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Abstract

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

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

INTRODUCTION

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

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

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

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

MATERIALS AND METHODS

Preparation of WPC hydrolysates

The 5% WPC solution was kept in incubator at 85 ºC for 15 min. After cooled to 50 ºC, the pH was adjusted to 5.5, 7.0 and 8.0 respectively for papain, neutrase and alcalase using 1 M NaOH or 1 M HCl and maintained during enzymatic treatment. The ratio of enzyme to substrate is 3000 U g^{-1} protein basis. WPC solution was kept at

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

In vitro digestion of WPC hydrolysates

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

Iron uptake by Caco-2 cells

Iron was fortified with $FeSO_4$ (iron 80 mg kg^{-1} dry basis) according to Chinese Hygienic Standard (GB14880-94). Caco-2 cells originating from human colorectal carcinoma were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and used in experiments at passage 30-50. Cells were seeded at a level of $1\sim1.5\times10^5$ cells/cm^2 in twelve-well transwell plates (Corning Inc.) and

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

Determination of iron contents and bioavailability

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

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

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

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

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

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

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

Analysis of free amino acids

The samples were deproteinized with 15% trichloroacetic acid (TCA), centrifuged at 5000 *g* for 20 min and then the supernatant filtered through a 0.45 μm membrane prior to the amino acid determination carried out on an amino acid analyzer (Hitachi

L-8800, Japan).

All glassware in this study was steeped in 30% HNO_3 overnight and then rinsed with deionized water before use in order to avoid mineral contamination.

Statistical analysis

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

RESULTS

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

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

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

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

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

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

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

Effect of hydrolysis on composition of WPC

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

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, β-Lg,
199 α-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, β-Lg, α-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.

DISCUSSION

The most important finding in the present study is that WPC hydrolysates by protease have different effect on absorption of iron by Caco-2 cells. Hydrolysates by neutrase strikingly increased the fortified iron absorption. Proteins of WPC could be degraded into peptides and/or amino acids when treated with proteolytic enzymes. With enzymatic degradation, occurrence of decreased molecular weight, increased charge, exposure of hydrophobic groups and disclosure of reactive amino acid side-chains will happen, which may results in changes of physiological and nutritional properties.

Iron dialysability is a primary step before iron can be absorbed in the small intestine (*23*). When evaluated with in vitro digestion system, the dialysabilities of fortified iron from WPC hydrolysates incubated with alcalase and neutrase reached almost 100%, which is well in accordance with Kim's report about alcalase hydrolysates (DH 13.60%) led iron solubility at 97.6%. However, dialysability of iron with papain hydrolysates was lower than 30%, which was significantly lower than hydrolysates by neutrase and alcalase, meant more than two thirds of fortified iron with papain treated hydrolysates could not penetrate the dialysis membrane. This indicated that binds of iron to papain treated hydrolysates rendered iron insoluble or complex between iron and protein and/or peptides was too large to pass into the membrane (*24*). We are surprised to find there was no significant correlation between dialysability and bioavailability of iron, them, conflicting with previous work that dialysability is a predictive indicator of iron absorption (*25*). This suggested that the affinity of a 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

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

Results of electrophoretic patterns of WPC hydrolysates (figures 3-5) give us another point. Figure 3 indicated a few remaining trace of BSA, IgG, β-Lg, α-La even after 5 h of incubation by neutrase, while figure 4 and 5 revealed complete removal of the major bands of whey protein by papain and alcalase. These results were well in correlation with the result of DH, suggesting that the hydrolysis capability of neutrase is weaker than that of papain and alcalase. Compared with hydrolysates by alcalase, the patterns of neutrase treated hydrolysates showed generation of new bands at the molecular weight (MW) of and lower than 10 KDa, while hydrolysates by papain had disperse in the area of MW at 10 KDa. For neutrase treated hydrolysates, a more distinct band around 10 KDa was exhibited. The generation of small molecular 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 (Fe^{3+}), keeping iron in its ferrous form
299 (Fe^{2+}) which is better for absorption (*6*). His may bind iron through imidazole
300 nitrogen (*28*). Argyri *et al*. reported hexapeptide PGPIPN (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 hydrolyasates 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: argnine; 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, β-Lg: β-lactoglobulin; α-La:
325 α-lactalbumin.
326

FIGURE CAPTIONS

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

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

Figure 2 Degree of hydrolysis (±SD) of whey protein concentrates by different enzymes.

neutrase (▯), papain (□), alcalase (○)

Figure 3 Tricine SDS-PAGE of WPC hydrolysates by neutrase

Figure 4 Tricine SDS-PAGE of WPC hydrolysates by papain

Figure 5 Tricine SDS-PAGE of WPC hydrolysates by alcalase

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		
	Neutrase	Papain	Alcalase	Neutrase	Papain	Alcalase	Neutrase	Papain	Alcalase	Neutrase	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

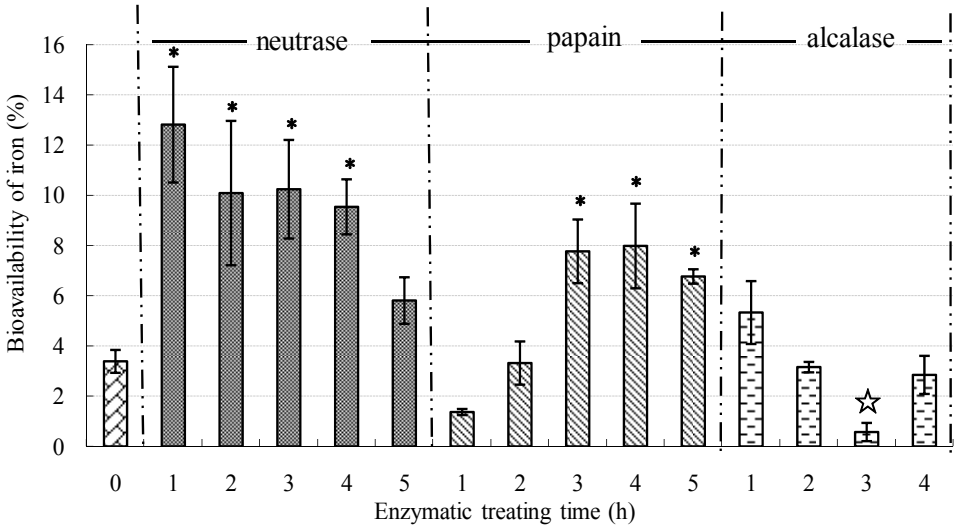

neutrase
papain
alcalase
Bioavailability of iron (%)
16
14
12
10
8
6
4
2
0
0 1 2 3 4 5 1 2 3 4 5 1 2 3 4
Enzymatic treating time (h)

Figure 2

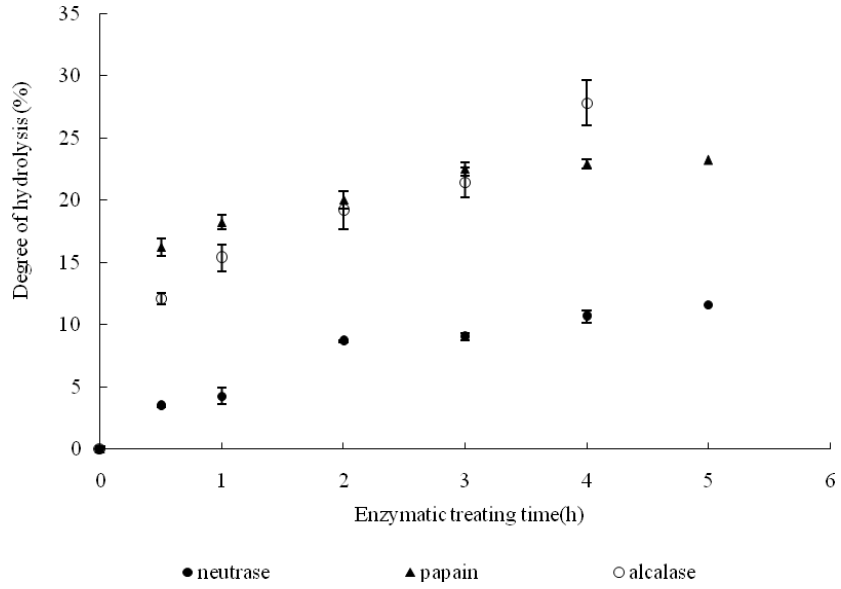

Degree of hydrolysis (%)
35
30
25
20
15
10
5
0
0
1
2
3
4
5
6
Enzymatic treating time(h)
neutrase
papain
alcalase

Figure 3 (neutrase)

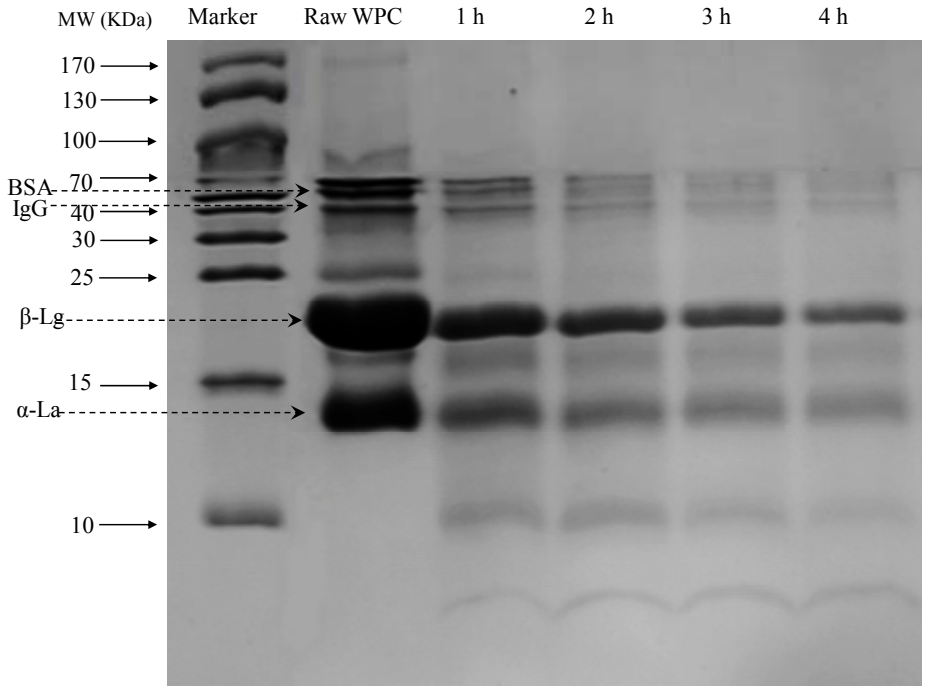


Figure 4 (papain)

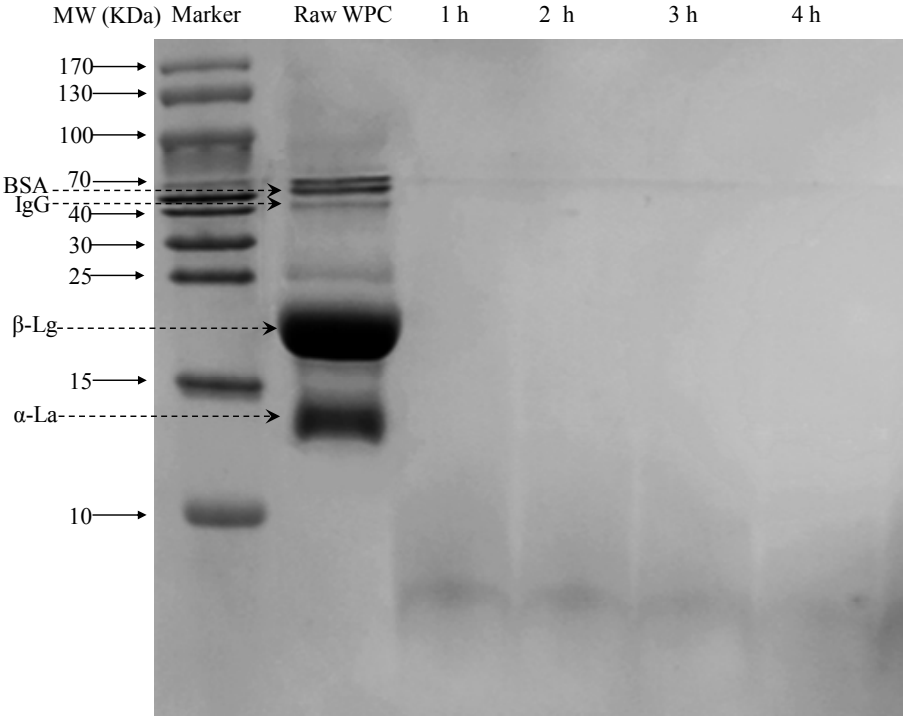
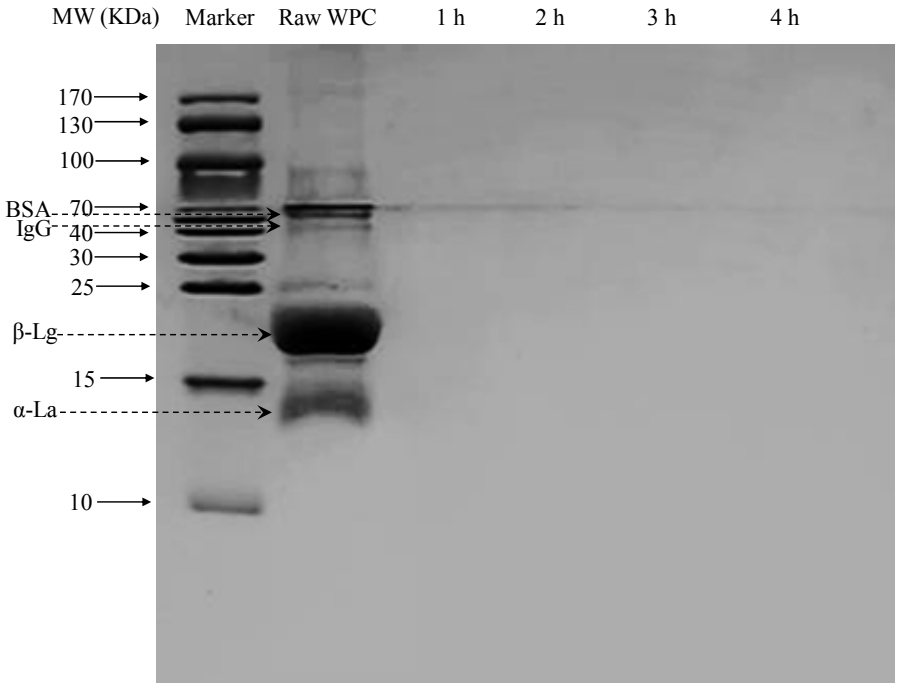


Figure 5 (alcalase)



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