African Journal of Biotechnology Vol. 8 (14), pp. 3314-3319, 20 July, 2009
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB09.262
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Limited hydrolysis of soybean protein concentrate and isolate with two proteases and the impact on emulsifying activity index of hydrolysates

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Accepted 5 May, 2009

Soy protein concentrate (SPC) and soy protein isolate (SPI) were limited hydrolyzed with trypsin or neutrase under controlled hydrolysis conditions. Eight soybean protein hydrolysates, 4 SPC hydrolysates and 4 SPI hydrolysates were prepared with degree of hydrolysis (DH) of 1 or 2%. SDS-PAGE was used to determine the hydrolysis profiles of soybean proteins in these hydrolysates. Emulsifying activity index of these hydrolysates were also evaluated. Analysis results from SDS-PAGE indicated that these hydrolysates had different peptide compositions due to the differences of enzyme specificity and degree of hydrolysis. SPC hydrolysates or SPI hydrolysates prepared with trypsin had more large peptides than that prepared with neutrase. The hydrolysates prepared with trypsin exhibited better emulsifying activity index than that with neutrase, especially when degree of hydrolysis of hydrolysates was 1%. Both protease used and DH obtained had influence on the emulsifying activity index of soybean protein hydrolysates prepared.

Keywords: Soy protein concentrate, soy protein isolate, limited enzymatic hydrolysis, emulsifying activity index.

INTRODUCTION

Soybean is a particularly valuable source of protein in the world, since its proteins have high biological value while its cost is relatively low. It is known that soybean proteins have several physiological functions such as cholesterol-lowering and body-fat reducing effects (Kito et al., 1993; Anderson et al., 1995; Aoyama et al., 2000). Soybean proteins now are widely used in processing foods as functional and nutritional ingredients (Hettiarachchy and Kalapathy, 1997; Liu, 2000). FDA had approved the health claim concerning the role of soybean proteins in reducing the risk of coronary heart disease (FDA, 1999). All these mentioned above have led to the increased interest in soybean protein-based foods.

In recent years, a significant growth of interest in new food ingredients could be observed. One of these is that some soybean protein products with special functional properties are available in market. Improvement in functional properties of soybean protein products may further increase their applications in foods, which might offer food producers more choice in production. One approach to improve the functional properties of soybean protein products is enzymatic treatment. It could be seen from literatures that limited proteolysis of soybean protein products offered a possibility to obtain hydrolysates with enhanced functional properties (Bernardi Don et al., 1991). The final improvement depended, however, not only on the enzyme and proteolysis conditions used, but also on the raw material. Puski (1975) had investigated the modification in functional properties of soybean protein isolate after treatment with *Aspergillus oryzae* with varying enzyme concentrations. Adler-Nissen and Olsen (1979) had shown considerable improvement in emulsifying capacity, solubility and foaming capacity of soybean protein isolate after limited proteolysis with alcalase or neutrase. Limited proteolysis of soybean protein isolate with pancreatin also has been shown to improve solubility and emulsifying activity (Qi et al., 1997). Pepsin, papain or trypsin had been shown to enhance the solubility and emulsifying properties of soybean protein isolates (Kim et al., 1990). Hettiarachchy and Kalapathy (1997) had obtained a hydrolysate from soybean protein isolate hydrolyzed

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Table 1. Hydrolysis conditions applied in the preparation of SPC and SPI hydrolysates.

Raw material	Product	Substrate concentration (%)	Protease[a] and addition (U/g proteins)	Temperature (°C)	pH	Hydrolysis Time (h)	DH (%)
SPC	SPC-N1[b]	10	N, 400	25	6.8	1	1
	SPC-N2[c]	10	N, 400	25		4	2
	SPC-T1[d]	10	T, 1700	35		1	1
	SPC-T2[e]	10	T, 1700	35		4	2
SPI	SPI-N1[f]	7.5	N, 250	25	7.2	1	1
	SPI-N2[g]	7.5	N, 250	25		2.5	2
	SPI-T1[h]	7.5	T, 610	35		1	1
	SPI-T2[i]	7.5	T, 610	35		3	2

[a]N, neutrase; T, trypsin.
[b]DH=1% SPC hydrolysate with neutrase.
[c]DH=2% SPC hydrolysate with neutrase.
[d]DH=1% SPC hydrolysate with trypsin.
[e]DH=2% SPC hydrolysate with trypsin.
[f]DH=1% SPI hydrolysate with neutrase.
[g]DH=2% SPI hydrolysate with neutrase.
[h]DH=1% SPI hydrolysate with trypsin.
[i]DH=2% SPI hydrolysate with trypsin.

with papain, with foaming property close to hen egg protein. Recently, a method was patented to obtain protein hydrolysates by continuous enzymatic proteolysis in an extruder (Weiss et al., 2002).

There are fewer reports on limited hydrolysis of soybean protein concentrate in the available literatures. Also, there is a need to reveal the relationship between mode of enzymatic hydrolysis and corresponding improvement in emulsifying activity index of soybean proteins. In our study, 2 soybean protein products, soybean protein concentrate (SPC) or soybean protein isolate (SPI), were hydrolyzed by neutrase or trypsin under controlled conditions to prepare soybean protein hydrolysates with low degree of hydrolysis. The influence of enzymatic hydrolysis of SPC or SPI on emulsifying activity index was evaluated. The hydrolysis profiles of SPC and SPI hydrolysates were determined with sodium dodecylsulfate-polyacrylamide gel electrophoresis, in order to reveal the relationship between mode of enzymatic hydrolysis and corresponding modification in emulsifying activity index.

MATERIALS AND METHODS

SPC and SPI

The raw material SPC was produced in the laboratory by ethanol treatment described by Baker et al. (1979) with some modifications. SPI was obtained from a local SPI manufacture. The crude protein content was 64.93% for SPC and 89.24% for SPI. Neutrase (70 units/mg) was purchased from Aoboxing chemical Co. (Beijing, China) and trypsin (300 units/mg) was purchased from Amresco Co. (USA). All reagents used were of analytical grade.

Limited enzymatic hydrolysis of SPC and SPI

A fixed substrate concentration was used in hydrolysis of SPC, 10% (w/w). Protease was added with enzyme-substrate ratio at 400 units /g proteins for neutrase, or 1700 units/g proteins for trypsin. After pH was adjusted to 6.8, the hydrolysis was carried at temperature 25°C for neutrase or 35°C for trypsin with continuous agitation. With 60 min hydrolysis, SPC hydrolysates with DH of 1% were prepared. With 240 min hydrolysis, SPC hydrolysates with DH of 2% were prepared. After hydrolysis, the hydrolysates were heated in boiling water bath to 95°C for 30 min to inactivate protease and then freeze-dried. Four SPC hydrolysates were prepared. The detailed hydrolysis conditions for SPC hydrolysate preparation were list in Table 1.

SPI was also hydrolyzed by neutrase or trypsin, with a fixed substrate concentration 7.5% (w/w). Neutrase was added at enzyme-substrate ratio 250 units/g proteins. After pH was adjusted to 7, the hydrolysis was carried out at 25°C with continuous agitation. SPI was hydrolyzed for 60 min to obtain SPI hydrolysates with DH 1%, or hydrolyzed for 150 min to obtain SPI hydrolysates with DH 2%. Trypsin was added at enzyme-substrate ratio 610 units/g proteins and pH 7. The hydrolysis was carried out at 35°C with continuous agitation. SPI was hydrolyzed for 60 min to obtain SPI hydrolysates with DH 1%, or hydrolyzed for 180 min to obtain SPI hydrolysates with DH 2%. After hydrolysis, the hydrolysates prepared were heated in boiling water bath to 95°C for 30 min to inactivate protease and then freeze-dried. Four SPI hydrolysates were also prepared. The detailed hydrolysis conditions for SPI hydrolysate preparation were list in Table 1.

SDS-PAGE analysis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis for 2 soybean protein products and their hydrolysates prepared was carried out following the method of Laemmli (1970). Samples for SPC or SPC hydrolysates (10 mg/mL) and for SPI or SPI hydrolysates (5 mg/mL), were prepared in Tris-HCl buffer (pH 6.8) containing 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.002% bromophenol blue. The samples and standard protein markers were all applied on the stacking gel slot. Electrophoresis was carried out at constant voltage of 80 V, then up to 120 V. After staining protein profiles with Coomassie brilliant blue R-250 and de-staining, the gels were analyzed using the Syngene Gel

documentation system.

Standard protein markers used and their molecular weights (in kDa) were as follow: egg albumin lysozyme (14.4), trypsin inhibitor (20.1), bovine carbonic anhydrase (31.0), ovalbumin (43.0) bovine serum albumin (66.2) phosphorylase b (97.4).

Evaluation of emulsifying activity index

Emulsifying activity index (EAI) is to express the emulsifying property of proteins in the oil/water interface area (m^2) (Pearce and Kinsella, 1978; Moure et al., 2005) and was employed in our study to show the emulsifying property of SPC, SPI and their hydrolysates. 20 ml of 0.1% (w/v) protein solution or hydrolysate solution in 0.05 mol/L phosphate buffer with pH 7.0 were homogenized with 6.60 ml refined soybean oil for 1 min in a high-speed blender at 10000 rpm. Immediately after homogenization, aliquots of 20 µl emulsion were diluted to 5 ml with 0.1% SDS. The absorbance of this mixture at 500 nm was recorded at a spectrophotometer. The EAI was calculated as follow:

$$EAI\ (m^2 / g) = 2T\left[\frac{(A \times D)}{(C \times \phi \times L \times 10)}\right]$$

where T = 2.303, A = observed absorbance, D = dilution factor, Φ = volume fraction of the dispersed phase (oil), C = weight of proteins per unit volume (g/L) of aqueous phase before the emulsion was formed, and L = path length of the cuvette (cm).

Determination of protein content and degree of hydrolysis

Protein content in SPC or SPI or hydrolysates was determined in duplicate by the Kjeldahl method as described in method 920.123 (AOAC, 2000) on a Kjeltec 2300 Analyzer (Foss, Sweden) and a conversion factor 6.25 was used. The content of free amino groups in SPC or SPI or hydrolysates was determined in triplicate by a formaldehyde titration method described in literature (Zhang et al., 2007) and expressed as mmol/g proteins. The DH for soybean protein hydrolysates was thereby calculated as follow:

$$DH\ (\%) = 100 \times \frac{FA_2 - FA_1}{7.8}$$

where 7.8 was the numbers of peptide bond in 1 g soybean proteins (mmol/g proteins), FA_2 and FA_1 represented the content of free amino groups in hydrolysates and raw soybean protein (SPC or SPI) (mmol/g proteins).

Enzyme activity assay

Enzyme activity of neutrase or trypsin was spectrophotometric assayed with a method described by Godfrey and Reichelt (1983) with some modifications. Casein was used as substrate. A reaction mixture of 3 mL of 15 mg/mL casein dissolved in 0.1 mol/L phosphate buffer (pH 7.0) and 0.6 mL of protease solution was incubated for 10 min at 37°C with constant shaking. The reaction was stopped by adding 1.2 mL of 17.5% (w/v) trichloroacetic acid. The precipitate was removed by filtration through Whatman no. 1 filter paper. Then, 1 mL of filtrate was mixed with 3 mL of 2% (w/v) Na_2CO_3 solution and 1 mL of 3-fold diluted Folin-Ciocalteu reagent. After vigorous mixing, the color was allowed to develop for 45 min at room temperature. The absorbance was analyzed at 700 nm, on the basis of tyrosine as standard. One enzyme activity unit was the amount of enzyme that would liberate 1 µmol of tyrosine per minute under the selected conditions of the assay (37°C, pH 7.0).

Statistical analysis

All data were expressed as means ± standard deviation from at least 3 independent experiments. Differences between the mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA). The excel version 2000 program was used.

RESULTS

Limited hydrolysis of SPC and SPI and hydrolysis profiles

SPC or SPI was enzymatic hydrolyzed under controlled conditions to prepare hydrolysates with low degree of hydrolysis in our study because extensive hydrolysis would destroy emulsifying property of proteins eventually. It was due to the difference in protein content in substrate, activity and enzyme specificity that different hydrolysis conditions were needed in hydrolysate preparation, the practical selections on hydrolysis conditions was not discuss here but listed in Table 1. Under selected conditions, 4 SPC hydrolysates and 4 SPI hydrolysates of DH 1% or 2% could be prepared.

SDS-PAGE is widely used to analyze the size distribution of the protein fractions in terms of molecular weight and was applied to show the hydrolysis profiles of soybean protein hydrolysates in our study. SDS-PAGE profiles for SPC and 4 SPC hydrolysates hydrolyzed by neutrase or trypsin were presented in Figure 1. It could be seen from Figure 1 that there were many low molecular weight peptide fractions in all 4 hydrolysates, with molecular weight less than 20 kD. Two storage proteins, β-conglycinin (fraction 7 S) and glycinin (fraction 11 S) exist in soybean (Marsman et al., 1997). It was shown clearly that these storage proteins were hydrolyzed by the action of neutrase or trypsin. When the DH of SPC hydrolysates was increased, hydrolysis was more complete, supported by the fact that more small peptides appeared. Compared to neutrase-catalyzed hydrolysate product SPC-N1 or SPC-N2, larger peptides were obtained in trypsin-catalyzed hydrolysate product SPC-T1 or SPC-T2

SDS-PAGE profiles for the SPI and 4 SPI hydrolysates hydrolyzed by neutrase or trypsin is presented in Figure 2, which were similar to that for SPC and SPC hydrolysates. It was also shown that 2 storage proteins (7 S and 11 S) in soybean were hydrolyzed by the action of neutrase or trypsin. When the DH of SPI hydrolysates was increased, hydrolysis was more complete, supported by the fact that more small peptides appeared. Larger peptides existed in trypsin-catalyzed hydrolysate product SPI-T1 or SPI-T2, comparing to neutrase-catalyzed hydrolysate product SPI-N1 or SPI-N2.

Emulsifying activity index of SPC or SPI hydrolysates

Figure 3 shows the EAI for SPC and 4 SPC hydrolysates and Figure 4 shows the EAI for SPI and 4 SPI hydroly-

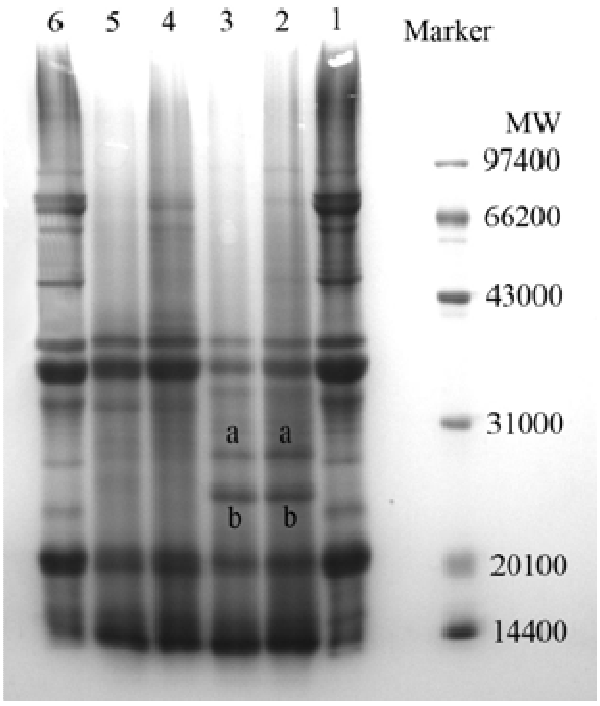

Figure 1. SDS-PAGE profiles of soybean protein concentrate (SPC) and four SPC hydrolysates. Lane 1 and Lane 6, SPC; Lane 2, DH = 1% SPC hydrolysates by neutrase; Lane 3, DH = 2% SPC hydrolysates by neutrase; Lane 4, DH = 1% SPC hydrolysates by trypsin; Lane 5, DH = 2% SPC hydrolysates by trypsin.

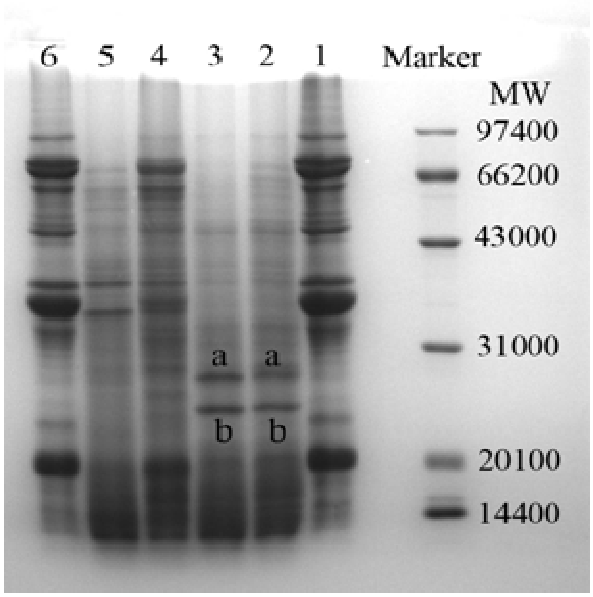

Figure 2. SDS-PAGE profiles of soybean protein isolate (SPI) and 4 SPI hydrolysates. Lane 1 and Lane 6, SPI; Lane 2, DH = 1% SPI hydrolysates by neutrase; Lane 3, DH = 2% SPI hydrolysates by neutrase; Lane 4, DH = 1% SPI hydrolysates by trypsin; Lane 5, DH = 2% SPI hydrolysates by trypsin.

sates. Compared to the EAI of intact SPC or SPI, it could be seen clearly that EAI for the 4 SPC hydrolysares or 4 SPI hydrolysates were improved significantly, as all the hydrolysates had a much higher EAI than intact SPC or SPI. When SPC was limited hydrolyzed to DH 1% by neutrase or trypsin, EAI of SPC hydrolysate was increased from 10 m^2/g proteins to 33.6 m^2/g proteins or 37.3 m^2/g proteins, respectively. When SPI was limited hydrolyzed to DH 1% by neutrase or trypsin, EAI of SPI hydrolysate would be increased from 36 m^2/g proteins to 53.4 m^2/g proteins or 64.5 m^2/g proteins, respectively. If SPC or SPI was hydrolyzed to DH 2% by neutrase or trypsin, similar increase in EAI occurred for SPC hydrolysates or SPI hydrolysates but with less extent. Limited hydrolysis of SPI by neutrase or trypsin had better improving result because the EAI of SPI-N1 and SPI-T1 was about 1.7 fold to that of SPC-N1 and SPC-T1. Emulsifying activity index of SPC-T1 was the highest in all 4 SPC hydrolysates and emulsifying activity index of SPI-T1 was also the highest in all 4 SPI hydrolysates. Also, trypsin was more suitable than neutrase to prepare SPC or SPI hydrolysates of high EAI, for SPC or SPI hydrolysates hydrolyzed by trypsin all had higher EAI than that hydrolyzed by neutrase.

With further comparison, it could be found that increase in degree of hydrolysis of SPC or SPI hydrolysate led to the reduction of its EAI, unfortunately. For example, SPC-T2 or SPC-N2 had smaller EAI than SPC-T1or SPC-N1 (27.2 or 35.9 m^2/g proteins in contrast to 33.6 or 37.3 m^2/g proteins, respectively) and SPI-T2 or SPI-N2 also had smaller EAI than SPI-T1or SPI-N1 (44.8 or 60.4 m^2/g proteins contrast to 53.4 m^2/g proteins or 64.5 m^2/g proteins, respectively). This confirmed that extensive hydrolysis of proteins would destroy their emulsifying property indeed.

DISCUSSION

Neutrase and trypsin are widely used proteases in protein hydrolysis or modification. It is well known that they have different catalyzation characteristics. Neutrase, which prefers to catalyze at hydrophobic amino acids, have many catalyzing sites to break soybean proteins into peptides. However, trypsin only catalyzes the bonds formed by carboxyl group of Lys or Arg, with higher specificity than neutrase. Hydrolysis of SPC or SPI with neutrase or trypsin would give different peptide compositions in the hydrolysates accordingly. By the action of neutrase, more bonds in soybean proteins were broken, which led to small peptides formed in neutrase-catalyzed hydrolysates. In contrast with neutrase, fewer bonds in soybean proteins were hydrolyzed by trypsin, which led to large peptides formed in trypsin-catalyzed hydrolysates. All these accounted for the different peptide profiles in SDS-PAGE analysis, as shown in Figures 1 and 2. Therefore there were more large peptides in trypsin-catalyzed hydrolysates (see distribution differences of protein bands

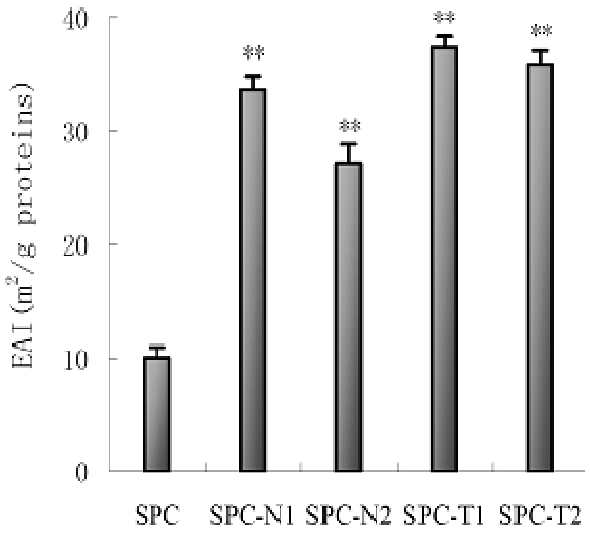

Figure 3. Emulsifying activity index of soybean protein concentrate (SPC) and 4 SPC hydrolysates. SPC-N1, DH = 1% SPC hydrolysates by neutrase; SPC-N2, DH = 2% SPC hydrolysates by neutrase; SPC-T1, DH=1% SPC hydrolysates by trypsin; SPC-T2, DH = 2% SPC hydrolysates by trypsin. The star mark sindicate that values were significantly different from that of SPC (**P < 0.01, n = 3).

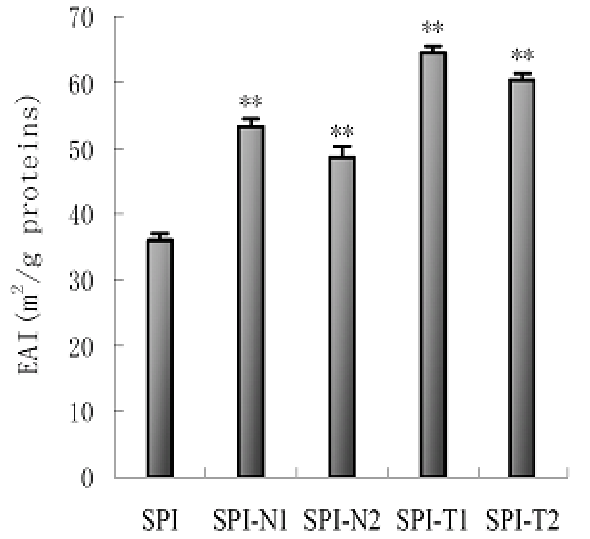

Figure 4. Emulsifying activity index of soybean protein isolate (SPI) and four SPI hydrolysates. SPI-N1, DH = 1% SPI hydrolysates by neutrase; SPI - N2, DH = 2% SPI hydrolysates by neutrase; SPI - T1, DH = 1% SPI hydrolysates by trypsin; SPI - T2, DH = 2% SPI hydrolysates by trypsin. The star marks indicate that values were significantly different from that of SPI (**P < 0.01, n = 3).

between Lane 4 and Lane 2, or distribution differences of protein bands between Lane 5 and Lane 3). It was interesting for us to see that there were 2 peptide fractions, labeled as a, b in Figures 1 and 2, existed in neutrase-catalyzed hydrolysates and did not existed in protein products or trypsin-catalyzed hydrolysates. These peptide fractions need to be identified in latter work.

Meanwhile, as hydrolysis progressed more peptide bonds were broken and much small peptides would be formed in hydrolysates with higher DH. It could be seen from Figures 1 and 2 clearly that there were more small peptides existing in hydrolysates with higher DH (see distribution differences of protein bands between Lane 2 and Lane 3, or distribution differences of protein bands between Lane 4 and Lane 5). Differences of peptide composition in SPC or SPI hydrolysates would lead to their different EAI.

Govindaraju and Srinivas (2006) obtained hydrolysates with low DH 3~5%, which were treated by papain, alcalase and fungal protease. Their results indicated that emulsifying property of hydrolysates was improved with low DH, but extensive hydrolysis resulted in remarkable reduction in emulsification. Neutrase could be used to prepare new soybean protein products with modified functional properties. Another study revealed that ex-truded when SPC was hydrolyzed mildly with neutrase, a clear improvement in EAI occurred, followed by a rapid decrease of the parameters with growing E:S ratio in the reaction mixture (Żmudziński and Surówka, 2004), which meant that an increase in DH impaired EAI of hydrolysates. The effect of proteolysis of extruded soybean flour with protamex on functional properties of hydrolysates had also been studied. Proteolysis could produce an enhancement in emulsifying properties, but increasing of the protamex to protein ratio in the reaction mixture led to a decrease of emulsifying properties, especially when the proteolysis was conducted for a prolonged time (Surówka et al., 2004).

Similar to these studies mentioned, EAI of 4 SPC hydrolysates was obviously improved at DH 1 or 2%. As DH of SPC hydrolysate was increased, it led to the decrease in EAI, for SPC-N2 or SPC-T2 had lower EAI than SPC-N1 or SPC-T1. The effect of DH on EAI was also found in 4 SPI hydrolysates with similar manner. The SDS-PAGE profiles (Figures 1 and 2) indicated that as DH was increased, more bonds in soybean proteins were hydrolyzed by proteases leading to smaller peptides formation. Because more large peptides existed in SPC-N1 or SPC-T1, they exhibited larger emulsifying activity index than SPC-N2 or SPC-T2. Also, SPI-N1 or SPI-T1 also exhibited larger emulsifying activity index than SPI-N2 or SPI-T2. Meanwhile, more small peptides were formed in neutrase-catalyzed hydrolysates than trypsin-catalyzed hydrolysates with same DH; therefore trypsin-catalyzed SPC hydrolysates or trypsin-catalyzed SPI hydrolysates had larger emulsifying activity index than neutrase-catalyzed SPC hydrolysates or neutrase-catalyzed SPI hydrolysates with same DH. All these meant that it was mode of enzymatic hydrolysis, mainly enzyme specificity and DH of hydrolysate that determined the improving extent in EAI.

Conclusions

With limited hydrolysis by neutrase or trypsin to DH 1 or 2%, soybean protein concentrates or soybean protein isolate could be modified to improve emulsifying activity clearly. SDS-PAGE analysis confirmed that more large peptides were present in SPC or SPI hydrolysates prepared with trypsin and more small peptides were present in SPC hydrolysate or SPI hydrolysate prepared with neutrase. Evaluation results showed that SPC hydrolysate or SPI hydrolysate prepared with trypsin had larger emulsifying activity index than those prepared with neutrase at same DH, which was related to the differences of peptide composition. Increase in DH of SPC or SPI hydrolysate would impair its emulsifying activity index. SPC hydrolysate prepared with trypsin to DH 1% had larger emulsifying activity index than SPC and other three SPC hydrolysates. SPI hydrolysates prepared with trypsin to DH 1% also had larger emulsifying activity index than SPI and other 3 SPI hydrolysates. It was the different specificity of enzyme used and different degree of hydrolysis that led to the different improvement in EAI of hydrolysates prepared. Mode of enzymatic hydrolysis (protease used and DH obtained) had important influence on the EAI of protein hydrolysates.

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