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1 **Effect of Neutrase, Alcalase and Papain Hydrolysis of Whey Protein**  
2 **Concentrates on Iron Uptake by Caco-2 Cells**

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21

22 **Abstract**

23 Effects of enzymatic hydrolysates of whey protein concentrates (WPC) on iron  
24 absorption were studied using *in vitro* digestion combined with Caco-2 cells models  
25 for improved iron absorption. Neutrase and papain treated WPC could improve iron  
26 absorption, especially hydrolysates by neutrase could significantly increase iron  
27 absorption to 12.8% compared to 3.8% in the control. Hydrolysates by alcalase had  
28 negative effects to the lowest at 0.57%. Two new bands at the molecular weight (MW)  
29 around and below 10 KDa occurred at tricine SDS-PAGE of hydrolysates by neutrase,  
30 and one new band at MW below 10 KDa in hydrolysates by papain. No new band was  
31 observed in hydrolysates by alcalase. Concentration of free amino acids indicated that,  
32 except for tyrosine and phenylalanine, amino acids in papain treated hydrolysates were  
33 higher than that of alcalase, and no cysteine and proline were found in hydrolysates  
34 by alcalase. The results suggested that hydrolysate by neutrase treated WPC is a  
35 promising facilitator for iron absorption. Peptides of MW around and lower than 10  
36 KDa and aspartic acid, serine, glutamic acid, glycin, cysteine, histidine and proline  
37 may be the attributors to enhancement.

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40 **Key words:** Iron absorption; enzymatic hydrolysates; *in vitro* digestion; Caco-2 cells

41

## 42 INTRODUCTION

43 Iron is one of the essential trace elements for human nutrition, and the only way for  
44 obtaining is dietary intake. Iron deficiency is prevalent caused by whether insufficient  
45 intake or presence of iron absorption inhibitors. In our previous studies, we have tried  
46 different ways to decrease contents of phytic acid, which is mentioned as one of  
47 important inhibitors of iron absorption, in rice for improved bioavailability of  
48 minerals. However, the efficacy of those approaches is limited (1, 2). Dietary iron  
49 fortification or supplementations is another way for better iron nutrition. Low  
50 bioavailability and poor taste of common supplementary substances hindered its  
51 application. In recent years, new supplements with relatively higher bioavailability  
52 and better edibility, such as ferrous ethylene diamine tetraacetic acid (Fe EDTA),  
53 ferrous glycinate and lactoferrin occurred. However these new sources are not popular  
54 because of economic aspects (3).

55 It was also reported that presence of some ingredients, such as ascorbic acids and  
56 some amino acids, could improve bioavailability of iron. Enhancement of protein,  
57 peptides and amino acids on bioavailability and absorption of iron is one of the most  
58 interested research fields. It was reported that some proteins, such as egg yolk protein  
59 and casein, inhibited bioavailability of iron, which was caused by formation of  
60 insoluble iron-protein complex, or presence of compete minerals (e.g. calcium in  
61 casein (4-7). Proteins and peptides, mainly from milk and meat, enhance iron  
62 absorption were also reported (8-10). It was summarized that these enhancers had  
63 similar characteristics as: 1) contain some of “specific” amino acids, such as Pro, Phe,

64 Met, His, Ser, Glu, Asp and Cys, though conclusions of different study were not well  
65 accordant and the exact effect and mechanism were uncertain yet (10, 11); 2) presence  
66 of sulphhydryl (-SH) groups. It was assumed that -SH could reduce  $Fe^{3+}$  to more  
67 soluble  $Fe^{2+}$  (12); 3) have low molecular weight, it was reported that most of  
68 enhancers had molecular weight (MW) lowered than 10 KDa (5, 10, 13, 14).

69 Studies on protein showed that alcalase and flavourzyme have extensive proteolytic  
70 activity for low molecular weight peptides and amino acids (13-15). However, effect  
71 and application of these hydrolysates on fortification of minerals is scarce. In order to  
72 achieve improved absorption of fortified iron, whey protein concentrates (WPC) ,  
73 which is by-product from the cheese-making and casein manufacture in the dairy  
74 industry and it grows as the same rate as milk (>2% per year) (16), was selected and  
75 enzymatic treated for hydrolysates containing peptides and different composition of  
76 amino acids. The objectives of this study were 1) to evaluate the possibility of  
77 application of enzymatic treated WPC for improvement of iron absorption using *in*  
78 *vitro* digestion combined with Caco-2 cells models; 2) to analyze the mechanisms of  
79 WPC hydrolysates on iron absorption.

## 80 **MATERIALS AND METHODS**

### 81 **Preparation of WPC hydrolysates**

82 The 5% WPC solution was kept in incubator at 85 °C for 15 min. After cooled to 50  
83 °C, the pH was adjusted to 5.5, 7.0 and 8.0 respectively for papain, neutrase and  
84 alcalase using 1 M NaOH or 1 M HCl and maintained during enzymatic treatment.

85 The ratio of enzyme to substrate is 3000 U g<sup>-1</sup> protein basis. WPC solution was kept at

86 50 °C for neutrase and alcalase, and 54°C for papain. During enzymatic treatment,  
87 samples were collected at intervals of every 30 min until 4-5 h. Enzymes were  
88 inactivated by heating the reaction mixture for 10 min at 100°C. The hydrolysates  
89 solutions were either lyophilized for further *in vitro* digestion or centrifuged at 4800 *g*  
90 for 15 min and the supernatants were taken for subsequent determination of degree of  
91 hydrolysis (DH). The DH was determined following the procedures used by  
92 Adler-Nissen, which is basically a spectrophotometric assay of the chromophore  
93 formed by the reaction of trinitro-benzene-sulfonic acid (TNBS) with primary amines  
94 (1979) (17).

#### 95 ***In vitro* digestion of WPC hydrolysates**

96 The *in vitro* digestion procedure described by Kiers *et al.* (18) was applied with small  
97 modification. About 1 g (accuracy 0.0001 g) lyophilized WPC hydrolysates were  
98 digested with pepsin and pancreatin to simulate digestion system of human body. The  
99 digests were subjected to centrifugation at 5000 *g* for 25 min at 4 °C and the  
100 supernatants were filtered with a 0.22 µm membrane for iron uptake experiment. The  
101 *in vitro* digestion was carried out in triplicates.

#### 102 **Iron uptake by Caco-2 cells**

103 Iron was fortified with FeSO<sub>4</sub> (iron 80 mg kg<sup>-1</sup> dry basis) according to Chinese  
104 Hygienic Standard (GB14880-94). Caco-2 cells originating from human colorectal  
105 carcinoma were obtained from the American Type Culture Collection (ATCC,  
106 Rockville, MD) and used in experiments at passage 30-50. Cells were seeded at a  
107 level of 1~1.5×10<sup>5</sup> cells/cm<sup>2</sup> in twelve-well transwell plates (Corning Inc.) and

108 maintained under conditions described by Glahn *et al.* (19). Iron uptake experiment  
109 was conducted when the activity of alkaline phosphatase could be detected and the  
110 transmonolayer electrical resistance was over  $500 \Omega \text{ cm}^2$ . The protocols used in the  
111 iron uptake were followed Glahn *et al.* (20). Briefly, growth medium was removed  
112 from transwell by aspiration, and the upper and lower chambers were rinsed with  
113 phosphate buffered saline (PBS). Then 1.5 mL Hanks' balanced salt solution (pH 6.7  
114 to 7.0) was filled in the lower chamber, while 0.5 mL *in vitro* digest was added to the  
115 upper chamber. Transport time was 1 h at 37 °C. Iron uptake was terminated by  
116 collecting the upper and lower chamber solutions separately and rinsing the upper  
117 chamber with three volume (0.5 mL) of stop solution (140 mM NaCl, 10 mM PIPES  
118 [piperazine-N, N'-bis (2-ethanesulfonic acid)], pH 6.8, 4°C). Next, 0.5 mL of removal  
119 solution (stop solution with 5 mM bathophenanthroline disulfonic acid and 5 mM  
120 sodium dithionite, pH 6.8, 4°C) was used to remove the non-specifically bound iron.  
121 Then, each monolayer received two more volume (0.5 mL) of stop solution. The  
122 outside of each insert was washed with 1.0 mL of stop solution (pH 6.8, 4°C). This  
123 rinse solution was added to the lower chamber. After collection of all solutions, cells  
124 were lysed and harvested by adding two volumes (0.5 mL) of 1 M NaOH to each  
125 insert (21). The experiment was carried out at least in quadruplicate.

#### 126 **Determination of iron contents and bioavailability**

127 Iron contents in WPC, solutions, dialysates and Caco-2 cells absorption were  
128 measured by inductively coupled plasma atomic emission spectrometry (ICP-AES)  
129 after microwave-accelerated digestion in triplicates, and the averages were used to

130 calculate the bioavailability and dialysability of iron, which were defined as follows.

$$131 \quad \text{Iron bioavailability (\%)} = \frac{\text{Iron}_{(\text{transport} + \text{retained})}}{\text{total iron}} * 100\%$$

$$132 \quad \text{Iron dialysability (\%)} = \frac{\text{dialysable iron}}{\text{total iron}} * 100\%$$

133 Where  $\text{Iron}_{(\text{transport} + \text{retained})}$  refers to the content of iron transported into the lower  
134 chamber solution plus that retained in the cells. Dialysable iron is the iron that could  
135 pass through the 0.22  $\mu\text{m}$  membrane after *in vitro* digestion. Total iron is the total  
136 content of iron in the *in vitro* digest.

### 137 **Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)**

138 Tricine SDS-PAGE analysis of peptides was performed with the methods described  
139 by Schagger and Von Jagow (22). The composition of acrylamide solutions and of all  
140 gels is defined by the letters T and C. T denotes the total concentration (%)  
141 acrylamide and bisacrylamide. C denotes the concentration (%) of the crosslinker  
142 relative to the total concentration T. Briefly, the gel was composed of a small-pore gel  
143 (16.5% T) overlaid by a 10% T, 3% C spacer gel that again was overlaid by the 4% T,  
144 3% C stacking gel. The anode buffer was 0.2 M Tris-HCl, pH 8.9, and the cathode  
145 buffer was 0.1 M Tris-HCl, pH 8.25, 0.1M tricine and 0.1% SDS. The electrophoresis  
146 runs started at 30 V, and changed to 100 V after about 1.5 h, when the sample had  
147 completely entered the stacking gel.

### 148 **Analysis of free amino acids**

149 The samples were deproteinized with 15% trichloroacetic acid (TCA), centrifuged at  
150 5000 *g* for 20 min and then the supernatant filtered through a 0.45  $\mu\text{m}$  membrane  
151 prior to the amino acid determination carried out on an amino acid analyzer (Hitachi



152 L-8800, Japan).

153 All glassware in this study was steeped in 30% HNO<sub>3</sub> overnight and then rinsed with  
154 deionized water before use in order to avoid mineral contamination.

### 155 **Statistical analysis**

156 All measurements were expressed as means±SD. The data were analyzed by SPSS  
157 10.0 one way analysis of variance (ANOVA). A multiple comparison procedure of the  
158 treatment means was performed by Duncan's new multiple range tests.

## 159 **RESULTS**

### 160 *Effects of enzymatic treated WPC on fortified iron absorption by Caco-2 cells*

161 Enzymes and treating periods applied significantly affected the effects of hydrolysates  
162 on bioavailability of fortified iron by Caco-2 cells ( $p<0.01$ ), as shown in Figure 1.

163 **Effect of neutrase:** Compared to control (none treated WPC), neutrase treated WPC  
164 significantly improved iron uptake by Caco-2 cells. The highest iron bioavailability  
165 occurred at hydrolysates obtained after 1 h treatment, which was 12.8% which is  
166 about 4-fold of the control (3.4%). The lowest (5.8%) was at 5 h treatment. Iron  
167 absorption with hydrolysates obtained after 1-4 h treatments were significantly higher  
168 than that of control ( $p<0.05$ ), and no statistical differences were observed among  
169 hydrolysates treated for 2 to 4 h.

170 **Effect of papain:** Depending on period, papain treated WPC exhibited improved or  
171 inhibitory effects on iron uptake by Caco-2 cells. Compared to control, WPC treated  
172 with papain for 3-5 h improved absorption of iron, while treated for 1-2 h inhibited  
173 iron absorption ( $p<0.05$ ). With prolonged period from 1 to 5 h, WPC treated with

174 papain increased iron absorption and reached relatively stable level (8%) after 3 h. No  
175 significant difference was observed among 3, 4 and 5 h.

176 **Effect of alcalase:** Differed from neutrase and papain, alcalase treated WPC had  
177 negative effect on iron absorption. Except for treatment for 1 and 2 h, which were at  
178 the compared level of control, hydrolysates from other periods decreased iron  
179 absorption, and hydrolysates from 3 h significantly lowered the iron absorption by  
180 83% of the control ( $p<0.05$ ).

181 *In vitro* dialysability of iron affected by WPC treated with neutrase, papain and  
182 alcalase for different periods were shown in table 1. Dialysability of iron were very  
183 closed to 100% for WPC hydrolysates by neutrase and alcalase, and no significant  
184 differences were observed among treating periods for both enzymes. However, papain  
185 hydrolysates significantly inhibited the amount of soluble iron ( $P<0.01$ ), which in the  
186 range of 21.3-28.6%.

#### 187 ***Effect of hydrolysis on composition of WPC***

188 **Degree hydrolysis (DH) of WPC:** Mean DH ( $\pm$ SD) of WPC by proteases was shown  
189 in figure 2. In our study design, we controlled DH of WPC in the range 0-30%. In  
190 intervals of 0.5 to 1 h, DH by papain was significantly higher than that of alcalase  
191 ( $P<0.01$ ). However, at the intervals of 2 and 3 h, they were at the same level ( $P>0.05$  ).  
192 At the same treatment intervals, DH by papain was 2.5 to 4.5 folds of neutrase. When  
193 looked DH by enzymes, it increasingly ordered as papain > alcalase > neutrase in the  
194 first 3 h. However, alcalase led the highest DH after 4 h treatment.

195 **Tricine SDS-PAGE:** The electrophoretic patterns of neutrase, papain, alcalase WPC

196 hydrolysates at each incubation time were shown in Figure 3-5. Compared to raw  
197 WPC, two new bands were observed at the molecular weight (MW) around and below  
198 10 KDa in hydrolysates by neutrase and main components such as BSA, IgG,  $\beta$ -Lg,  
199  $\alpha$ -La were degraded to different extent. And the band around 10 KDa became lighter  
200 with treating time. Differed from the pattern of hydrolysates by neutrase, figure 4 and  
201 5 illustrated a complete removal of BSA, IgG,  $\beta$ -Lg,  $\alpha$ -La in all papain and alcalase  
202 hydrolysates of WPC at each time of incubation. Papain hydrolysates showed  
203 dispersion lower than 10 KDa, while neither band nor dispersion were observed for  
204 alcalase hydrolysates in this area.

205 **Free amino acids:** Free amino acids composition of WPC hydrolysates by neutrase,  
206 papain and alcalase at each time of enzymatic incubation were listed in table 2. With  
207 the prolonged enzymatic treating time, contents of most amino acids for each  
208 hydrolysates increased. In general, the content of amino acid in papain WPC  
209 hydrolysates was the highest. Compare papain and alcalase, except for Tyr and Phe,  
210 higher content of other 15 type of amino acids were detected in papain than alcalase at  
211 each incubation time. No Cys and Pro were found in hydrolysates by alcalase, and no  
212 Cys, Leu, His and Pro by neutrase.

213 Table 3 presented the free amino acid composition of WPC hydrolysate by neutrase,  
214 papain and alcalase after in vitro digestion. Contents each free amino acid increased at  
215 corresponding time when compared with Table 2. And digests from papain  
216 hydrolysates showed higher contents of Asp, Ser, Glu, Gly, Cys, His, and Pro than  
217 alcalase.

218 **DISCUSSION**

219 The most important finding in the present study is that WPC hydrolysates by protease  
220 have different effect on absorption of iron by Caco-2 cells. Hydrolysates by neutrase  
221 strikingly increased the fortified iron absorption. Proteins of WPC could be degraded  
222 into peptides and/or amino acids when treated with proteolytic enzymes. With  
223 enzymatic degradation, occurrence of decreased molecular weight, increased charge,  
224 exposure of hydrophobic groups and disclosure of reactive amino acid side-chains  
225 will happen, which may results in changes of physiological and nutritional properties.  
226 Iron dialysability is a primary step before iron can be absorbed in the small intestine  
227 (23). When evaluated with in vitro digestion system, the dialysabilities of fortified  
228 iron from WPC hydrolysates incubated with alcalase and neutrase reached almost  
229 100%, which is well in accordance with Kim's report about alcalase hydrolysates (DH  
230 13.60%) led iron solubility at 97.6%. However, dialysability of iron with papain  
231 hydrolysates was lower than 30%, which was significantly lower than hydrolysates by  
232 neutrase and alcalase, meant more than two thirds of fortified iron with papain treated  
233 hydrolysates could not penetrate the dialysis membrane. This indicated that binds of  
234 iron to papain treated hydrolysates rendered iron insoluble or complex between iron  
235 and protein and/or peptides was too large to pass into the membrane (24). We are  
236 surprised to find there was no significant correlation between dialysability and  
237 bioavailability of iron, them, conflicting with previous work that dialysability is a  
238 predictive indicator of iron absorption (25). This suggested that the affinity of a  
239 compound for iron and the type of complex formed may be equally important in

240 determination of effect on nonheme iron absorption. If bound tightly, donation of iron  
241 to the mucosal cell may not occur, whereas if weakly bound, iron may dissociate and  
242 precipitate. This in turn illustrated that iron was taken up by Caco-2 cells as the form  
243 of cation. Therefore, the affinity of undigested or partially digested proteins for iron  
244 and the size of the compounds formed could determine the dialysability of iron (24).  
245 These finding on bioavailability and dialysability of iron further encouraged the  
246 investigation of characteristics of these WPC hydrolysates.

247 In order to reveal the mechanisms of WPC hydrolysates on iron bioavailability, the  
248 DH of hydrolysates, gel electrophoresis and composition of amino acids of raw and in  
249 vitro digested WPC hydrolysates was analysed. For three enzymes, DH of WPC  
250 increased with time and trends of DH were not similar to trends of iron bioavailability.  
251 Iron absorption decreased as DH increased by neutrase could be explained by  
252 production of some iron-binding peptides, which could bind minerals through  
253 carboxyl oxygens in peptide chain and improve iron absorption (26), at starting period  
254 of hydrolysis, and however, these peptides may subsequently be degraded as the  
255 hydrolysis was carried on and thus resulted in progressively lower bioavailability of  
256 iron at higher DH. While on the other hand, changes of composition of amino acids  
257 may be related to increase of iron bioavailability during hydrolysis. In agreement with  
258 previous work, more extensive hydrolysis of WPC was observed by alcalase than by  
259 neutrase (26), which may be contributed by considerable capacity of alcalase on  
260 breakage of large numbers of peptide bonds. A relatively crude alcalase, which was  
261 extraction of *B. Licheniformis*, contains several proteases with different specificities

262 (27). Similar DH of WPC by alcalase did not show certain levels of iron absorption as  
263 neutrase or papain. For alcalase treated hydrolysates, iron absorption was less than  
264 20% of the control when DH at 21.4%. Our results disagreed with the results of effect  
265 of bovine milk protein on iron absorption in human, which was DH of whey protein at  
266 36% had higher iron absorption than that at 16%. This may be due to discrepancy in  
267 application of proteases which resulted in different binding iron cation properties of  
268 WPC hydrolysates. Differences of iron absorption between hydrolysates by neutrase,  
269 papain and alcalase signified the importance of active sites on protein of enzymes and  
270 the DH since they markedly affect the composition of hydrolysates, which may  
271 determine iron-binding capacity. Of them, the active sites of enzymes seem more  
272 important.

273 Results of electrophoretic patterns of WPC hydrolysates (figures 3-5) give us another  
274 point. Figure 3 indicated a few remaining trace of BSA, IgG,  $\beta$ -Lg,  $\alpha$ -La even after 5 h  
275 of incubation by neutrase, while figure 4 and 5 revealed complete removal of the  
276 major bands of whey protein by papain and alcalase. These results were well in  
277 correlation with the result of DH, suggesting that the hydrolysis capability of neutrase  
278 is weaker than that of papain and alcalase. Compared with hydrolysates by alcalase,  
279 the patterns of neutrase treated hydrolysates showed generation of new bands at the  
280 molecular weight (MW) of and lower than 10 KDa, while hydrolysates by papain had  
281 disperse in the area of MW at 10 KDa. For neutrase treated hydrolysates, a more  
282 distinct band around 10 KDa was exhibited. The generation of small molecular  
283 peptides (<10 kDa) may be responsible for enhancing effect on iron absorption. Our

284 results supported the results that filtrate (MW < 10 kDa) of cow milk whey obtained  
285 after ultra-filtration with 10 kD membrane caused markedly greater Caco-2 cell  
286 ferritin formation than retentate (components > 10 kDa) (5). Study on human milk  
287 also showed that low-molecular-weight components, which may include  
288 carbohydrates, salts, peptides and vitamins, enhance iron uptake (5).

289 Although except for dispersion area of MW lower than 10 KDa occurring in papain  
290 WPC hydrolysates, a high similarity was observed in the electrophoretic patterns for  
291 papain and alcalase WPC hydrolysates, distinctions were found in composition of  
292 amino acids, either before or after in vitro digestion. Higher contents of Asp, Ser, Glu,  
293 Gly, Cys, His and Pro in papain hydrolysate may be also the reason for its promotion  
294 iron uptake by Caco-2 cells. Storcksdieck *et al* reported that Asp, Glu, Gly and Pro in  
295 the gel filtration fraction from the pepsin/pancreatin digests of meat protein were  
296 responsible for iron binding. Both Asp and Glu are known to form stable iron chelates  
297 with a putative tridentate structure (10). Many investigators have pointed out that Cys  
298 could exert a reducing effect on the ferric form ( $\text{Fe}^{3+}$ ), keeping iron in its ferrous form  
299 ( $\text{Fe}^{2+}$ ) which is better for absorption (6). His may bind iron through imidazole  
300 nitrogen (28). Argyri *et al.* reported hexapeptide PGPIP<sub>N</sub> (Pro-Gly-Pro-Ile-Pro-Asp)  
301 rich in Pro exerted an enhancing effect on iron uptake in Caco-2 cells in a  
302 dose-reponsive manner (29). It has been suggested that Pro induces structural bends in  
303 proteins (30) and thus might aid peptides in assuming a conformation that favors  
304 stable iron-binding. Additionally, the stability of these complexes might be further  
305 enhanced by the resistance of Pro-rich peptides to digestion due to steric hindrance

306 (10). Only at the brush border are Pro bonds cleaved by dipeptidyl peptidase IV and  
307 related enzymes (31). Although neutrase treated hydrolysates exhibited the most  
308 promoting effect on iron absorption, it did not have a high content of the amino acids  
309 promising to enhance iron bioavailability. We hypothesized that relatively low MW  
310 (<10 KDa) peptides instead of amino acids served as the major facilitators of iron  
311 bioavailability.

312

313

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319

#### 320 **Abbreviations:**

321 Ala: alanine; Arg: arginine; Asp: aspartic acid; Cys: cystine; Glu: glutamic acid; Gly:  
322 glycine; His: histidine; Ile: isoleucine; Leu: leucine; Lys: lysine; Met: methionine; Phe:  
323 phenylalanine; Pro: proline; Ser: serine; Thr: threonine; Trp: tryptophan; Tyr: tyrosine;  
324 BSA: bovine serum albumin; IgG: gimmunoglobulin,  $\beta$ -Lg:  $\beta$ -lactoglobulin;  $\alpha$ -La:  
325  $\alpha$ -lactalbumin.

326



327 **FIGURE CAPTIONS**

328 Figure 1 Bioavailability of iron with WPC hydrolysates treated with neutrase, papain  
329 and alcalase

330 \* :significantly increase iron absorption (to control,  $p<0.01$ ); ☆: significantly  
331 decrease iron absorption (to control,  $p<0.01$ )

332

333 Figure 2 Degree of hydrolysis ( $\pm$ SD) of whey protein concentrates by different  
334 enzymes.

335 neutrase (⊠), papain (□), alcalase (○)

336

337 Figure 3 Tricine SDS-PAGE of WPC hydrolysates by neutrase

338

339 Figure 4 Tricine SDS-PAGE of WPC hydrolysates by papain

340

341 Figure 5 Tricine SDS-PAGE of WPC hydrolysates by alcalase

342

343

344 Table 1 Dialysability (%) of iron affected by WPC treated with neutrase, papain and  
345 alcalase for different periods

Treating time (h)	Neutrase	Papain	Alcalase
1	93.52±5.81a	24.27±6.28a	103.75±35.09a
2	90.15±8.61a	28.62±4.74b	112.68±2.05a
3	103.71±5.20a	24.03±1.50a	93.83±14.10a
4	107.44±18.87a	28.51±2.31b	83.97±7.62a
5	90.35±6.35a	21.35±1.67a	-

346

Table 2 Contents of free amino acids in enzymatic hydrolysates (mg/100g, dry basis)

	1 h			2 h			3 h			4 h		
	Neutrased	Papain	Alcalase	Neutrased	Papain	Alcalase	Neutrased	Papain	Alcalase	Neutrased	Papain	Alcalase
Asp	10.2	413.8	1.0	11.7	387.9	1.9	12.2	379.3	2.3	13.9	440.4	4.0
Thr	7.1	75.2	0.0	8.2	76.2	62.6	7.0	80.2	64.3	8.3	180.8	84.8
Ser	1.3	194.8	65.8	1.6	200.3	59.1	1.5	207.3	67.3	2.2	259.6	81.5
Glu	3.6	94.8	32.6	4.5	93.9	76.5	4.7	103.5	114.3	5.7	117.8	177.8
Gly	5.8	366.6	0.0	8.2	302.2	0.0	8.3	404.6	2.0	9.8	359.4	2.7
Ala	0.9	69.1	63.4	0.7	76.3	134.2	0.0	76.3	148.6	0.9	100.4	181.1
Cys	0.0	195.5	0.0	0.0	187.7	0.0	0.0	188.2	0.0	0.0	162.1	0.0
Val	23.9	68.7	15.3	25.5	59.2	21.4	23.2	70.6	27.1	28.2	81.0	40.0
Met	9.4	67.1	0.0	12.0	71.9	2.7	11.0	73.0	5.0	13.8	92.8	9.0
Ile	22.1	45.7	0.0	32.5	42.7	0.0	34.1	43.0	4.2	38.0	48.7	9.0
Leu	0.0	448.8	66.1	0.0	480.7	128.3	0.0	490.7	190.9	0.0	607.9	292.8
Tyr	183.0	268.1	299.9	195.6	209.3	360.9	172.0	257.1	417.1	194.2	234.0	510.5
Phe	36.3	125.9	498.4	60.2	137.6	609.0	53.9	145.7	662.6	67.2	176.0	816.5
Lys	6.9	190.1	4.4	7.7	207.8	7.9	8.2	215.2	11.5	9.5	275.9	24.7
His	0.0	48.2	2.4	0.0	49.9	3.7	0.0	51.8	4.7	0.0	64.1	7.4
Arg	20.5	143.4	6.8	34.5	149.4	11.3	30.1	152.5	15.4	36.1	190.8	21.8
Pro	0.0	4.3	0.0	0.0	4.0	0.0	0.0	4.0	0.0	0.0	5.0	0.0

Table 3 Contents of free amino acids in enzymatic hydrolysates after *in vitro* digestion (mg/100g, dry basis)

	1 h			2 h			3 h			4 h		
	Neutrase	Papain	Alcalase	Neutrase	Papain	Alcalase	Neutrase	Papain	Alcalase	Neutrase	Papain	Alcalase
Asp	187.1	427.6	70.0	174.6	371.3	64.0	197.1	554.4	64.5	192.0	464.4	58.3
Thr	490.2	612.0	644.9	472.2	489.1	725.4	579.2	727.2	737.9	510.0	614.4	656.8
Ser	266.0	415.6	213.1	267.2	352.2	234.1	313.8	522.7	232.9	295.8	448.6	214.6
Glu	358.4	575.4	526.9	381.9	497.4	391.4	434.4	707.2	407.8	377.1	588.3	400.9
Gly	85.7	398.0	65.7	88.5	327.3	72.0	103.9	476.4	81.1	84.6	413.9	68.1
Ala	260.7	314.0	432.6	256.8	272.2	265.6	304.2	395.3	289.0	279.6	332.8	265.1
Cys	128.9	152.6	0.0	131.1	127.9	0.0	152.7	197.6	0.0	148.4	168.8	0.0
Val	331.1	390.6	469.0	298.4	304.9	429.2	387.4	455.3	450.2	294.0	376.3	395.7
Met	153.1	234.9	200.1	154.2	200.5	202.4	190.1	300.1	214.6	170.2	251.7	199.6
Ile	415.3	364.3	392.3	387.6	300.7	332.0	485.4	415.4	330.6	425.7	344.8	278.6
Leu	1286.7	1399.5	1691.0	1277.2	1316.9	1635.7	1449.6	1623.5	1713.2	1338.1	1418.9	1567.3
Tyr	923.3	784.9	1237.0	940.4	654.4	1042.4	1074.8	964.7	1095.8	1026.6	782.7	991.1
Phe	675.8	674.3	1034.6	634.1	555.8	1027.8	730.7	825.6	1091.6	669.4	671.9	1017.5
Lys	1895.9	1572.8	1641.9	1908.3	1342.9	1486.5	2116.5	1870.9	1483.7	2034.8	1595.3	1325.5
His	185.7	216.0	157.5	181.4	177.1	142.7	220.3	268.5	147.7	197.9	223.9	133.1
Arg	529.5	561.1	704.1	502.1	374.0	653.1	611.4	579.3	665.1	549.4	500.5	594.3
Pro	73.6	54.1	29.0	66.9	41.4	26.4	78.4	62.8	25.1	74.7	52.5	24.7

Figure 1

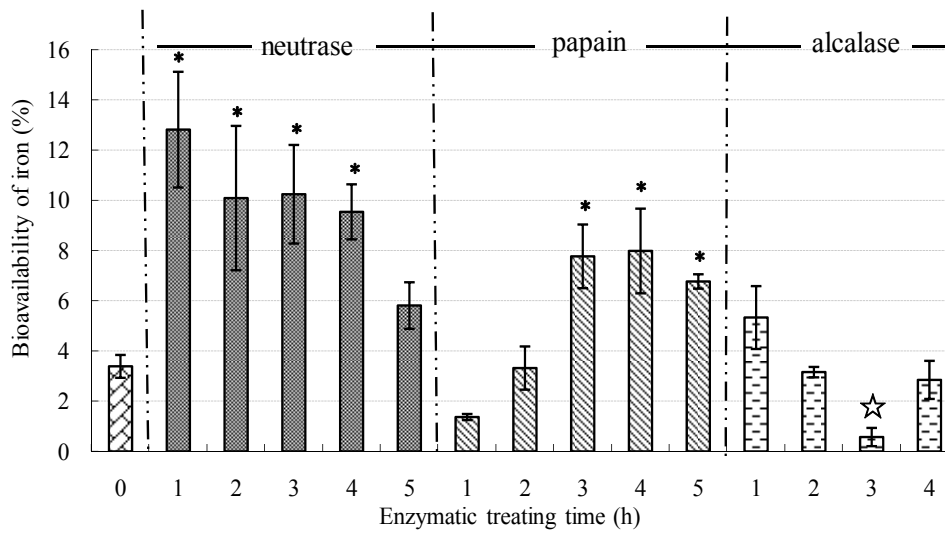


Figure 2

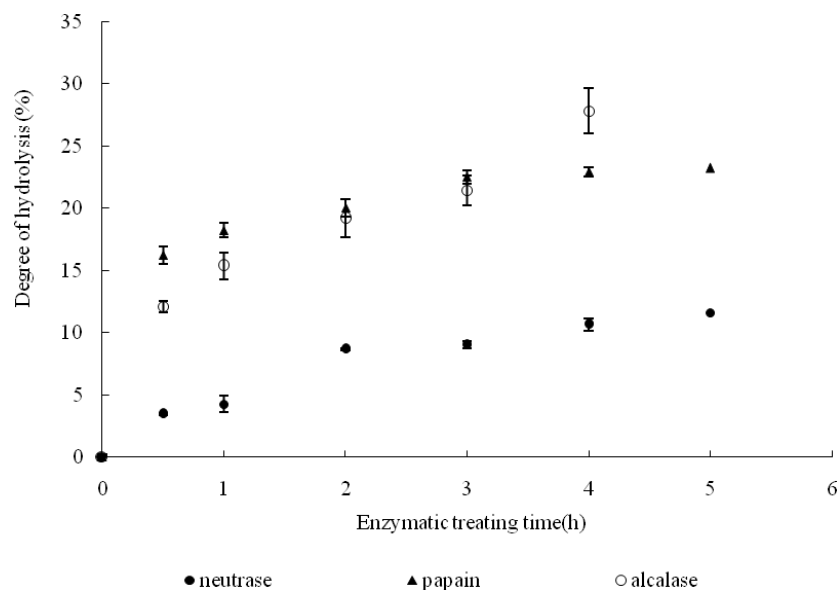


Figure 3 (neutrase)

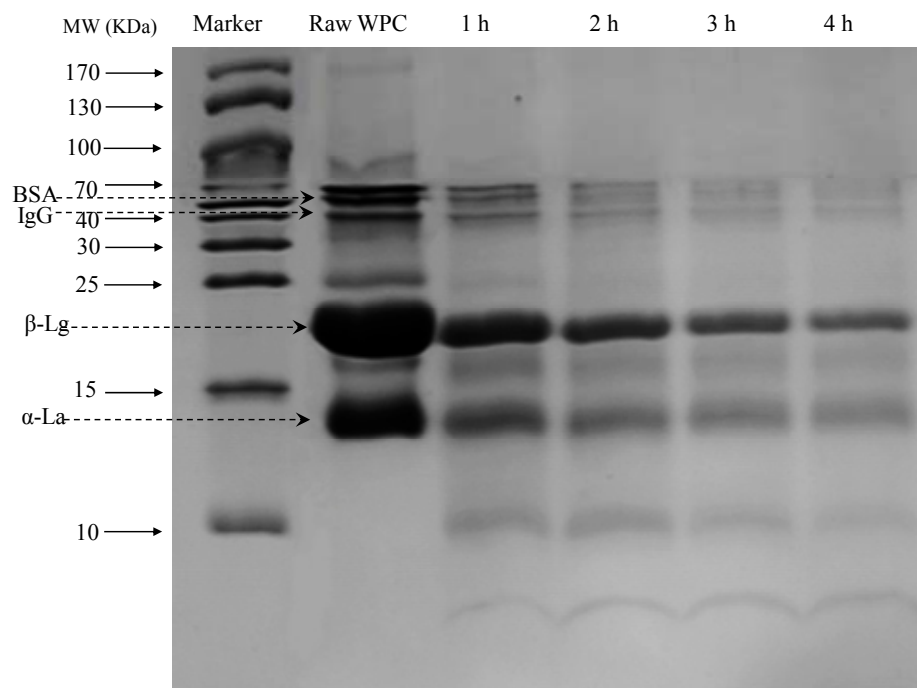


Figure 4 (papain)

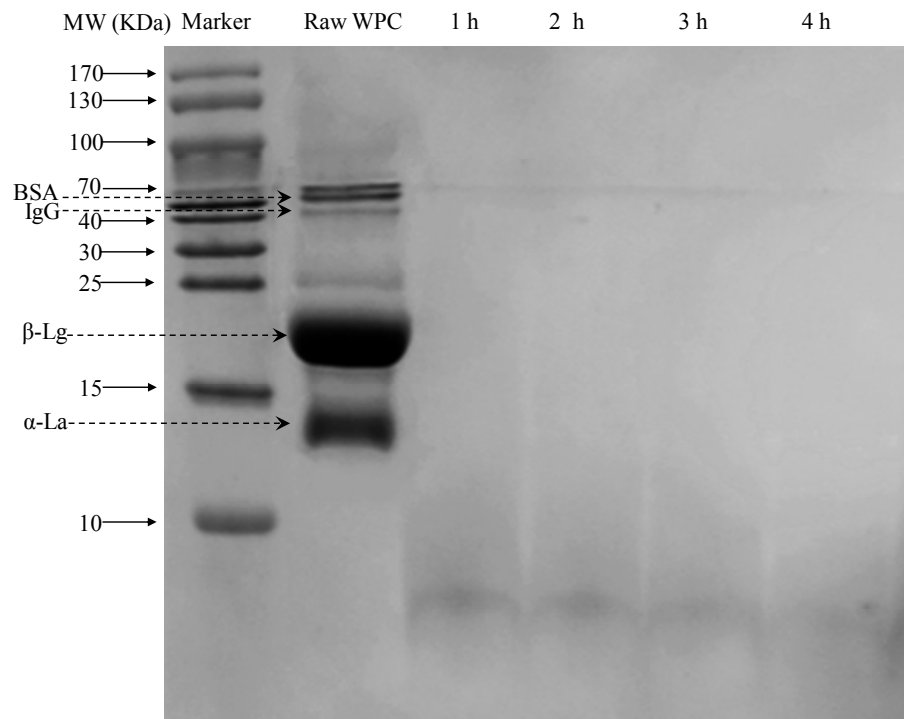
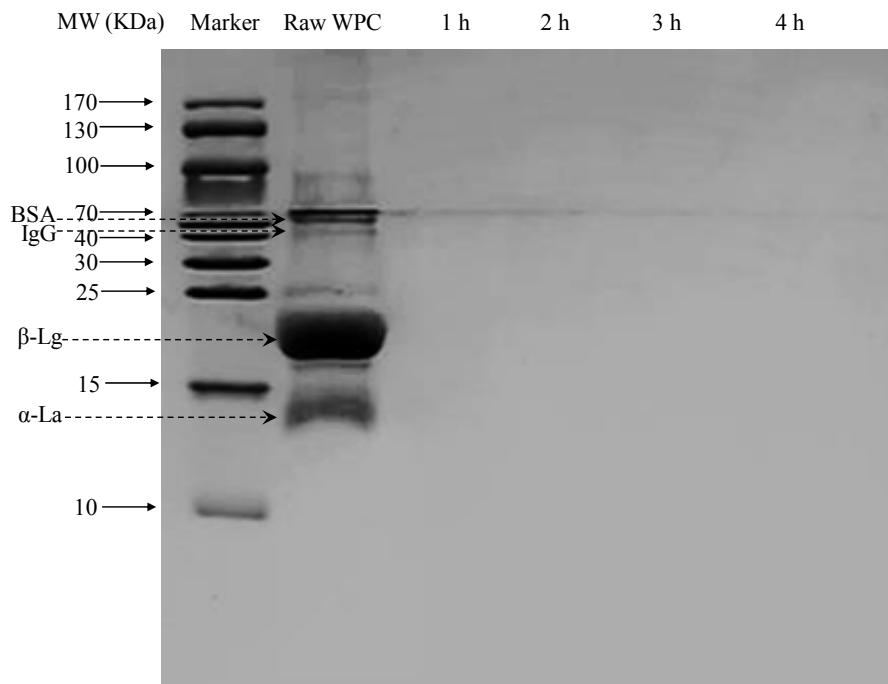




Figure 5 (alcalase)



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