Carbohydrate Polymers 89 (2012) 1244–1249

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Laccase mediated conjugation of heat treated β-lactoglobulin and sugar beet pectin

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ARTICLE INFO

Article history:
Received 22 November 2011
Received in revised form 5 March 2012
Accepted 11 April 2012
Available online 21 April 2012

Keywords:
Sugar beet pectin
β-Lactoglobulin
Laccase
Conjugation
Solubility

ABSTRACT

Laccase, an oxidative enzyme, was used to catalyze the hetero and homo covalent conjugation between ferulic acid in sugar beet pectin (SBP) and tyrosine in heated β-lactoglobulin (H_BLG). The conjugation of SBP and H_BLG was confirmed by peak position using size exclusion chromatography, multi angle laser light scattering, refractive index, and UV detection. H_BLG, pre-treated with laccase, eluted at an earlier volume with greater UV280 absorbance than non-laccase treated dispersions. Tyrosine decreased in H_BLG that contained laccase treated SBP samples. Heat enhanced exposure of tyrosine in BLG and improved conjugation with SBP by laccase. H_BLG·SBP conjugates with laccase had improved solubility than laccase untreated dispersions at pH values near the isoelectric point of BLG.

1. Introduction

Much interest has focused on the complexes between proteins and polysaccharides because these complexes show enhanced functional properties, such as solubility and gelling as well as foaming and emulsifying stability than lone polymers (Dickinson & Galazka, 1991; Guzey & McClements, 2007; Suzuki & Kondo, 1982). In addition, these complexes are considered as potential encapsulation and delivery systems for functional and bioactive components for medical and cosmetic products (Goldberg, Langer, & Jia, 2007; Madene, Jacquot, Scher, & Desobry, 2006; Renard et al., 2002), as well as the food industry, such as fat replacer, cream substitute, and oil substitute (Kloser & Sheuring, 1954; Pettersson, Johnsson, Brannas, & Pickova, 2009). Complex formation between proteins and polysaccharides can be achieved through electrostatic interaction, hydrogen bonds, hydrophobic interactions and covalent interactions (Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). Among those interactions, covalent conjugation has more advantages through maintenance of molecular integrity and solubility over a wide range of conditions. It can also contribute to improvement of functional properties and increase resistance to heat and proteolytic attack under various conditions (Dickinson & Galazka, 1991; Hattori, Okada, & Takahashi, 2000). With chemical activating agents, Maillard reaction and enzymatic conjugation have been investigated for covalent cross-linking of proteins and polysaccharides (Flanagan & Singh, 2006; Hattori et al., 2000; Kato, Wada, Kobayashi, Seguro, & Motoki, 1991; Kato, 2002; Kim, Choi, Shin, & Moon, 2003; Selinheimo, Lampila, Mattinen, & Buchert, 2008; Walsh, Cleary, McCarthy, Murphy, & FitzGerald, 2003). However, possible forming of toxic side-products and undesirable hazard materials could occur in chemical and Maillard reactions. An enzymatic method is preferred because enzymes are specific, non-toxic food ingredients (Flanagan & Singh, 2006; James & Simpson, 1996).

A variety of enzymes form conjugates and improve functionality of protein and polysaccharide heteroconjugates (Boeriu et al., 2004; Chen, Vazquez-Duhalt, Wu, Bentley, & Payne, 2001; Faergemand, Otte, & Qvist, 1997; Faergemand, Otte, & Qvist, 1998; Kuuva, Lantto, Reinikainen, Buchert, & Autio, 2003; Littoz & McClements, 2008; Selinheimo et al., 2008; Tianhong Chen, Wu, & Payne, 2002; Walsh et al., 2003). Transglutaminase catalyzes the acyl transfer between a λ-carboxyamide of a protein bound glutamine (as a donor) and lysine (as an acceptor) (Fork & Finlayson, 1977). It has been reported that transglutaminase induced the successful covalent cross-linking between gum arabic containing protein and sodium caseinate (Flanagan & Singh, 2006). The conjugation of BLG and cationic polysaccharide was catalyzed by microbial transglutaminase and improved the emulsifying properties (Ikeuchi, Aoki, Yoshida, Takahashi, & Hattori, 2008). Laccase and tyrosinase were also used to form conjugates between phenolic compounds present in proteins and polysaccharides (Chen et al., 2001; Flanagan & Singh, 2006; Selinheimo et al., 2008).

Oxidative crosslinking of homopolymers, like SBP or protein, has been shown to improve functional properties. Laccase and

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0144-8617/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2012.04.028

peroxidase form stable gels in SBP via oxidative coupling and covalent cross-links between beet pectin molecules (Kuuva et al., 2003; Micard & Thibault, 1999). SBP extracted by acid and autoclaving can form gels through oxidative cross-linking by peroxidase at low 1.5% concentration (Oosterveld, Beldman, & Voragen, 2000). Emulsions stabilized by BLG and SBP showed improved stability if SBP were cross-linked by laccase after adsorption to BLG, resulting from layer-by-layer stabilization (Littoz & McClements, 2008). Emulsion oil droplets coated by electrostatic deposition of laccase treated SBP and BLG showed better stability than at pH 4.5 with NaCl addition non laccase treated deposition (Chen, McClements, Gray, & Decker, 2010; Littoz & McClements, 2008). In previous work from this laboratory, SBP that was conjugated by laccase prior to emulsion formation, showed improved emulsion stability, smaller $d_{4,3}$ diameter and uniform droplet during 30 days storage at 37 °C, compared to control SBP (Jung & Wicker, 2012a). The improved stability was attributed to the initial adsorption of the protein moiety of SBP and formation of a thick stabilizing layer via cross-linked SBP.

Laccase can also oxidize phenolic compounds in proteins, such as tyrosine, cysteine and tryptophan containing peptides (Gianfreda, Xu, & Bollag, 1999; Mattinen et al., 2005, 2006; Micard & Thibault, 1999). While BLG was reported as a poor substrate for laccase since tyrosine residues are buried in the 3-dimensional structure of BLG (Mattinen et al., 2006; Uhrinova et al., 2000), we observed conjugation of heated BLG with laccase (Jung & Wicker, submitted for publication). In addition, laccase incubation with BLG resulted in change from spherical to more rod like structure (Jung & Wicker, 2012b). Heated BLG could enhance the hetero-conjugation with SBP and BLG.

Residues available for hetero-conjugation include tyrosines in BLG and the protein moiety of SBP, and ferulic acid in SBP. Our previous work established that laccase catalyzes homo-conjugation of SBP and that either laccase or heat caused a structural change in BLG that exposed residues that would serve as sites for conjugation. The aim of this study was to determine if covalent conjugation between SBP and BLG pre treated by heat or laccase could be catalyzed by addition of laccase and to determine the effect on some functional properties of the putative conjugates.

2. Materials and methods

2.1. Materials

Low heat dry powder β-lactoglobulin (BLG) was provided by Davisco Foods International (Lot no. JE 003-6-922, Le Sueur, MN) and contained 93.6% undenatured BLG out of 97.8% total protein as stated by the manufacturer. Sugar beet pectin (SBP) was donated by Herbstreith & Fox KG (Lot no. 0 05 03 024, Elmsford, NY). Degree of esterification of SBP was 59% and 73% galacturonic acid contents described by manufacturer. Laccase (*Rhus vernificera*, EC 1.10.3.2, *p*-diphenol oxidase), syringaldazine was purchased from Sigma–Aldrich Co. (St. Louis, MO). All analytical grade reagents were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Stock preparation

SBP and BLG dispersions were prepared by separately dispersing 30 mg/ml dry power in 100 mM sodium phosphate buffer (pH 6.5), stirring at room temperature for at least 2 h and storing overnight at 4 °C for complete hydration. Both dispersions were centrifuged for 30 min at 5000 × *g*, 4 °C prior to use (Sorvall RC 6Plus, Thermo Scientific Products).

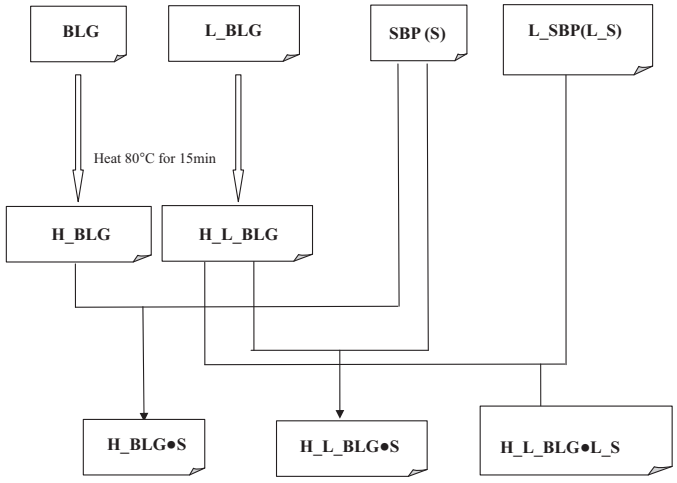

Fig. 1. Flow diagram of laccase mediated H_BLG and SBP mixture preparation. BLG: β-lactoglobulin; S: sugar beet pectin; L_BLG: β-lactoglobulin with laccase treatment; L_S: sugar beet pectin with laccase treatment; H_BLG: heat treated β-lactoglobulin for 15 min at 80 °C; H_L_BLG: heat treated β-lactoglobulin for 15 min at 80 °C, with laccase treatment; H_BLG·S: mixture of heated BLG & S; H_L_BLG·S: mixture of heated and laccase treated BLG & S; H_L_BLG·L_S: mixture of heated and laccase treated BLG & laccase treated S.

2.3. Laccase activity measurement

Laccase activity was measured through a modified procedure (Jung & Wicker 2012a) of the previously described method using syringaldazine as substrate at 30 °C (Ride, 1980). The loss in absorbance at 530 nm per unit of time was used to quantify activity.

2.4. Laccase mediated SBP and H_BLG conjugation

The sample preparation is presented in Fig. 1 and is adapted from previous work with SBP (Jung & Wicker 2012a). The centrifuged SBP and BLG dispersions were equilibrated with and without laccase in a water bath (30 °C) for 20 h with mild agitation on an orbital shaker (PR 70 Red Rotor Hofer Scientific, MO). An aliquot of 1 unit laccase/mg total sample solid was added to the dispersions. Activity of the laccase was measured immediately before addition. The laccase treated and non-laccase treated BLG dispersions were heated at 80 °C for 15 min and then mixed with or without laccase treated SBP. These SBP and BLG mixtures were equilibrated for another 20 hr at ambient temperature. Acronyms to denote treatments are described in legend of Fig. 1.

2.5. Particle size measurement

Particle size distributions of H_BLG and SBP and heat treated BLG with laccase were determined by Malvern Mastersizer (Model MSS, Malvern Instruments Limited, Worcestershire, UK) and the presentation code was 3 OHD.

2.6. Size exclusion chromatography, multi-angle laser light scattering (HPSEC-MALLS)

Size exclusion chromatography was combined with multi angle laser light scattering (DAWN DSP-F), refractive index (RI, Optilab 903, Wyatt Technology, USA), and UV detectors (Thermo Separation Scientific Products) to analyze the samples as described (Jung & Wicker 2012a).

2.7. Determination of tyrosine

The procedure to determine tyrosine in samples was adapted from a previous method (Hassan, 1975) as described by Jung and Wicker (2012b) using absorbance measured at 360 nm and 430 nm with a UV–visible spectrophotometer (UV-1700, Shimadzu, Columbia, MD, USA) to estimate the concentration of tyrosine (μg/ml).

2.8. Solubility

The samples were diluted with Milli-Q water at 1:10 ratio and then pH was adjusted to the pH 2, 4, 5, 7 for each sample with 1 M, 0.5 M HCl and 0.1 M NaOH solutions as required. The pH adjusted samples were under moderate stirring at 37 °C for 1 h. The pH was re-checked and re-adjusted as needed in the middle of stirring. The samples were centrifuged (Sorvall RC 6 Plus, Thermo Scientific Products) for 30 min at 10,000 × *g* at 20 °C. The supernatants were collected and the soluble protein was measured by the bicinchoninic acid (BCA) method at 560 nm using microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). Solubility was expressed as the percentage of protein content in supernatant relative to untreated protein content.

2.9. Statistical analysis

Values reported were the result of at least duplicate conjugations. Statistical differences estimated by one-way analysis of variance and Student's *t*-test ($p < 0.05$).

3. Results

3.1. Conjugate properties

The UV elution profiles monitored at 280 nm and 325 nm for the presence of BLG and SBP in mixed samples are presented in Fig. 2a and b, respectively. The UV profile was divided into four zones based on peak position of H_BLG and SBP. H_BLG alone eluted as two peaks between 8.2–9.0 ml (zone 2) and 9.5–10.1 ml (zone 4) volume; the first peak was attributed to BLG aggregates and the second peak was attributed to dimeric BLG. SBP eluted with two peaks at zone1 and zone 3 (Fig. 2b, UV_{325} elution profile). H_BLG mixed with SBP with and without laccase eluted between 6.8 and 10.1 ml, covering a broader and more extended (zones 1, 2, 3, and 4) elution volume than H_BLG (Fig. 2a). The elution profiles indicate that protein was eluted with SBP at 6.8–8.4 ml (zone 1) corresponding to a reduction of absorbance where H_BLG eluted (8.2–10.1 ml, zones 3, 4) (Fig. 2a). It suggests that some parts of H_BLG were associated with SBP and eluted at zone 1 due to formation of complexes with SBP. Since SBP and BLG were mixed at pH 6.5, above pI of BLG, both BLG and SBP are net negatively charged. Therefore, electrostatic interaction between BLG and SBP is not favored. Nevertheless, some non-covalent adsorption was noticed by the increased absorbance in the elution volume between 6.8 and 8.4 ml in H_BLG·S sample without laccase (Fig. 2a). It is possible that soluble complexes are formed by the interaction between negatively charged polysaccharide and partially positively charged polypeptide regions on unfolded protein (Cai & Arntfield, 1997; Cooper, Dubin, Kayitmazer, & Turksen, 2005). However, the peak height was relatively small compared to laccase treated samples (Fig. 2a).

As observed in the UV_{280} elution profile, H_L_BLG·S, the mixture of laccase treated H_BLG and SBP, had the biggest peak in the 6.8–8.2 ml (zone 1) elution range (Fig. 2a) where SBP elutes (Fig. 2b, SBP at UV_{325}). The smallest peak was observed where H_BLG eluted in 8.4–10.1 ml elution volume (Fig. 2a). It indicated that heat and laccase treatments promoted interaction between BLG and SBP, and

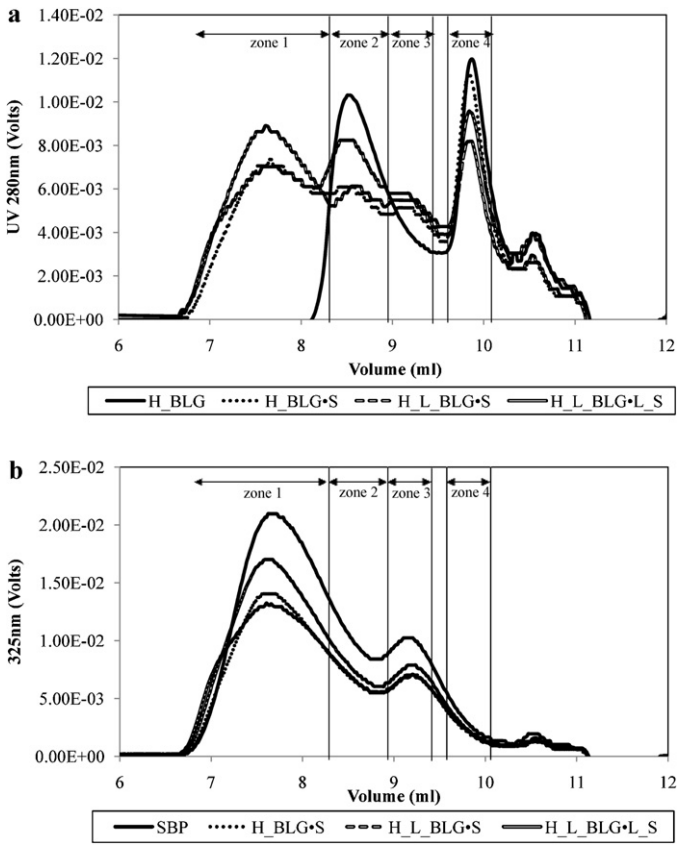

Fig. 2. UV elution profile at 280 nm (a) and 325 nm (b) of H_BLG and H_BLG & SBP with and without laccase. See legend in Fig. 1 for code of abbreviations.

complexes are mostly concentrated in zone 1. H_L_BLG·L_S, in which BLG and SBP were independently incubated with laccase prior to mixing, showed a bigger peak at 8.2–8.9 ml (zone 3) compared to other mixtures. Previously, it was reported that homo-conjugation was induced between H_L_BLG if BLG was incubated with laccase and heat treated (Jung & Wicker, submitted for publication). Furthermore, the peak height of H_L_BLG·L_S at 6.8–8.2 ml 9 (zone 1) was about the same height peak with H_BLG-S but the first peak was more polydisperse at 6.8–7.0 ml. It suggests that homo oxidative cross-linking of SBP molecules through the ferulic acids was favored since laccase was incubated with SBP prior to mixing with H_L_BLG (Fig. 2a). These results showed that the conjugation was encouraged by laccase pre-treated BLG in the presence of SBP, whether it was homo- or hetero-conjugation. Flanagan and Singh (2006) reported the conjugated sample between gum arabic and sodium caseinate by transglutaminase, eluted earlier than sodium caseinate alone, monitored by HPSEC-MALLS. Also, the conjugate showed an increased molecular weight.

The UV_{325} elution profile denoted the elution of SBP that contains ferulic acid (Fig. 2b), and is the site for oxidative coupling between ferulic acid via laccase (Kuuva et al., 2003; Mattinen et al., 2006). Untreated SBP alone showed the highest peak and the peak height was reduced when mixed with heated BLG with and without laccase treatment (Fig. 2b). The first peaks in H_L_BLG·L_S and H_L_BLG·S showed extended width on the left part of the peak (6.8–7.0 ml), suggesting larger aggregates were formed. All mixed samples showed lower ferulic acid contents than SBP alone sample, and H_L_BLG·S had the highest ferulic acid contents among mixed samples (zone 1, Fig. 3b). A larger peak in H_L_BLG·S indicated that oxidative coupling between ferulic acids was due to hetero cross-linking between ferulic acid in SBP and tyrosine in BLG. Another possibility is hetero cross-linking between tyrosine

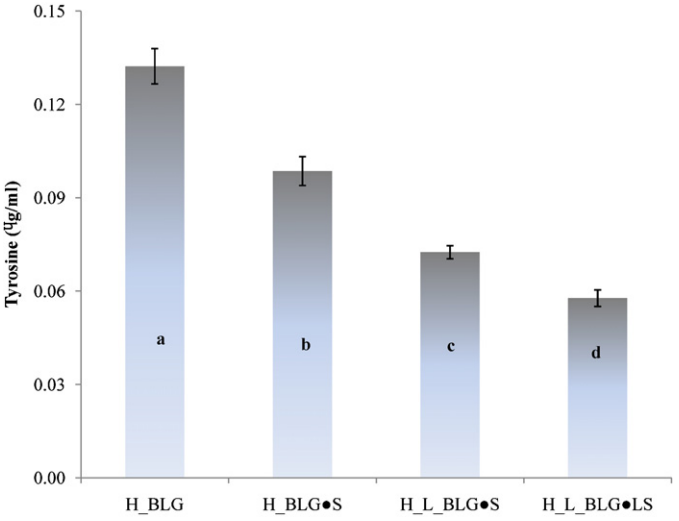

Fig. 3. Changes of tyrosine residue in H_BLG, and H_BLG & SBP with and without laccase. [a,b,c,d]: Same letters are not significantly different among samples ($p < 0.05$). See legend in Fig. 1 for code of abbreviations.

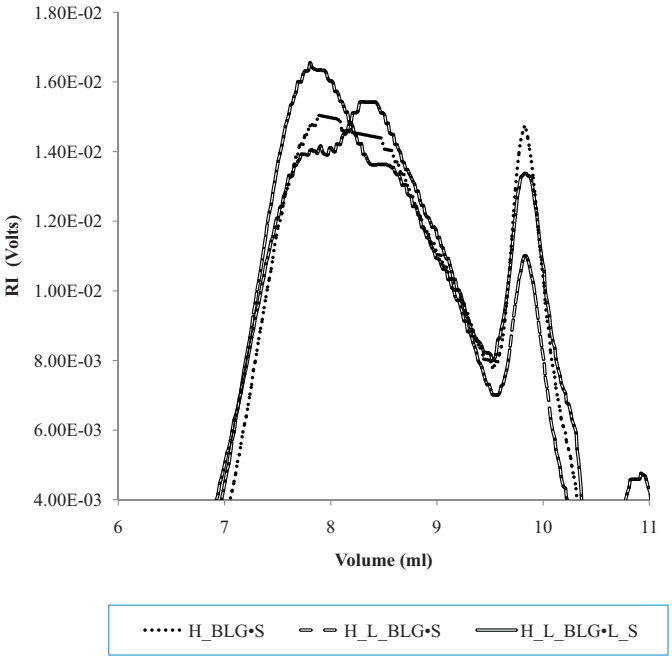

Fig. 4. RI elution profile of H_BLG & SBP with and without laccase. See legend in Fig. 1 for code of abbreviations.

in protein moiety of SBP and tyrosine in BLG was favored. More homo-conjugations in H_L_BLG·L_S could be suggested due to the lowest peak height of ferulic acid resulting from oxidative coupling within SBP molecules. This homo-conjugation might have preferred during SBP incubation with laccase prior to mixing with BLG.

The amount of tyrosine residues in H_BLG was 0.13 μg/ml and decreased ($p < 0.05$) when mixed with SBP and laccase (Fig. 3). Tyrosine in H_L_BLG·S and H_L_BLG·L_S were reduced by as much as ~26% and ~41%, respectively, compared to H_BLG-S. H_L_BLG·L_S showed the lowest concentration, 0.058 μg/ml (Fig. 3). This indicated that dityrosine between two tyrosine residues or intermolecular cross-links via dityrosine were formed whether they occurred by homo-polymer or hetero-polymer formation (Gross & Sizer, 1959). The decrease in tyrosine residues was in agreement with the result of UV_{280} profile, which demonstrated more distinctive polymerization in zone 2 in H_L_BLG·L_S (Fig. 2a). In addition, the reduction of the tyrosine concentration supported that cross-links between tyrosine in BLG, and ferulic acid or tyrosine in SBP might be encouraged through laccase (Fig. 3). The measured tyrosine residue in SBP was no more than 0.01 μg/ml (data not shown), and thus may not be a dominant factor for cross-linking. However, Boeriu et al. (2004) reported that tyrosine residues in the protein part of polysaccharide could be catalyzed by the oxidoreductase enzyme, so tyrosine residues present in SBP cannot be excluded as one of the substrates for laccase. The contribution of hydroxyl groups (–OH) in phenolic compounds to conjugation via laccase was confirmed by FTIR spectra measurement. The C—O—C stretching vibration was formed while phenolic (C—OH) stretching vibration disappeared while tyrosine containing peptide was incubated with laccase (Mattinen et al., 2005). It is likely that tyrosine was consumed by oxidative coupling via laccase forming cross-linking whether homo- or hetero-conjugation (Fig. 3). The lowest tyrosine concentration in H_L_BLG·L_S could be additional evidence that the homo-conjugation was more favored within heated BLG through tyrosine (Fig. 3). The RI detector records the concentration of all substances found in the sample whereas UV at 280 or 325 nm monitors the concentration of the specific substances detected at the given wavelength. In this study, RI elution profile showed a similar trend with the UV_{280} profile. H_L_BLG·S had a higher peak at 6.8–8.4 ml and H_L_BLG·L_S showed a higher peak around 8.4–9.0 ml elution volume with a reduction in peak which BLG eluted (9.5–10.1 ml), as described earlier in the elution profile of protein monitored by UV at 280 nm (Figs. 2a and 4).

The particle size distribution of H_BLG, and heat treated BLG with or without laccase treated SBP are presented in Fig. 5. H_BLG had a single peak around 150 μm. Peyron, Mouecoucou, Fremont, Sanchez, & Gontard (2006) reported heat treated BLG (pH 7.0, 1.7%, w/v) at 80 °C for 25 min showed two particle size populations at 0.7 μm and 600 μm. The range of reported particle sizes may result from differences in heating conditions and polymer concentration. Mixed samples of SBP and heated BLG showed smaller and more polydisperse peaks than H_BLG alone (Fig. 5). Moreover,

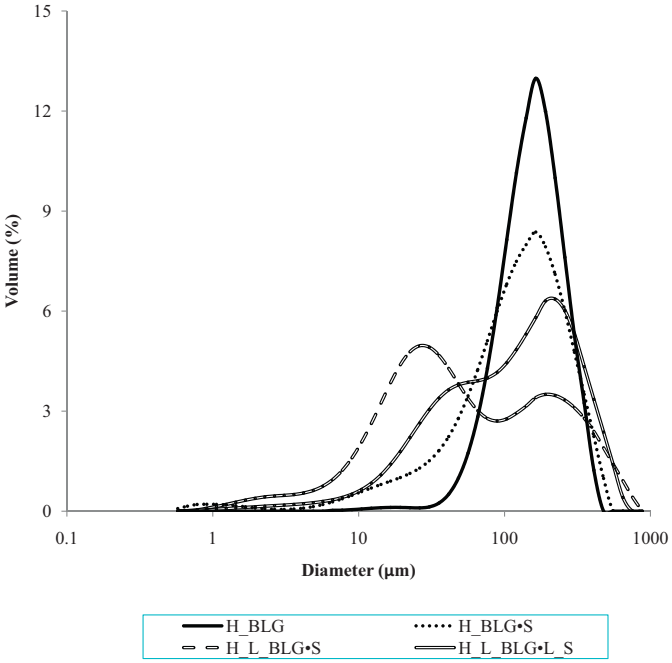

Fig. 5. Particle size distribution of H_BLG, and H_BLG & SBP with and without laccase. See legend in Fig. 1 for code of abbreviations.

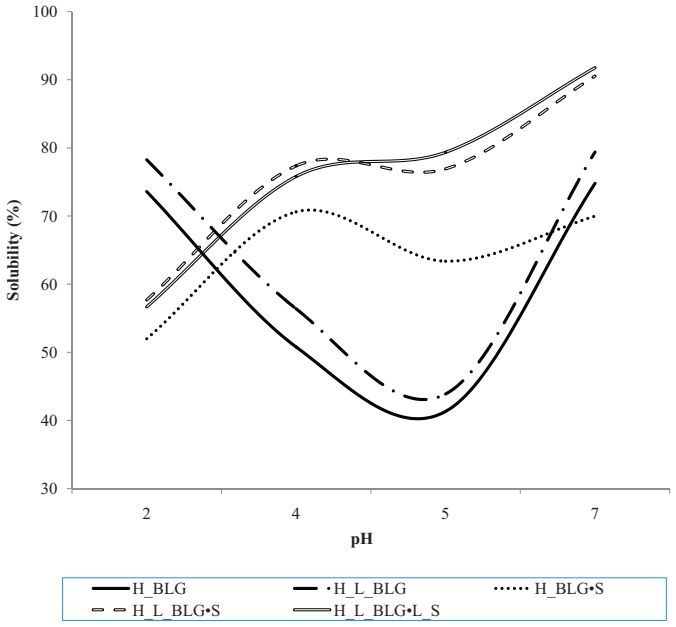

Fig. 6. Soluble protein concentration of H_BLG, and H_BLG & SBP with and without laccase. See legend in Fig. 1 for code of abbreviations.

laccase treated H_L_BLG·L_S and H_L_BLG·S demonstrated progressively smaller size population between 10 μm and 100 μm with reduction in the peak at 150 μm (Fig. 5). H_L_BLG·S and H_L_BLG·L_S showed two poorly resolved peaks compared to H_BLG-S. H_L_BLG·S exhibited greater volume % on the first peak, smaller size population, and the smaller peak in the second, larger population peak. Although H_L_BLG·L_S had a smaller peak in the larger size population than H_BLG·S, d_{43} was bigger than other mixed sample (Data not shown). It has been reported that pectin helps prevent protein aggregation (Cooper et al., 2005; Peyron et al., 2006). In this study, addition of SBP or laccase treatment of BLG or SBP reduced apparent particle size. Possible explanations include interruption of aggregation of H_BLG by SBP or laccase treated SBP, dissociation of aggregates of H_BLG or change in shape of aggregates due to conjugation.

3.2. Functional properties

The solubility at different pH values (pH 2, 4, 5, 7) is presented in Fig. 6 and expressed as soluble protein concentration in the supernatant after centrifugation. Soluble protein % was calculated based on the total protein measured in unheated BLG. H_BLG showed significantly lower solubility ~40% at pH values close to the isoelectric point (pI, ~pH 5.1) of BLG. Protein has the lowest solubility at its pI occurring protein precipitation by formation of net electrical charge on the surface of protein. On either side of the pI, solubility increased to ~80% at acidic pH and pH 7 (Fig. 6). Chevalier, Chobert, Popineau, Nicolas, & Haertl (2001) also reported that heated protein showed around 50% solubility in the range of pH 4.0–5.5, and a constant decrease solubility was observed as heat temperature increased from 60 °C to 90 °C. H_L_BLG and BLG pre-treated with laccase, had a higher solubility over the entire range of pH than H_BLG (Fig. 6). In addition, the amount of soluble protein increased more significantly in the presence of SBP at pH above 4 (Fig. 6). It was also reported that solubility improved in the range of pI of BLG and sugars conjugates produced through Maillard reaction for 72 h at 60 °C (Chevalier et al., 2001). Protein aggregates might be interrupted by the presence of pectin, and some soluble complexes between BLG and SBP were produced (Jones, Decker, & McClements, 2009; Peyron et al., 2006). However, lower solubility was observed in samples mixed with SBP than H_BLG alone at pH 2; pH value below the pKa of pectin. At pH values above the pKa, negatively charge SBP might form soluble complexes with positively charged BLG by electrostatic interaction (Jourdain, Leser, Schmitt, Michel, & Dickinson, 2008) and contribute to formation of soluble complexes. Laccase treated mixed samples exhibited a higher solubility than H_BLG and H_BLG·S at pH greater than 4 (Fig. 6). At pH 7, solubility was more than 90% but there was no significant difference between H_L_BLG·S and H_L_BLG·L_S ($p < 0.05$). These results clearly showed that soluble complexes formed between heated BLG and SBP, and prevented the precipitation of the protein at pH around pI and it was enhanced by laccase treatment.

4. Conclusions

Hetero-conjugation was preferred in H_L_BLG·S as evidenced by more protein elution with SBP at UV_{280} and more ferulic acid elution at UV_{325} than observed in other mixed samples. Laccase mediated cross-linking through ferulic acid was interrupted by interacting with tyrosine. Homo-conjugation was more dominant in H_L_BLG·L_S within SBP through ferulic acids and within BLG through tyrosine residue. Conjugated samples had higher solubility over a wide pH range and had ~40% better solubility than H_BLG at pH 5. It was likely that soluble conjugates between SBP and BLG prevented protein precipitation.

References

Boeriu, C. G., Oudgenoeg, G., Spekking, W. T. J., Berendsen, L. B. J. M., Vancon, L., Boumans, H., et al. (2004). Horseradish peroxidase-catalyzed cross-linking of feruloylated arabinoxylans with casein. *Journal of Agricultural and Food Chemistry*, *52*(21), 6633–6639.

Cai, R., & Arntfield, S. D. (1997). Thermal gelation in relation to binding of bovine serum albumin polysaccharide systems. *Journal of Food Science*, *62*(6), 1129–1134.

Chen, B. C., McClements, D. J., Gray, D. A., & Decker, E. A. (2010). Stabilization of soybean oil bodies by enzyme (laccase) cross-linking of adsorbed beet pectin coatings. *Journal of Agricultural and Food Chemistry*, *58*(16), 9259–9265.

Chen, T. H., Vazquez-Duhalt, R., Wu, C. F., Bentley, W. E., & Payne, G. F. (2001). Combinatorial screening for enzyme-mediated coupling. Tyrosinase-catalyzed coupling to create protein–chitosan conjugates. *Biomacromolecules*, *2*(2), 456–462.

Chevalier, F. i, Chobert, J.-M., Popineau, Y., Nicolas, M. G., & Haertl, T. (2001). Improvement of functional properties of beta-lactoglobulin glycated through the Maillard reaction is related to the nature of the sugar. *International Dairy Journal*, *11*(3), 145–152.

Cooper, C. L., Dubin, P. L., Kayitmazer, A. B., & Turksen, S. (2005). Polyelectrolyte–protein complexes. *Current Opinion in Colloid & Interface Science*, *10*(1–2), 52–78.

Dickinson, E., & Galazka, V. B. (1991). Emulsion stabilization by ionic and covalent complexes of β-lactoglobulin with polysaccharides. *Food Hydrocolloids*, *5*, 281–296.

Faergemand, M., Otte, J., & Qvist, K. B. (1997). Enzymatic cross-linking of whey proteins by a Ca^{2+}-independent microbial transglutaminase from *Streptomyces lydicus*. *Food Hydrocolloids*, *11*(1), 19–25.

Faergemand, M., Otte, J., & Qvist, K. B. (1998). Cross-linking of whey proteins by enzymatic oxidation. *Journal of Agricultural and Food Chemistry*, *46*(4), 1326–1333.

Flanagan, J., & Singh, H. (2006). Conjugation of sodium caseinate and gum arabic catalyzed by transglutaminase. *Journal of Agricultural and Food Chemistry*, *54*(19), 7305–7310.

Fork, J. E., & Finlayson, J. S. (1977). ε-(γ-glutamyl) lysine cross-link and the catalytic role of transglutaminase. *Advances in Protein Chemistry*, *31*, 2–120.

Gianfreda, L., Xu, F., & Bollag, J.-M. (1999). Laccases: A useful group of oxidoreductive enzymes. *Bioremediation Journal*, *3*, 1–25.

Goldberg, M., Langer, R., & Jia, X. Q. (2007). Nanostructured materials for applications in drug delivery and tissue engineering. *Journal of Biomaterials Science-Polymer Edition*, *18*(3), 241–268.

Gross, A. J., & Sizer, I. W. (1959). Oxidation of tyramine, tyrosine, and related compounds by peroxidase. *Journal of Biological Chemistry*, *234*(6), 1611–1614.

Guzey, D., & McClements, D. J. (2007). Impact of electrostatic interactions on formation and stability of emulsions containing oil droplets coated by beta-lactoglobulin–pectin complexes. *Journal of Agricultural and Food Chemistry*, *55*(2), 475–485.

Hassan, S. S. M. (1975). New spectrophotometric method for simultaneous determination of tryptophan and tyrosine. *Analytical Chemistry*, *47*(8), 1429–1432.

Hattori, M., Okada, Y., & Takahashi, K. (2000). Functional changes in beta-lactoglobulin upon conjugation with carboxymethyl cyclodextrin. *Journal of Agricultural and Food Chemistry*, *48*(9), 3789–3794.

Ikeuchi, T., Aoki, T., Yoshida, T., Takahashi, K., & Hattori, M. (2008). Functional improvements to beta-lactoglobulin by preparing an edible conjugate with cationic saccharide using microbial mransglutaminase (MTGase). *Bioscience Biotechnology and Biochemistry*, *72*(5), 1227–1234.

James, J., & Simpson, B. K. (1996). Application of enzymes in food processing. *Critical Reviews in Food Science and Nutrition*, *36*(5), 437–463.

Jones, O. G., Decker, E. A., & McClements, D. J. (2009). Formation of biopolymer particles by thermal treatment of [beta]-lactoglobulin-pectin complexes. *Food Hydrocolloids*, *23*(5), 1312–1321.

Jourdain, L., Leser, M. E., Schmitt, C., Michel, M., & Dickinson, E. (2008). Stability of emulsions containing sodium caseinate and dextran sulfate: Relationship to complexation in solution. *Food Hydrocolloids*, *22*(4), 647–659.

Jung, J., & Wicker, L. (2012a). Laccase mediated conjugation of sugar beet pectin and the effect on emulsion stability. *Food Hydrocolloids*, *28*(1), 168–173.

Jung, J., & Wicker, L. (2012b). Laccase modification of β-lactoglobulin and interaction with sugar beet pectin (submitted for publication).

Jung, J., & Wicker, L. The study of conformational changes in β-lactoglobulin upon heating and laccase, and laccase catalyzed conjugation of β-lactoglobulin and sugar beet pectin. *Food Research International* (submitted for publication).

Kato, A. (2002). Industrial applications of Maillard-type protein–polysaccharide conjugates. *Food Science and Technology Research*, *8*(3), 193–199.

Kato, A., Wada, T., Kobayashi, K., Seguro, K., & Motoki, M. (1991). Ovomucin food protein conjugates prepared through the transglutaminase reaction. *Agricultural and Biological Chemistry*, *55*(4), 1027–1031.

Kim, H. J, Choi, S. J., Shin, W. S., & Moon, T. W. (2003). *Emulsifying properties of bovine serum albumin-galactomannan conjugates*, *51*(4), 1049–1056.

Kloser, G. M., & Sheuring, J. J. (1954). Reliability of the reichert-meissl and polenske methods for detecting substitute fats in ice cream. *Journal of Dairy Science*, *37*(6), 647-647.

Kuuva, T., Lantto, R., Reinikainen, T., Buchert, J., & Autio, K. (2003). Rheological properties of laccase-induced sugar beet pectin gels. *Food Hydrocolloids*, *17*(5), 679–684.

Littoz, F., & McClements, D. J. (2008). Bio-mimetic approach to improving emulsion stability: Cross-linking adsorbed beet pectin layers using laccase. *Food Hydrocolloids*, *22*(7), 1203–1211.

Madene, A., Jacquot, M., Scher, J., & Desobry, S. (2006). Flavour encapsulation and controlled release – A review. *International Journal of Food Science and Technology*, *41*(1), 1–21.

Mattinen, M. L, Hellman, M., Permi, P., Autio, K., Kalkkinen, N., & Buchert, J. (2006). Effect of protein structure on laccase-catalyzed protein oligomerization. *Journal of Agricultural and Food Chemistry*, *54*(23), 8883–8890.

Mattinen, M. L., Kruus, K., Buchert, J., Nielsen, J. H., Andersen, H. J., & Steffensen, C. L. (2005). Laccase-catalyzed polymerization of tyrosine-containing peptides. *FEBS Journal*, *272*(14), 3640–3650.

Micard, V., & Thibault, J. F. (1999). Oxidative gelation of sugar-beet pectins: Use of laccases and hydration properties of the cross-linked pectins. *Carbohydrate Polymers*, *39*(3), 265–273.

Oosterveld, A., Beldman, G., & Voragen, A. G. J. (2000). Oxidative cross-linking of pectic polysaccharides from sugar beet pulp. *Carbohydrate Research*, *328*(2), 199–207.

Pettersson, A., Johnsson, L., Brannas, E., & Pickova, J. (2009). Effects of rapeseed oil replacement in fish feed on lipid composition and self-selection by rainbow trout (*Oncorhynchus mykiss*). *Aquaculture Nutrition*, *15*(6), 577–586.

Peyron, S., Mouecoucou, J., Fremont, S., Sanchez, C., & Gontard, N. (2006). Effects of heat treatment and pectin addition on beta-lactoglobulin allergenicity. *Journal of Agricultural and Food Chemistry*, *54*(15), 5643–5650.

Renard, D., Robert, P., Lavenant, L., Melcion, D., Popineau, Y., Gueguen, J., et al. (2002). Biopolymeric colloidal carriers for encapsulation or controlled release applications. *International Journal of Pharmaceutics*, *242*(1–2), 163–166.

Ride, J. P. (1980). The effect of induced lignification on the resistance of wheat cell-walls to fungal degradation. *Physiological Plant Pathology*, *16*(2), 187.

Schmitt, C., Sanchez, C., Desobry-Banon, S., & Hardy, J. (1998). Structure and technofunctional properties of protein–polysaccharide complexes: A review. *Critical Reviews in Food Science and Nutrition*, *38*(8), 689–753.

Selinheimo, E., Lampila, P., Mattinen, M. L., & Buchert, J. (2008). Formation of protein–oligosaccharide conjugates by laccase and tyrosinase. *Journal of Agricultural and Food Chemistry*, *56*(9), 3118–3128.

Suzuki, S., & Kondo, T. (1982). Interactions of gelatin – Acacia microcapsules with surfactants. *Colloids and Surfaces*, *4*(2), 163–171.

Tianhong Chen, H. D. E., Wu, L.-Q., & Payne, G. F. (2002). In vitro protein–polysaccharide conjugation: Tyrosinase-catalyzed conjugation of gelatin and chitosan. *Biopolymers*, *64*(6), 292–302.

Uhrinova, S., Smith, M. H., Jameson, G. B., Uhrin, D., Sawyer, L., & Barlow, P. N. (2000). Structural changes accompanying pH-induced dissociation of the beta-lactoglobulin dimer. *Biochemistry*, *39*(13), 3565–3574.

Walsh, D. J., Cleary, D., McCarthy, E., Murphy, S., & FitzGerald, R. J. (2003). Modification of the nitrogen solubility properties of soy protein isolate following proteolysis and transglutaminase cross-linking. *Food Research International*, *36*(7), 677–683.