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TITLE PAGE
- Food Science of Animal Resources -
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ARTICLE INFORMATION	Fill in information in each box below
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Article Title	Preparation of Hypoallergenic Whey Protein Hydrolysate by a Mixture of Alcalase and Prozyme and Evaluation of its Digestibility and Immunoregulatory Properties
Running Title (within 10 words)	Digestibility and Immunoregulatory Properties of Hypoallergenic Whey Protein Hydrolysate
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10
11 **Abstract (within 250 words)**

12 Whey protein (WP) has nutritional value, but the presence of β -lactoglobulin (β -LG)
13 and α -lactalbumin (α -LA) cause allergic reactions. In this study, hypoallergenic whey
14 protein hydrolyate (HWPH) was prepared by decomposing β -LG and α -LA of WP using
15 exo- and endo-type proteases. The enzyme mixing ratio and reaction conditions were
16 optimized using response surface methodology (RSM). Degradation of α -LA and β -LG
17 was confirmed through gel electrophoresis, and digestion, and absorption rate, and
18 immunostimulatory response were measured using *in vitro* and *in vivo* systems.

19 Through RSM analysis, the optimal hydrolysis conditions for degradation of α -LA and
20 β -LG included a 1:1 mixture of Alcalase and Prozyme reacted for 10 h at a 1.0%
21 enzyme concentration relative to substrate. The molecular weight of HWPH was <5
22 kDa, and leucine was the prominent free amino acid. Both *in vitro* and *in vivo* tests
23 showed that digestibility and intestinal permeability were higher in HWPH than in WP.

24 In BALB/c mice, as compared to WP, HWPH reduced allergic reactions by inducing
25 elevated Type 1/Type 2 helper T cell ratio in the blood, splenocytes, and small intestine.
26 Thus, HWPH may be utilized in a variety of low allergenicity products intended for
27 infants, adults, and the elderly.

28 **Keywords:** hydrolysate; whey protein; response surface methodology; hypoallergenic;
29 infant nutrition

31 **Introduction**

32 Whey, a by-product resulting from the production of cheese, is a generic term for the
33 remaining water-soluble components other than cheese (milk concentrate curd). With
34 the exception of casein, which is involved in cheese production, whey is valued highly
35 nutritionally and physiologically because of the presence of active ingredients including
36 proteins, lactose, minerals, vitamins, and inorganic components (Boscaini et al., 2023;
37 Yiğit et al., 2023).

38 Whey protein (WP) not only boosts immunity due to the presence of β -lactoglobulin
39 (β -LG), α -lactalbumin (α LA), bovine serum albumin, lactoferrin (LF), immunoglobulin
40 (Ig), enzymes, and glycomacropeptide, and is also a source of essential amino acids
41 (Marshall, 2004). Though it is obvious that WP is an excellent nutritive food, some of
42 its components have been linked to allergic conditions in infants and young children
43 (Jaiswal and Worku, 2022; Wright et al., 2022). Among the above mentioned proteins,
44 the major antigens include casein, β -LG, and α LA, and it has been reported that most
45 patients allergic to milk allergy have specific IgE antibodies against at least two
46 antigens (Cohen et al., 2022; Savilahti and Kuitunen, 1992; Sicherer and Sampson,
47 1999). Among WPs, β -LG is known to be more allergenic than α LA. In human milk, in
48 particular, β -LG is present in smaller amounts ranging from 1-150 μ g/mL (Exl and
49 Fritsché, 2001), and its content is lower than that of casein, another allergen of milk,
50 which comprises approximately 7 to 12% of milk protein.

51 To lessen milk allergies, β -LG is hydrolyzed using digestive enzymes, and
52 hypoallergenic milk powder prepared using proteolytic enzymes is commercially
53 available (Lynch and Buckin, 2022). To reduce milk allergic reactions, it is necessary to
54 provide a diet that restricts the causative milk or provided in a form that lowers the
55 antigenicity of milk. Different treatment methods, such as heat, ultra-high pressure

56 (Landim et al., 2023), and enzymes, can be used to reduce the antigenicity of proteins.
57 Because one of the main allergens in milk, casein, is heat-resistant, the reduction in
58 antigenicity by heat treatment is insignificant (Bu et al., 2013; Vandenplas et al., 2022).
59 Thus, milk proteins containing both structural and sequential epitopes are mainly
60 hydrolyzed by enzymes. Although it doesn't exist in human milk and has a lower
61 content than α LA, the primary allergen of WP, β -LG, shows resistance during digestion
62 in the stomach and penetrates the intestines to cause milk allergies.

63 Therefore, this study aimed to prepare WP hydrolysates (WPH) with reduced
64 antigenicity of α -LA and β -LG allergens as target substrates. Optimal hydrolysis
65 conditions for degrading antigenic WPs using a mixture of endo- and exo-type enzymes
66 were selected using response surface methodology (RSM). By preparing a
67 hypoallergenic whey protein hydrolysate (HWPH), we developed a milk protein
68 manufacturing technology that can be fed to milk-allergic infants. The digestibility and
69 immune stimulation of the prepared hydrolysate were also evaluated.

70

71 **Materials and Methods**

72 **Materials**

73 In this study, whey protein concentrate (WPC; Agri-mark, FL, USA) with a protein
74 content of 80% (dry basis) was used as the raw WP. A 75% α -LA and a 95% β -LG from
75 bovine milk (Samyoung Innovation, Pyeongtaek, Republic of Korea) were used as the
76 allergen standards. Alcalase (Novozymes, Copenhagen, Denmark), protamex
77 (Novozymes), flavourzyme (Novozymes), and collupulin (DSM, Heerlen, Netherlands)
78 were employed as endo-type proteolytic enzymes, whereas prozyme (DuPont,
79 Delaware, USA) was used as an exo-type enzyme. Pepsin and pancreatin (Sigma-

80 Aldrich, St. Louis, USA) were used as the artificial digestive enzymes. L-leucine was
81 purchased from Sigma-Aldrich.

82 **Preparation of Whey Protein Hydrolysate**

83 To prepare the whey protein hydrolysate, a mixture of endo- and exo-type protease
84 enzymes was added to a 10% suspension of WP at a concentration of 1.0% (v/w)
85 relative to the substrate solution. This was then hydrolyzed for 8 h at 50°C at pH 7.8
86 with shaking at 100 rpm. Each reaction solution was treated at 90°C for 10 min to
87 inactivate protease and then centrifuged at 8,000 rpm for 20 min to separate the
88 supernatant. A WP solution without protease treatment was used as a control. The
89 reaction conditions and enzyme characteristics used for WP hydrolysis are listed in
90 Table S1.

91 **Identification of β -LG and α -LA in Whey Protein Hydrolysate Using sodium** 92 **dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)**

93 HWP (10 mg) was dissolved in 1 mL of sample buffer [0.5 M trihydrochloric acid
94 (HCl), glycerol, 10% SDS, 0.1% bromophenol blue, pH 6.8], heat-treated at 100°C for 5
95 min, centrifuged, and the supernatant separated. A 20 μ L of this was run on a 12%
96 polyacrylamide gel at 135 V for approximately 90 min. To confirm the presence of α -
97 LA and β -LG proteins, the gel was stained with Coomassie Brilliant Blue R-250 for 6 h,
98 and then destained using a solution containing 10% methanol and 10% acetic acid until
99 the protein bands were clearly visible.

100 **Amino-nitrogen (A-N) contents and degree of hydrolysis (DH) Calculation**

101 The A-N content of HWP was measured using the 2,4,6-trinitrobenzenesulfonic acid
102 (TNBS) method (Nielsen et al., 2001). Briefly, 125 μ L of the sample, 400 μ L of 0.212
103 M phosphate buffer (pH 8.2), and 0.4 mL of 0.1% TNBS were mixed and incubated in
104 the dark at 50°C for 60 min. Next, 0.8 mL of 100 mM HCl was added and left for 20

105 min. After another 10 min, 0.8 mL of distilled water was added, and the absorbance was
106 measured at 340 nm. The amount of A-N was calculated using L-leucine as a standard.

107 The DH of proteins following enzyme treatment was calculated using the following
108 equation by estimated the A-N content of whey protein hydrolysates (Benjakul and
109 Morrissey, 1997). The total A-N content was measured by neutralizing WP with sodium
110 hydroxide (NaOH) after hydrolysis at 100°C for 24 h using 6 N HCl.

$$111 \quad \text{DH} = (L_t - L_0)/(L_{\text{max}} - L_0) \times 100$$

112 Where L_t is the A-N content after hydrolysis at time t , while L_0 is the A-N content
113 before hydrolysis, and L_{max} is the A-N content after acid hydrolysis with 6 N HCl.

114 **Establishment of Optimal Mixing Ratio of Alcalase and Prozyme and of Optimal** 115 **Reaction Conditions Using RSM**

116 The RSM was used to select the optimal mixing ratio of Alcalase and Prozyme. A
117 total of 15 combinations were composed of three repetitions of the experiment at 10%
118 substrate, 50°C, pH 7.8, with the mixing ratio of the two enzymes, Alcalase and
119 Prozyme, as an independent variable (Table S2). The optimal mixing ratio of the two
120 enzymes was selected by measuring the A-N of the hydrolysate corresponding to the
121 experimental combination. RSM was used to analyze the experimental results, and the
122 regression equation representing the optimal mixing ratio of Alcalase and Prozyme was
123 as follows: $aX + bY + cXY = 32.29x + 68.87y + 96.58xy$, where X is Alcalase (%)
124 and Y is Prozyme (%).

125 A total of 39 combinations were composed of three repetitions of the experiment at
126 10% substrate, 50°C, pH 7.8, with the amount of enzyme added and hydrolysis time by
127 the mixed enzyme of Alcalase and Prozyme as independent variables (Table S2). The
128 A-N of the hydrolysate corresponding to the experimental combination was measured to
129 select the optimal enzyme addition amount and hydrolysis time. RSM was used to

130 analyze the experimental results, and the regression equation representing the optimal
131 conditions for the amount of enzyme added and hydrolysis time was as follows: $A + aX$
132 $+ bY + cX^2 + dY^2 + eXY = 29.6286 - 0.9220 X + 4.5520 Y + 0.4706 X^2 + 8.3643 Y^2 -$
133 $2.0389 XY$, where X is the hydrolysis time and Y is the amount of the enzyme added.

134 **Amino Acid Composition by High-Performance Liquid Chromatography (HPLC)**

135 **Analysis**

136 Amino acids were derivatized using AccQ-Tag reagent (Waters, Milford, MA, USA)
137 according to the manufacturer's instructions and then analyzed using an HPLC system
138 (Waters). An AccQ-Tag column (3.9 mm × 150 mm, Waters) was used with the
139 following parameters: temperature, 37°C; flow rate of the mobile phase [Water AccQ-
140 Tag Eluent A (acetate-phosphate buffer), acetonitrile, and Milli-Q Water], 1.0 mL/min,
141 and a fluorescence detector (250 nm of excitation and 395 nm of emission) was used to
142 analyze the data.

143 The analysis for total amino acids was performed after acid hydrolysis. Briefly, 10
144 mL of 6 N HCl solution was added to 0.5 g of the samples taken in a test tube, which
145 was sealed and hydrolyzed at 110°C for 24 h. The filtrate obtained was centrifuged, and
146 the supernatant concentrated at 50°C to completely evaporate the acid and water, and
147 then diluted to 5 mL using 20 mM HCl (pH 2.2). The dissolved solution was filtered
148 through a 0.45 μm membrane, and the filtrate was used as a sample for HPLC analysis.

149 ***In vitro* Digestibility and Intestinal Permeability Assay**

150 *In vitro* digestibility was determined as described by Garrett et al. Garrett et al. (1999)
151 using pepsin and pancreatin. After dissolving 1,000 mg of the sample in distilled water,
152 the pH was adjusted to 2.0. Pepsin (2.5% of the substrate) was added, reacted at 37°C
153 for 1 h, and the pH was then adjusted to 5.3 with sodium bicarbonate and further
154 adjusted to 7.5 with 5 N NaOH. Pancreatin was added at 4% per substrate, reacted at

155 37°C for 2 h, and was then heated at 90°C for 10 min to inactivate the digestive enzyme.
156 A-N (mg) was measured in the supernatant obtained by centrifugation (12,000 rpm, 10
157 min), and digestibility (%) was calculated using the following formula:
158 Digestibility (%) = [A-N in digested sample (mg) – A-N in sample before digestion
159 (mg)] / [Total A-N in sample before digestion (mg) – A-N in sample before digestion
160 (mg)] × 100. (Total A-N in sample before digestion: A-N content after acid hydrolysis).

161 A Caco-2 Ready 24-well plate (Komabiotec, Seoul, Republic of Korea) was used
162 for the permeability analysis of WP and HWP (Van Breemen and Li, 2005). The plate
163 was incubated in a 5% CO₂ incubator (37°C) for 4 h, thawed, and then replaced with
164 Dulbecco's Modified Eagle's Medium containing 1 g/L glucose, 10% fetal bovine
165 serum, and 1% glutamine. The assay was conducted 48 h after the medium was
166 replaced. For the intestinal permeability analysis, WP and HWP prepared using
167 digestive enzymes (pepsin and pancreatin) were diluted 10-fold with HBSS buffer.
168 After washing the Caco-2 plate with a complete monolayered film three times using
169 HBSS buffer, 250 and 750 µL of the diluted sample and HBSS buffer were dispensed
170 into the insert and receiver plates, respectively. The buffer on the receiver plate was
171 recovered over time (20, 40, 60, and 120 min). After analyzing the protein content of
172 the sample initially distributed in the insert and the sample collected over time using the
173 bicinchoninic acid assay, the permeability of the sample was analyzed using the
174 following formula (Park et al., 2021): Permeability (%) = [Total protein (mg) in the
175 receiver plate] / [Initial protein (mg) added to the insert] × 100.

176 **Evaluation of Absorption Rate by High-Dose Single Oral Administration**

177 SD rats (6-week-old, male) were purchased from Oriental Bio (Seongnam, Republic
178 of Korea). The animals were housed in a breeding room at 21 ± 1°C, a relative humidity
179 of 50~55%, and a light-dark cycle of 12 h. Drinking water and feed were provided ad

180 libitum. After acclimation for a week, four SD-rats were randomly assigned per
181 experimental group. WP and HWPB were orally administered at 500 mg/kg, sacrificed
182 according to the sample administration times (20, 40, 60, and 120 min), and blood was
183 collected. Blood samples were collected from the control group at 0 min. Following
184 centrifugation (3,000 rpm, 15 min, 4°C), serum was collected to measure the total A-N
185 content. All animal experiments were approved by the Korea University Animal
186 Experiment Ethics Committee (KUIACUC-2022-0095).

187 **Molecular Weight Distribution of Hydrolysates**

188 To measure the molecular weight distribution of the hydrolysate using HPLC, the
189 sample was filtered (microfilter, 0.45 µm) and 20 µL was injected into a Superdex G-75
190 column (10 × 300 mm, GE Healthcare, Anaheim, CA, USA). Subsequently, absorbance
191 was measured at 220 nm, and the column was eluted with 50 mM ammonium formate
192 buffer (pH 5.5) (0.5 mL/min). The molecular weight distribution of the hydrolysate was
193 measured according to the molecular weight distribution curve prepared using
194 glutathione (MW 307), aprotinin (MW 6,512), cytochrome c (MW 12,384), enolase
195 (MW 67,000), lactate dehydrogenase (MW 142,000), and glutamate dehydrogenase
196 (MW 290,000) as standard proteins, which were purchased from Sigma-Aldrich.

197 **Evaluation of Immunological Responses of Hypoallergenic Hydrolysates**

198 A specific pathogen free male BALB/c mice aged 8 weeks were purchased from
199 Daehan Biolink Co., Ltd. (Chungbuk, Republic of Korea) and experimented under
200 aseptic management (breeding room temperature, 25°C; humidity, 55%; and sterile
201 distilled water supply). Mice were divided into groups administered 250 and 500 mg/kg
202 of WP and HWPB, respectively, and a control group that was administered isotonic
203 sodium chloride solution. Eight mice were included in each group. Gastric intubation
204 was performed five times per week for 4 weeks. Body weight was measured twice a

205 week at 3-day intervals. After administration, blood was collected and centrifuged at
206 3,000 rpm for 10 min to separate the serum.

207 After sacrifice, aseptically excised splenocytes for cell culture were dispensed into 24
208 well-culture plates and cultured in a CO₂ incubator for 48 h. Subsequently,
209 phytohemagglutinin (Sigma, 5 µg/L × 10⁶ cells) was added as a polyclonal stimulator
210 for in vitro activation. The concentration of cytokines (INF-γ and IL4), antibodies
211 (IgG2a and IgG1) and IgE in the cell culture medium were measured using an ELISA
212 kit purchased from BD Bioscience (San Diego, CA, USA). For the small intestine
213 cytokines, 2 mL of PBS was added to 0.5 g of duodenum aseptically extracted after
214 sacrifice, vortexed, and left at 4°C for 2 days. Thereafter, the supernatant obtained by
215 centrifugation was analyzed for cytokines and IgE using the ELISA kits.

216 **Statistical Analysis**

217 Data were statistically analyzed using SPSS (ver. 12.0; SPSS Inc., Chicago, IL,
218 USA), and the mean and standard deviation were calculated for all measured values.
219 The significance between the experimental groups was tested by ANOVA, followed by
220 Tukey's multiple range test at p<0.05 level. RSM analysis was performed using Minitab
221 17 software (Minitab Inc., State College, PA, USA) to confirm the suitability of the
222 model to understand the relationship between the independent and dependent variables.

223

224 **Results and Discussion**

225 **Selection of Enzyme Combinations for the Preparation of HWP**

226 WP accounts for approximately 20% of the total milk protein. β-LG and α-LA are the
227 major allergens, and serum albumin, Ig, and LF also act as allergens (Hochwallner et
228 al., 2014). α-LA and β-LG are more effective in reducing allergic reactions using
229 proteolytic enzymes due to their acid-resistant and heat-resistant structures (Stănciuc

230 and Rapeanu, 2010; Yuan et al., 2018). Proteolytic enzymes under mild conditions
231 convert proteins to shorter chain peptides and amino acids, yielding minimal
232 byproducts. It is documented that enzymatic protein hydrolysis can alter the protein
233 structure without compromising its nutritional value, thereby reducing the functional
234 properties or allergens (Cui et al., 2023; Kheroufi et al., 2022).

235 To select enzymes that could degrade α -LA and β -LG, endo-type enzymes (Alcalase,
236 Collupulin, and Protamex) and exo-type enzymes (Prozyme) were mixed, and
237 hydrolysis was performed for 8 h. A combination of enzymes in which α -LA and β -LG
238 were degraded was selected by SDS-PAGE. Following electrophoresis, the α -LA and β -
239 LG bands were not seen in the hydrolysate prepared using the mixture of Alcalase and
240 Prozyme, revealing that the two allergens were decomposed (Figure S1). Figure 1
241 shows the A-N and DH in the hydrolysate obtained after treatment with the enzyme
242 mixture. The levels of A-N and DH in the HWPBs obtained from by mixing Alcalase
243 and Prozyme were significantly higher than those in the other hydrolysates. Thus, this
244 mixture was the most suitable enzyme combination for the hydrolysis of α -LA and β -
245 LG.

246 Proteolytic enzymes have different properties depending on the order of hydrolysis
247 and the combination of endo-type enzymes that hydrolyze proteins roughly and exo-
248 type enzymes that degrade proteins from the end (Bautista Palomas et al., 1999).
249 Alcalase, a serine protease (endo-type), cleaves the internal chains of proteins and
250 peptides as well as peptide bonds following Glu, Met, Leu, Tyr, Lys, and Gln (Adamson
251 and Reynolds, 1996). Prozymes are fungal exo-proteases from *Aspergillus oryzae*
252 suitable for producing HWPB (Suh et al., 2017). Among the hydrolysates prepared
253 using exo- and endo-type enzymes, α -LA and β -LG were not detected in the

254 hydrolysates prepared using Alcalase and Prozyme, and their A-N content and DH were
255 the highest (Figures 1 and S1).

256 **Mixing Ratio of Alcalase and Prozyme for the Preparation of HWPB**

257 According to the 15 combinations recommended by the RSM, hydrolysates were
258 prepared to choose the appropriate mixing ratio of Alcalase and Prozyme for the
259 production of HWPB (Table S2A). DH was calculated by measuring the A-N content of
260 the hydrolysate (Figure 2). The A-N content and DH of the hydrolysate obtained by
261 mixing Alcalase and Prozyme at 1:1 and 1:3, respectively, were significantly higher
262 than those of the hydrolysate obtained at other mixing ratios ($p < 0.05$). The regression
263 equation for the A-N content obtained from the multiple regression analysis is as
264 follows: $A-N \text{ (mg/mL)} = 32.29 x + 68.87 y + 96.58 xy$ [X : Alcalase (%), Y : Prozyme
265 (%), $R^2 = 97.54\%$]

266 As a result of predicting the optimal ratio of an enzyme mixture using RSM, when
267 Alcalase and Prozyme were mixed 1:1, the A-N content was expected to be the highest
268 at 50.42 mg/mL. The A-N content of the hydrolysate produced by Alcalase and
269 Prozyme mixed in a 1:1 ratio, as predicted by RSM, was 51.52 mg/mL, and the actual
270 measured value (50.42 mg/mL) was close to this predicted value. Thus, for the
271 preparation of HWPB, the combination of Alcalase and Prozyme at 0.5/0.5 or 0.25/0.75
272 was most appropriate (Figure 2).

273 Hydrolysis conditions such as temperature, pH, and enzyme–substrate ratio also
274 affect protein hydrolysis (Diniz and Martin, 1997; Vieira et al., 1995). When enzymes
275 are to be mixed, the mixing ratio plays an important role in determining the
276 hydrolysate's properties. The hydrolysate produced by the mixture of Alcalase and
277 Prozyme at a ratio of 1:1 showed the highest A-N and DH (Figure 2).

278 **Optimization of Enzyme Amount and Hydrolysis Time for the Preparation of**
279 **HWPH by Alcalase-Prozyme Mixture**

280 To select the optimal enzyme concentration and the hydrolysis time for HWPH
281 production by the Alcalase-Prozyme mixture, the A-N content of the hydrolysate was
282 measured according to the 39 combinations suggested by the RSM (Table S2B). The
283 regression equation for the A-N content obtained from the multiple regression analysis
284 is as follows: $A-N \text{ (mg/mL)} = 0.471 X^2 + 8.364 Y^2 - 2.039 XY - 0.922 X + 4.552 Y +$
285 29.62 [X: hydrolysis time (h), Y: enzyme addition amount (%), $R^2 = 96.80\%$]

286 With an increase in the amount of enzyme and the hydrolysis time, the A-N content
287 also increased (Figure 2). The highest amount of A-N was observed when hydrolysis
288 was performed for 10 h with the addition of 1.0% enzyme compared to the substrate.
289 The expected amount of A-N at the optimal enzyme concentration and hydrolysis time
290 predicted by RSM was 60.00 mg/mL, and the actual measured value of 59.58 mg/mL
291 was close to this, with the R^2 value being 0.968. To produce a HWPH by the Alcalase-
292 Prozyme mixture, hydrolysis with 1.5% enzyme addition was found to be most suitable
293 compared to the substrate for 8 h.

294 Under the optimal reaction conditions of the WPH to produce low allergens, when
295 WP and allergen-inducing substances α -LA and β -LG were hydrolyzed as substrates,
296 the decomposition of these substances was confirmed through gel electrophoresis
297 (Figure 3). The bands for these allergens appeared dark before hydrolysis (0 h), and
298 faded after 4 h of hydrolysis. After an 8 h hydrolysis, almost no bands were visible,
299 confirming that α -LA and β -LG were decomposed by the mixed enzyme treatment.

300 **Amino Acids Composition of HWPH**

301 The amino acid composition of HWPH treated with Alcalase and Prozyme enzymes
302 (Table 1) revealed the composition of free amino acids as $2,089.72 \pm 76.18 \mu\text{mole/g}$ and

303 that of total amino acids as $4,987.21 \pm 93.44$ $\mu\text{mole/g}$. In all, 17 types of free amino
304 acids were detected, with leucine being the most prominent (16.8%, 351.73 ± 4.62
305 $\mu\text{mole/g}$), followed by valine (13.8%), lysine (11.2%), and isoleucine (8.7%). As high
306 levels of these amino acids cause bitterness, the bitterness in the sample was consistent
307 with their presence.

308 **Digestibility and Intestinal Permeability by Caco-2 Cell**

309 Changes in A-N content and digestibility were measured when WP and HWPB were
310 treated with the artificial digestive enzymes pepsin and pancreatin (Figures. 4A-B). As
311 the digestion of pepsin and pancreatin progressed, the A-N content and digestibility
312 tended to increase. WP and HWPB were more hydrolyzed by pancreatin treatment, and
313 the increase in A-N content and digestibility was significantly higher than that by pepsin
314 treatment. The amount of A-N at each stage of the digestive enzyme treatment was
315 significantly higher in HWPB than in WP (Figure 4A). As for the change in
316 digestibility, HWPB, which had a high A-N content before hydrolysis, showed lower
317 digestibility than WP in the pepsin treatment stage but a higher level of digestibility
318 increase than WP in the pancreatin treatment stage was noted (Figure 4B).

319 By measuring the intestinal permeability of Caco-2 cells (Figures 4C-F), it was
320 confirmed that the permeability of HWPB was significantly higher than that of the WP
321 sample at the indicated treatment time points of 20, 40, 60, and 120 min. The intestinal
322 permeability of Caco-2 cells at each stage of treatment with pepsin and pancreatin was
323 also significantly higher in HWPB than in WP. Thus, it was confirmed that the
324 digestibility and intestinal permeability of HWPB determined by Alcalase and Prozyme
325 were higher than those of WP.

326 The size of the peptides constituting the protein also affects the digestibility and
327 absorption. Peptides of ≤ 500 Da have higher bioavailability than peptides of $\geq 2,000$

328 Da (Feng and Betti, 2017; Wang and Li, 2017). Hydrolysates composed of small
329 peptides are absorbed into the bloodstream through the intestinal wall and confer their
330 effects (Chatterjee et al., 2018). WP and HWPH are converted into smaller peptides by
331 pepsin and pancreatin and absorbed through the intestinal mucosa. During *in vitro*
332 digestion, pancreatin treatment resulted in higher A-N content and digestibility than
333 pepsin treatment (Figure 4).

334 **Evaluation of Absorption Rate of WP and HWPH in Sprague-Dawley (SD) Rats**

335 Changes in the amount of A-N in the blood were measured after the oral
336 administration of WP and HWPH to SD rats (Figure 5). Twenty min after oral
337 administration, the highest amount of A-N was observed in the blood, which decreased
338 gradually thereafter. After 20 and 40 min, as compared to WR, the administration of
339 HWPH resulted in a significantly higher amount of A-N in the blood ($p < 0.001$).
340 HWPH, a hypoallergenic whey protein hydrolysate produced by Alcalase-Prozyme, is
341 composed of smaller peptides than WP and is easily digested and absorbed; therefore,
342 the amount of A-N in the blood may have been high at the beginning of oral
343 administration.

344 Pancreatin contains endopeptidases (trypsin and chymotrypsin) and exopeptidases
345 (carboxypeptidase) (Andriamihaja et al., 2013), which can hydrolyze WPs into smaller
346 peptides than pepsin. Since β -LG is resistant to digestive enzymes (Battaglin Villas
347 Boas et al., 2015), HWPH, in which β -LG is decomposed by Alcalase/Prozyme
348 treatment, showed higher digestibility than WP. The measurement of the absorption rate
349 through Caco-2 cells and animal experiments (Figures 4 and 5) showed that the
350 hydrolysate (HWPH) had better permeability than before hydrolysis (WP). Animal
351 experiments showed a difference in the absorption rate within 40 min of oral
352 administration.

353 **Molecular Weight Distribution of HWPH**

354 The molecular weight distributions of the WP and HWPH were determined using a
355 Superdex G-75 column (Figure 6). WP was noted to contain several peptides with
356 molecular weights of 18 and 5.8 kDa. In contrast, HWPH produced by Alcalase and
357 Prozyme mainly consists of peptides of 5 kDa or less, and the main peaks were
358 approximately 384 Da and 214 Da, respectively. WP was degraded into small peptides
359 of 2.6 , 1.3, and 0.491 kDa when treated with pepsin, and following pancreatin
360 treatment, it was hydrolyzed into peptides with smaller molecular weights than when
361 treated with pepsin (Figures 6B-C). The molecular weight distribution of HWPH was
362 similar after pepsin and pancreatin treatments. Since the HWPH had already been
363 hydrolyzed by the enzyme (Figures 6E-F), the effect of pepsin and pancreatin
364 treatments on the molecular weight distribution seems to be minimal.

365 Allergens can also be identified by their molecular weights. Enzymatic hydrolysis of
366 milk proteins can prevent allergic sensitization by minimizing the number of sensitizing
367 protein epitopes through the decomposition of allergenic sites or reduction in protein
368 size (<1,500 Da) (Crittenden and Bennett, 2005; Fiocchi et al., 2003). WP is mainly
369 composed of 1,800 and 5,800 Da peptides, but Alcalase/Prozyme hydrolysate (HWPH)
370 was composed of short peptides of 384 and 214 Da (Figure 6). Therefore, the peptides
371 constituting HWPH are small in size, which increases the absorption and bioavailability,
372 but will be limited in antibody production due to their size.

373 **Effect of HWPH Administration on Cellular Immunity of mice**

374 The content and ratio of cytokines (INF- γ and IL-4) and antibodies (IgG2a and IgG1)
375 corresponding to Type 1 helper T (Th1) and Type 2 helper T (Th2) effector cells in the
376 blood, spleen cells, and small intestine are closely related to the immune response
377 caused by allergens. INF- γ and IL-4 in the blood of mice administered with WP and

378 HWPH tended to marginally increase as compared to mice in the control group, but this
379 difference was not significant (Table 2). Although not statistically significant, the ratio
380 of Th1/Th2 (INF- γ /IL-4) also showed an increase with HWPH treatment. The ratio of
381 IgG2a/IgG1 between WP and HWPH was significantly increased by the oral
382 administration of 250 and 500 mg/kg HWPH.

383 CD4⁺ helper T (Th) cells play a central role in the immune response and secrete
384 various cytokines that affect the function of B cells and the immune system. Depending
385 on the secreted cytokines, they are divided into Th1 and Th2 effector cells (Herzyk et
386 al., 2001; Lavigne et al., 1998) which exhibit characteristic cytokine profiles (MacLeod
387 et al., 2008). Cytokines (INF- γ) and antibodies (IgG2a) secreted from Th1 cells have an
388 antagonistic action with cytokines (IL-4) and antibodies (IgG1) secreted from Th2 cells.
389 The ratio of INF- γ /IL-4 in the splenocytes decreased significantly in the group
390 administered with 500 mg/kg of WP compared to that in the control group ($p < 0.01$).
391 The ratio of IgG2a/IgG1 (Th1:Th2) was similar or higher in the HWPH-administered
392 groups than in the control group, whereas the WP orally administered group showed a
393 lower ratio, but the difference was not significant. There was a significant difference in
394 the IgG2a/IgG1 ratios between the HWPH and WP groups.

395 Cytokines and antibodies specific to Th1 and Th2 cells in the small intestine of mice
396 orally administered WP and HWPH were analyzed (Table 2). The IL-4 content in the
397 groups orally administered with WP and HWPH was significantly higher than that in
398 the control group ($p < 0.05$). The ratio of INF- γ /IL-4, corresponding to the Th1/Th2 ratio,
399 tended to increase as the dose of HWPH increased, and the 500 mg/kg oral
400 administration group showed a significantly higher level than the control group
401 ($p < 0.05$). IgG2a levels in the small intestine in the two groups were significantly higher
402 than in the control group. The ratio of IgG2a/IgG1 and the ratio of Th1 to Th2 cells

403 were significantly higher in the HWPB-administered group than in the control group
404 ($p < 0.001$). There was a significant difference in the IgG2a/IgG1 ratio between the WP
405 and HWPB groups in terms of the dose ($p < 0.001$).

406 Abnormalities in the maintenance of Th1 and Th2 cell homeostasis are known to lead
407 to various immunopathological abnormalities. When Th2 is relatively dominant, it
408 promotes decreased defense against microbial infections, such as bacteria and viruses,
409 allergic development, and some autoimmune diseases (Prigent et al., 1995; Umetsu and
410 DeKruyff, 1997), and excessive immune responses (cell-mediated immunity) of Th1
411 cells are associated with auto-immune diseases (Gans et al., 2003; Margalit et al., 2005).
412 As depicted in Table 2, the ratio of IFN- γ /IL-4 was higher for HWPB than for WP,
413 indicating that the allergic reaction induced by Th2 was somewhat suppressed. This is
414 due to the fact that IgG2a isotype switching is induced by IFN- γ , and isotype switching
415 of IgG1 or IgE is induced by IL-4, which inhibits the function or production of IFN- γ
416 (Kühn et al., 1991; Snapper and Paul, 1987). The IgG2a/IgG1 ratio was higher in the
417 HWPB than in the WP, suggesting that the Th1 response was relatively stimulated
418 rather than the Th2 response, and thus, antibody conversion was induced, leading to a
419 significant suppression of the allergic reaction (Table 2).

420 Oral administration of HWPB increased the levels of Th1 cytokines and antibodies in
421 the blood, spleen, and small intestine, whereas the levels of Th2 cytokines and
422 antibodies were lower than those in the WP group. In addition, the IgE content in the
423 blood, splenocytes, and small intestine was significantly different between the two
424 groups (Figure 7). HWPB, in which α -LA and β -LG had been decomposed, had a
425 lower level of allergen-related intracellular immune response than WP, and thus
426 presumed to have a lower allergenic potential due to the hydrolysis of the allergen-
427 inducing substances. The allergic response to WP is representative of type I immune

428 hypersensitivity involving IgE (Martorell-Aragonés et al., 2015). Oral administration of
429 HWPB resulted in a significantly lower IgE production than that of WP (Figure 7).
430 HWPB, a hydrolysate produced by Alcalase and Prozyme, seems to reduce allergic
431 reactions as it hydrolyzes LG and LA, the two allergens in WP.

432

433 **Conclusion**

434 The mixing ratio of Alcalase and Prozyme, and the enzyme treatment conditions for
435 HWPB production were optimized using RSM. The mixed treatment of Alcalase and
436 Prozyme effectively degraded α -LA and β -LG, the major allergens of WP. Because
437 HWPB is converted into small peptides by enzyme treatment and has a low molecular
438 weight, its digestibility and intestinal permeability are higher than those of WP. In
439 addition, it was confirmed that HWPB suppressed the allergic immune response by
440 stimulating the Th1 response, but not the Th2 response, and by inducing antibody
441 conversion. Thus, HWPB can be expected to be useful in a variety of products with low
442 allergenicity targeting infants, adults, and the elderly.

443

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579 **Table 1. Composition of amino acids in hypoallergenic whey protein hydrolysates**

Contents ($\mu\text{mole/g}$)	Free amino acids	Total amino acids
Aspartic acid (Asp)	46.96 \pm 0.33	516.35 \pm 4.63
Serine (Ser)	151.70 \pm 0.86	308.41 \pm 2.31
Glutamine (Glu)	169.00 \pm 2.30	720.73 \pm 9.91
Glycine (Gly)	25.27 \pm 0.90	182.34 \pm 3.57
Histidine (His)	61.55 \pm 4.06	95.91 \pm 3.63
Arginine (Arg)	79.38 \pm 0.91	394.24 \pm 21.16
Threonine (Thr)	57.53 \pm 28.23	72.17 \pm 13.46
Alanine (Ala)	48.65 \pm 35.25	288.05 \pm 5.65
Proline (Pro)	100.55 \pm 2.67	388.43 \pm 6.84
Cysteine (Cys)	10.91 \pm 1.14	282.89 \pm 8.71
Tyrosine (Tyr)	73.76 \pm 0.93	93.46 \pm 2.77
Valine (Val)	288.57 \pm 6.28	396.82 \pm 4.46
Methionine (Met)	66.35 \pm 0.28	78.32 \pm 2.07
Lysine (Lys)	234.71 \pm 1.66	308.88 \pm 5.78
Isoleucine (Ile)	182.55 \pm 3.47	249.27 \pm 5.98
Leucine (Leu)	351.73 \pm 4.62	469.35 \pm 4.30
Phenylalanine (Phe)	140.56 \pm 3.47	141.59 \pm 0.99
Total	2089.72 \pm 76.18	4987.21 \pm 93.44

580 Values are mean \pm standard deviation for each group, n = 3.

581 **Table 2. Changes in cytokines and antibodies in the blood, spleen cells and small intestine after oral administration of WP and HWPH**

Sample	(mg/kg)	Serum					
		INF- γ (pg/mL)	IL-4 (pg/mL)	INF- γ /IL-4	IgG2a (ng/mL)	IgG1 (ng/mL)	IgG2a/IgG1
CON		8.87 \pm 1.50 ^{ns}	5.30 \pm 0.21 ^{ns}	1.65 \pm 0.24 ^{ns}	52.62 \pm 2.18	26.32 \pm 0.65	2.01 \pm 0.13
WP	250	8.82 \pm 2.07	7.73 \pm 0.72	1.12 \pm 0.32	136.70 \pm 1.76 ^{***}	69.29 \pm 1.57	1.97 \pm 0.02
	500	9.95 \pm 1.71	10.53 \pm 2.90	1.16 \pm 0.35	103.27 \pm 2.98 ^{**}	500.69 \pm 36.01 ^{***}	0.21 \pm 0.01 ^{***}
HWPH	250	10.70 \pm 1.89	7.64 \pm 1.16	1.55 \pm 0.47	155.63 \pm 5.22 ^{***,#}	64.15 \pm 3.33	2.43 \pm 0.12 [#]
	500	11.66 \pm 0.81	8.94 \pm 2.61	1.63 \pm 0.33	339.82 \pm 13.33 ^{***,###}	63.96 \pm 3.20 ^{###}	5.35 \pm 0.44 ^{***,###}
Sample	(mg/kg)	Spleen					
		INF- γ (μ g/g)	IL-4 (μ g/g)	INF- γ /IL-4	IgG2a (μ g/g)	IgG1 (μ g/g)	IgG2a/IgG1
CON		45.37 \pm 0.89	2.85 \pm 0.28	16.74 \pm 1.72	0.36 \pm 0.08	0.32 \pm 0.04	1.18 \pm 0.30
WP	250	44.30 \pm 0.85	3.68 \pm 0.28	12.37 \pm 0.87	0.40 \pm 0.05	0.82 \pm 0.04 ^{***}	0.50 \pm 0.08
	500	45.13 \pm 0.63	4.57 \pm 0.51 [*]	10.50 \pm 1.15	0.48 \pm 0.05	1.89 \pm 0.08 ^{***}	0.26 \pm 0.03
HWPH	250	44.30 \pm 0.62	3.51 \pm 0.98	13.28 \pm 1.21 [*]	0.70 \pm 0.08 ^{*,#}	0.53 \pm 0.02 ^{*,###}	1.35 \pm 0.16 ^{*,##}
	500	52.19 \pm 0.92 ^{***,###}	3.85 \pm 0.16	13.67 \pm 1.71	0.77 \pm 0.08 ^{*,#}	0.72 \pm 0.01 ^{***,###}	1.08 \pm 0.12 ^{###}
Sample	(mg/kg)	Small intestine					
		INF- γ (μ g/g)	IL-4 (μ g/g)	INF- γ /IL-4	IgG2a (μ g/g)	IgG1 (μ g/g)	IgG2a/IgG1
CON		1.92 \pm 0.34	3.17 \pm 0.003	0.61 \pm 0.11	0.28 \pm 0.01	6.08 \pm 0.25	0.05 \pm 0.002
WP	250	1.91 \pm 0.79	3.97 \pm 0.01 ^{***}	0.48 \pm 0.20	0.39 \pm 0.01 ^{***}	11.18 \pm 0.18 ^{***}	0.04 \pm 0.001
	500	3.77 \pm 0.17	5.37 \pm 0.02 ^{***}	0.70 \pm 0.03	0.73 \pm 0.03 ^{***}	14.83 \pm 0.13 ^{***}	0.05 \pm 0.002
HWPH	250	4.54 \pm 1.04	3.54 \pm 0.02 ^{***,###}	1.281 \pm 0.30	0.94 \pm 0.04 ^{***,###}	7.19 \pm 0.06 ^{*,###}	0.13 \pm 0.01 ^{###}
	500	6.85 \pm 0.62 [*]	4.58 \pm 0.01 ^{***,###}	1.49 \pm 0.14 ^{*,##}	1.68 \pm 0.03 ^{***,###}	6.81 \pm 0.22 ^{###}	0.25 \pm 0.01 ^{###}

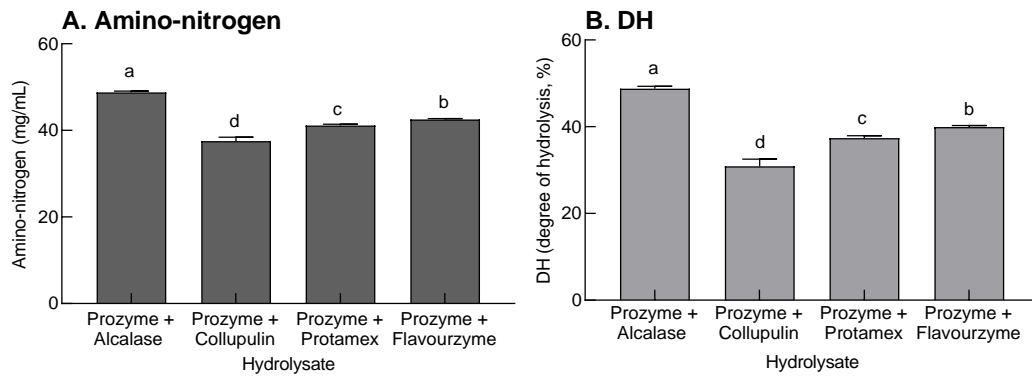
582 Values are presented as the mean \pm standard error of the mean (SEM) for each group, n = 8. *p<0.05, **p<0.01 and ***p<0.001 indicate
583 significant difference from the control group (CON) based on Tukey's multiple test. #p<0.05, ##p<0.01, and ###p<0.001 indicate significant
584 differences between groups (WP vs. HWPH) at the same concentration by Student's t-test. ns, no significant difference between the groups. WP,
585 whey protein before hydrolysis; HWPH, hypoallergenic whey protein hydrolysate.

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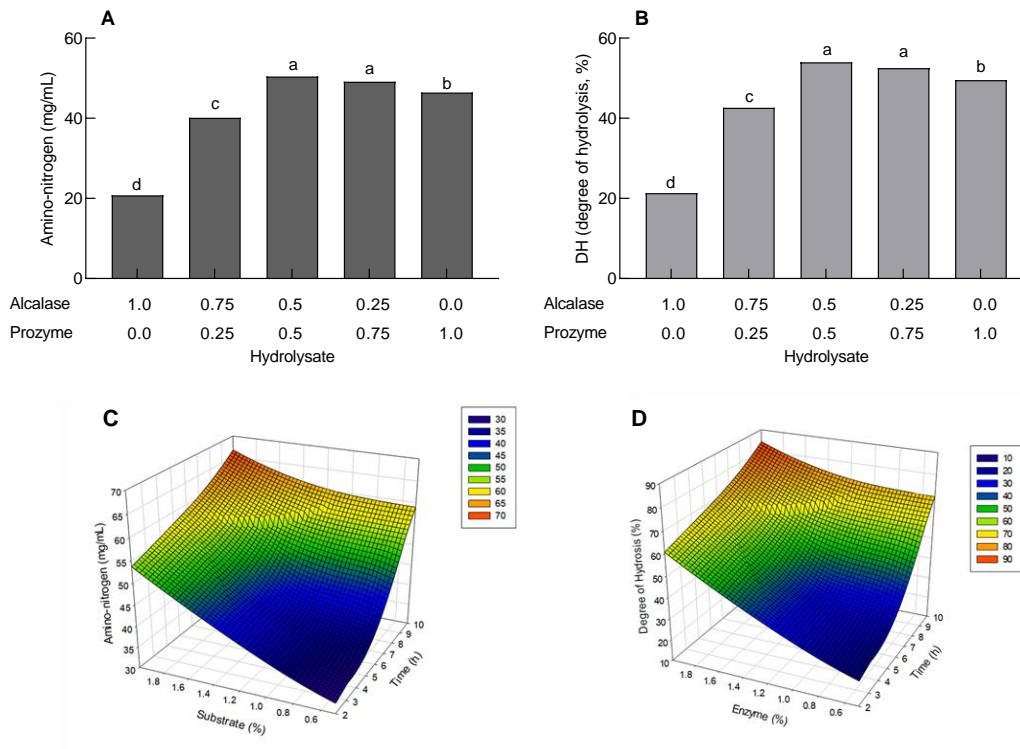
Figures



589

590 **Fig. 1. Amino-nitrogen (A-N) (A) and degree of hydrolysis (DH) (B) of whey**
591 **protein hydrolysate by mixing endo- and exo-types protease.** Values are mean \pm
592 standard deviation for each group, n = 3. Different letters represent significant
593 difference at $p < 0.05$ based on Tukey's multiple range test.

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595

596 **Fig. 2. A-N content (A, C) and DH (B, D) of whey protein hydrolysate optimized**

597 **using response surface methodology (RSM).** (a-b) RSM conditions according to the

598 mixing ratio of Alcalase and Prozyme. Whey protein hydrolysis was performed using a

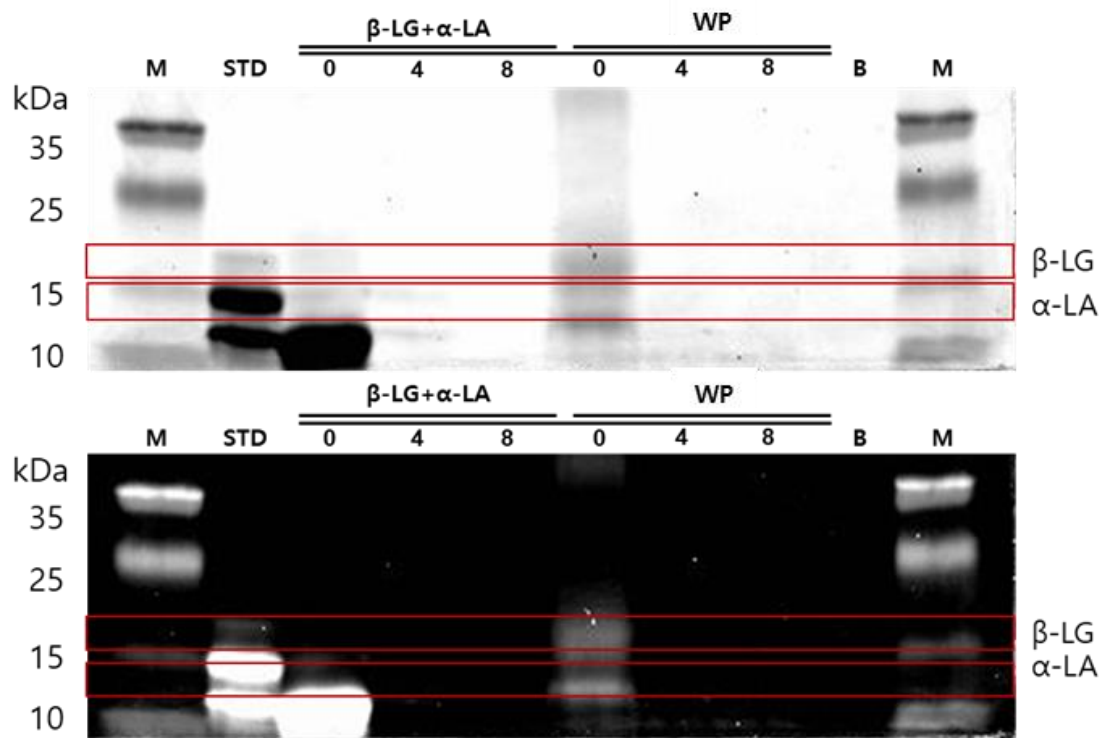
599 mixture of Alcalase and Prozyme in 10% substrate for 8 at 50°C and pH 7.8. (c-d) RSM

600 conditions according to the addition amount and reaction time of the mixed enzymes.

601 Values are mean \pm standard deviation for each group, n = 3. Other letters are

602 significantly different at $p < 0.05$ by Tukey's multiple range test.

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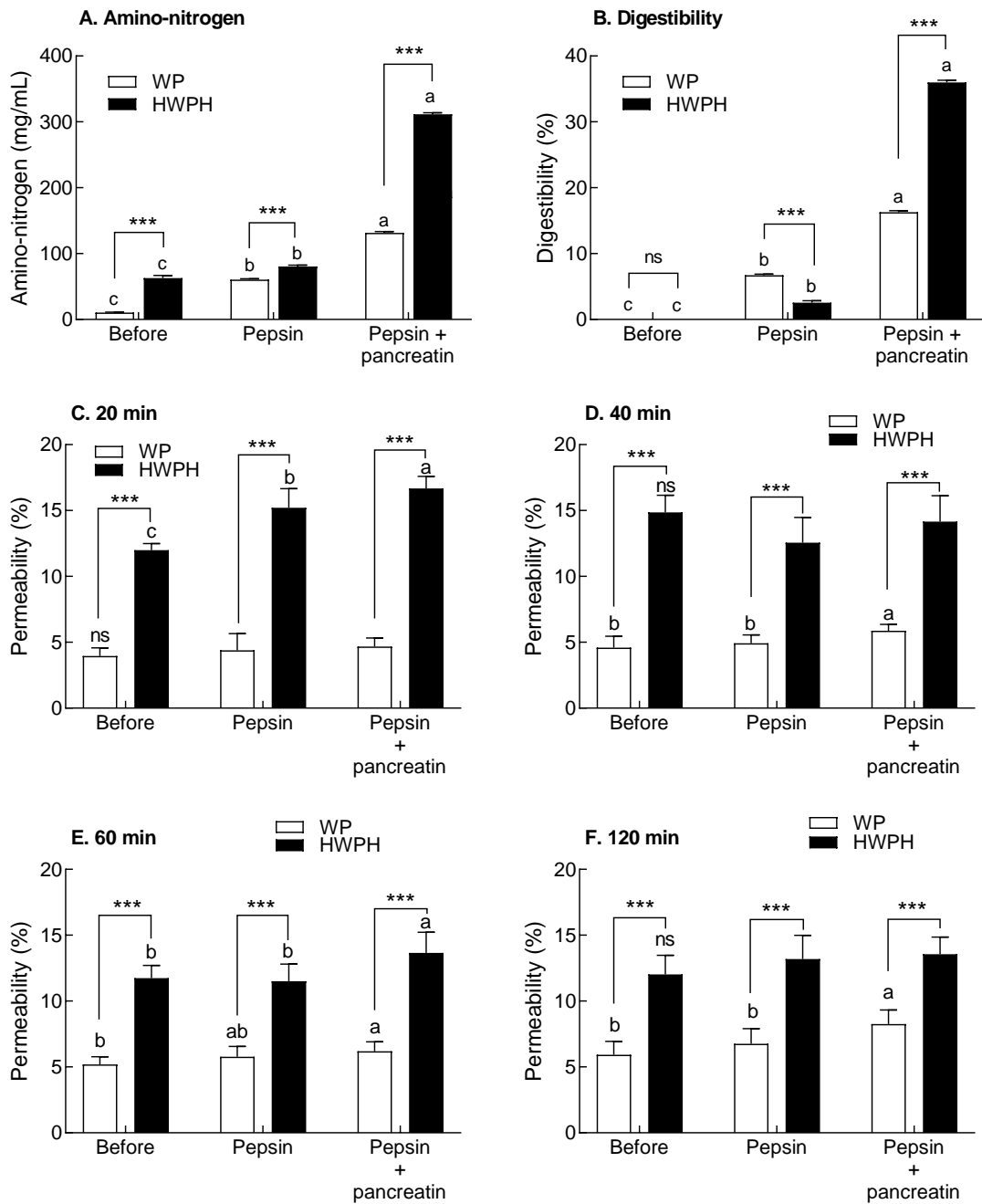


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605 **Fig. 3. Bands of α -lactalbumin (α -LA) and β -lactoglobulin (β -LG) as seen in gel**

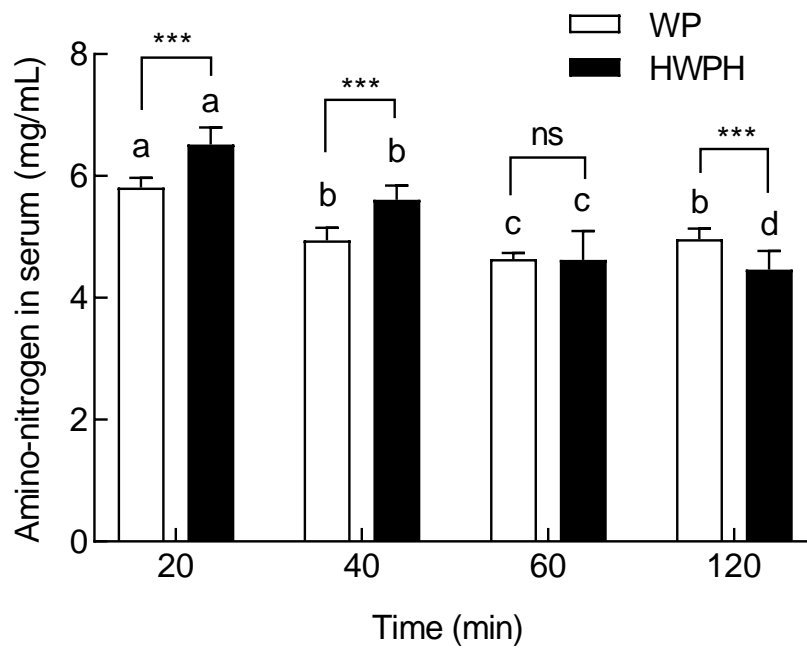
606 **electrophoresis according to hydrolysis time of whey protein (WP).**

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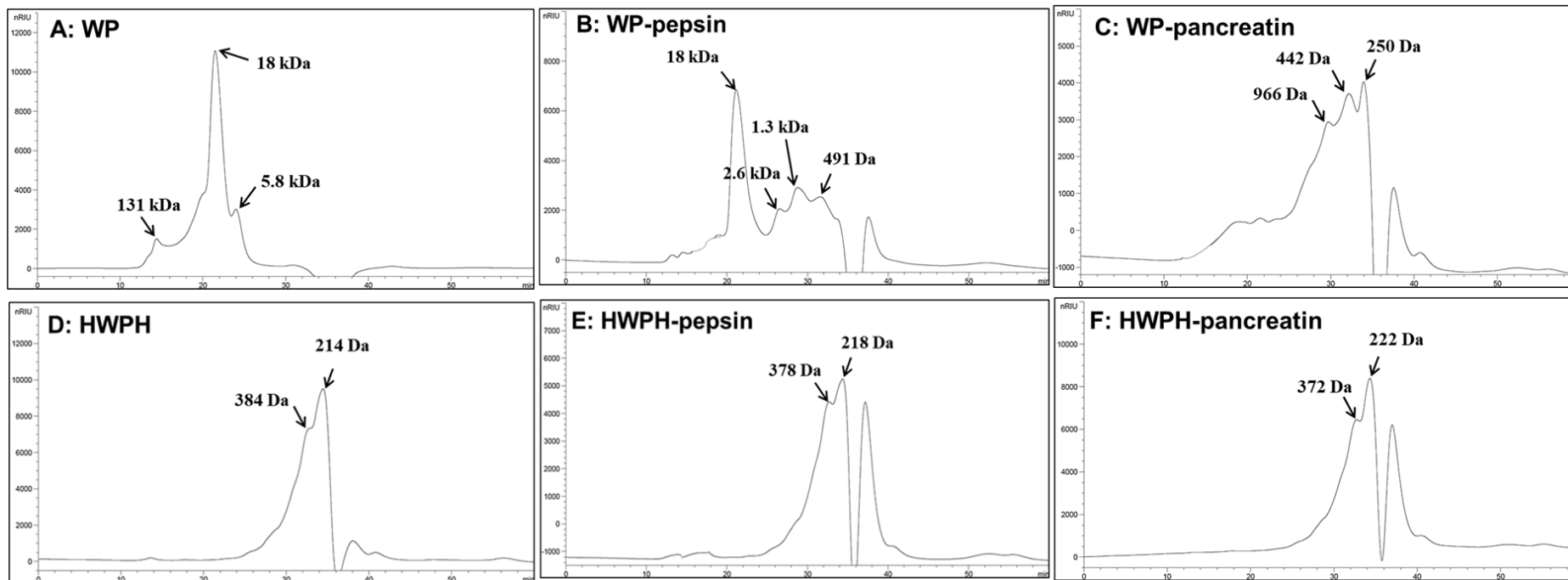
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609 **Fig. 4. Changes in (A) A-N content, (B) digestibility, and (C-F) intestinal**
 610 **permeability according to time in Caco-2 cells exposed to WP and hypoallergenic**
 611 **wey protein hydrolysate (HWPH) treated with *in vitro* digestive enzymes.** Values
 612 are mean \pm standard deviation for each group, n = 3. Different letters represent
 613 significant differences at p<0.05 based on Tukey's multiple range test. *** p<0.001 for
 614 HWPH as compared to WP. ns, not significant.



615

616 **Fig. 5. A-N changes in serum of Sprague-Dawley (SD) rats following oral**
 617 **administration of WP and HWPH before and after hydrolysis of WP with mixed**
 618 **enzymes.** Values are mean \pm standard deviation for each group, n = 4. Different letters
 619 represent significant difference at $p < 0.05$ based on Tukey's multiple range test.
 620 *** $p < 0.001$ for HWPH compared to WP. ns, not significant.



621

622 **Fig. 6. Molecular weight distribution of WP (A, B, C) and HWPB (D, E, F).** Absorbance was measured at 220 nm while eluting with 50 mM
 623 ammonium formate buffer (pH 5.5). Glutathione (MW 307), aprotinin (MW 6512), cytochrome c (MW 12,384), enolase (MW 67,000), lactate
 624 dehydrogenase (MW 142,000), and glutamate dehydrogenase (290,000) were used as molecular weight standard proteins.

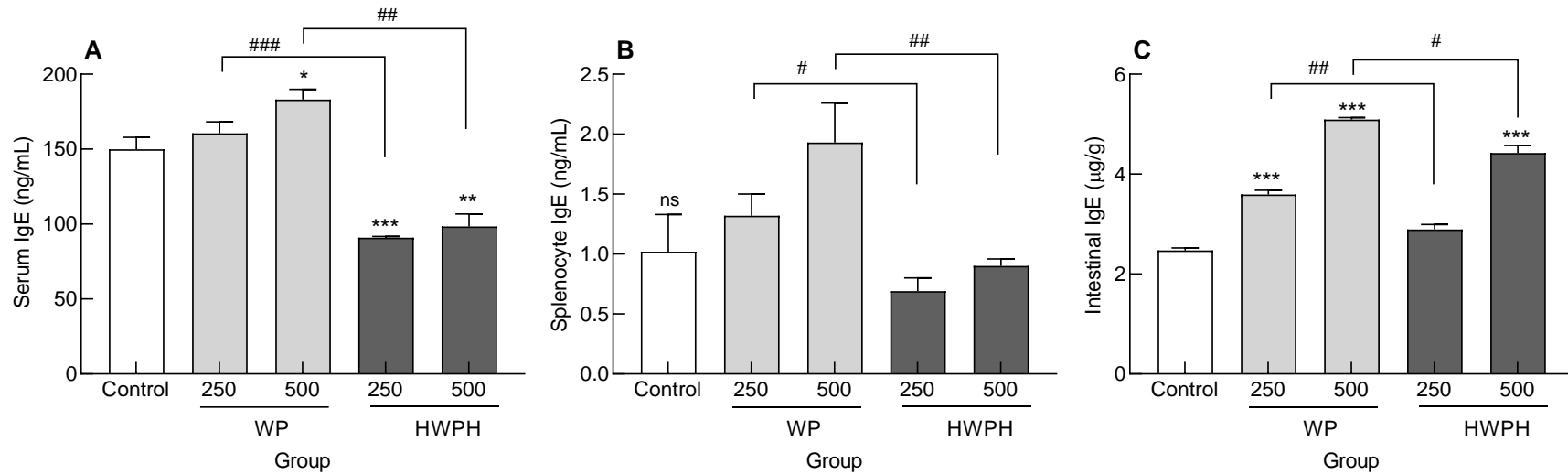


Fig. 7. Changes in immunoglobulin (Ig) E in the blood, spleen cells, and small intestine in mice after oral administration of WP and HWPH. Values are presented as the mean \pm SEM for each group, $n = 8$. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ as compared to the control group based on Tukey's multiple test. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ between groups of the same concentration using student t-test. ns, no significant difference between the groups.

Supplementary Data

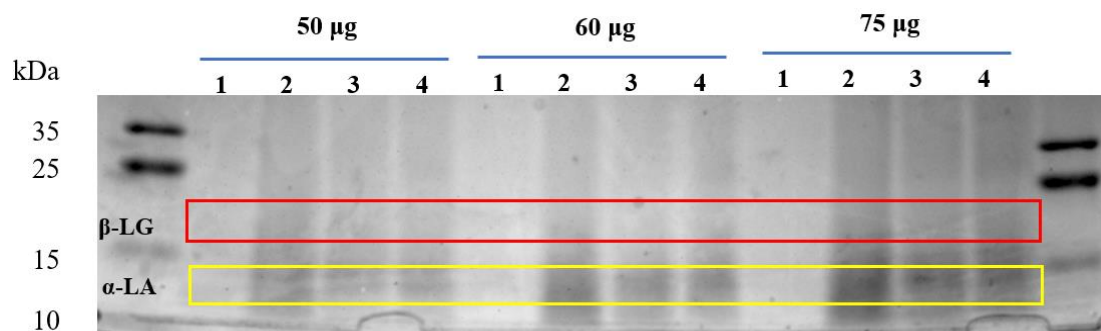


Fig. S1. Gel electrophoresis of whey protein hydrolysis for the analysis of α -lactalbumin and β -lactoglobulin. Lane 1: hydrolysate prepared by treatment with prozyme and alcalase, Lane 2: hydrolysate prepared by treatment with prozyme and collupulin, Lane 3: hydrolysate prepared by treatment with prozyme and protamex, Lane 4: hydrolysate prepared by treatment with prozyme and flavourzyme. Protein concentration (50, 60, and 70 μ g) of samples loaded on gel.

Table S1. Characteristics of the various proteases

Enzyme	Main activity	Source	Optimum conditions	
			Temp. (°C)	pH
Alcalase® 2.4 L FG	Endo-protease	<i>Bacillus</i> sp.	50	7.8
Collupulin MG	Endo-protease	<i>Carica papaya</i>	50-70	5.0-7.5
Protamex	Broad-spectrum endo-protease	<i>Bacillus</i> sp.	35-60	5.5-7.5
Flavourzyme® 500 MG	Protease (endo & exo)	<i>Aspergillus oryzae</i>	50	5.0-7.0
Prozyme 2000P	exo-peptidase	<i>Aspergillus oryzae</i>	50-60	6.0-9.0

Table S2. Combination of experimental runs based on RSM. A: mixing ratio of enzymes, B: Reaction conditions (enzyme addition amount and hydrolysis time) for mixed enzymes

A. Mixing ratio of enzymes			
RUN	Substrate (%)	Alcalase (%)	Prozyme (%)
1	10	0.5	0.5
2		1	0
3		0.5	0.5
4		0.75	0.25
5		0.75	0.25
6		1	0
7		0.5	0.5
8		0	1
9		0.25	0.75
10		0	1
11		1	0
12		0.25	0.75
13		0.25	0.75
14		0	1
15		0.75	0.25

B. Reaction conditions for mixed enzymes			
RUN	Substrate (%)	Enzyme (%)	Time (h)
1	10	2	6
2		2	6
3		1	6
4		1.5	4
5		1	2
6		1	10
7		1.5	8
8		1	2
9		1.5	8
10		0.5	6
11		2	6
12		1	6
13		1	6
14		1	6
15		1	6
16		1.5	4
17		0.5	6
18		1	6
19		0.75	8
20		0.75	4
21		0.5	6
22		1	6
23		1	10
24		1	6
25		0.75	8
26		1	6
27		0.75	4
28		1	2
29		1.5	4
30		0.75	4
31		1	6
32		1	6
33		1	6
34		1	6
35		1.5	8
36		1	6
37		1	10

38		1	6
39		0.75	8