Emulsifying Characteristics of Gelatin Hydrolysate from Tilapia Skin Covalently Attached with N-hydroxysuccinimide Esters of Fatty Acids

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ABSTRACT

This study aims to combine two modification methods, namely enzymatic hydrolysis and covalent attachment with hydrophobic groups, to increase the emulsifying properties of gelatin. The experiment was conducted by using a completely randomized design with three replicates. Enzymatic hydrolysis of gelatin resulted in higher contents of free amino groups, which could be attached to hydrophobic groups. Gelatin hydrolysates covalently attached with the N-hydroxysuccinimide esters of C14:0 and C18:0 fatty acids at a molar ratio of 3.0 showed high emulsifying activity but low stability. Among the samples obtained, gelatin hydrolysate covalently attached with C18:0 at a molar ratio of 3.0 revealed the highest emulsifying activity; however, this sample cannot be considered the best emulsifier among the samples because of its low stability.

Keywords: Covalent attachment; emulsion; gelatin; hydrolysis

INTRODUCTION

An emulsion refers to a mixture of two immiscible liquids that are thermodynamically unstable (Jain et al., 2012). These two liquids consist of a liquid phase in the form of fine droplets dispersed in another immiscible liquid phase (Fustier et al., 2010). During food processing and storage, emulsions often become unstable, which causes a significant change in product characteristics (Muray, 2008). Several additives, such as a stabilizer, are needed to maintain the quality of emulsion systems in food. Two types of stabilizers, namely, emulsifiers and texture modifiers, have been developed (Mclements, 2016). Emulsifiers are substances usually added to emulsion systems to decrease the interfacial tension of the two phases. A texture modifier enhances the stability of an emulsion by retarding the movement of droplets.

Gelatin, the protein resulting from the hydrolysis of collagen to varying degrees (Haug and Draget, 2011), is extracted commercially from bovine and porcine skin and bone. Marine gelatin was recently developed as an alternative gelatin to address concerns related to bovine spongiform encephalopathy and religious and social issues (Aewsiri et al., 2011). Gelatin presents functional properties related to its structure, amino acid composition, and molecular weight distribution. For example, the protein may be a suitable texture modifier for stabilizing emulsions because of its viscosity and gelling properties. It may also act as a barrier to stabilize thin water films separating oil droplets. Gelatin consists of hydrophilic and hydrophobic amino acids, both of which confer it with surface active properties that could enable its use as an emulsifying agent. However, although gelatin can stabilize emulsions, it still presents lower stability compared with other proteins, such as casein, and surfactants (Jain et al., 2018).

Gelatin has lower surface activity compared with other types of emulsifiers because it has fewer hydrophobic groups. Enzymatic hydrolysis can increase the hydrophobicity and free amino group contents of gelatin. Aewsiri et al. (2011) also reported that modification of cuttlefish gelatin by the N-hydroxsuccinimide esters of fatty acids could improve the surface hydrophobicity and surface activity of the protein.

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During covalent attachment, hydrophobic groups react with gelatin via N-terminal amino groups or the amino groups of lysine. Gelatin is mostly composed of glycine, proline, and hydroxyproline and contains very small amounts of lysine. Increasing free amino group contents is necessary to improve the reactivity between gelatin and hydrophobic groups. Hydrolysis of peptide bonds is a method that can be used to increase free amino group contents. The present study aims to investigate the effect of the combination of enzymatic hydrolysis and covalent attachment with hydrophobic groups on the emulsifying characteristics of gelatin hydrolysate from tilapia skin.

RESEARCH METHODS

Materials

Commercial tilapia skin (FGS 230) and scale gelatin were purchased from Nippi Collagen Industries, Co., Ltd. (Tokyo, Japan). Alcalase was purchased from EMD Millipore Corp. (Tokyo, Japan). Stearic acid, myristic acid, N-hydroxysuccinimide, and dicyclocarbodiimide were purchased from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan). Capric acid was obtained from Sigma Aldrich, Co., Ltd. (Tokyo, Japan).

Apparatus

Dialysis membranes with molecular weight cutoff values (MWCOs) of 3.5, 6, and 14 kDa (Spectra/Por, Shiga, Japan) were used in this research. A freeze dryer (Eyela FDU 2100I Tokyo, Japan), spectrophotometer (Jasco V630, Tokyo, Japan), vortex mixer (Delta Mixer SE-08 Taitec, Tokyo, Japan), and ACE homogenizer (AM-8 Nissei; Tokyo, Japan) were also employed.

Hydrolysis with alcalase to obtain low-molecular weight gelatin

Hydrolysis was carried out by using the method of Gimenez et al. (2009) with slight modifications. Enzymatic hydrolysis of gelatin previously obtained in our laboratory was conducted with alcalase under controlled conditions (pH, temperature, enzyme concentration). Gelatin (25% w/v) was dissolved in 10 mM phosphate buffer (pH 8) and submitted to enzymatic hydrolysis using alcalase (3.03 U/mL) at 50 °C for 3 h under an enzyme/substrate ratio of 1:20 (w/w). The pH of the reaction was kept constant by addition of 1 N NaOH solution to the reaction medium when necessary. The enzyme was inactivated by heating the mixture to 90 °C for 10 min. The mixture was then centrifuged at 3800 rpm for 15 minutes at 4 °C. The supernatant corresponding to the hydrolysate from gelatin was dialyzed by using 3.5, 6, and 14 kDa MWCO membranes. Samples were freeze-dried in a freeze dryer (Eyela FDU 2100I, Tokyo, Japan) and stored at −80 °C in a freezer.

Covalent attachment of gelatin hydrolysate with N-hydroxysuccinimide esters of fatty acids

Covalent attachment of fatty acids was carried out by reacting the N-hydroxysuccinimide esters of different fatty acids with the amino groups of gelatin hydrolysate according to the method of Wierenga et al. (2003) with a slight modification. Gelatin hydrolysate (MW, 3.5–<14 kDa) was dissolved in 100 mM Na2CO3 buffer (pH 8.5) to obtain a final concentration of 1% protein as determined by the biuret method (Robinson and Hodgson, 1940). N-Hydroxysuccinimide esters of different fatty acids were dissolved in dimethyl sulfoxide to obtain solutions of different concentrations. Solutions of the N-hydroxysuccinimide esters of different fatty acids (80 mL) were then added to 20 mL of gelatin to obtain final molar ratios of N-hydroxysuccinimide esters/gelatin of 2.0 and 3.0. The pH of the mixture was 10, which is too high for most food applications. Thus, 0.1 M HCl was added to the mixtures to neutralize the pH. The mixtures were stirred continuously for 18 h at room temperature. Thereafter, the samples were dialyzed by using 6 KDa MWCO dialysis membranes for 24 h against a 20-fold volume of water to remove free fatty acids and N-hydroxysuccinimide esters not bound to the protein. The dialysate was freeze-dried, and the resulting gelatin powder was stored at 20 °C.

Analytical methods

The parameters analyzed included free amino group content and emulsion activity index (EAI).

Free amino group content

Free amino group content analysis was carried out according the method of Church et al. (1983). OPA reagent was prepared by mixing 80 mg of o-phthalaldehyde, 2 mL of methanol, 50 mL of 0.1 M Na2B4O7, 5 mL of 20% (w/v) SDS, and 0.2 mL of β-mercaptoethanol. Then, 150 μL of a sample or standard solution was mixed with 3.0 mL of OPA reagent. The mixture was allowed to stand for 2 min at room temperature, and then its absorbance was measured at 340 nm using a spectrophotometer (Jasco V630; Tokyo, Japan). Degree of modification was calculated as follows:

\[
\text{Degree of modification} = \frac{\text{Initial free amino acid amount} - \text{number of free amino acids after modification}}{\text{Initial free amino acid amount}} \times 100\%
\]
Emulsion activity index

The EAI and emulsion stability index of the gelatin samples were determined according to the method of Pearce and Kinsella (1978) with slight modifications. Soybean oil (2 mL) and gelatin solution (1% gelatin, 6 mL) were homogenized at a speed of 20,000 rpm for 1 min. Emulsions were then pipetted out at 0 and 10 min and 100-fold diluted with 0.1% SDS. The mixture was mixed thoroughly for 10 s using a vortex mixer (Delta Mixer SE-08 Taitec; Tokyo, Japan). The absorbance of the resulting dispersion was measured at 500 nm using a spectrophotometer (Jasco V630; Tokyo, Japan). EAIs at 0 and 10 min were calculated by using the following formula (Aewsiri et al., 2009):

$$\text{EAI (m}^2/\text{g}) = (2 \times 2.303 \times A \times DF)/løC$$

where $A = A_{500}$, $DF =$ dilution factor (100), $l =$ path length of the cuvette (m), $\phi =$ oil volume fraction, and $C =$ protein concentration in the aqueous phase (g/m$^3$).

Experimental design and statistical analysis

The experiment was carried out by using a completely randomized design with three replicates. The data were assessed by using one-way analysis of variance (ANOVA) to determine significant differences and two-way ANOVA to understand interaction effects. Significant differences were declared at a 5% significance level.

RESULTS AND DISCUSSION

Free amino group content

The results of free amino group content of gelatin hydrolysate fractions were presented in Table 1. Free amino group content of gelatin was determined by O-phthalaldehyde (OPA) method. OPA in conjunction with reduced sulfhydryl groups reacts with primary amines to form fluorescent moieties (Held, 2001). Gelatin hydrolysate with molecular weight (MW) <3.5 kDa had greater number of free amino group (46.17 mM) followed by MW 3.5 kDa to <6 kDa (38.83 mM), MW 6 kDa up to <14 kDa (32.23 mM) and native gelatin (11.22 mM). Enzymatic hydrolysis caused the cleavage of peptide bond. Alcalase is an enzyme with broad specificity that can hydrolyse most peptide bond in protein molecule. Lower molecular peptide that can be produced will give higher number of primary amine.

The free amino group contents of gelatin hydrolysates covalently attached with the $N$-hydroxysuccinimide esters of different fatty acids at various molar ratios are presented in Table 2. The gelatin hydrolysate attached with the $N$-hydroxysuccinimide esters of fatty acids had lower free amino group contents compared with gelatin hydrolysate. These results indicate that $N$-hydroxysuccinimide esters of fatty acids react with gelatin hydrolysate via amino groups of the N-terminal or ε-amino group of lysine (Aewsiri et al., 2011).

Among the samples obtained, GH-C10:0 (gelatin hydrolysate-C10:0) and GH-C14:0 (gelatin hydrolysate-C14:0) at a molar ratio of 3.0 showed similar free amino group contents, while GH-C14:0 at a molar ratio of 2.0 revealed the highest free amino group content (11.59 mM). These results are different from previous research. Gelatin attached with longer chains of fatty acids and higher molar ratios between gelatin and fatty acid have lower free amino group contents (Lin et al., 2012; Aewsiri et al., 2011). Such different results may be attributed to differences in the original source (protein), mixture of amino acids, and accessibility of free amino groups, all of which can affect the effectiveness of acylation (Matemu et al., 2012).

Compared with those of other samples, the free amino group content of GH-C18:0 (gelatin hydrolysate-C18:0) at a molar ratio of 3.0 was the lowest. This result indicates that C18:0 at a molar ratio of 3.0 is more effective than C10:0 0 and C14:0 in decreasing free amino group contents, likely due to its longer fatty acid chains and high molar ratio (Aewsiri et al., 2011).

Two-way ANOVA was used to analyze the effect and interaction between molar ratio and fatty acid chain length on free amino group contents. Molar ratio and fatty acid chain length played important roles in determining free amino group contents. Aewsiri et al. (2011) and Magdassi et al. (1996) demonstrated that different chain lengths and molar ratios affect the reactivity between the $N$-hydroxysuccinimide esters of fatty acids and gelatin hydrolysate. The results also showed that interactions between molar ratio and chain length on free amino group contents were significant (Table 2).

Table 1. Free amino group content of gelatin with different molecular weight (gelatin concentration 1%)

| Molecular weight (kDa) | Free amino group (mM) |
|------------------------|-----------------------|
| >100                   | 11.22±0.14a           |
| <3.5                   | 46.17±1.65d           |
| 3.5-<6                 | 38.83±1.35c           |
| 6-<14                  | 32.23±0.34b           |

Mean ± SD (n = 3). * Different letters in the same column indicate significant differences (p < 0.05).
length determine free amino group contents to some extent. The degree of modification was expressed as a percentage of the number of covalently attached amino groups relative to the total number of free amino groups in the control gelatin. Increases in degree of modification reflect protein unfolding, which increases the availability of reactive residues (Schwenke et al., 2001). Acylation initiates the unfolding of the protein structure and exposes buried amino groups to interact with interface. Wanasundara and Shahidi (1997) reported that acylation blocks off reactive amino groups by increasing acyl residues. Higher molar ratios increase the degree of modification because more hydrophobic groups can covalently attached with free amino groups.

**Emulsion properties**

The emulsion properties of gelatin hydrolysates covalently attached with the N-hydroxysuccinimide esters of different fatty acids at various molar ratios are presented in Table 3. The emulsion activity of gelatin hydrolysate (0.72 m²/g) was lower than that of native gelatin (MW > 100 kDa). This result is different from the findings of Matemu et al. (2011), who revealed that tofu whey hydrolysate (<3 kDa) has higher emulsion activity compared with native soy protein. Protein hydrolysis can enhance the number of charged groups and hydrophobic residues of protein molecules by exposing their interior (Ma and Wood 1987).

The low EAI of gelatin hydrolysate may be affected by the hydrophilic and hydrophobic balance of gelatin. Gelatin is relatively hydrophilic when its amino acid sequences are mainly composed of glycine, proline, and hydroxyproline (Chen et al., 2015). Therefore, hydrolysis is inadequate to increase the emulsion activity of gelatin. According to Surh et al. (2005), large droplets and destabilized oil are uncommon in high-molecular weight (HMW) gelatin because the thickness of adsorbed gelatin membranes increases with increasing molecular weight.

| Treatment                | Free amino group | Degree of Modification |
|--------------------------|------------------|------------------------|
| Gelatin hydrolysate (GH) | 34.85±1.59d      | 0a                     |
| GH-C10:0 (2.0)           | 7.08±0.30b       | 79.64±1.78c            |
| GH-C10:0 (3.0)           | 7.53±1.36b       | 78.25±4.87c            |
| GH-C14:0 (2.0)           | 11.59±1.12c      | 66.75±2.34b            |
| GH-C14:0 (3.0)           | 7.77±0.91b       | 77.64±3.25c            |
| GH-C18:0 (2.0)           | 6.03±0.12ab      | 82.69±0.79cd           |
| GH-C18:0 (3.0)           | 4.38±0.37a       | 87.45±0.52d            |

Mean ± SD (n = 3). * Different letters in the same column indicate significant differences (p < 0.05).

| Samples            | EAI (0 min) | EAI (10 min) | ΔEAI    |
|--------------------|-------------|--------------|---------|
| Gelatin            | 1.92±0.20d  | 0.42±0.02b   | 1.49±0.20d |
| GH                 | 0.72±0.02b  | 0.52±0.01c   | 0.19±0.02ab |
| GH-C10:0 (2.0)     | 0.31±0.01a  | 0.22±0.01a   | 0.10±0.01a  |
| GH-C10:0 (3.0)     | 0.95±0.01c  | 0.64±0.03d   | 0.31±0.03b  |
| GH-C14:0 (2.0)     | 0.80±0.07bc | 0.43±0.03b   | 0.36±0.06b  |
| GH-C14:0 (3.0)     | 3.42±0.04g  | 2.48±0.04g   | 0.94±0.08c  |
| GH-C18:0 (2.0)     | 2.19±0.05e  | 0.44±0.06b   | 1.76±0.09e  |
| GH-C18:0 (3.0)     | 2.15±0.13f  | 5.85±0.13h   | 3.70±0.01h  |

Mean ± SD (n = 3). * Different letters in the same column indicate significant differences (p < 0.05).
weight. GH-C10:0 at a molar ratio of 2.0 had lower emulsion activity (0.31 m²/g) than gelatin hydrolysate. The EAI of GH-C10:0 at a molar ratio of 3.0 and GH-C14:0 at a molar ratio of 2.0 were not significantly different compared with that of gelatin hydrolysate. Free amino group analysis of the modified gelatin revealed that free amino group contents significantly decreased relative to that of gelatin hydrolysate after modification. This finding indicates that EAI would increase as the result of a higher degree of modification. The different results may be attributed to differences in surface hydrophobicity and hydrophilic lipophilic balance (HLB). Modification with C10:0 and C14:0 at a molar ratio of 2.0 did not increase the surface hydrophobicity, HLB, or surface activity of gelatin hydrolysate.

The emulsion activity of GH-C14:0 at a molar ratio of 3.0 (3.42 m²/g) was nearly identical to that of BSA. Compared with GH-C14 at a molar ratio of 2.0, GH-C14:0 at a molar ratio of 3.0 had a higher EAI. This result may be attributed to the lower degree of modification of GH-C14:0 at a molar ratio of 2.0 compared with that of GH-C14:0 at a molar ratio of 3.0. Although GH-C14:0 at a molar ratio of 3.0 had a degree of modification similar to that of C10:0, the higher EAI observed may be caused by longer hydrophobic chains in the modification process. GH-C18:0 at a molar ratio of 2.0 had an EAI lower than that of GH-C18:0 at a molar ratio of 3.0. This result could also be correlated with the degree of modification of GH-C18:0 at molar ratios of 2.0 and 3.0. The highest EAI was obtained from GH-C18:0 at a molar ratio of 3.0.

Incorporation of the N-hydroxysuccinimide ester of fatty acids enhances the surface hydrophobicity of modified gelatin. Increases in surface hydrophobicity usually result in better surface activity, which can improve the emulsifying properties of gelatin (Aewsiri et al., 2011). The length of the hydrophobic chain in covalently attached gelatin increases as the length of the fatty acid applied increases. Longer chains of hydrophobic carbon decrease surface tension quickly because more amphiphilic molecules are present in the solution surface (Lin and Chen, 2006). Increases in molar ratio also result in higher surface hydrophobicity, therefore facilitated gelatin to localize and rearrange in interface, therefore reduced surface tension rapidly (Aewsiri et al., 2011).

The EAIs of gelatin hydrolysate, GH-C10:0 at molar ratios of 2.0 and 3.0, and GH-C14:0 at a molar ratio of 2.0 did not change significantly after 10 minutes. GH-C10:0 at a molar ratio of 3.0 and C14:0 at a molar ratio of 2.0 had similar ΔEAI that were lower than those of other samples. This result shows that gelatin hydrolysate, GH-C10:0 at molar ratios of 2.0 and 3.0, and GH-C14:0 at a molar ratio of 2.0 have higher emulsion stability compared with other samples. Low-molecular weight gelatin is fairly stable because non-adsorbed gelatin promotes depletion–flocculation in HMW gelatin emulsions. The strength of depletion–flocculation increases as the size of droplets and molecular weight of non-adsorbed gelatin increase (Mclements, 2016). The emulsion of GH-C10:0 was also stable because C10:0 is a medium-length fatty acid that provides suitable amphiphilic characteristics and facilitates rearrangement at the oil–water interface (Matemu et al., 2011). GH-C14:0 at a molar ratio of 3.0 showed a ΔEAI of 0.94 m²/g and was the second most stable emulsion among the samples. GH-C18:0 (3.0) showed a ΔEAI of 2.15 m²/g but was less stable compared with other samples. The emulsion with GH-C18:0 as an emulsifier had high activity but low stability because C18:0, as a long fatty acid chain, results in poor interactions between the oil and protein, thereby causing flocculation (Matemu et al., 2011).

Covalent attachment of gelatin with longer fatty acid chains and higher molar ratios resulted in higher emulsifying activity. Longer fatty acid chains and higher molar ratios increase the surface hydrophobicity of gelatin. Therefore, gelatin could easily be adsorbed on the interface and increased gelatin surface pressure. Increases in surface pressure cause lower interfacial tension, which, in turn, leads to higher emulsifying activity (Aewsiri et al., 2011). Covalently attached gelatin with longer fatty acid chains and higher molar ratios tended to have lower emulsifying ability. Longer fatty acid chains and higher molar ratios increase the surface hydrophobicity of gelatin as well as hydrophobic interactions between fatty acid chains exposed to the aqueous phase. Over time, this hydrophobic interaction tends to promote flocculation, eventually resulting in coalescence of droplets (Monahan et al., 1996, Demetriades et al., 1997).

Two-way ANOVA was used to analyze the effect and interaction between molar ratio and fatty acid chain length on the emulsifying properties of modified gelatin. Molar ratio and fatty acid chain length played significant roles in determining the emulsifying properties of modified gelatin. Higher molar ratios and fatty acid chain lengths increased emulsification activity but decreased emulsifying stability. The results also showed significant differences among the emulsifying properties of the samples and that the interaction between molar ratio and chain length determined free amino group contents.
CONCLUSION

Enzymatic hydrolysis of gelatin resulted in higher contents of free amino groups to which hydrophobic group could attach. However, increases in free amino group content do not necessarily increase emulsifying activity. Modification of free amino group contents with hydrophobic compounds was important in increasing emulsification properties. Covalent attachment of gelatin hydrolysate with \(N\)-hydroxysuccinimide esters of C14:0 and C18:0 fatty acids at a molar ratio of 3.0 produced higher emulsifying activity but lower emulsifying stability. While gelatin attached with \(N\)-hydroxysuccinimide esters of C18:0 fatty acids at a molar ratio of 3.0 had the highest emulsifying activity compared with other samples, it cannot be considered the best emulsifier among the samples because of its low stability.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

REFERENCES

Aewsiri, T., Benjakul, S., & Visessanguan, W. 2009. Functional properties of gelatin from cuttlefish (Sepia pharaonis) skin as affected by bleaching using hydrogen peroxide, Food Chemistry, 115 (1): 243-249. doi.org/10.1016/j.foodchem.2008.12.012

Aewsiri, T., Benjakul, S., Visessanguan, W., Wierenga, P. A., & Gruppen, H. 2011. Improvement of foaming properties of cuttlefish skin gelatin by modification with \(N\)-hydroxysuccinimide esters of fatty acid, Food Hydrocolloids, 25(5): 1277-1284. doi.org/10.1016/j.foodhyd.2010.11.027

Chen, S., Tang, L., Su, W., Weng, W., Osako, K., & Tanaka, M. 2015. Separation and characterization of alpha-chain subunits from tilapia (Tilapia zillii) skin gelatin using ultrafiltration, Food Chemistry, 188: 350–356. doi.org/10.1016/j.foodchem.2015.04.084

Church, F. C., Swaisgood, H. E., Porter, D. H., & Catignani, G. L. 1983. Spectrophotometric assay using o-phthalaldehyde for determination of proteinolysis in milk and isolated milk proteins, Journal of Dairy Science, 66(6): 1219-1227. doi.org/10.3168/jds.S0022-0302(83)81926-2

Demetriades, K., Coupland, J. N., & McClements, D. J. 1997. Physical Properties of whey protein stabilized emulsions as related to pH and NaCl, Journal of Food Science 62(2): 342-347. doi.org/10.1111/j.1365-2621.1997.tb03997.x

Fustier, P., Taherian, A. R., & Ramaswamy, H. S. 2010. Emulsion delivery systems for functional foods. In Smith, J. & Charter, E., editors, Functional Food Product Development, Wiley Blackwell Publishing House, United States.

Haug, I. J. & Draget, K. I. 2011. Gelatin. In Philips, G. O. & Williams, P. A., editors, Handbook of Food Proteins, 1st Ed., Woodhead Publishing, Cambridge, pp 92-105.

Held, P. 2001. Total protein quantification. https://www.biotwk.com/resources/application-notes/total-protein-quantification-using-opa/ October 2016.

Jain, G. K., Ahmad, F. J., & Khar, R. K. 2012. Pharmaceutical Emulsions (Chapter 9). In Smith, A.L., editor, Theory and Practice of Physical Pharmacy. 1st Ed. Elsevier, New Delhi, India, pp 223-248.

Jain, S., Dhakar, D., & Anal, A. K. 2017. Proteins and peptides derived from chicken processing by products and waste (Chapter 16.3). In Anal, A.K., editor, Food Processing By-Products and their Utilization, John Wiley & Sons Ltd. United Kingdom, 372-386.

Lin, L.H., & Chen, K. M. (2006). Preparation and surface activity of gelatin derivative surfactants. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 272(1-2): 8–14. doi.org/10.1016/j.colsurfa.2005.07.006

Lin, L. H., Chen, K. M., Liu, H. J., Chu, H. C., Kuo, T. C., Hwang, M. C., & Wang, C. F. (2012). Preparation and surface activities of modified gelatin–glucose conjugates. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 408: 97–103. doi.org/10.1016/j.colsurfa.2012.05.036

Ma, C-Y., & Wood, D. F. 1987. Functional properties of oat proteins modified by acylation, trypsin hydrolysis or linoleate treatment, Journal of the American Oil Chemists’ Society, 64 (12): 1726-1731.

Magdassi, S., Toledano, I.O., & Zakay-Rones, Z. 1996. Solubilization in colloidal immunoclusters, Journal of Colloid and Interface Science, 184(2): 360-364. doi.org/10.1006/jcis.1996.0630

Matemu, A.O, Kayahara, H., Murasawa, H., Katayama, S., & Nakamura, S. 2011. Improved emulsifying properties of soy proteins by acylation with saturated fatty acids, Food Chemistry, 124 (2): 596–602. doi.org/10.1016/j.foodchem.2010.06.081

Matemu, A.O., Kayahara, S., Katayara, H., Murasawa, H., & Nakamura, S. 2012. Improving Surface Functional Properties of Tofu Whey-Derived Peptides by Chemical
Modification with Fatty Acids, Journal of Food Science, 77(4): C333-9. Doi: 10.1111/j.1750-3841.2012.02631.x

Monahan, F. J., McClements, D. J., & German, J. B. 1996. Disulfide-mediated polymerization reactions and physical properties of heated WPI-stabilized emulsions, Journal of Food Science, 61(3), 504-509. Doi.org/10.1111/j.1365-2621.1996.tb13143.x

Murray, B. S. 2008. Controlling emulsion stability: microstructural and microrheological origins of flocculating systems in Gums and Stabilisers for the Food Industry 14 (Williams, P. A. and Philips, G. O), Royal Society of Chemistry, United Kingdom: 211-220.

Pearce, K. N., & Kinsella, J. E. (1978). Emulsifying properties of proteins: evaluation of a turbidimetric technique. Journal of Agricultural and Food Chemistry, 26 (3): 716-723. Doi.org/10.1021/jf60217a041

Robinson, H. W., & Hogden, C. G. 1940. The biuret reaction in the determination of serum proteins: I. A study of the conditions necessary for the production of a stable color which bears a quantitative relationship to the protein concentration, Journal of Biological Chemistry, 135: 707-726.

Schwenke, K. D., Knopfe, C., Seifert, A., Gornitz, E., & Ziwer, D. 2001. Acetylation of faba bean legumin: Conformational changes and aggregation, Journal of the Science of Food and Agriculture, 81 (1): 126-134. Doi.org/10.1002/1097-0010(20010101)81:1<126::AID-JSFA788>3.0.CO;2-Y

Surh J., Gu, Y. S., Decker, E. A. & McClements, D. J. 2005. Influence of environmental stresses on stability of a/w emulsions containing cationic droplets stabilized by SDS–fish gelatin membranes, Journal of Agricultural and Food Chemistry, 53 (10): 4236-4244. Doi.org/10.1021/jf047944i

Wanasundara, P.K.J.P.D. & Shahidi, F. 1997. Functional properties of acylated flax protein isolates. Journal of Agriculture and Food Chemistry, 45(7):2431–41. doi.org/10.1021/jf9607829