

Original article

Egg yolk protein modification by controlled enzymatic hydrolysis for improved functionalities

Guang Wang & Tong Wang*

Department of Food Science and Human Nutrition, Iowa State University, Ames, IA 50011-1061, USA

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Summary Delipidated egg yolk protein (EYP) is produced as a co-product of egg yolk lecithin extraction. This EYP is expected to have poor functionality because of protein denaturation caused by ethanol treatment during lecithin extraction. Two food grade endo-proteases were used to produce EYP hydrolysates (EYPh) with two degrees of hydrolysis (DH), 3% and 6%. Protein solubility was improved as DH increased. Solubility profiles for both EYP and EYPh were relatively less pH-dependent compared with soy protein. Except for foaming capacity, EYPh showed improvement in foam stability, foaming speed, and foam density. Emulsion stability was improved for all EYPhs. Treatments at DH of 6% showed significant increase in emulsification capacity. We have shown quantitatively how controlled enzymatic hydrolysis can be applied to ethanol-treated lipid-free EYP to increase protein solubility, and thus to improve foaming and emulsification properties.

Keywords Delipidation, egg yolk protein, emulsification, enzymatic hydrolysis, foaming properties, solubility.

Introduction

Egg yolk is a good source of phospholipids, especially phosphatidylcholine and phosphatidylethanolamine. We have developed a method for total lipid extraction and phospholipid fractionation from fresh egg yolk (Palacios & Wang, 2005). In the process of delipidation by using ethanol and hexanes sequentially, lipid-free egg yolk protein (EYP) is produced as co-product. However, this delipidated EYP is alcohol denatured. It is generally accepted that alcohol denatured proteins have greatly reduced functionalities. Therefore, it is critically important to determine and improve functional properties of such EYP to make this value-added egg processing and utilization economically feasible for the egg industry.

Egg yolk protein is a high quality protein. Sakanaka *et al.* (2000) reported that lipid-free EYP had amino acid score of 100. *In vitro* test for digestibility showed that EYP had faster digestion than milk casein when treated with pepsin and pancreatin. In the test of protein efficiency ratio, the criteria to determine the nutritional value of proteins, EYP showed significantly higher value than that of milk casein. However, applying this lipid-free EYP as functional ingredient in food may be challenging because of its predicted poor protein func-

tionality as a result of alcohol denaturation. Polar solvents can irreversibly cause conformational changes of protein structure, and lead to aggregation and precipitation of proteins (Lapanje, 1978). Ethanol can access the hydrated protein and displace the bound water that hydrates the protein, thus denature the protein (Khmelnitsky *et al.*, 1991). Plant proteins, such as soy protein, subjected to hexane extraction still show good solubility and functionalities, however, the ethanol washed soy protein concentrate has significantly reduced functionalities. Our ethanol treated EYP is expected to have reduced water solubility and therefore reduced foaming and emulsification properties.

It has been shown that controlled enzymatic hydrolysis is an effective method in modifying functionalities of food proteins, and this method has been used in various soy proteins (Jung *et al.*, 2005; Lamsal *et al.*, 2006) and whey proteins (Severin & Xia, 2006). Enzymatic hydrolysis of protein causes breakdown of protein molecules and increase in protein solubility. Solubility of protein is essential for most proteins to provide good functionalities, such as foaming and emulsification. For EYP, Sakanaka *et al.* (2004) reported that EYP could be completely hydrolysed and it showed antioxidant activity in a linoleic acid oxidation system. However, there is little information on the effect of controlled enzymatic hydrolysis of EYP on its foaming and emulsification properties. We hypothesised that controlled enzymatic hydrolysis

*Correspondent: Fax: 515 294 8181;
e-mail: tongwang@iastate.edu

can improve functionality of ethanol-treated lipid-free EYP and we need to obtain the quantitative data.

In this study, two food grade proteases were used to hydrolyse ethanol-denatured EYP at two different degrees of hydrolysis (DH). Protein solubility, emulsification property, and foaming property of the corresponding hydrolysates were studied to examine the effectiveness of such hydrolysis treatments on functionality improvement.

Materials and methods

Materials and preparation of egg yolk protein

Ethanol-treated EYP was obtained as the co-product of egg lecithin extraction (Palacios & Wang, 2005). Briefly, fresh eggs purchased from a local store were broken and egg white was carefully removed. Fresh EYP, 400 g, was mixed with 400 mL of 100% ethanol for dehydration and polar lipid extraction. The mixture was centrifuged for 15 min at $4900 \times g$ and 20 °C by using a Sorvall RC 3B plus centrifuge (International Equipment Company, Needham Heights, MA, USA). Liquid phase was removed, and this process was repeated another two times. The ethanol-insoluble fraction was then mixed with 270 mL hexanes for three times, and the mixture centrifuged at the same condition as for ethanol extraction. The solid fraction after air drying at ambient temperature was extracted again by adding another 140 mL of 100% ethanol followed by centrifugation to ensure complete extraction of polar lipids. The solid fraction was lipid-free EYP, and it was air-dried overnight followed by vacuum oven drying for 24 h at ambient temperature. All protein samples were ground by using a laboratory Wiley mill (Arthur H. Thomas Co., Philadelphia, PA, USA) with a 40-mesh filter and sealed with polyethylene ziplock bag and stored in refrigerator (4 °C) until use.

Total residual lipid in EYP was extracted with ten times (volume to weight) of chloroform:methanol (2:1, v/v). Solvent was evaporated and this residual lipid was put in a vacuum oven overnight to remove any residual solvent. The residual oil content was measured as 4.76%.

SPI (Profam-974[®]) was received as a generous gift from Archer Daniels Midland Company (Decatur, IL, USA). All chemicals and solvents used were reagent grade.

Enzymes and protein hydrolysis treatment

The two food-grade enzymes used in this study were liquid Protex 7L and solid Protamex 1.5. Both enzymes were obtained as gifts from the manufactures and enzyme concentration varied from 1–10%. Protex 7L (Genencor International, Rochester, NY, USA) is a bacterial neutral endoprotease originated from *Bacillus amyloliquefaciens* and its effective component is protease neutral (E.C. 3. 4. 24. 28) with a minimum activity of

1600 azocasein g⁻¹. Enzyme Protamex 1.5 (Novozymes N/A, Franklinton, NC, USA) is also an endo-protease originated from *B. amyloliquefaciens* and *Bacillus licheniformis* with effective components of E.C. 3. 4. 21. 62 and E.C. 3. 4. 24. 28, and its activity is 1.5 AU g⁻¹. Both enzymes are non-specific proteases and have an optimal pH of about 7 when temperature is 25–60 °C (Adler-Nissen, 1986; DSM Nutrafacts, 2009). Besides these two endoproteases that were used in final experimental design, an exoprotease was also tested in our preliminary experiment. However, under the equivalent hydrolysis condition, it was not as effective as the endoproteases and therefore, was not used.

The pH-STAT method was used to determine degree of hydrolysis (DH). According to this method that was established by (Adler-Nissen (1986)), the amount of base consumed in mole is proportional to the amino groups liberated in mole during the hydrolysis process. Degree of hydrolysis was calculated using Adler-Nissen's equation:

$$\text{DH}\% = 100 * V_B * N_B / (\alpha * M_p * h_{\text{tot}}),$$

where α is degree of dissociation of α -amino group, M_p is the mass of protein (g), h_{tot} is the total number of peptide bonds in the protein (meq g⁻¹ protein), and V_B and N_B are volume (mL) and concentration (normality) of alkaline added. At fixed temperature and constant pH environment, α is a constant, and h_{tot} varies based on specific proteins. The pH and temperature used in our study were 7.0 and 50 °C, respectively, and α is 0.44 under this condition (Adler-Nissen, 1986). The value of h_{tot} for EYP is unknown currently and it was assumed to be 8 based on a reference suggestion (Cherry & Barford, 1988). This value is similar to the value for soy protein which is 7.8. Therefore, the resulting DH% values shown in this study were estimates rather than absolute DH, and such relative measurement should not significantly interfere with addressing the questions as this experiment was designed to.

The hydrolysis reaction was operated in a 250 mL jacketed glassware with a pH-STAT automatic titration (Model 718, Brinkmann, Switzerland) connected with an iso-thermal water bath. Egg yolk protein slurry of 10% (w/w) was prepared and stirred for 30 min to fully disperse the protein. The protein suspension was preheated to 50 °C and pH was adjusted to 7 by using 2 N sodium hydroxide solution. Based on above equation, fixed amount of enzymes (Table 1) for achieving DH of 0%, 3%, and 6% were added to the suspension. To determine the optimal dose of enzyme, EYPs were previously treated with a series of enzyme doses for hydrolysis, and DH change vs. reaction time was plotted. If the trend of the curve became relatively flat within certain time, we then chose this dose condition as the optimum. Regarding reaction time at each level of DH%, we wanted the two enzymatic treatments to have

Table 1 Parameters used in and obtained from enzymatic hydrolysis of egg yolk protein

Treatment	EaDH3	EaDH6	EbDH3	EbDH6
Enzyme	Protex	Protex	Protamax	Protamax
Desired DH, %	3	6	3	6
EYP, g	10	10	10	10
Water, g	90	90	90	90
E/S, %	0.05	0.15	0.08	0.20
Enzyme amount	166.7 μ L	499.5 μ L	150 mg	400 mg
NaOH consumed, 2 N, mL	0.515	1.03	0.515	1.03
Reaction time, min	32.4 \pm 0.6c	80.4 \pm 5.0b	41.0 \pm 6.0c	94.0 \pm 8.5a

Different letters in reaction time represent significant difference at 95% confidence level.

E/S, %: amount of enzyme to substrate (g/g). Concentration of enzyme in Protex was estimated as 3% and Protamax was estimated as 5% to calculate E/S. EaDH3: Protex, DH = 3%; EaDH6: Protex, DH = 6%; EbDH3: Protamax, DH = 3%; EbDH6: Protamax, DH = 6%. DH: degree of hydrolysis.

similar time to avoid any possible difference caused by incubation time. The reaction was carried out until the set DH value was reached. Inactivation of the enzymes was done by acidifying hydrolysates to pH 4.0–4.2 immediately after titrations were finished (Adler-Nissen, 1986). Egg yolk protein hydrolysates were then placed in a freezer (-20°C) until functionality tests were performed. Being one of the most common methods of enzyme inactivation, the heat inactivation was also examined by heating EYPH for 15 min in water bath at about 90°C for Protex 7L (Genencor International)-treated EYPH. All EYPHs were neutralised to pH 7 by using 1 N sodium hydroxide before functional properties were determined.

Water solubility profile

Water solubility curve was established in the pH range of 2–10 with one unit interval. The 10% EYPH dispersion was diluted to 2% with deionised water and gently agitated for 1 h, and pH was adjusted at 30 and 60 min using 1 N sodium hydroxide or 1 N hydrochloric acid. All samples were then centrifuged at $10\,000 \times g$ for 10 min at 20°C (Jung *et al.*, 2005). Protein content in the supernatant was determined by using the Biuret protein assay (Gornall *et al.*, 1949). Duplicate protein quantifications were done for each treatment. Bovine serum albumin (BSA) (Sigma Chemical Co., St Louis, MO, USA) was used to establish the standard curve.

Foaming properties

The 10% EYPH dispersion was diluted to obtain 100 mL of 0.5% protein concentration and pH 7. The dispersion was placed into a 400 mL glass column fitted

with a medium size fritted glass disk at the bottom according to a method of Wang *et al.* (2004). Nitrogen gas was purged at a speed of 100 mL min^{-1} to make a final foam volume of 300 mL from 100 mL of 0.5% protein solution. Three measurements were made: time to reach final volume of 300 mL, t_f in seconds; volume of liquid sample consumed at the end of foaming, V_{\max} in mL; and time used for half of the incorporated liquid to drain back, $t_{1/2}$ in seconds. Three foaming property parameters were calculated based on these measurements (Wang *et al.*, 2004):

(1) Foaming capacity, mL of foam formed per mL of N_2 purged,

$$F_c = \frac{300\text{ mL}}{100\text{ mL min}^{-1} \times t_f \times 1\text{ min } 60\text{ s}^{-1}}, \text{ in mL mL}^{-1}$$

(2) K-value, the specific rate constant for liquid drainage, used as the foam stability measurement,

$$K = \frac{1}{V_{\max} \times t_{1/2}}, \text{ in mL}^{-1}\text{ s}^{-1}$$

(3) Foaming speed, the speed of liquid being incorporated into foams,

$$V_i = \frac{V_{\max}}{t_f}, \text{ in mL s}^{-1}$$

Emulsification properties

Egg yolk protein hydrolysate suspension of 25 mL in 2% concentration prepared from the 10% stock hydrolysate was mixed with dyed (Sudan Red 2B, about 4 ppm) soybean oil at constant rate of oil addition, 5 mL min^{-1} . A Bamix brand hand-held blender (Switzerland) was used to continuously mix oil and EYPH dispersion at 'Low' speed during oil addition until apparent phase inversion was observed, at which point the oil-in-water emulsion system lost its viscosity. The amount of oil added till phase inversion was used to calculate emulsification capacity (EC, g oil g protein $^{-1}$).

Emulsion stability was measured by following method: dyed vegetable oil, 8 mL, was added into 32 mL 2% EYPH and mixed for 1 min by using the same hand-held blender at 'High' speed setting. From the resulting emulsion, 10 mL was transferred into 15 mL plastic centrifuge tube that has a marker interval of 0.5 mL. Emulsion stability (ES, %) was calculated by dividing non-separated volume by total volume after 1 day standing at ambient temperature (20°C). The calculation is shown as following:

$$\text{ES}(\%) = \frac{\text{Volume, non - separated, mL}}{\text{Volume, total, mL}} \times 100$$

Statistical analysis

All treatments were duplicated, and data analyses were done using SAS program (version 9.1, SAS Institute Inc., Cary, NC, USA). One-way Analysis of Variance (ANOVA) was used and least significant differences was calculated at $P < 0.05$.

Results and discussion

Enzymatic hydrolysis

Because the two enzymes used showed different catalytic activities, different amounts of enzyme had to be applied in order to obtain the same DH within similar time frame. The least amount of enzyme used in each treatment was determined as that at the end of the reaction, hydrolysis speed (represented as slope of DH% vs. time) was low as indicated by the gradually flattened curves (Fig. 1). The two enzymes used did not show significantly different reaction time in producing EYPs with DH of 3%, however, Protamex-treated EYP had significantly longer reaction time than Protex for DH 6% (Table 1). All four treatments showed good hydrolysis reproducibility as shown in Fig. 1.

Water solubility profile

Bovine serum albumin was used as the standard to establish standard equation for protein solubility determination. The equation is:

Absorbance at 540 nm = 0.0206 * (mg protein mL⁻¹) + 0.0025, $R^2 = 0.9999$.

The solubility curves of EYP and corresponding EYPs are shown in Fig. 2. Solubility curve of a commercial SPI was determined and also shown in Fig. 2 as a comparison. EYP and its hydrolysates showed relatively flat curves across the pH range, and there were no obvious isoelectric points. On the contrary, SPI showed the expected U-shape profile as pH increased from 2 to 10, which is consistent with other reports (Jung *et al.*, 2005; Lamsal *et al.*, 2006). Kong *et al.* (2007) reported similar pH-independent solubility curve of wheat gluten hydrolysates, though the non-hydrolyzed wheat gluten was pH-dependent. The low solubility of untreated EYP could be the effect of protein denaturation by ethanol during lipid extraction. Ethanol may have caused exposure of hydrophobic core of native EYP, a protein unfolding, hydrophobic interaction and aggregation. Enzymatic hydrolysis was expected to break protein molecules or aggregates into smaller pieces, thus increasing solubility. The evidence of high solubility at DH of 6% and low solubility at DH of 3% from both enzyme treatments supports this hypothesis. Solubility profile at even higher DH value such as 10% was also measured in our study, and it showed a maximal solubility of >60% (data not shown). However, its foaming properties were significantly impaired. This decrease in foaming properties indicates that DH of 10% may have been excessive for optimal protein functionality.

It's worth mentioning that the overall solubility of SPI is lower than expected. This may be caused by the

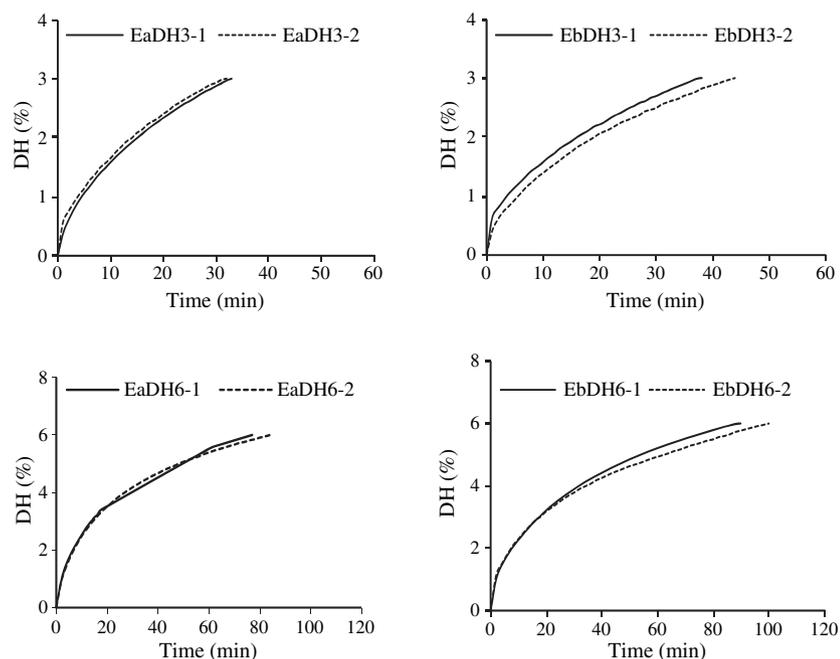


Figure 1 Duplicated hydrolysis of egg yolk protein at pH 7 and 50 °C to achieve 3% and 6% hydrolysis by using two proteases as measured by pH-STAT. EaDH3 and EaDH6: Protex, DH = 3% and 6%, respectively; EbDH3 and EbDH6: Protamex, DH = 3% and 6%, respectively.

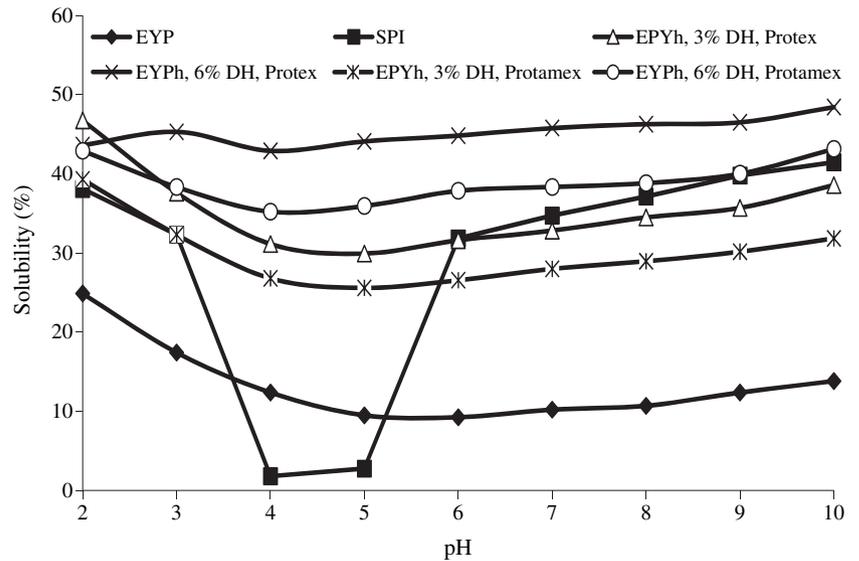


Figure 2 Water solubility profiles of egg yolk protein hydrolysates, egg yolk protein control, and soy protein isolate. DH, degree of hydrolysis.

experimental conditions, especially for centrifugation, which may be slightly different from that used by others. It is also possible that the SPI used was not freshly prepared and the solubility may be partially lost because of storage and natural protein degradation.

Various factors may influence protein solubility, such as the extent of protein denaturation, amino acid composition, hydrogen bonding, ionic strength, aromatic/aliphatic ratio, and hydrophobicity. These factors usually are interactive and influence each other. Among these factors, the effects of amino acid net charge and protein hydrophobicity on protein solubility are most studied (Hayakawa & Nakai, 1985; Dill, 1990; Shaw *et al.*, 2001). Tanford (1961) suggested that the solubility of a protein is proportional to the square of the net charge on the protein. The net charge would also affect how the solubility is affected by pH change. To evaluate the general net charge of soy and yolk proteins, we used amino acid composition of soy protein and EYP from

USDA protein database as shown in Table 2. The net charge ratio is expressed as the negative charge (sum of glutamic acid and aspartic acid) divided by positive charge (sum of arginine and lysine). Soy protein has a net charge ratio of 2.9, whereas the value for EYP is only 1.5. This difference in net charge may in part explain why solubility profile of EYP was less sensitive to pH change.

Foaming properties

As shown in Table 3, no significant difference was observed for foaming capacity (Fc) among all EYPs and the two controls. For K-value, which is the rate of liquid drainage and therefore is a measure of foam stability, the three hydrolyzed treatments (EaDH3, EbDH3, and EbDH6) showed significantly lower values than that of EYP control, but not for the EaDH6 sample. The lower K-values indicate that the foams created from

Table 2 Charged amino acid content in egg yolk protein and soy protein^a

Amino acid	Charge	Soy protein		Egg yolk protein	
		g 100 g ⁻¹	mole 100 g ⁻¹	g 100 g ⁻¹	mole 100 g ⁻¹
Aspartic acid	(-)	10.23	0.09	3.35	0.03
Glutamic acid	(-)	17.45	0.14	4.34	0.03
Arginine	(+)	6.67	0.04	2.44	0.02
Lysine	(+)	5.32	0.04	2.72	0.02

^aData obtained from USDA national nutrient database for standard reference, <http://www.nal.usda.gov/fnic/foodcomp/search/> (accessed on June 2007).

Net charge ratio for each protein is calculated by dividing sum of negative mole by sum of positive mole. Therefore, the net charge for SPI and EYP is 2.9 and 1.5, respectively.

Table 3 Foaming properties of egg yolk protein hydrolysates and controls

Treatment	Fc, mL mL ⁻¹	Kx10 ³ , 1 (mL*s) ⁻¹	V _i x10 ³ , mL s ⁻¹	V _{max} , mL
SPI	1.43 ± 0.02	8.25 ± 0.74 a	5.3 ± 0.3 d	40.0 ± 2.8 e
EYP (DH0)	1.45 ± 0.01	5.73 ± 0.06 b	7.0 ± 0.0 c	52.0 ± 0.0 d
EaDH3	1.42 ± 0.00	2.34 ± 0.01 d	12.5 ± 0.0 a	95.0 ± 0.0 b
EaDH6	1.32 ± 0.01	8.04 ± 0.38 a	9.0 ± 0.1 b	74.0 ± 1.4 c
EbDH3	1.41 ± 0.01	2.40 ± 0.02 d	12.4 ± 0.1 a	95.0 ± 0.0 b
EbDH6	1.37 ± 0.02	2.55 ± 0.07 c	12.7 ± 0.2 a	100.0 ± 0.0 a
LSD _{0.05}	0.25	0.80	0.4	3.0

Different letters in the same column indicate significant difference at 95% confidence level. See Table 2 footnote for treatment abbreviations.

Fc, foaming capacity; K, foam stability; V_i, foaming speed; V_{max}, foam density; EYP, egg yolk protein; SPI, soy protein isolate; LSD, least significant difference.

these treatments were more stable. Meanwhile, all hydrolysis treatments showed significantly higher foaming speed (V_i) than EYP and SPI. This indicates that the formation of foam by the hydrolysates was much faster in comparison with the EYP control. The possible reason for this improvement is that the small and more dispersed peptide chains are more mobile to migrate onto the liquid–air interface and easier to change molecular or structural conformation to form protein film enclosing air. Parameter V_{max} represents the maximum liquid volume consumed to create 300 mL foam during purging process and indicates the density of the foam. All treatments created higher density of foam than EYP and SPI controls. This means all EYPs produced smaller bubbles and finer foam than did the controls. Therefore, we can generally conclude that controlled hydrolysis of EYP improved the foaming properties of ethanol-treated delipidated EYP.

Emulsification properties

Whole egg yolk is a good natural emulsifier and it has been used in mayonnaise-making for a long time because of their high emulsification capacity (EC) and high stability. Emulsification capacity is the amount of oil that can be emulsified under specific conditions using 1 g of protein and emulsion stability (ES) describes the ability of protein to keep an emulsion without phase separation over time at a given temperature and gravitational field (Panyam & Kilara, 1996). Mean EC and ES for EYP and its hydrolysates are presented in Table 4.

The two treatments (EaDH6, EbDH6) with DH of 6% showed significantly higher EC than EYP and SPI. But EaDH3 and EbDH3, with DH of 3%, had significant lower EC values than did EYP. This result indicates that higher DH (6%) is more desirable in increasing emulsification capacity. Literature review by Lamsal *et al.* (2006) suggested that soy protein hydrolysates had increased EC with DH up to 5%. However,

Table 4 Emulsification properties of egg yolk protein hydrolysates

Treatment	Emulsification capacity, g oil g ⁻¹ protein	Emulsion stability, %
SPI	551.0 ± 1.3 d	30 ± 0 b
EYP (DH0)	661.0 ± 7.3 c	25 ± 1 c
EaDH3	495.0 ± 9.8 d	31 ± 1 b
EaDH6	935.0 ± 48.4 a	38 ± 3 a
EbDH3	396.0 ± 6.5 e	32 ± 1 b
EbDH6	739.0 ± 23.0 b	36 ± 1 a
LSD _{0.05}	69.0	3

Different letters in the same column indicate significant difference at 95% confidence level.

See table 1 and table 3 footnotes for abbreviations of treatments and terms.

this is not shown in our study for EYP hydrolysis. Emulsion stability of EYP was significantly improved by enzymatic hydrolysis for both DHs and for both enzyme treatments. Again, higher DH gave better emulsion stability.

Another finding from this study is that heat inactivation of the enzymes after hydrolysis had marked detrimental effect on EC of EYP hydrolysates. After the enzymatic hydrolysis with DH of 6%, we tried to terminate the hydrolysis reaction by heating the hydrolysates at 90 °C for 15 min. The EC change is shown in Table 5. Heated hydrolysate had decreased EC by three-folds compared with the one that was not heated. The heated protein had EC even lower than EYP control. The EC difference caused by heat inactivation indicates that EYP and EYPs are heat sensitive, as majority of other proteins are. This result is consistent with another study done on emulsion properties of whole egg yolk after heating (Guilmineau & Kulozik, 2006).

Effect of enzyme type and DH% on functional properties of EYP hydrolysates

In this experiment, we found that the two enzymes showed similar hydrolysis trend with time. However, generally the hydrolysates created by using Protex showed higher solubility than by Protamex at the same DH value as shown in Fig. 2. This difference in solubility indicates the peptide bonds they attacked during hydrolysis may be different, though they are both non-specific proteases. In general, treatments with lower DH showed slightly better foaming performance, but they had poorer emulsification properties than the higher DH treatments. Therefore, the two functionalities are not positively correlated.

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Table 5 Emulsification capacity as affected by heat inactivation of the protease Protex 7L

Treatment	EYP, DH 0%	EYP _h , DH 6%, not-heated	EYP _h , DH 6%, heated
EC, g oil/ g protein	509 ± 25.6 b	918 ± 67.2 a	300 ± 3.5 c

EYP, egg yolk protein control (no hydrolysis treatment); EYP_h, DH 6%, not-heated, Protex hydrolysed EYP with DH of 6%, freeze-dried.

EYP_h, DH 6%, heated, Protex hydrolysed EYP with DH of 6%, inactivation by heating for 15 min at 90 °C.

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