015. Ginseng marc-derived low-molecular-weight
371 oligosaccharide inhibits the growth of skin melanoma cells via activation RAW 264.7 cells. Int
372 Immunopharmacol 29:344-353.
373 Shanura Fernando IP, Asanka Sanjeewa KK, Samarakoon KW, Lee WW, Kim HS, Ranasinghe P,
374 Gunasekara UKDSS, Jeon YJ. 2018. Antioxidant and anti-inflammatory functionality of ten Sri Lankan
375 seaweed extracts obtained by carbohydrase assisted extraction. Food Sci Biotechnol 27:1761-1769.
376 Sharma JN, Al-Omran A, Parvathy SS. 2007. Role of nitric oxide in inflammatory diseases.
377 Inflammopharmacol 15:252-9.
378 Sun X, Chakrabarti S, Fang J, Yin Y, Wu J. 2016. Low-molecular-weight fractions of alcalase
379 hydrolyzed egg ovomucin extract exert anti-inflammatory activity in human dermal fibroblasts through
380 the inhibition of tumor necrosis factor-mediated nuclear factor κB pathway. Nutr Res 36:648-657.
381 Vis EH, Plinck AF, Alink GM, van Boekel MAJS. 1998. Antimutagenicity of heat-denatured ovalbumin,
382 before and after digestion, as compared to caseinate, BSA, and soy protein. J Agric Food Chem 46:3713-
383 3718.

384

ACCEPTED

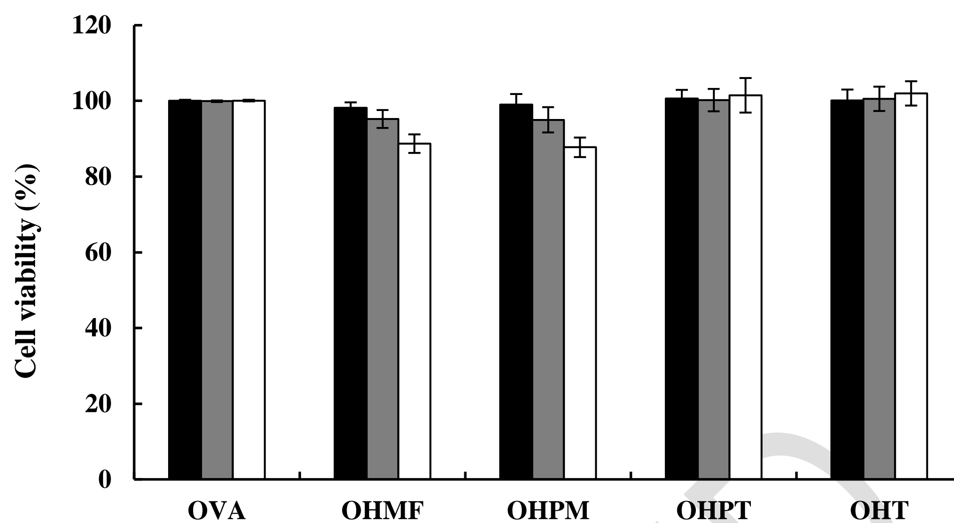


385 **Fig. 1. SDS-PAGE of OVA and OVA hydrolysates.** M, marker; lane 1, standard OVA (Sigma-Aldrich);
386 lane 2, OVA; lane 3, OHMF (hydrolysates treated with Multifect 14L); lane 4, OHPM (hydrolysates
387 treated with Promod 278P); lane 5, OHPT (hydrolysates treated with Protex 6L); lane 6, OHT
388 (hydrolysates treated with trypsin).

389

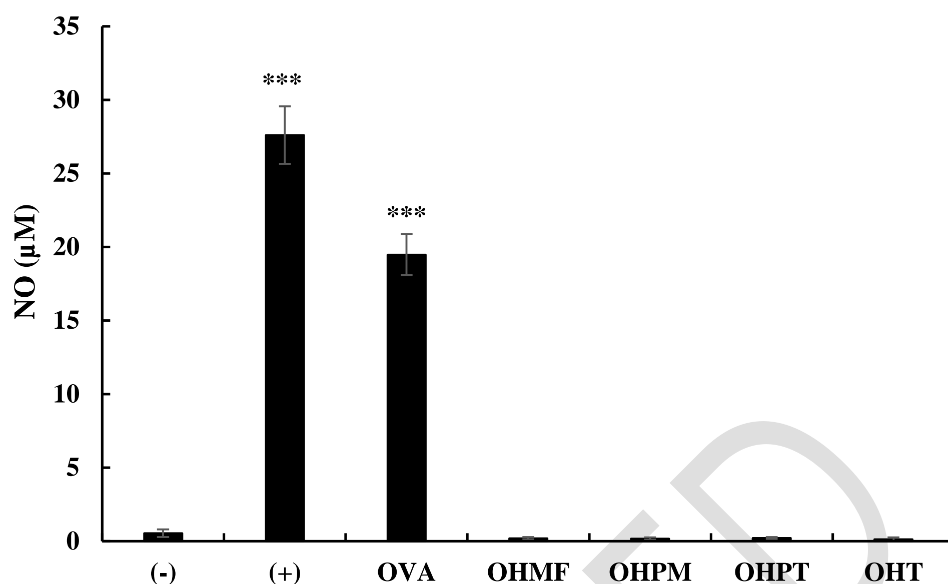
390

ACCEPTED



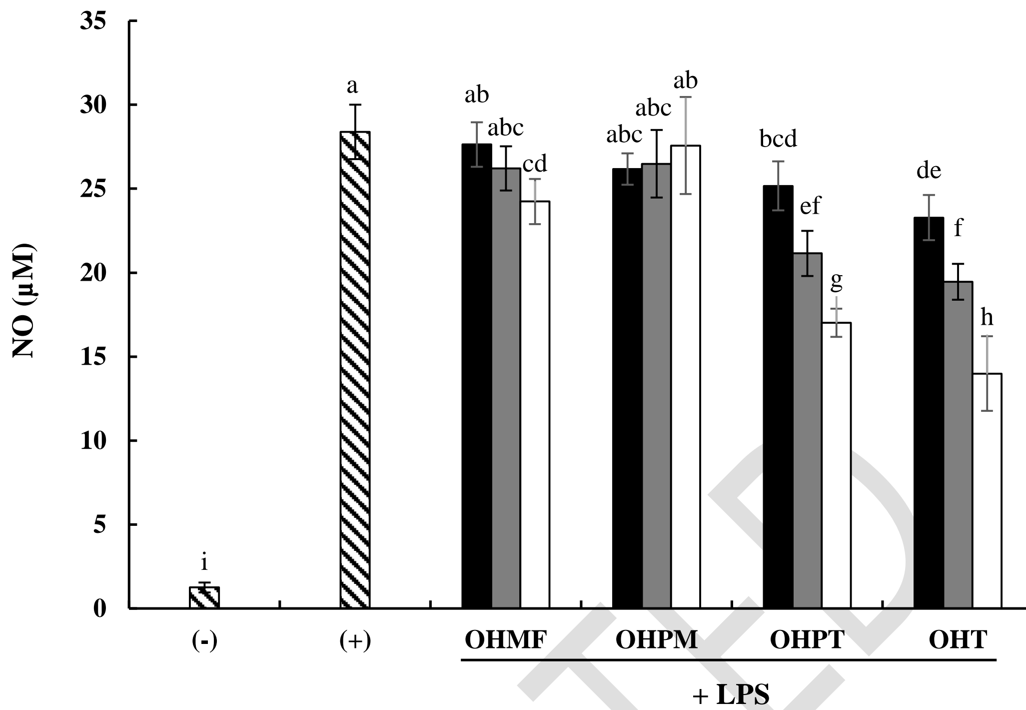
391 **Fig. 2. Cell viability of RAW 264.7 cells treated with OVA and OVA hydrolysates.** ■: 0.5 mg/mL,
392 ▩: 1 mg/mL, □: 2 mg/mL. All values are represented as mean±S.D. of triplicate experiments. OVA:
393 ovalbumin, OHMF: hydrolysates treated with Multifect 14L, OHPM: hydrolysates treated with Promod
394 278P, OHPT: hydrolysates treated with Protex 6L, OHT: hydrolysates treated with trypsin.
395

ACCEPTED



396 **Fig. 3. Nitric oxide production in RAW 264.7 cells.** All values represent the mean ± S.D. of triplicate
397 experiments. Bars labeled with different letters represent significant differences. *** represents a
398 statistically significant difference of $p<0.001$ (Student's *t*-test) compared with the negative control. (-):
399 distilled water, (+): LPS 100 ng/mL, OVA: ovalbumin, OHMF: hydrolysates treated with Multifect 14L,
400 OHPM: hydrolysates treated with Promod 278P, OHPT: hydrolysates treated with Protex 6L, OHT:
401 hydrolysates treated with trypsin. All extracts were used at a concentration of 2 mg/mL.
402
403

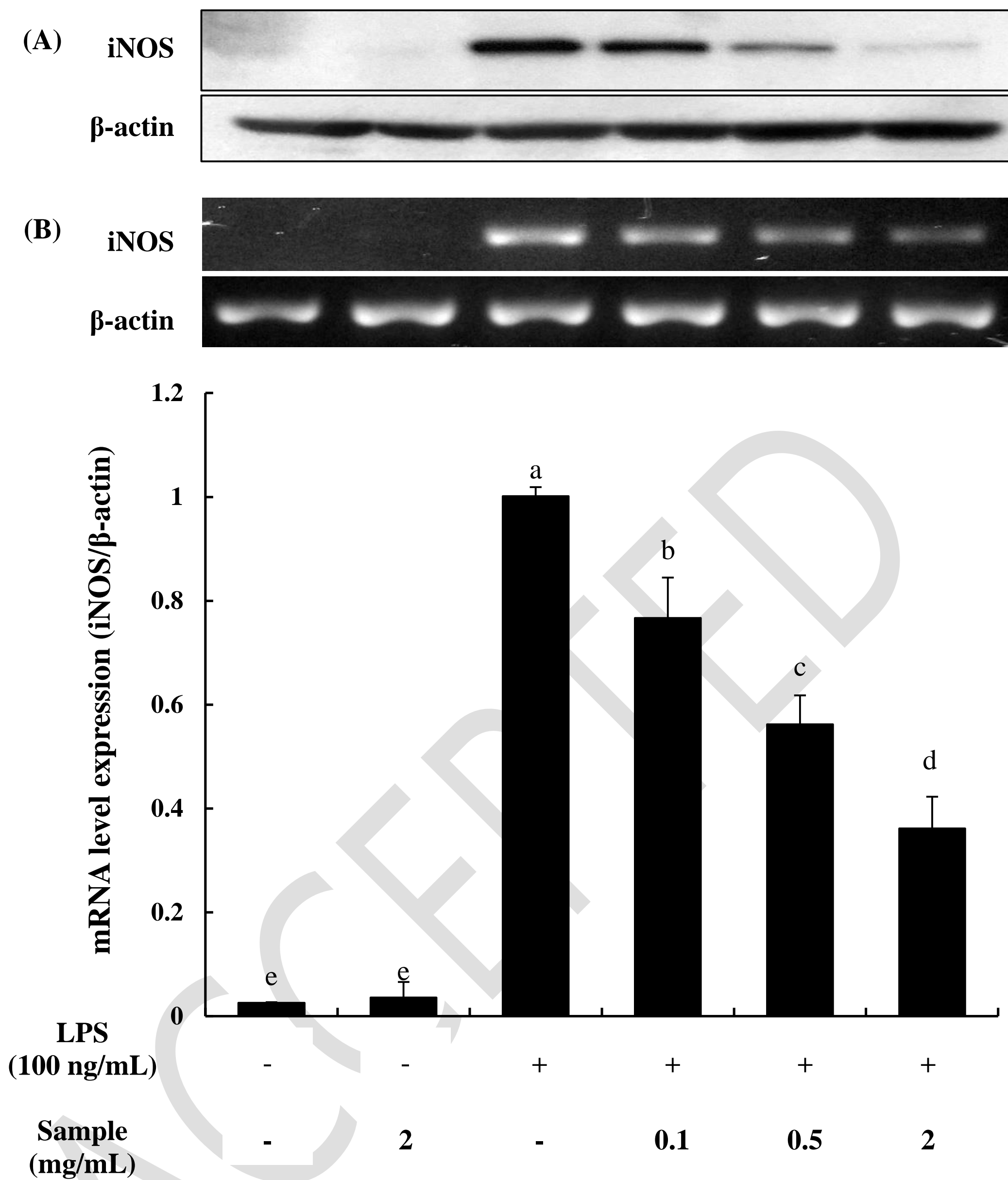
ACCEPTED



404 **Fig. 4. Inhibitory effects of OVA hydrolysates on nitric oxide production in LPS-stimulated RAW**
405 **264.7 cells.** ■: 0.5 mg/mL, ■: 1 mg/mL, □: 2 mg/mL. All values represent the mean ± S.D. of triplicate
406 experiments. Bars labeled with different letters represent statistically significant differences.
407 Significance was evaluated using one-way ANOVA and Duncan's multiple range test ($p<0.05$). (-):
408 distilled water, (+): LPS 100 ng/mL, OVA: ovalbumin, OHMF: hydrolysates treated with Multifect 14L,
409 OHPM: hydrolysates treated with Promod 278P, OHPT: hydrolysates treated with Protex 6L, OHT:
410 hydrolysates treated with trypsin.

ACCEPTED

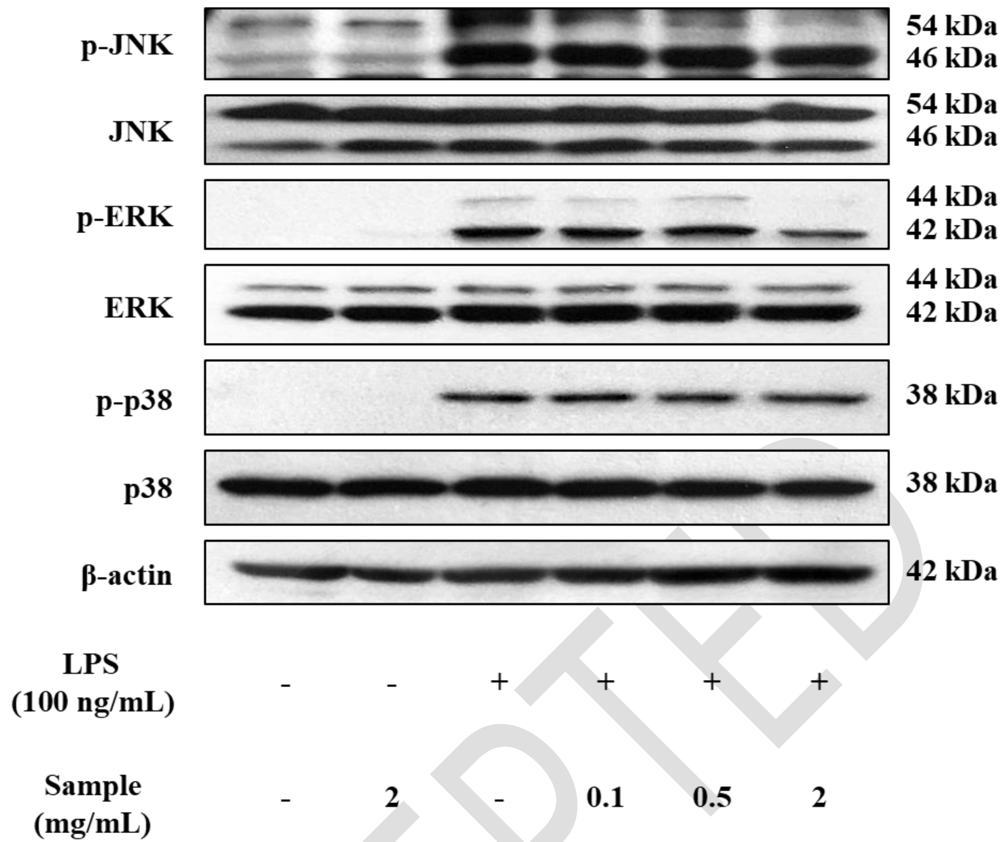
411



412 **Fig. 5. Effects of ovalbumin tryptic hydrolysate (OHT) on the expression of iNOS protein (A), and**
413 **gene transcription (B) in LPS-induced RAW 264.7 cells.** All values represent the mean ± S.D. of
414 triplicate experiments. Bars labeled with different letters represent significant differences. Significance
415 was evaluated using a one-way ANOVA and Duncan's multiple range test ($p<0.05$).

416

ACCEPTED



417 **Fig. 6. Effects of ovalbumin tryptic hydrolysate (OHT) on phosphorylation of MAPK genes (p38,**

418 **ERK, and JNK) in LPS-induced RAW 264.7 cells.**

ACCEPTED