

LACCASE MEDIATED CONJUGATION AND IMPROVED FUNCTIONAL PROPERTIES
OF SUGAR BEET PECTIN AND β -LACTOGLOULIN

by

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(Under the Direction of Louise Wicker)

ABSTRACT

Laccase, an oxidative enzyme, oxidizes phenolic compounds with broad substrate specificity, including amino acids such as tyrosine and cysteine in protein. Ferulic acid in sugar beet pectin (SBP) and tyrosine in β -lactoglobulin (BLG) are potential substrates for laccase catalyzed formation of homo- and hetero- conjugates. The aim of this work was to determine if laccase catalyzes the conjugation of SBP and BLG, to characterize SBP-BLG conjugates, and to determine the effect on the functional properties of homo- and hetero-conjugates. Separately prepared SBP dispersion (3%, w/v) and BLG dispersion (3%, w/v) in 100 mM sodium phosphate buffer (pH 6.5), were equilibrated with or without laccase at 30°C for 20 hours. Then, BLG dispersions were heated at 80 °C for 15 min and mixed with SBP treated with or without laccase. Size exclusion chromatography combined with multi angle laser light scattering, refractive index, and UV detection was performed to confirm the formation of homo- and hetero-conjugates.

Laccase treated SBP had a larger molecular weight and developed a more compact, branched structure based on smaller RMS (Root Mean Square radius) as compared to the larger molecular weight than non-treated SBP. Emulsions prepared with conjugated SBP retained smaller (d_{43}) and more uniform particle size (light microscopy) during 30 days storage than non-conjugated SBP. Conformational change in laccase treated BLG, was manifested by smaller and broader distribution of RMS, changed polymer chain shape and larger particle size, compared to non-treated BLG. Laccase catalyzed polymerization in BLG after heat treatment, which suggests that laccase might work after exposing more tyrosine when heated. Furthermore, hetero-conjugation between heat treated BLG and SBP with laccase was confirmed by UV 280: the laccase treated BLG peak eluted earlier and at greater absorbance than laccase untreated dispersions. Laccase mediated SBP-BLG conjugates showed ~35 % higher solubility at the isoelectric point of BLG and relatively constant G'' (loss modulus) against shear force compared to non-treated SBP-BLG samples.

INDEX WORDS: Laccase, Sugar beet pectin, β -lactoglobulin, Hetero- Homo- Conjugation, HPSEC-MALLS, Emulsion, Solubility

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DEDICATION

This dissertation is dedicated to my parents, Taewhan Jung and Jung soon, Lim, to my husband, Taesik Yun and to my twins, Jangho and Taeyoung Yun for all of their love and support.

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TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS.....	v
LIST OF TABLES	vii
LIST OF FIGURES	viii
CHAPTER	
1 INTRODUCTION	1
2 LITERATURE REVIEW.....	10
3 LACCASE MEDIATED CONJUGATION OF SUGAR BEET PECTIN AND THE EFFECT ON EMULSION STABILITY	43
4 LACCASE MEDIATED CONJUGATION OF β -LACTOGLOBULIN AND SUGAR BEET PECTIN	68
5 LACCASE MEDIATED CONJUGATION OF HEAT TREATED β -LACTOGLOBULIN AND SUGAR BEET PECTIN	99
6 CONCLUSION	128

LIST OF TABLES

	Page
Table 3-1: Mw, RMS, recovery % and δ –potential of SBP and L-SBP samples	61
Table 3-2: δ -potential of SBP and L-SBP emulsions for 30 incubation day	66
Table 4-1: Physical properties of BLG and LBLG (laccase treated BLG).	91
Table 4-2: Physical properties of HBLG and HLBLG	93

LIST OF FIGURES

	Page
Figure 2-1: Possible cross-links forms between α -casein and hOSX	24
Figure 3-1: Elution profiles of representative SBP and L-SBP samples monitored by light scattering, refractive index and UV at 325 nm.	59
Figure 3-2: UV elution profiles of SBP (solid blue line) and L-SBP (dashed red line) at 325 nm	60
Figure 3-3: Cumulative molecular weight fractions of SBP and L-SBP samples.	62
Figure 3-4: Size distributions of SBP (solid blue line) and L-SBP (dashed red line) samples.	63
Figure 3-5: d_{43} diameter of SBP and L-SBP emulsions for 30 incubation days	64
Figure 3-6: d_{43} size distribution of SBP and L-SBP emulsions at 0 day (a), 10 day (b) and 30 day (c)	65
Figure 3-7: Light microscopy images of SBP, L-SBP emulsions at 0, 15 and 30 day	67
Figure 4-1: Flow diagram of laccase mediated SBP and BLG sample preparation	89
Figure 4-2: UV elution profile of BLG, heat treated BLG (HBLG) and laccase pre-treated BLG with heat treatment (HLBLG) monitored at 280nm.....	90
Figure 4-3: Differential RMS distribution of BLG and LBLG	92
Figure 4-4: Changes of Surface SH groups of BLG treated with laccase and heat treatment	94
Figure 4-5: Changes of tyrosine residue of BLG treated with laccase and heat treatment.	95
Figure 4-6: RI (Refractive Index) elution profile of complexes of SBP and BLG pre-incubated with and without laccase.....	96

Figure 4-7: Cumulative Mw distribution of complexes between SBP and BLG pre-incubated with and without laccase	97
Figure 4-8: UV elution profile monitored at 325 nm of SBP and SBP-BLG interacted sample with and without laccase	98
Figure 5-1: Flow diagram of laccase mediated HBLG and SBP mixture preparation	120
Figure 5-2: UV elution profile of HBLG, and HBLG & SBP with and without laccase monitored at 280nm.....	121
Figure 5-3: UV elution profile of SBP, and HBLG & SBP with and without laccase monitored at 325nm	122
Figure 5-4: Changes of tyrosine residue in HBLG, and HBLG & SBP with and without laccase	123
Figure 5-5: RI elution profile of HBLG & SBP with and without laccase	124
Figure 5-6: Particle size distribution of HBLG, and HBLG & SBP with and without laccase ..	125
Figure 5-7: Soluble protein concentration of HBLG and HBLG & SBP with and without laccase	126
Figure 5-8: G'' on shear force for HBLG and HBLG & SBP with and without laccase at 0.1HZ frequency	127

CHAPTER 1

INTRODUCTION

Proteins and polysaccharides are widely used in the food industry controlling structure and texture of food products. The functionality of proteins and polysaccharides, such as solubility, gelling, forming and emulsifying stability, can be synergistically enhanced when they interact, over that of proteins and polysaccharides alone (Dickinson & Galazka, 1991; Guzey & McClements, 2007; Suzuki & Kondo, 1982). Additionally, complexes have been investigated to be used as potential encapsulation substances for bioactive nutrients in delivery system. Application is gaining interest in the cosmetic and pharmaceutical industries, as well as the food industry (Madene, Jacquot, Scher, & Desobry, 2006; Renard, Robert, Lavenant, Melcion, Popineau, Gueguen, et al., 2002; Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998).

Protein and polysaccharide complexes could be obtained by various means using chemically, enzymatically, and thermally induced molecular bonds and electrostatic interactions. Enzymatic method is preferred to form covalent interaction between protein and polysaccharide due to minimal production of possible toxic by-products from chemical cross-linking (James & Simpson, 1996). Moreover, covalent conjugation is favored in maintaining molecular integrity and solubility, over a wide range of conditions and attributes that improve functional properties and increase resistance to heat and proteolytic attack under various conditions (Dickinson & Galazka, 1991; Hattori, Okada, & Takahashi, 2000).

Transglutaminase, peroxidase and laccase are the most common enzymes that have been used to induce enzymatic conjugation between protein and polysaccharide conjugation. (Boeriu,

Oudgenoeg, Spekking, Berendsen, Vancon, Boumans, et al., 2004; T. H. Chen, Vazquez-Duhalt, Wu, Bentley, & Payne, 2001; Faergemand, Otte, & Qvist, 1997, 1998; Kuuva, Lantto, Reinikainen, Buchert, & Autio, 2003; Littoz & McClements, 2008; Selinheimo, Lampila, Mattinen, & Buchert, 2008; Tianhong Chen, 2002; Walsh, Cleary, McCarthy, Murphy, & FitzGerald, 2003). Transglutaminase works primarily on protein molecules by catalyzing acyl transfer between a λ -carboxyamide of a protein bound glutamine (as a donor) and lysine (as an acceptor) (Fork, 1977). However, recently, the conjugation between sodium caseinate and gum arabic has been successfully produced by transglutaminase through the protein moiety of gum arabic (Flanagan & Singh, 2006). Cross-linked milk protein and soy protein by transglutaminase improved viscosity and gelation (Faergemand, Otte, & Qvist, 1997; Sakamoto, Kumazawa, & Motoki, 1994) and solubility (Walsh, Cleary, McCarthy, Murphy, & FitzGerald, 2003). Laccase and peroxidase form cross-links as they catalyze phenolic compounds including ferulic acid in polysaccharide and tyrosine in protein by transfer of electrons from substrate to molecular oxygen (Faergemand, Otte, & Qvist, 1998; Figueroa-Espinoza & Rouau, 1998; Matheis & Whitaker, 1984; Oudgenoeg, Hilhorst, Piersma, Boeriu, Gruppen, Hessing, et al., 2001).

Sugar beet pectin (SBP) has poor gelling ability which is different from non beet pectins traditionally used in the food industry as gelling, thickening and stabilizing agents (May, 1990; Voragen AGJ, 1995). The poor gelling ability of SBP is due to the large amount of acetyl groups, high content of neutral sugars and ferulic acid, which is ester linked to arabinose and galactose side chains in the hairy region (Oosterveld, Beldman, & Voragen, 2000; Rombouts & Thibault, 1986). It has been reported that laccase (p-diphenol oxidase, E.C. 1.10.3.2) improves gelling ability in SBP by the covalent cross-linking through the ferulic acids, the phenolic compounds present in SBP (Kuuva, Lantto, Reinikainen, Buchert, & Autio, 2003; Micard & Thibault, 1999).

The degree of gelling ability could be controlled by type of SBP, incubation time and concentration of laccase but above a certain activity, laccase restricts gelling and rate of cross-linking (Kuuva, Lantto, Reinikainen, Buchert, & Autio, 2003).

SBP is an emulsifying food hydrocolloid, since protein and acetyl groups contribute to the hydrophobic properties and the carbohydrate fraction contributes to the hydrophilic properties (Guillon, 1988; Leroux, Langendorff, Schick, Vaishnav, & Mazoyer, 2003; Nakauma, Funami, Noda, Ishihara, Al-Assaf, Nishinari, et al., 2008). The hydrophobic fraction covers droplets by adsorbing onto the oil surface quickly, and linear pectin chains bound to protein contribute stability by increasing viscosity and steric stabilization in O/W emulsion (Leroux, Langendorff, Schick, Vaishnav, & Mazoyer, 2003; Nakauma, et al., 2008). Ferulic acid in SBP also increases the emulsifying property by adsorbing onto the surface of the oil droplets (Siew & Williams, 2008; Williams, Sayers, Viebke, Senan, Mazoyer, & Boulenguer, 2005). SBP was verified as an electrostatically effective emulsifier, showing bigger negative δ -potential in o/w emulsion compared to gum arabic and soybean soluble polysaccharide (Nakauma, et al., 2008; Siew & Williams, 2008). Emulsion prepared with cross-linked SBP and β -lactoglobulin (BLG) as layer by layer method had better stability than non laccase treated emulsion at pH 4.5 with NaCl addition (B. C. Chen, McClements, Gray, & Decker; Littoz & McClements, 2008).

Laccase also catalyzed cross-links between tyrosine and tyrosine-containing peptides leading to extensive polymerization observed with MALDI-TOF and SDS-PAGE (Mattinen, Kruus, Buchert, Nielsen, Andersen, & Steffensen, 2005). Laccase mediated polymerization was enhanced in the presence of the low molecular weight phenolic substance, ferulic acid (Faergemand, Otte, & Qvist, 1998; Selinheimo, Lampila, Mattinen, & Buchert, 2008). Thus, not only the homo-conjugation but also hetero-conjugation could be suggested between tyrosine in

the protein and ferulic acid in the polysaccharide (Mattinen, Kruus, Buchert, Nielsen, Andersen, & Steffensen, 2005; Selinheimo, Lampila, Mattinen, & Buchert, 2008). Hetero-conjugation between oligosaccharide and α -casein by laccase was shown by size exclusion chromatography (Mattinen, Kruus, Buchert, Nielsen, Andersen, & Steffensen, 2005; Selinheimo, Lampila, Mattinen, & Buchert, 2008). It was suggested that protein such as BSA and BLG are poor substrates for laccase since most of tyrosine residues are buried in 3 dimensional structure and laccase are inaccessible to protein (Uhrinova, Smith, Jameson, Uhrin, Sawyer, & Barlow, 2000).

The aim of this study was to determine if laccase catalyzed the individual and collective conjugation of SBP and BLG. The effect of laccase and heat treatment to facilitate covalent conjugation between SBP and BLG, was determined and effect on functional properties was measured. BLG is one of the major whey proteins and it has 36kDa Mw as a dimer at neutral pH with two disulfide groups, and one free, buried cysteine residue (Walstra, Wouters, & Geurts, 2006). Buried cysteine and hydrophobic groups are exposed when the dimer dissociates into a monomer by heating over 70°C, due to partial unfolding (Fox & McSweeney, 2003; Qi, Holt, McNulty, Clarke, Brownlow, & Jones, 1997; Relkin, 1998). BLG's structure and mechanism are well known, thus it was a suitable model protein in this research.

Conjugated particles with modified functionality are substrates that can be applied to improve encapsulation technology or in a delivery system. Naturally made conjugated polymer particles might be the most suitable material that can be used in human health, with higher bioavailability and stability against enzymatic hydrolysis and pH changes protecting bioactive nutrients in the human body.

References

- Boeriu, C. G., Oudgenoeg, G., Spekking, W. T. J., Berendsen, L. B. J. M., Vancon, L., Boumans, H., Gruppen, H., vanBerkel, W. J. H., Laane, C., & Voragen, A. G. J. (2004). Horseradish Peroxidase-Catalyzed Cross-Linking of Feruloylated Arabinoxylans with Casein. *Journal of Agricultural and Food Chemistry*, 52(21), 6633-6639.
- Chen, B. C., McClements, D. J., Gray, D. A., & Decker, E. A. 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.
- 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.
- Figueroa-Espinoza, M. C., & Rouau, X. (1998). Oxidative Cross-Linking of Pentosans by a Fungal Laccase and Horseradish Peroxidase: Mechanism of Linkage Between Feruloylated Arabinoxylans¹. *Cereal Chemistry*, 75(2), 259-265.
- 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.
- Fox, P. F., & McSweeney, P. L. H. (2003). *Advanced dairy chemistry* (3rd ed.). New York: Kluwer Academic/Plenum.
- Guillon, F., & Thibault, J. -F. (1988). Further characterization of acid- and alkali-soluble pectins from sugar beet pulp. *Lebensmittel Wissenschaft und Technologie*, 21, 198-205.
- 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.
- 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.
- James, J., & Simpson, B. K. (1996). Application of enzymes in food processing. *Critical Reviews in Food Science and Nutrition*, 36(5), 437-463.
- 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.
- Leroux, J., Langendorff, V., Schick, G., Vaishnav, V., & Mazoyer, J. (2003). Emulsion stabilizing properties of pectin. *Food Hydrocolloids*, 17(4), 455-462.
- 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.

- Matheis, G., & Whitaker, J. R. (1984). Modification of Proteins by Polyphenol Oxidase and Peroxidase and their products. *Journal of Food Biochemistry*, 8(3), 137-162.
- 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.
- May, C. D. (1990). Industrial pectins: Sources, production and applications. *Carbohydrate Polymers*, 12(1), 79-99.
- 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.
- Nakauma, M., Funami, T., Noda, S., Ishihara, S., Al-Assaf, S., Nishinari, K., & Phillips, G. O. (2008). Comparison of sugar beet pectin, soybean soluble polysaccharide, and gum arabic as food emulsifiers. 1. Effect of concentration, pH, and salts on the emulsifying properties. *Food Hydrocolloids*, 22(7), 1254-1267.
- 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.
- Oudgenoeg, G., Hilhorst, R., Piersma, S. R., Boeriu, C. G., Gruppen, H., Hessing, M., Voragen, A. G. J., & Laane, C. (2001). Peroxidase-mediated cross-linking of a tyrosine-containing peptide with ferulic acid. *Journal of Agricultural and Food Chemistry*, 49(5), 2503-2510.
- Qi, X. L., Holt, C., McNulty, D., Clarke, D. T., Brownlow, S., & Jones, G. R. (1997). Effect of temperature on the secondary structure of beta-lactoglobulin at pH 6.7, as determined by CD and IR spectroscopy: A test of the molten globule hypothesis. *Biochemical Journal*, 324, 341-346.

- Relkin, P. (1998). Reversibility of heat-induced conformational changes and surface exposed hydrophobic clusters of beta-lactoglobulin: their role in heat-induced sol-gel state transition. *International Journal of Biological Macromolecules*, 22(1), 59-66.
- Renard, D., Robert, P., Lavenant, L., Melcion, D., Popineau, Y., Gueguen, J., Duclairoir, C., Nakache, E., Sanchez, C., & Schmitt, C. (2002). Biopolymeric colloidal carriers for encapsulation or controlled release applications. *International Journal of Pharmaceutics*, 242(1-2), 163-166.
- Rombouts, F. M., & Thibault, J. F. (1986). Enzymatic and Chemical Degradation and the Fine-Structure of Pectins from Sugar-Beet Pulp. *Carbohydrate Research*, 154, 189-203.
- Sakamoto, H., Kumazawa, Y., & Motoki, M. (1994). Strength of Protein Gels Prepared with Microbial Transglutaminase as Related to Reaction Conditions. *Journal of Food Science*, 59(4), 866-871.
- 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.
- Siew, C. M., & Williams, P. A. (2008). Role of protein and ferulic acid in the emulsification properties of sugar beet pectin. *Journal of Agricultural and Food Chemistry*, 56(11), 4164-4171.
- Suzuki, S., & Kondo, T. (1982). Interactions of gelatin--acacia microcapsules with surfactants. *Colloids and Surfaces*, 4(2), 163-171.

- Tianhong Chen, H. D. E., Li-Qun Wu Gregory F. Payne. (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.
- Voragen AGJ, P. W., Thibault JF, Axelos MAV, Renard CMGC. (1995). Pectins. In *Food polysaccharides and their Applications*, (pp. 287-339). New York: Marcel Dekker Inc.
- 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.
- Walstra, P., Wouters, J. T. M., & Geurts, T. J. (2006). *Dairy science and technology* (2nd ed.). Boca Raton: CRC/Taylor & Francis.
- Williams, P. A., Sayers, C., Viebke, C., Senan, C., Mazoyer, J., & Boulenguer, P. (2005). Elucidation of the Emulsification Properties of Sugar Beet Pectin. *Journal of Agricultural and Food Chemistry*, 53(9), 3592-3597.

CHAPTER 2

LITERATURE REVIEW

Pectin

Pectin is used commonly in the food industry as a gelling, thickening and stabilizing agent (May, 1990; Rombouts & Thibault, 1986). Pectin, an anionically charged structural plant polysaccharide, found in the cell walls, primarily consists of a linear backbone of α (1-4)-D-galacturonic acid residues, with varying degrees of methyl esters (smooth region). The homogalacturonic backbone is interrupted with 1, 2 linked L-rhamnose residues, which has side chains of neutral sugars, such as D-galactose, L-arabinose, D-mannose and D-glucuronic acid. These neutral sugars are linked to rhamnose or some of the galacturonic residues, but they are not evenly distributed on the backbone and the neutral sugar rich regions are referred to as a hairy region (May, 1990; Voragen AGJ, 1995). Pectin is further classified based on the degree of esterification (DE), which is defined as the moles of methoxyl groups per 100 moles of galacturonic acid residues, as high-methoxyl pectin (HMP) classified at DE > 50%, and low-methoxyl pectins (LMP) classified at < 50%. Besides degree of esterification, the functionalities of pectin are related with their structure and determined depend on the distribution of the methoxyl group, acetyl group, molecular weight, the amount of galacturonic acid and neutral sugar (De Vries, Hansen, Soderberg, Glahn, & Pedersen, 1986).

Depending on methoxyl group, LMP (DE < 50%) and HMP (DE >50%) have different gelling mechanisms. HMP requires acidic medium (pH 3.5) and water soluble solute, typically sucrose (65%) for gelling and occurs through hydrogen bonds and hydrophobic interaction

(Oakenfull and Scott 1984). LMP gelling can be induced through ionic interactions of polyvalent cations, such as calcium, with free carboxyl groups on the pectin (Powell, Morris et al. 1982). Pectins are often modified to develop their functional properties with chemical, enzymatic or physical manipulation. Modified pectins show different gelling activity and calcium binding capabilities (Limberg, Korner, Buchholt, Christensen, Roepstorff, & Mikkelsen, 2000; Willats, Knox, & Mikkelsen, 2006). Enzymatic de-esterification, especially plant pectin methylesterase (PME, E. C. 3.1.1.11) showed a blockwise distribution and created calcium sensitive pectin in which HMP can gel in the presence of Ca^{2+} without the addition of sucrose (Hunter, Thomas, De Haseth, & Wicker, 2006; Joye & Luzio, 2000). Not only the methoxyl contents, but also the distribution pattern of free and esterified carboxyl groups, and the length of unsubstituted galacturonan backbone have an effect on the gelling activity (Y. Kim, Teng, & Wicker, 2005; Lee, Garti, Rivner, Urbauer, & Wicker, 2008)

Gelation in sugar beet pectin

Currently, apple pomace and citrus are considered the most commercially feasible raw material for pectin, and sugar beet pulp is gaining interest due to distinguishing characteristics (M.-C. Ralet, Crepeau, Buchholt, & Thibault, 2003; Savary, Yoo, Liu, & Nunez, 2003). Sugar beet pectin has a different chemical structure from non beet pectins in that consisting of 1) a shorter galacturonan chain 2) a larger amount of neutral sugar and acetyl group at galacturonic acid residues, and 3) a feruloyl group which are ester-linked to C-2 of the arabinose and C-6 of the galactose in the side chains (M. C. Ralet, Thibault, J.F., Faulds, C. B., & Williamson, G., 1994; Rombouts & Thibault, 1986). Additionally, sugar beet pectin has significantly 4) a larger protein content (10.4%), compare to other polysaccharides (J. F. Thibault, 1988). However, sugar beet pectin has relatively poor gelling ability compared to other pectin due to a greater

proportion of neutral sugars and acetyl groups (Keenan, Belton, Matthew, & Howson, 1985; Phippen, 1950). The gelling ability in sugar beet pectin could be improved by removing arabinose residue with arabinofuranosidase, which results in greater accessibility of ferulic acid and improved gel formation (Keenan, Belton, Matthew, & Howson, 1985; Oosterveld, Beldman, & Voragen, 2000). It was verified that gel was formed through the oxidative cross-linking using oxidoreductases (e.g. laccase, peroxidase) and ammonium persulfate, followed by increasing in molecular weight and viscosity (J.-F. Thibault & Rombouts, 1986). It has been documented in several studies that oxidative coupling via the oxidative enzymes, such as laccase and peroxidase, increased the gelling ability in the presence of ferulic acid in sugar beet pectin (Kuuva, Lantto, Reinikainen, Buchert, & Autio, 2003; Micard & Thibault, 1999). The degree of cross-linking and incubation time needed for gelation was controlled by the sugar beet pectin extraction method and the type of laccase and concentration (Micard & Thibault, 1999). Kuuva et al. (2003) reported improved gelling ability was seen at longer incubation time and lower laccase activity, and gelling and rate of cross-linking were restricted above a certain activity of laccase. In addition, it was found that the more acetylated sugar beet pectin showed a poor gelling ability in spite of higher ferulic acid contents (Kuuva, Lantto, Reinikainen, Buchert, & Autio, 2003). It is very difficult to predict the gelling behavior since the chemical composition, characteristics of pectin used, the presence of salt and Ca^{2+} affect the laccase induced gelation (Kuuva, Lantto, Reinikainen, Buchert, & Autio, 2003; J. F. Thibault, Garreau, & Durand, 1987).

Destabilization mechanism in emulsion system

An emulsion is defined as a mixture of two immiscible liquids, with one liquid dispersed in another, forming small droplets. Emulsion stability has been great interesting in industry due to application to functional foods, medicine, and cosmetics as well as food product. Water and oil

are predominant liquid used in the emulsion system which is classified O/W (oil in water) and W/O (water in oil) emulsion. The former is the emulsion that oil droplets dispersed in water (e.g. milk, ice-cream, dressings) while the latter is that water droplets dispersed in oil (e.g. butter and margarine) (McClements, 2007). However, an emulsion is thermodynamically unstable due to unfavorable conditions, including mechanical forces, temperature and pH. Physical instability could be generated through various mechanisms such as gravitational separation (e.g. creaming, sedimentation), flocculation, coalescence and Ostwald ripening (Dickinson, 1992; McClements, c2005).

Gravitational separation is caused by the differences in density between continuous phase and droplets, discontinuous phase. When droplets have lower density than the surrounding continuous phase, droplets move upward and which is referred as creaming. Whereas sedimentation occurs when droplets move downward due to higher density than the continuous phase. Creaming is more prevalent in W/O emulsion because edible oil is normally lighter than water (McClements, 2007). Gravitational separation could be predicted by the following Stokes' Law:

$$V_s = - \frac{2gr^2(\rho_2 - \rho_1)}{9\eta_1}$$

g : gravity acceleration ρ : density 1 : water
 r : droplet radius η : viscosity 2 : oil

According to Stokes' Law, gravitational instability could be hindered by a smaller droplet size, by a reduction in the density difference between oil and water phases or by the increase of viscosity in continuous phase.

Flocculation occurs when attractive interactions are stronger than repulsive interactions between droplets, and leads droplets association but individual integrities are maintained. Gravitational separation is accelerated as the size increases in droplets by forming flocculation.

Microscopy and the measurement of droplet size are the most common methods to provide flocculation shape and size (Bushell, Yan, Woodfield, Raper, & Amal, 2002; Murphy, 2001). Coalescence is a process which is associated with the combination of two or more droplets merging into a single large droplet. A larger size droplet causes the rapid formation of oil layer on top of the emulsion. When the large droplets grow at the expense of small droplets ($< 1\mu\text{m}$), and they convert into the continuous phase as oil droplets are disappeared due to mass transport (Kabalnov, 2001; Kabalnov & Weers, 1996).

To improve the stability of emulsion, there are several approaches which include increasing viscosity in the continuous phase (Ozer, Muguet, Roy, Grossiord, & Seiller, 2000), adding emulsifiers (Ogawa, Decker, & McClements, 2003; Walsh, Cleary, McCarthy, Murphy, & FitzGerald, 2003), controlling emulsifier and oil ratio (Dickinson, Golding, & Povey, 1997) as well as pH and ionic strength (Guzey, Kim, & McClements, 2004). A type of emulsifier is one of the most critical factors, which can improve a long-term stability of an emulsion system by producing smaller droplets, by a lowering surface tension by preventing droplet from aggregation and by generating repulsive forces between droplets (Walstra, 1993). Furthermore, diverse emulsifiers can apply in different optimum applications with their own character since the type of emulsifier can vary from synthetic to natural food ingredients (Stauffer, 1999). Natural biopolymers such as polysaccharides and proteins have been investigated to replace synthetic emulsifiers. They have been also modified and applied with various strategies to overcome emulsion instability. Covalent conjugates of sodium caseinate and maltodextrin, formed by dry heating, showed improved emulsion stability at 45 °C for 7 days compared to sodium caseinate used alone (O'Regan & Mulvihill, 2010). Guezy et al. (2004) demonstrated that an emulsion prepared with conjugates of β -lactoglobulin and pectin by electrostatic interaction

was stable at pH 3 which induced opposite charge on each polymer and led electrostatic interaction between the two polymers.

Emulsification in polysaccharides

In order to be an effective emulsifier for polymers, it should be surface active and surfactant to lower surface tension at formed emulsion droplets. It can protect droplets against flocculation and coalescence via repulsive interactions, binding strongly and creating a stabilizing layer on the surface of emulsion droplets (Dickinson, 2003; Dickinson, 2009; McClements, c2005). Polysaccharides are conventionally considered as a stabilizer, due to its high hydrophilic nature and thickening properties which impart the long term stability by adsorption onto emulsion droplets. Proteins are favored as an emulsifier because hydrophobic region in adhere can adhere rapidly to the interface of the oil droplets covering and it has relatively small molecular weight and high solubility (Dickinson, 2003). However, protein is labile to heat and lacks long term stability under unfavorable conditions. Since long term stability mainly depends on electrostatic repulsion between charged droplets, stability is disrupted easily when ionic strength is too high or pH becomes close to the pI (Dickinson, 2008). Hydrophilic portions and large molecules in polysaccharides generate sufficient barriers between droplets by the steric repulsion against flocculation over a wide range of unfavorable conditions. It is necessary for the hydrophobic groups to be adsorbed efficiently on the interface of oil to achieve surface layer saturation (Beysseriat, Decker, & McClements, 2006). A hydrophobically modified cellulose and starch have been applied to emulsion system and verified that modified potato starch worked as an efficient emulsifier generating high surface load (Nilsson & Bergenstahl, 2007). Some polysaccharides that have protein moiety (e.g. gum arabic, sugar beet pectin, galactomannan) have been investigated to prove their functionality as polysaccharide

emulsifiers (Garti, 1993). The protein fraction linked to the polysaccharide contributes hydrophobic properties by adsorbing onto the oil droplet, and the polysaccharide loops bounded to protein forms exterior wall material in an O/W emulsion (Leroux, Langendorff, Schick, Vaishnav, & Mazoyer, 2003; Nakauma, Funami, Noda, Ishihara, Al-Assaf, Nishinari, et al., 2008).

The structure of sugar beet pectin is characterized by larger amount of acetyl group contents which are linked to C2 and C3 position of the D-galacturonic acid backbone, and by the 'hairy region' containing more neutral sugars which are linked to rhamnose or galacturonic acid residues. The ferulic acids attach to arabinose residue and galactose residue in hairy region (Micard, Renard, & Thibault, 1996). It has been reported that the ferulic acids, the acetyl groups and the proteins in sugar beet pectin were the elements that contribute to emulsifying and stabilizing in an emulsion (Siew & Williams, 2008; Williams, Sayers, Viebke, Senan, Mazoyer, & Boulenguer, 2005). Nakauma et al. (2008) compared the surface-volume mean diameter ($d_{3,2}$) of emulsions prepared with sugar beet pectin, soybean soluble polysaccharide and gum arabic. The sugar beet pectin exhibited more effective stability showing the smallest ($d_{3,2}$) diameter, and the order of ability as an emulsifying agent was sugar beet pectin > soybean soluble polysaccharide > gum arabic (Nakauma, et al., 2008). An emulsion prepared with the sugar beet pectin was used as wall material to encapsulate fish oil and improved encapsulation efficiency and oxidative stability (Drusch, 2007).

β -lactoglobulin (BLG)

β -lactoglobulin (BLG) is the one of the major whey proteins and has been interested in the food industry due to its unique functionality such as gelling, foaming and surface active, as well as characteristics that can attribute of texture and palatability in a food system (Kontopidis, Holt, & Sawyer, 2004). It exists in mammalian species and has several different genetic variants.

A and B are the most common bovine BLG variant that have significant properties in milk industry processing (Hill, 1996). BLG is a small, soluble and globular protein of 162 amino acid residues with 18.4 kDa molecular weight (Kinsella, 1989). BLG's three dimensional structure consists of nine strands of the antiparallel β -sheet, which form a hydrophobic barrel on one side, with a three turn α -helix on the outer surface. BLG is more stable at acidic pH, and evidenced maximum stability temperature ($T_{\max}$) increased as pH decreased (Kella & Kinsella, 1988). At physiological pH, BLG mainly exists as the dimeric form, which is associated with hydrophobic interactions and hydrogen bonds, with a molecular weight of 36,400 Da (Kinsella, 1989). Isoelectric point of BLG is around 5.2 (Kinsella, 1989). The dimer dissociates to a monomer at high temperatures and at pH values below 3.5 or pH values above 7.5 (McKenzie, 1971).

BLG belongs to lipocalin protein family, which binds small hydrophobic ligands (Flower, North, & Sansom, 2000; Harold, Michael, & Judith, 1987; Kavanagh, Clark, & Ross-Murphy, 2000). More than one binding sites have been studied in several studies (Lange, Kothari, Patel, & Patel, 1998; Wang, Allen, & Swaisgood, 1997, 1999). The central cavity in a conical shape seems the main site that provides the ligand binding, and is made of β -strands and the lining of the pocket is very hydrophobic (Lange, Kothari, Patel, & Patel, 1998; Sawyer, 1998). BLG's binding function is inhibited at acidic conditions because it becomes closed structure, at low pH while allows ligand to go through hydrophobic binding site at high pH due to becoming open structure (Kontopidis, Holt, & Sawyer, 2004). Linear molecules like small fatty acid and retinol with cyclohexenyl ring structure are suitable to bind with BLG (Kontopidis, Holt, & Sawyer, 2004). Medical and nutritional useful food ingredients or modified hydrophobic polymer could have been applied within binding site (Skerra, 2000). Retinol, cholesterol, ω -3 polyunsaturated fatty acids and vitamin D including various aromatic compounds have been investigated as a

model of hydrophobic functional food loaded at BLG in a delivery system as well as complexes of BLG and pectin.

Aggregation in BLG by heat treatment

BLG denatures by pH, alkali, heat, cold, pressure, organic compounds or metal ions, and the denaturation is processed by initially dissociation of dimer to monomer, followed by a change in the polypeptide chain conformation and subsequent aggregation (Bauer, Carrotta, Rischel, & Ogendal, 2000; Sava, Van der Plancken, Claeys, & Hendrickx, 2005). The dissociation of dimer to monomeric form was confirmed through FT-IR by disappearance of a 1623 cm^{-1} band upon heating and appearance of 1618 cm^{-1} and 1682 cm^{-1} bands that involved in aggregation when heated at $80\text{ }^{\circ}\text{C}$ (Lefevre & Subirade, 1999). It was also found that pH affected the degree of unfolding and the aggregation showing different spectra at pH 4.4 and pH 7.4 by FT-IR,

While dimer dissociates to monomer, unfolding and sulfhydryl reactivity increases rapidly which leads to disulphide interchange and the aggregation (Fox & McSweeney, 2003). BLG has 5 cysteine residues but only one cysteine group (Cys¹²¹) is free and other cysteine groups are present as disulfide bonds (Kinsella, 1989). Free thiol group, Cys¹²¹ is buried and masked in the hydrophobic interior of the native protein (Bryant & McClements, 1998; K. Shimada & Cheftel, 1989). However, buried hydrophobic groups and the free Cys¹²¹ are exposed and initiate the aggregation via thiol-disulfide exchange when dimer dissociates at temperatures above $65\text{ }^{\circ}\text{C}$. Then, it leads further aggregation and gelation via hydrophobic association which is inaccessible in the native state (Creamer, Bienvenue et al. 2004; Lowe, Anema et al. 2004). Participation of disulfide bonds in aggregation kinetics was confirmed by lack of disulfide-linkage in the presence of the free thiol (Cys¹²¹) blocking groups, N-

ethylmaleimide (Hoffmann & vanMil, 1997). The characteristics of aggregation in BLG were determined by size exclusion chromatography combined with light scattering. It was observed that small oligomers ranging from dimers to tetramers were formed at an early stage of heating and bigger aggregates were built up via disulfide bond with decreasing in small size oligomer's concentration (Bauer, Carrotta, Rischel, & Ogendal, 2000). Two steps in aggregation: formation of primary aggregates and subsequent of aggregation of the primary aggregates, were also monitored by atomic force microscopy (Ikeda, 2003). Besides, disulfide bond, hydrogen bond and hydrophobic interaction were suggested in the mechanisms of heat induced aggregation in protein (Manderson, Creamer, & Hardman, 1999). More aggregates can be formed with increasing of ionic strength or at the isoelectric point where the hydrophobic interactions are the strongest (Ikeda, 2003; Van Dijk & Smit, 2000).

Thermally induced gelation in BLG

Heat induced aggregation in whey protein is mainly contributed by the disulfide bridge formation between free thiol group (SH) and one of the two disulfide bridges (S-S), confirmed by the reaction between free SH groups and the Ellman's reagent, DTNB (K. Shimada & Cheftel, 1989). These aggregates become gel when protein concentration exceeds a critical level, and different types of gel could be formed depending on pH conditions and controlling the electrostatic repulsion (Ikeda & Li-Chan, 2004).

A fine stranded thick gel, with transparent and rubbery texture, is formed when the intermolecular electrostatic repulsion is predominant and relatively slow heat is applied. A white opaque, particulate gel is produced from larger aggregates, if BLG is heated near the isoelectric point or if intermolecular repulsion is screened by shifting of pH or by increasing ionic strength (Clark, 1998; Ikeda & Foegeding, 1999a; Ikeda, Foegeding, & Hagiwara, 1999). Fine-stranded

transparent gel could be formed at low (acidic) pH and also at neutral pH, but mechanical properties are quite different depending on pH conditions. A gel formed at the low pH exhibited shear thinning and low strain properties and stabilized with hydrogen bonds instead of intermolecular disulfide bond (Kazuko Shimada & Cheftel, 1988). Gels formed at neutral pH, showed deformation with a large strain and a small stress (Langton & Hermansson, 1992; Manderson, Creamer, & Hardman, 1999). It suggests that intermolecular disulfide bonds may influence the gel elasticity. Opaque gel seemed to create a large aggregate of protein but a liquid phase was separated from a gel, easily (Aymard, Nicolai, Durand, & Clark, 1999; Gimel, Durand, & Nicolai, 1994). The protein gel network can be managed by the controlling pH, ionic strength and the type of co-existing ion.

The characterization of the gel network can be determined by structural changes at the molecular level, as well as the appearance, water retention and mechanical properties. In general, α -helix structure is unfolded completely when heated over 70 °C whereas β -sheet is quite stable by heat treatment (Belloque & Smith, 1998; Panick, Malessa, & Winter, 1999). Ikeda et al. (2003) confirmed that α -helix decreased more in a fine stranded gel than an opaque gel, but β -sheet remained on both gels, monitored by Raman spectroscopy. In addition, it was found that hydrophobic interactions were involved significantly to form the opaque gel, evidenced by observing broad peak of 1345 cm^{-1} that assigned to C-H bending vibration, hydrophobic side chains, in Raman spectroscopy (Ikeda & Li-Chan, 2004).

Protein and polysaccharide complexes

Complex formation between proteins and polysaccharides can be achieved by electrostatic interactions, hydrogen bonds, hydrophobic interactions and covalent bonds (Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). Electrostatic interactions are formed by oppositely

charged macromolecules, using simple processing operations such as pH adjustment and mixing. However, the electrostatic interactions are affected strongly by physicochemical parameters and readily disrupted by modification of environmental conditions such as pH, ionic strength, charge density, molecular weight of biopolymer, biopolymer weight ratio and concentration (Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). Therefore, industrial application, such as encapsulation of functional food components, may be limited because molecular complexes formed by electrostatic interactions are highly sensitive to process conditions (Hong & McClements, 2007).

Covalent cross-linking by Maillard reaction between proteins and polysaccharides has advantages due to molecular integrity and solubility over a wide range of conditions and improved functional properties, and increased resistance to heat and proteolysis are observed (Dickinson & Galazka, 1991; Hattori, Okada, & Takahashi, 2000). However, covalent conjugation by the Maillard reaction is restrictive in terms of the time (5-15days) and the temperature (50-70°C), and possible formation of unknown toxic side-products, loss in nutritional value and loss of color or flavor (Flanagan & FitzGerald, 2002; Flanagan & Singh, 2006; Hattori, Okada, & Takahashi, 2000; Kato, 2002; H. J. Kim, Choi, Shin, & Moon, 2003; Pawlowski, Kallénius, & Svenson, 1999).

Polysaccharides interacted with proteins by the electrostatic attraction form more stable hydrogel particles by successive heat treatment. (Hong & McClements, 2007; Yu, Hu, Pan, Yao, & Jiang, 2006). These hydrogel particles showed stable gel size over a wide pH range (pH 2 – 10.5) without dissociation (Yu, Hu, Pan, Yao, & Jiang, 2006).

Enzyme mediated conjugation of protein and polysaccharide

Covalent conjugation with enzymes has been investigated as a new approaches for utilization and further application of functional components in delivery systems and foods ingredients (Flanagan & Singh, 2006; Kato, Wada, Kobayashi, Seguro, & Motoki, 1991; Tianhong Chen, 2002). Enzymes are protein that catalyze biochemical reactions under mild conditions of the temperature and the pH, and have been know as natural and non-toxic food components that improve food nutritional quality, safety and alternative applications for several food products (James & Simpson, 1996).

Various enzymes have been used to conjugate proteins and polysaccharide, and improved functionalities of conjugation have been verified. Transglutaminase catalyzes acyl transfer between a λ -carboxamide of a protein bound glutamine (as a donor) and lysine (as an acceptor) (Fork, 1977). Whey protein (Faergemand, Otte, & Qvist, 1997), soy protein (Walsh, Cleary, McCarthy, Murphy, & FitzGerald, 2003) and sodium caseinate (Flanagan & Singh, 2006) are the most common substrates that have been used to investigate the cross-linking reaction through the transglutaminase, and lead to polymerization in protein with a subsequent increase in molecular weight. Flanagan et al. (2006) studied the transglutaminase mediated conjugation between sodium caseinate and protein residue present in gum arabic and its cross-linking was verified by decreased elution time followed by increased molecular weight, determined in the results of size exclusion chromatography. Cross-linked milk proteins via transglutaminase enhanced the viscosity and the gelation (Faergemand, Otte, & Qvist, 1997; Sakamoto, Kumazawa, & Motoki, 1994), and improved solubility was observed in cross-linked soy protein via transglutaminase with alcalase hydrolysis treatment (Walsh, Cleary, McCarthy, Murphy, & FitzGerald, 2003)

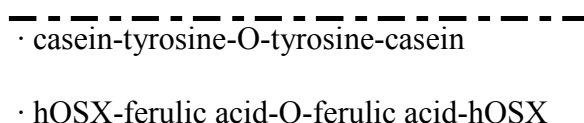
The oxidoreductase family (e.g. laccase, peroxidase and monoamine oxidase) forms

cross-linkages as they catalyze phenolic compounds such as ferulic acid in polysaccharides and also tyrosine in proteins (Faergemand, Otte, & Qvist, 1998; Figueroa-Espinoza & Rouau, 1998; Matheis & Whitaker, 1984; Oudgenoeg, Hilhorst, Piersma, Boeriu, Gruppen, Hessing, et al., 2001). Oxidative coupling reaction occurs by transferring one electron from substrate to molecular oxygen (Yaropolov, Skorobogatko, Vartanov, & Varfolomeyev, 1994). Tyrosinase mainly catalyzes tyrosine residues in protein, whereas peroxidase and laccase act more broadly on various phenolic compounds present in protein and polysaccharides (Buchert, 2007).

Tyrosinase oxidizes phenols and subsequent oxidation of diphenol to quinone, and quinone induces heterogeneous polymers by non-enzymatic reactions. The formation of covalent cross-linking via laccase is processed through the active radical couplings under non enzymatic reaction which is occurred with the loss of single electrons (Lerch, 1983; Thurston, 1994). Peroxidase has a wider range of substrates than laccase, but requires hydrogen peroxide as a hydrogen acceptor (Boeriu, Oudgenoeg, Spekking, Berendsen, Vancon, Boumans, et al., 2004; Oudgenoeg, et al., 2001). Laccase is capable of using oxygen present in the sample for cross-linking without additional mediator such as hydrogen peroxide (Norsker, Jensen, & Adler-Nissen, 2000). It has been reported that laccase induced oxidative cross-linking through ferulic acids in sugar beet pectin forming gel (Kuuva, Lantto, Reinikainen, Buchert, & Autio, 2003; Micard & Thibault, 1999; Norsker, Jensen, & Adler-Nissen, 2000). Laccase also can oxidize some amino acids (tyrosine, cysteine) and leads polymerization in protein, and hetero-conjugation between polysaccharides and proteins. Covalent cross-links between α -casein and oligosaccharide or hydrolyzed oat spelt xylan (hOSX) through ferulic acid in polysaccharides and tyrosine in protein has been reported (Mattinen, Kruus, Buchert, Nielsen, Andersen, & Steffensen, 2005; Selinheimo, Lampila, Mattinen, & Buchert, 2008). Possible hetero- and homo cross-links

between hOSX and α -casein through phenol groups (-OH) are presented in below scheme (Selinheimo, Lampila, Mattinen, & Buchert, 2008) (Fig. 2-1).

Homo cross- linking



Hetero cross-linking

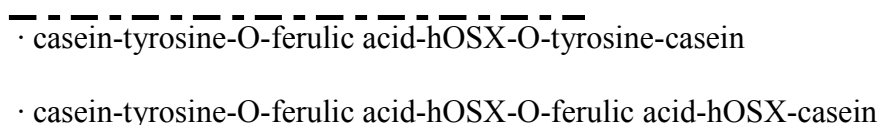


Fig. 2-1. Possible cross-linking between α -casein and hOSX.

Adapted from Selinheimo et al. 2008.

Functionality in conjugated protein with polysaccharides

Proteins and polysaccharides are considered as functional food ingredients which have been used widely in the food products, controlling structural and textural properties due to their aggregation and gelling behavior (Dickinson, 2003). The functionality of each biopolymer can be affected by the presence of other ingredients. In fact, they exhibit improved functional properties, such as solubility, viscosity, gelation, emulsifying and foaming, when they interact with other polymers than used alone (Bryant & McClements, 1998; Chevalier, Chobert, Popineau, Nicolas, & Haertl, 2001; O'Regan & Mulvihill, 2009; Suzuki & Kondo, 1982; Xie & Hettiarachchy, 1997).

In general, β -lactoglobulin (BLG) is highly soluble, but demonstrates a low solubility when heated and/or around the isoelectric point (pI). The solubility is expressed as the percentage of the initial untreated protein concentration, and determined by measuring the

absorbance at 280nm using UV-spectrophotometer or by various protein assay methods with supernatant after removing insoluble protein by centrifugation. BLG exhibited a constant decrease in solubility when heated, with elevated temperatures, and the lowest solubility was shown when BLG heated around pH 5, near the isoelectric point (Chevalier, Chobert, Popineau, Nicolas, & Haertl, 2001). However, it has been reported that solubility and emulsifying properties were improved in conjugated biopolymers when protein conjugated with polysaccharides (Chevalier, Chobert, Popineau, Nicolas, & Haertl, 2001; Jimenez-Casta, Lopez-Fandi, Olano, & Villamiel, 2005; Walsh, Cleary, McCarthy, Murphy, & FitzGerald, 2003). Chevalier et al. (2001) reported that glycated BLG with monosaccharides showed improved the solubility at pH 5 with heat treatment and glycated with sugars may protect proteins from heat denaturation (Jou & Harper, 1996). Conjugated protein with polysaccharide often exhibited the lowest solubility at different pH (generally at acidic pH) instead of pI. The pI might be shifted or anionic polysaccharides might form insoluble complexes resulting from interacting with positive charge of protein by strong electrostatic (Jimenez-Casta, Lopez-Fandi, Olano, & Villamiel, 2005; Jourdain, Leser, Schmitt, Michel, & Dickinson, 2008).

The complexes of proteins and polysaccharides give an advantage in emulsion system in that polysaccharides interacted with the protein provide a long term stability by providing a long-range steric repulsion in the adsorbed layer between two oil droplets (R. Ettelaie, 2005). Flocculation is considered as the major problem in instability mechanism of the emulsion system and occurs by droplet collisions when the attractive force is more dominant than the repulsive force between oil droplets (McClements, 2007). Theoretically, it has been found that a large hydrophilic polysaccharide entity (>5-10kDa) is enough to offer the steric stabilization to overcome the attractive force that occurred between protein layers in an emulsion under

unfavorable conditions (e.g. pH, ionic strength) (Ettelaie, Akinshina, & Dickinson, 2008). When polysaccharides are not enough to saturate protein surface, it leads to bridging flocculation whereas depleting flocculation happens when non-adsorbed polysaccharides exceed a certain level (Dickinson, Golding, & Povey, 1997; McClements, c2005).

Complexes of protein and polysaccharide form layers at the surface of emulsion droplets with two different ways. The first one is the way pre-mixed protein and polysaccharide deposit on the oil droplets together which is denoted as 'mixed emulsion'. For the alternative method, primary emulsion is prepared by the formation of protein layer as single emulsifier at first, and subsequently charged polysaccharide adsorbed onto the primary emulsion producing secondary layer which is termed as 'layer by layer emulsions' (Jourdain, Leser, Schmitt, Michel, & Dickinson, 2008). Layer by layer system could be achieved by the electrostatic interaction between primary layer and secondary layer by controlling pI of the protein and pK of the polysaccharide (Guzey, Kim, & McClements, 2004; Hong & McClements, 2007; Nilsson & Bergenstahl, 2007). This electrostatic method has limitations in that interactions between the layers might be disrupted easily when pH or ionic strength is changed. It has been reported that mixed system is more convenient to prepare and has better stability than layer by layer systems (R. Ettelaie, 2005). The complex multilayer system, more than three layers, has been attempted to overcome the limitation of layer by layer system and to protect emulsion against harsh environmental conditions. Furthermore, multi layer system could be considered as a potential encapsulation wall material for lipid nutrients with another emulsion system (Guzey, Kim, & McClements, 2004).

Application of protein and polysaccharides complexes in delivery system

Interacted molecular complexes of various proteins and polysaccharides have been studied to be used at industrial applications. Encapsulation of bioactive nutrients forming wall material is gaining a great interest in cosmetic and pharmaceutical as well as food industry (Madene, Jacquot, Scher, & Desobry, 2006; Renard, Robert, Lavenant, Melcion, Popineau, Gueguen, et al., 2002; Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). Several methods such as spray drying and freeze drying, as well as forming conjugated particle (microgel) and emulsion have been investigated. Emulsion system is the most commonly developed methods for functional lipophilic ingredients in encapsulation technology (Acarturk & Takka, 1999; Arimoto, Ichikawa, & Fukumori, 2004; Given, 2009; M Jimenez, 2006; Ramadas, Paul, Dileep, Anitha, & Sharma, 2000; Weinbreck, Minor, & De Kruif, 2004).

Encapsulation technology has a role of protecting nutrient molecules from harsh environmental conditions like enzymatic hydrolysis and pH changes in gastrointestinal tract, and chemical degradation that may be happened during preparation and storage. Additionally, it is important to deliver functional materials to the target site controlling release (Luzzi, 1970). Alginate-chitosan gel particles formed by CaCl_2 , showed less degradation around pH 7 (Acarturk & Takka, 1999). Alginate-chitosan gel, which encapsulate had increased drug absorption and bioavailability under simulated intestinal fluids and stomach acid (Ramadas, Paul, Dileep, Anitha, & Sharma, 2000). Entrapment of the major functional lipophilic nutrient like ω -3 polyunsaturated fatty acid is popular due to its beneficial effect on human health. However, it is highly sensitive to heat, pH and oxidation, resulting in undesirable odors and a reduction in health benefit. Therefore, multilayer emulsions have been applied to improve bioavailability of fatty acid and to prevent degradation in delivery systems. Multilayer emulsion containing ω -

fatty 3 fatty acid was made by homogenizing fish oil (ω -3 fatty acid) with an anionic lecithin for primary layer then subsequently cationic chitosan was added as secondary layer to encapsulate fish oil physically within lecithin and chitosan interaction (Klinkesorn, Sophanodora, Chinachoti, McClements, & Decker, 2005). It was reported that DHA loaded in BLG and low methoxyl pectin complexes showed better stability and protection against storage than unprotected DHA (Zimet & Livney, 2009). It has been investigating to evaluate and to improve the utilization of protein and polysaccharides complexes at encapsulation system as potential wall materials within in-vitro and in-vivo mechanism.

References

- Acarturk, F., & Takka, S. (1999). Calcium alginate microparticles for oral administration: II effect of formulation factors on drug release and drug entrapment efficiency. *Journal of Microencapsulation*, 16(3), 291-301.
- Arimoto, M., Ichikawa, H., & Fukumori, Y. (2004). Microencapsulation of water-soluble macromolecules with acrylic terpolymers by the Wurster coating process for colon-specific drug delivery. *Powder Technology*, 141(3), 177-186.
- Aymard, P., Nicolai, T., Durand, D., & Clark, A. (1999). Static and dynamic scattering of beta-lactoglobulin aggregates formed after heat-induced denaturation at pH 2. *Macromolecules*, 32(8), 2542-2552.
- Bauer, R., Carrotta, R., Rischel, C., & Ogendal, L. (2000). Characterization and isolation of intermediates in beta-lactoglobulin heat aggregation at high pH. *Biophysical Journal*, 79(2), 1030-1038.
- Belloque, J., & Smith, G. M. (1998). Thermal denaturation of beta-lactoglobulin. A H-1 NMR study. *Journal of Agricultural and Food Chemistry*, 46(5), 1805-1813.

- Beysseriat, M., Decker, E. A., & McClements, D. J. (2006). Preliminary study of the influence of dietary fiber on the properties of oil-in-water emulsions passing through an in vitro human digestion model. *Food Hydrocolloids*, 20(6), 800-809.
- Boeriu, C. G., Oudgenoeg, G., Spekking, W. T. J., Berendsen, L. B. J. M., Vancon, L., Boumans, H., Gruppen, H., vanBerkel, W. J. H., Laane, C., & Voragen, A. G. J. (2004). Horseradish Peroxidase-Catalyzed Cross-Linking of Feruloylated Arabinoxylans with Casein. *Journal of Agricultural and Food Chemistry*, 52(21), 6633-6639.
- Bryant, C. M., & McClements, D. J. (1998). Molecular basis of protein functionality with special consideration of cold-set gels derived from heat-denatured whey. *Trends in Food Science & Technology*, 9(4), 143-151.
- Buchert, J. S., E.;Kruus, K.;Mattinen, M-L.;Lantto, R.;Autio, K. . (2007). *Cross-linking enzymes in food processing*. Cambridge, U.K., : Woodhead Publishing
- Bushell, G. C., Yan, Y. D., Woodfield, D., Raper, J., & Amal, R. (2002). On techniques for the measurement of the mass fractal dimension of aggregates. *Advances in Colloid and Interface Science*, 95(1), 1-50.
- 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.
- Clark, A. H. (1998). *Gelation of globular proteins*. Gaintherburg: Aspen Publishers Inc.
- De Vries, J. A., Hansen, M., Soderberg, J., Glahn, P. E., & Pedersen, J. K. (1986). Distribution of methoxyl groups in pectins. *Carbohydrate Polymers*, 6(3), 165-176.
- Dickinson, E. (1992). *An Introduction to Food Colloids*. Oxford: Oxford Science.
- Dickinson, E. (2003). Hydrocolloids at interfaces and the influence on the properties of dispersed

- systems. *Food Hydrocolloids*, 17(1), 25.
- Dickinson, E. (2008). Interfacial structure and stability of food emulsions as affected by protein-polysaccharide interactions. *Soft Matter*, 4(5), 932-942.
- Dickinson, E. (2009). Hydrocolloids as emulsifiers and emulsion stabilizers. *Food Hydrocolloids*, 23(6), 1473-1482.
- Dickinson, E., & Galazka, V. B. (1991). Emulsion stabilization by ionic and covalent complexes of β -lactoglobulin with polysaccharides. *Food Hydrocolloids*, 5, 281-296.
- Dickinson, E., Golding, M., & Povey, M. J. W. (1997). Creaming and Flocculation of Oil-in-Water Emulsions Containing Sodium Caseinate. *Journal of Colloid and Interface Science*, 185(2), 515-529.
- Drusch, S. (2007). Sugar beet pectin: A novel emulsifying wall component for microencapsulation of lipophilic food ingredients by spray-drying. *Food Hydrocolloids*, 21(7), 1223-1228.
- Ettelaie, R., Akinshina, A., & Dickinson, E. (2008). Mixed protein-polysaccharide interfacial layers: a self consistent field calculation study. *Faraday Discussions*, 139, 161-178.
- 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.
- Figueroa-Espinoza, M. C., & Rouau, X. (1998). Oxidative Cross-Linking of Pentosans by a Fungal Laccase and Horseradish Peroxidase: Mechanism of Linkage Between Feruloylated Arabinoxylans¹. *Cereal Chemistry*, 75(2), 259-265.

- Flanagan, J., & FitzGerald, R. J. (2002). Physicochemical and nitrogen solubility properties of *Bacillus* proteinase hydrolysates of sodium caseinate incubated with transglutaminase pre- and post-hydrolysis. *Journal of Agricultural and Food Chemistry*, 50(19), 5429-5436.
- 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.
- Flower, D. R., North, A. C. T., & Sansom, C. E. (2000). The lipocalin protein family: structural and sequence overview. *Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology*, 1482(1-2), 9-24.
- 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.
- Fox, P. F., & McSweeney, P. L. H. (2003). *Advanced dairy chemistry* (3rd ed.). New York: Kluwer Academic/Plenum.
- Garti, N., Reichman, D. (1993). Hydrocolloids as food emulsifiers and stabilizers. *Food Microstructure*, 12, 411-426.
- Gimel, J. C., Durand, D., & Nicolai, T. (1994). Structure and Distribution of Aggregates formed after Heat-induced denaturation of Globular-proteins. *Macromolecules*, 27(2), 583-589.
- Given, P. S. (2009). Encapsulation of Flavors in Emulsions for Beverages. *Current Opinion in Colloid & Interface Science*, 14(1), 43-47.
- Guzey, D., Kim, H. J., & McClements, D. J. (2004). Factors influencing the production of o/w emulsions stabilized by beta-lactoglobulin-pectin membranes. *Food Hydrocolloids*, 18(6), 967-975.
- Harold, M. F., Michael, J. B., & Judith, A. E. (1987). Binding of p-Nitrophenyl Phosphate and Other Aromatic Compounds by β -Lactoglobulin. *Journal of Dairy Science*, 70(2), 252-

- 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.
- Hill, J. P., Boland, M. J., Creamer, L. K., Anema, S.G., Otter, D.E., Paterson, G.R., Lowe, R., Motion, R. L., Thresher, W. C., . (1996). *Macromolecular Interactions in Food Technology*. Washington, DC.: American Chemical Society.
- Hoffmann, M. A. M., & vanMil, P. (1997). Heat-induced aggregation of beta-lactoglobulin: Role of the free thiol group and disulfide bonds. *Journal of Agricultural and Food Chemistry*, 45(8), 2942-2948.
- Hong, Y. H., & McClements, D. J. (2007). Formation of Hydrogel Particles by Thermal Treatment of & Beta-Lactoglobulin-Chitosan Complexes. *Journal of Agricultural and Food Chemistry*, 55(14), 5653-5660.
- Hunter, J. L., Thomas, A. G. A., De Haset, J. A., & Wicker, L. (2006). Valencia Orange Pectinmethylesterase Modified Pectin characterized by Fourier Transform Infrared Spectroscopy, Charge Fractionation and Gelling *Journal of Food Quality*, 29(5), 479-491.
- Ikeda, S. (2003). Atomic force microscopy and Raman scattering spectroscopy studies on heat-induced fibrous aggregates of beta-lactoglobulin. *Spectroscopy-an International Journal*, 17(2-3), 195-202.
- Ikeda, S., & Foegeding, E. A. (1999a). Effects of lecithin on thermally induced whey protein isolate gels. *Food Hydrocolloids*, 13(3), 239-244.
- Ikeda, S., Foegeding, E. A., & Hagiwara, T. (1999). Rheological study on the fractal nature of the protein gel structure. *Langmuir*, 15(25), 8584-8589.

- Ikeda, S., & Li-Chan, E. C. Y. (2004). Raman spectroscopy of heat-induced fine-stranded and particulate beta-lactoglobulin gels. *Food Hydrocolloids*, 18(3), 489-498.
- James, J., & Simpson, B. K. (1996). Application of enzymes in food processing. *Critical Reviews in Food Science and Nutrition*, 36(5), 437-463.
- Jimenez-Casta, L., Lopez-Fandi, R., Olano, A., & Villamiel, M. (2005). Study on beta-lactoglobulin glycosylation with dextran: effect on solubility and heat stability. *Food Chemistry*, 93(4), 689-695.
- Jou, K. D., & Harper, W. J. (1996). Effect of di-saccharides on the thermal properties of whey proteins determined by differential scanning calorimetry (DSC). *Milchwissenschaft-Milk Science International*, 51(9), 509-512.
- 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.
- Joye, D. D., & Luzio, G. A. (2000). Process for selective extraction of pectins from plant material by differential pH. *Carbohydrate Polymers*, 43(4), 337-342.
- Kabalnov, A. (2001). Ostwald ripening and related phenomena. *Journal of Dispersion Science and Technology*, 22(1), 1-12.
- Kabalnov, A., & Weers, J. (1996). Kinetics of mass transfer in micellar systems: Surfactant adsorption, solubilization kinetics, and ripening. *Langmuir*, 12(14), 3442-3448.
- 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.
- Kavanagh, G. M., Clark, A. H., & Ross-Murphy, S. B. (2000). Heat-induced gelation of globular proteins: part 3. Molecular studies on low pH beta-lactoglobulin gels. *International Journal of Biological Macromolecules*, 28(1), 41-50.
- Keenan, M. H. J., Belton, P. S., Matthew, J. A., & Howson, S. J. (1985). A ¹³C-n.m.r. study of sugar-beet pectin. *Carbohydrate Research*, 138(1), 168-170.
- Kella, N. K., & Kinsella, J. E. (1988). Enhanced Thermodynamic Stability of Beta-Lactoglobulin at Low pH - a Possible Mechanism. *Biochemical Journal*, 255(1), 113-118.
- 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.
- Kim, Y., Teng, Q., & Wicker, L. (2005). Action pattern of Valencia orange PME de-esterification of high methoxyl pectin and characterization of modified pectins. *Carbohydrate Research*, 340(17), 2620-2629.
- Kinsella, N., D.M. Whitehead (1989). Proteins in whey: Chemical, physical and functional properties. *Advances in Food Nutrition Research*, 33, 343-438.
- Klinkesorn, U., Sophanodora, P., Chinachoti, P., McClements, D. J., & Decker, E. A. (2005). Stability of spray-dried tuna oil emulsions encapsulated with two-layered interfacial membranes. *Journal of Agricultural and Food Chemistry*, 53(21), 8365-8371.
- Kontopidis, G., Holt, C., & Sawyer, L. (2004). Invited Review: beta-lactoglobulin: Binding properties, structure, and function. *Journal of Dairy Science*, 87(4), 785-796.
- 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.
- Lange, D. C., Kothari, R., Patel, R. C., & Patel, S. C. (1998). Retinol and retinoic acid bind to a

- surface cleft in bovine [beta]-lactoglobulin: a method of binding site determination using fluorescence resonance energy transfer. *Biophysical Chemistry*, 74(1), 45-51.
- Langton, M., & Hermansson, A. M. (1992). Fine-Stranded and Particulate Gels of Beta-Lactoglobulin and Whey-Protein at Varying pH. *Food Hydrocolloids*, 5(6), 523-539.
- Lee, H., Garti, N., Rivner, J., Urbauer, J. L., & Wicker, L. (2008). De-esterification pattern of Valencia orange pectinmethylesterases and characterization of modified pectins. *Journal of the Science of Food and Agriculture*, 88(12), 2102-2110.
- Lefevre, T., & Subirade, M. (1999). Structural and interaction properties of beta-Lactoglobulin as studied by FTIR spectroscopy. *International Journal of Food Science and Technology*, 34(5-6), 419-428.
- Lerch, K. (1983). Neurospora tyrosinase: structural, spectroscopic and catalytic properties. *Molecular Cellular Biochemistry*, 52, 125-138.
- Leroux, J., Langendorff, V., Schick, G., Vaishnav, V., & Mazoyer, J. (2003). Emulsion stabilizing properties of pectin. *Food Hydrocolloids*, 17(4), 455-462.
- Limberg, G., Korner, R., Buchholt, H. C., Christensen, T. M. I. E., Roepstorff, P., & Mikkelsen, J. D. (2000). Analysis of different de-esterification mechanisms for pectin by enzymatic fingerprinting using endopectin lyase and endopolygalacturonase II from *A. Niger*. *Carbohydrate Research*, 327(3), 293-307.
- Luzzi, L. A. (1970). Microencapsulation. *Journal of Pharmaceutical Sciences*, 59(10), 1367.
- M Jimenez, H. S. G. C. I. B. (2006). Spray-dried encapsulation of Conjugated Linoleic Acid (CLA) with polymeric matrices. *Journal of the Science of Food and Agriculture*, 86(14), 2431-2437.
- 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.
- Manderson, G. A., Creamer, L. K., & Hardman, M. J. (1999). Effect of heat treatment on the circular dichroism spectra of bovine beta-lactoglobulin A, B, and C. *Journal of Agricultural and Food Chemistry*, 47(11), 4557-4567.
- Matheis, G., & Whitaker, J. R. (1984). Modification of Proteins by Polyphenol Oxidase and Peroxidase and their products. *Journal of Food Biochemistry*, 8(3), 137-162.
- 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.
- May, C. D. (1990). Industrial pectins: Sources, production and applications. *Carbohydrate Polymers*, 12(1), 79-99.
- McClements, D. J. (2007). Critical review of techniques and methodologies for characterization of emulsion stability. *Critical Reviews in Food Science and Nutrition*, 47(7), 611-649.
- McClements, D. J. (c2005). *Food emulsions : principles, practices, and techniques* (2nd ed.). Boca Raton CRC Press.
- McKenzie, H. A. (1971). *β-lactoglobulins*. New York: Academic Press.
- Micard, V., Renard, C., & Thibault, J. F. (1996). Enzymatic saccharification of sugar-beet pulp. *Enzyme and Microbial Technology*, 19(3), 162-170.
- 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.
- Murphy, D. B. (2001). *Fundamentals of Light Microscopy and Electronic Imaging*. New York: John Wiley and Sons.
- Nakauma, M., Funami, T., Noda, S., Ishihara, S., Al-Assaf, S., Nishinari, K., & Phillips, G. O.

- (2008). Comparison of sugar beet pectin, soybean soluble polysaccharide, and gum arabic as food emulsifiers. 1. Effect of concentration, pH, and salts on the emulsifying properties. *Food Hydrocolloids*, 22(7), 1254-1267.
- Nilsson, L., & Bergenstahl, B. (2007). Emulsification and adsorption properties of hydrophobically modified potato and barley starch. *Journal of Agricultural and Food Chemistry*, 55(4), 1469-1474.
- Norsker, M., Jensen, M., & Adler-Nissen, J. (2000). Enzymatic gelation of sugar beet pectin in food products. *Food Hydrocolloids*, 14(3), 237-243.
- O'Regan, J., & Mulvihill, D. M. (2009). Preparation, characterisation and selected functional properties of sodium caseinate-maltodextrin conjugates. *Food Chemistry*, 115(4), 1257-1267.
- O'Regan, J., & Mulvihill, D. M. (2010). Sodium caseinate-maltodextrin conjugate stabilized double emulsions: Encapsulation and stability. *Food Research International*, 43(1), 224-231.
- Ogawa, S., Decker, E. A., & McClements, D. J. (2003). Production and characterization of O/W emulsions containing cationic droplets stabilized by lecithin-chitosan membranes. *Journal of Agricultural and Food Chemistry*, 51(9), 2806-2812.
- 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.
- Oudgenoeg, G., Hilhorst, R., Piersma, S. R., Boeriu, C. G., Gruppen, H., Hessing, M., Voragen, A. G. J., & Laane, C. (2001). Peroxidase-mediated cross-linking of a tyrosine-containing peptide with ferulic acid. *Journal of Agricultural and Food Chemistry*, 49(5), 2503-2510.
- Ozer, O., Muguet, V., Roy, E., Grossiord, J. L., & Seiller, M. (2000). Stability study of W/O/W

- viscosified multiple emulsions. *Drug Development and Industrial Pharmacy*, 26(11), 1185-1189.
- Panick, G., Malessa, R., & Winter, R. (1999). Differences between the pressure- and temperature-induced denaturation and aggregation of beta-lactoglobulin A, B, and AB monitored by FT-IR spectroscopy and small-angle X-ray scattering. *Biochemistry*, 38(20), 6512-6519.
- Pawlowski, A., Kallenius, G., & Svenson, S. B. (1999). A new method of non-cross-linking conjugation of polysaccharides to proteins via thioether bonds for the preparation of saccharide-protein conjugate vaccines. *Vaccine*, 17(11-12), 1474-1483.
- Pippen, E. L., McCready, M. C., & Owens, H. S. (1950). Gelation properties of partially acetylated pectins. *Journal of the American Chemical Society*, 72, 813-816.
- R. Ettelaie, E. D. a. B. S. M. (2005). *Interactions, Microstructure and Processing*. Cambridge: Royal Society of Chemistry.
- Ralet, M.-C., Crepeau, M.-J., Buchholt, H.-C., & Thibault, J.-F. (2003). Polyelectrolyte behaviour and calcium binding properties of sugar beet pectins differing in their degrees of methylation and acetylation. *Biochemical Engineering Journal*, 16(2), 191.
- Ralet, M. C., Thibault, J.F., Faulds, C. B., & Williamson, G. (1994). Isolation and purification of feruloylated oligosaccharides from cell walls of sugar-beet pulp. *Carbohydrate Research*, 263, 227-241.
- Ramadas, M., Paul, W., Dileep, K. J., Anitha, Y., & Sharma, C. P. (2000). Lipoinsulin encapsulated alginate-chitosan capsules: intestinal delivery in diabetic rats. *Journal of Microencapsulation*, 17(4), 405-411.
- Renard, D., Robert, P., Lavenant, L., Melcion, D., Popineau, Y., Gueguen, J., Duclairoir, C.,

- Nakache, E., Sanchez, C., & Schmitt, C. (2002). Biopolymeric colloidal carriers for encapsulation or controlled release applications. *International Journal of Pharmaceutics*, 242(1-2), 163-166.
- Rombouts, F. M., & Thibault, J. F. (1986). Enzymatic and Chemical Degradation and the Fine-Structure of Pectins from Sugar-Beet Pulp. *Carbohydrate Research*, 154, 189-203.
- Sakamoto, H., Kumazawa, Y., & Motoki, M. (1994). Strength of Protein Gels Prepared with Microbial Transglutaminase as Related to Reaction Conditions. *Journal of Food Science*, 59(4), 866-871.
- Sava, N., Van der Plancken, I., Claeys, W., & Hendrickx, M. (2005). The kinetics of heat-induced structural changes of beta-lactoglobulin. *Journal of Dairy Science*, 88(5), 1646-1653.
- Savary, B. J., Yoo, S. H., Liu, L. S., & Nunez, A. (2003). Pectin acetyl esterase - analysis and application for sugar beet pectin utilization [electronic resource]. *1st joint IIRB-ASSBT Congress, 2th Feb. - 1st March 2003, San Antonio (USA)*, 921-924.
- Sawyer, L., S. Brownlow, I. Polikarpov, and S.Y.Wu. . (1998). β -lactoglobulin: Structural studies, biological clues. *International Dairy Journal*, 8(65), 65-72.
- 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.
- Shimada, K., & Cheftel, J. C. (1988). Texture characteristics, protein solubility, and sulfhydryl group/disulfide bond contents of heat-induced gels of whey protein isolate. *Journal of*

- Agricultural and Food Chemistry*, 36(5), 1018-1025.
- Shimada, K., & Cheftel, J. C. (1989). Sulfhydryl-group Disulfide bond Interchange Reactions during Heat-induced Gelation of Whey-Protein Isolate. *Journal of Agricultural and Food Chemistry*, 37(1), 161-168.
- Siew, C. M., & Williams, P. A. (2008). Role of protein and ferulic acid in the emulsification properties of sugar beet pectin. *Journal of Agricultural and Food Chemistry*, 56(11), 4164-4171.
- Skerra, A. (2000). Lipocalins as a scaffold. *Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology*, 1482(1-2), 337-350.
- Stauffer, C. E. (1999). *Emulsifiers*. St Paul, MN: Eagen Press.
- Suzuki, S., & Kondo, T. (1982). Interactions of gelatin--acacia microcapsules with surfactants. *Colloids and Surfaces*, 4(2), 163-171.
- Thibault, J.-F., & Rombouts, F. M. (1986). Effects of some oxidising agents, especially ammonium peroxydisulfate, on sugar-beet pectins. *Carbohydrate Research*, 154(1), 205-215.
- Thibault, J. F. (1988). Characterization and Oxidative Crosslinking of Sugar-beet Pectins Extracted from Cossettes and Pulps under Different Conditions. *Carbohydrate Polymers*, 8(3), 209-223.
- Thibault, J. F., Garreau, C., & Durand, D. (1987). Kinetics and mechanism of the reaction of ammonium persulfate with ferulic acid and sugar-beet pectins. *Carbohydrate Research*, 163(1), 15-27.
- Thurston, C. F. (1994). The Structure and Function of Fungal Laccases. *Microbiology-Uk*, 140, 19-26.

- Tianhong Chen, H. D. E., Li-Qun Wu Gregory F. Payne. (2002). In vitro protein-polysaccharide conjugation: Tyrosinase-catalyzed conjugation of gelatin and chitosan. *Biopolymers*, 64(6), 292-302.
- Van Dijk, J. A. P. P., & Smit, J. A. M. (2000). Size-exclusion chromatography-multiangle laser light scattering analysis of [beta]-lactoglobulin and bovine serum albumin in aqueous solution with added salt. *Journal of Chromatography A*, 867(1-2), 105-112.
- Voragen AGJ, P. W., Thibault JF, Axelos MAV, Renard CMGC. (1995). Pectins. In *Food polysaccharides and their Applications*, (pp. 287-339). New York: Marcel Dekker Inc.
- 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.
- Walstra, P. (1993). Principles of Emulsion Formation. *Chemical Engineering Science*, 48(2), 333-349.
- Wang, Q. W., Allen, J. C., & Swaisgood, H. E. (1997). Binding of vitamin D and cholesterol to beta-lactoglobulin. *Journal of Dairy Science*, 80(6), 1054-1059.
- Wang, Q. W., Allen, J. C., & Swaisgood, H. E. (1999). Binding of lipophilic nutrients to beta-lactoglobulin prepared by bioselective adsorption. *Journal of Dairy Science*, 82(2), 257-264.
- Weinbreck, F., Minor, M., & De Kruif, C. G. (2004). Microencapsulation of oils using whey protein/gum arabic coacervates. *Journal of Microencapsulation*, 21(6), 667-679.
- Willats, W. G. T., Knox, P., & Mikkelsen, J. D. (2006). Pectin: new insights into an old polymer are starting to gel. *Trends in Food Science & Technology*, 17(3), 97-104.
- Williams, P. A., Sayers, C., Viebke, C., Senan, C., Mazoyer, J., & Boulenguer, P. (2005).

- Elucidation of the Emulsification Properties of Sugar Beet Pectin. *Journal of Agricultural and Food Chemistry*, 53(9), 3592-3597.
- Xie, Y. R., & Hettiarachchy, N. S. (1997). Xanthan Gum Effects on Solubility and Emulsification Properties of Soy Protein Isolate. *Journal of Food Science*, 62(6), 1101-1104.
- Yaropolov, A. I., Skorobogatko, O. V., Vartanov, S. S., & Varfolomeyev, S. D. (1994). Laccase - Properties, Catalytic Mechanism, and Applicability. *Applied Biochemistry and Biotechnology*, 49(3), 257-280.
- Yu, S., Hu, J., Pan, X., Yao, P., & Jiang, M. (2006). Stable and pH-Sensitive Nanogels Prepared by Self-Assembly of Chitosan and Ovalbumin. *Langmuir*, 22(6), 2754-2759.
- Zimet, P., & Livney, Y. D. (2009). Beta-lactoglobulin and its nanocomplexes with pectin as vehicles for [omega]-3 polyunsaturated fatty acids. *Food Hydrocolloids*, 23(4), 1120-1126.

CHAPTER 3

LACCASE MEDIATED CONJUGATION OF SUGAR BEET PECTIN

AND THE EFFECT ON EMULSION STABILITY¹

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Abstract

Laccase catalyzes intermolecular cross-links between ferulic acid of sugar beet pectin (SBP). Conjugation of SBP by laccase was confirmed by increased molecular weight, monitored by size exclusion chromatography, combined with multi angle laser light scattering (HPSEC-MALLS), by reduced ferulic acid concentration detected at UV 325 nm, as well as by increased particle size. In addition, cross-linked SBP developed a more compact, branched structure as verified by a smaller root mean square (RMS) when molecular weight increased. Emulsions prepared with conjugated SBP had significantly smaller $d_{4,3}$ diameter and uniform droplet size, with more negative ζ -potential than non-treated SBP, during 30 days storage at 37 °C. The $d_{4,3}$ diameter was 5.3 μm and 3.8 μm for SBP and conjugated SBP, respectively. Covalently conjugated SBP has increased functionality and improved emulsion stability, most likely attributable to development of thick layer at the oil interface.

INDEX WORDS: Sugar beet pectin, Laccase, Ferulic acid, Cross-linking, Emulsion

Introduction

Pectin is an anionically charged structural plant polysaccharide consisting of a linear backbone of α (1-4)-D-galacturonic acid residue, with side chains of neutral sugars. It has traditionally been used in the food industry as a gelling, thickening and stabilizing agent (May, 1990; Voragen AGJ, 1995). Sugar beet pectin (SBP) is different from non beet pectin in that it has protein and greater amounts of acetyl groups at the C2 and C3 positions of the galacturonic acid residue. Also, SBP contains greater amounts of neutral sugars and ferulic acid which are ester linked to arabinose and galactose side chains in the hairy region (Oosterveld, Beldman, & Voragen, 2000; Rombouts & Thibault, 1986). About 50-60 % ferulic acid is linked to the backbone of the arabinan side chains while the rest of the ferulic acid is linked to galactose residues (Ralet, 1994).

SBP is known as a food emulsifying agent since it has approximately 10% protein which plays a key role in emulsification properties. The carbohydrate fraction gives hydrophilic properties attributing stabilization by increasing viscosity and steric stabilization (Guillon, 1988; Nakauma, Funami, Noda, Ishihara, Al-Assaf, Nishinari, et al., 2008). Atomic force microscopy showed that the protein fraction within SBP was linked to the linear pectin chain (Kirby, MacDougall, & Morris, 2008). The protein fraction adsorbs onto the oil droplet and polysaccharide loops are extended from the oil surface and forms exterior wall material in O/W emulsions (Leroux, Langendorff, Schick, Vaishnav, & Mazoyer, 2003; Nakauma, et al., 2008). Acetyl groups also contribute emulsifying ability in SBP, since acetylated citrus pectin showed better emulsion stability than de-acetylated SBP (Leroux, Langendorff, Schick, Vaishnav, & Mazoyer, 2003).

Emulsions prepared with SBP exhibited more effective stability showing more negative

ζ -potential and slightly lower surface tension than the gum arabic emulsions, which are also a protein rich polysaccharide (Nakauma, et al., 2008; Siew & Williams, 2008). The protein, ferulic acid, and the proportion of acetyl ester groups affect the emulsifying property and stability of oil droplets (Siew & Williams, 2008; Williams, Sayers, Viebke, Senan, Mazoyer, & Boulenguer, 2005). It has been suggested that higher molecular weight fractions adsorbed onto the emulsion droplet preferentially during emulsification and high molecular weight materials might contain more protein and/or ferulic acid (Siew & Williams, 2008). As an application of SBP stabilized emulsions, SBP, used as wall material to encapsulate fish oil, had better encapsulation efficiency, and required lower quantities, and had good stability against oxidation compared to other wall materials like gum arabic (Drusch, 2007).

SBP also forms stable gels via oxidative enzymes, such as laccase and peroxidase, by the oxidative coupling and covalent cross-links between beet pectin molecules (Kuuva, Lantto, Reinikainen, Buchert, & Autio, 2003; Micard & Thibault, 1999). The degree of cross-linking and gelling ability was controlled by SBP extraction method and type, and type of laccase (Micard & Thibault, 1999). Kuuva et al (2003) found that improved gelling ability was seen at longer incubation time and increased concentration of laccase but above certain activity of laccase restricts gelling and rate of cross-linking (Kuuva, Lantto, Reinikainen, Buchert, & Autio, 2003).

Laccase is a copper containing polyphenol oxidase (p-diphenol oxidase, E.C. 1.10.3.2) derived from bacteria, fungi and plant origins (Thurston, 1994). It oxidizes various mono-, di-, polyphenols, methoxy phenols as well as aromatic compounds such as thiols and aromatic amines, by reducing molecular oxygen to water (Gianfreda, 1999; Micard & Thibault, 1999). The oxidation mode catalyzed by laccase generates free radicals (typically phenoxy radicals) with loss of single electrons and reactive radicals lead to form quinone and polymerization based

on radical coupling (Archibald, Bourbonnais, Jurasek, Paice, & Reid, 1997). Laccase is a more suitable oxidase enzyme for further application relating to food processing and industry due to the wider substrate specificity (Selinheimo, Lampila, Mattinen, & Buchert, 2008). Covalently cross-linked pectin may have a different behavior and may affect the different functionality of pectin. The purpose of this research is to determine if laccase conjugates SBP, and to compare the stability of emulsions prepared by SBP and laccase mediated SBP conjugation.

Materials & Methods

Materials

Sugar beet pectin (SBP) was obtained from Herbstreith & Fox KG (Elmsford, NY). Laccase (*Rhus vernificera*, E.C. 1. 10. 3. 2, p-diphenol oxidase), syringaldazine were purchased from Sigma – Aldrich Co. (St. Louis, MO).

Conjugation properties

Stock preparation

Sugar beet pectin (SBP) was prepared by dispersing 3 % (w/v) dry power in 100 mM sodium phosphate buffer (pH 6.5), stirred at room temperature at least for 2 hours and stored overnight at 4 °C for complete hydration. Dispersions were centrifuged for 30 min at 5000 g, 4 °C to remove insoluble material. The centrifuged SBP was equilibrated with and without laccase in a water bath at 30 °C for 20 hours with mild agitation on an orbital shaker (PR 70 Red Rotor Hofer scientific, MO). An aliquot of 1 unit laccase/ mg pectin was added to the dispersion for laccase mediated SBP dispersion. An aliquot of water was added to the control SBP dispersions. The samples were diluted 10 times with Milli-Q water to slow down enzyme activity. The suppressed enzyme activity was confirmed by measuring the changes of the optical density at 325 nm, which is for determination of ferulic acid in SBP (Faergemand, Otte, & Qvist, 1998).

Laccase activity measurement

Laccase activity was measured by modified procedure of a previous study which used syringaldazine as substrate at 30 °C (Ride, 1980). Laccase was dissolved in cold, deionized water and stirred vigorously for 10 min, filtered through 5 µm PVDF (Polyvinylidene difluoride) syringe filters (Millipore, Bedford, MA). The change in slope of absorbance at 530 nm per unit of time was used to assess activity (Units/ml enzyme).

Size Exclusion Chromatography with Multi-Angle Laser Light Scattering (HPSEC-MALLS)

HPSEC-MALLS system consisting of a Waters P500 pump with a degasser (Waters, Milford, MA), three detectors, multi-angle light scattering detectors, refractive index detector and UV detectors were present in line. The multi-angle light scattering detector (DAWN DSP-F) was equipped with a P10 flow cell and a He-Ne laser light source ($\lambda=633$ nm). Refractive index detector (Optilab 903, Wyatt Technology, Santa Barbara, CA) was operated at 633 nm and UV detector (Thermo Separation Scientific Products) was operated at 325 nm to determine the presence of ferulic acid in samples (Synytsya, Copikova, Jankovska, Matejka, & Machovic, 2003). Samples were filtered through 0.8 µm syringe filters (Millipore, Bedford, MA) and injected by the Spectrasystem autosampler (AS 3000, Thermoseparation Products, San Jose, CA) with a 50 µl loop.

The separation was achieved by a PL-Aquagel-OH guard column (50×7.5 mm) and PL-Aquagel-OH mix columns (300×7.5 mm) (Polymer Laboratories, Inc., Amherst, MA) with a flow rate of 0.5 ml/min, with 10 mM of Na-Phosphate and 100 mM of Na-Nitrate, pH 7 as an elution buffer. The eluent buffer was filtered through 0.2 and 0.1 µm filters. Specific refractive index increment (dn/dc) values were used 0.131 for SBP (Corredig & Wicker, 2001). Elution data was processed using ASTRA software (vs. 4.73.03) and weight average molecular weight

(Mw), root mean square (RMS) radius were calculated. All measurements were carried out at least in duplicate with three prepared samples.

Particle size and Zeta potential measurement

The particle size and size distributions of SBP and laccase mediated SBP dispersion (L-SBP) were performed using a Particle Size Analyzer with the BI-Zeta option (90 Plus, Brookhaven Instruments Corporation, Holtsville, NY) with a 50 mV diode laser (90° angle) and a BI-9000AT correlator. The samples were diluted to 0.05 wt% with Milli-Q water prior to analysis. Multimodal size distribution was reported and it was calculated as volume & weight diameter. Volume & weight average diameter is defined as $\sum nd^4 / \sum nd^3$ with diameter d and number of particles n . The zeta-potential was performed subsequently after the particle size determination for the same sample using an electrophoresis instrument (ZetaPlus, Brookhaven Instruments Corporation, Holtsville, NY). The measurements were carried out in duplicate with 5 runs. Zeta-potential was calculated from the average of 5 readings. All measurements were carried out at least in duplicate, with three prepared samples.

Emulsifying properties

Emulsion preparation

Emulsion was prepared by mixing 10 % vol. Tetradecane (Fisher Scientific, NJ) and 1 % wt Laccase treated and non treated SBP in 100 mM phosphate buffer (pH 6.5). The mixtures were pre-mixed using a PRO300A homogenizer (Pro Scientific Inc., Oxford, Ct) for 1 min. They were subsequently homogenized by 3 passes at 40 MP in a high pressure homogenizer (Emulsi-Flex C5, Avestin Inc., Ottawa, ON, CA) on ice. The emulsions were sealed in a graduate conical test tube and stored at incubator (37 °C , VWR, Batavia, IL).

Particle size distribution

Particle size distributions of emulsion prepared by SBP and L-SBP were measured using Malvern Mastersizer (Model MSS, Malvern Instruments Limited, Worcestershire, U.K.) and the presentation code was 3NAD. The refractive index used was 1.445 and 1.330 for n-tetradecane and water, respectively. Particle size was reported as volume weighted average diameter $d_{4,3} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3}$, where n_i is the number of particles and d_i is the diameter of particle. The measurements were carried out every 5 days for 30 incubation days and samples were mixed gently with a vortex, prior to assay.

ζ-potential

The sample was diluted to 0.005 % with Milli-Q water and the ζ-potential was measured using Zeta-Plus (Brookhaven Instruments Corporation, Holtsville, NY). The measurements were calculated from the average of 5 readings taken on the same sample and it was conducted in duplicate. All measurements were carried out at least in duplicate with three prepared samples.

Light microscopy

The stability and size of emulsions were observed by upright light microscopy (Leica DM LB, Leica Microsystems Inc., Bannockburn, USA). A drop of emulsion was placed on the microscope glass slide and covered with a glass cover slip and examined with a 20 x magnification lens. The sample images were acquired by digital camera (Spot idea, Diagnostic Instruments Inc, MI USA).

Statistical analysis

One-way analysis of variance and student's t-test were used to determine the differences between SBP and L-SBP in the conjugation properties and emulsion stability.

Results

Conjugation properties

Representative elution profiles of SBP and L-SBP monitored by light scattering, refractive index and UV detectors are given in Fig.3-1. LS, RI, and UV peaks do not overlay, indicating SBP is a heterogeneous polymer in size and composition. The earlier eluting slices are large, but small in concentration relative to later eluting slices. In this study, two separate peaks were selected based on the given UV elution profile clearly detecting ferulic acid at 325 nm. Higher molecular weight peak elutes between 6.8 ml and 8.7 ml and the second mass peak elutes from 9.0 ml to 9.6 ml. Both peaks contain ferulic acid and the second peak has smaller concentrations of ferulic acid relative to the first peak, as shown on the UV chromatograph profile. The ferulic acid is a phenolic compound which forms covalent cross-linking in SBP via laccase. A decrease in the UV chromatogram, at 325 nm, was observed in the SBP sample with laccase and is attributed to cross-linking of SBP via ferulic acid (Fig.3-2). This result is in agreement with Littoz et al. (2008) who studied the absorbance changes at 325 nm over time with the addition of laccase to SBP.

The molecular weight (Mw), root mean square (RMS) radius and recovery % of each peak analyzed by HPSEC-MALLS and ζ -potential of SBP and L-SBP are presented in Table 3-1. The L-SBP had a significantly higher average Mw than SBP in the both of the first and second peak ($p < 0.05$) (Table 3-1). Mw changes could be more clearly visualized in the molecular weight cumulative distribution (Fig.3-3). With the addition of laccase, the cumulative molecular weight curve slightly shifted to the right side and had a steeper slope and narrower distribution (Fig. 3-3). It is conceivable that L-SBP would have twice Mw as two SBP moieties were conjugated together. However, greater difference in Mw was observed in smaller Mw fractions rather than

larger Mw SBP fractions, which suggests that laccase preferentially cross-links smaller Mw fractions of SBP.

Despite the overall greater Mw in L-SBP, the RMS radius was lower than in SBP, especially for the second peak (Table 3-1). This suggests that conjugated L-SBP might be more compact and have more branched structure than SBP. In addition, the chain conformation information generated by the slope of log-log plots of RMS versus Mw exhibited SBP and L-SBP had very low slope value which indicates they are a spherically branched polymer (Beri, Walker, Reese, & Rollings, 1993). It suggests that L-SBP has a higher branching structure as shown by lower slope value than SBP (data is not shown). In the study of two acacia gum exudates, Acacia seyal which has highly branched galactan framework showed higher molecular weight and smaller hydrodynamic volume (Elmanan, Al-Assaf, Phillips, & Williams, 2008; Flindt, Al-Assaf, Phillips, & Williams, 2005).

The ζ -potential of L-SBP was less negative than that of SBP. The structure of the SBP, confirmed by the atomic force microscopy showed that it has extended and linear backbone mostly and a small branched molecular fraction (Kirby, MacDougall, & Morris, 2008). The change in ζ -potential from -40.5 mV to -32.5 mV, in the presence of laccase might be to burying of negative carboxyl groups within a more highly branched conjugated SBP (Table 3-1).

Particle size

Size distribution of SBP and L-SBP is presented at Fig.3-4. The SBP exhibited dominant diameter population around 30 nm and smaller populations around 500 nm and greater than 1000 nm. The main population of L-SBP was seen on the second peak around 800 nm. The larger diameter populations are consistent with conjugation. However, there is evidence of smaller volumes of larger and smaller diameter populations.

Dynamic light scattering calculates the particle size from the measured diffusion coefficient by scattered light intensity from particles moving based on their random Brownian motion. Multimodal size distribution obtained by the intensity, volume and number of particles were calculated using software. Size distribution by volume is preferred, since it gives a more realistic view, especially in samples having more than two peaks according to the manufacturer. Since size distribution by intensity is proportional to the square of the molecular weight, small amount of aggregated or larger particles can dominate and induce misleading results in the distribution. Therefore, in this research, multimodal size distribution by volume was selected.

Emulsion stability

Emulsion stability is reported in Fig. 3-5, which shows the measured average volume weight diameter (d_{43}) of emulsions prepared with SBP and L-SBP. $d_{4,3}$ is the most sensitive diameter to monitor the presence of large particles such as aggregation and flocculation (McClements, 2007). Fig. 3-5 shows selected typical droplet size distribution of samples. Both samples showed almost the same droplet size, approximately 1.1 μm at day 0 and diameters increased over the storage time. The rate of increase in d_{43} diameter was greater in SBP after 10 days, whereas L-SBP showed more moderate increases. The differences in droplet size between SBP and L-SBP stabilized emulsions were significant after 15 days ($p < 0.05$) (Fig. 3-5). This observation is also seen in distribution graph (Fig. 3-6).

The droplet size distribution of SBP and L-SBP emulsion samples almost overlapped at 0 day (Fig 3-6a) and distribution was shifted to a larger diameter in both with longer storage times. At 30 d, the distribution of SBP stabilized emulsion showed lower and broader distribution than L-SBP (Fig 3-6c). This indicates that L-SBP stabilized emulsions were more stable when stored for longer periods of time. Also, L-SBP stabilized emulsions showed more uniformly dispersed

droplets without the second population. The improved effect may be due to increased steric repulsion. In layer by layer deposition of protein, SBP, followed by cross-linking by laccase, emulsions showed better stability against NaCl and creaming and it was suggested that thick polymer layer decreased van der Waals attraction with increasing steric repulsion between droplets (Littoz & McClements, 2008).

The ζ -potential of SBP and L-SBP emulsion was conducted to determine charge of emulsions droplets and stability during 30 days incubation. Freshly made emulsions showed around -37 mV and longer incubation days result in a more positive charge on both samples (Table 3-2). L-SBP shows relatively smaller reduction rate from -36.8 mV to -34 mV compared to SBP which showed a difference from -36.5 mV to -28.5 mV. These changes were consistent with differences in droplet size diameter. The charge is responsible for determination of its interaction with other droplets in emulsions and emulsifier is critical factor which prevent flocculation within droplets by adsorbing onto the oil droplet due to electrostatic repulsion (Dickinson, 1992; McClements, c2005). Larger particles can attribute the reduction of the electrostatic repulsion between the droplets resulting in decreased ζ -potential. The ζ -potential was significantly different after 20 incubation days between SBP and L-SBP.

The changes in droplet size and distribution were confirmed by microscopy images (Fig. 3-7). It was found that noticeably large droplets and some flocculation were produced at 30 days in SBP stabilized emulsions, which were not detected by size distribution. Since commercial particle size instrument requires sample dilution and stirring to make homogeneous solutions, small weak flocculation might be disrupted. L-SBP which becomes highly branched and has a higher molecular weight might be more concentrated with protein and ferulic acid, which may be the reason that attributes more stable stability in the emulsion during storage. It was suggested

that higher molecular weight fraction adsorbed onto the oil droplet preferentially during emulsification due to having more protein and ferulic acid (Siew & Williams, 2008).

Conclusion

This present study reports that laccase induced the conjugation in SBP through ferulic acid and conjugation was confirmed by measuring the reduced ferulic acids in L-SBP. Laccase mediated conjugation SBP leads to larger molecular weight, smaller RMS which indicates having more packed and highly branched structure. Emulsion coated with cross-linked SBP showed significant smaller d_{43} diameter and stronger electrostatic repulsion during incubation. Its stability was confirmed as laccase mediated SBP emulsion had smaller and well dispersed droplets in the microscope image. It is suggested that more highly branched cross linked SBP improves long term emulsion stability covering oil droplets sufficiently.

References

- Archibald, F. S., Bourbonnais, R., Jurasek, L., Paice, M. G., & Reid, I. D. (1997). Kraft pulp bleaching and delignification by *Trametes versicolor*. *Journal of Biotechnology*, 53(2-3), 215-236.
- Beri, R. G., Walker, J., Reese, E. T., & Rollings, J. E. (1993). Characterization of chitosans via coupled size-exclusion chromatography and multiple-angle laser light-scattering technique. *Carbohydrate Research*, 238, 11-26.
- Corredig, M., & Wicker, L. (2001). Changes in the molecular weight distribution of three commercial pectins after valve homogenization. *Food Hydrocolloids*, 15(1), 17-23.
- Dickinson, E. (1992). *An Introduction to Food Colloids*. Oxford: Oxford Science.
- Drusch, S. (2007). Sugar beet pectin: A novel emulsifying wall component for microencapsulation of lipophilic food ingredients by spray-drying. *Food Hydrocolloids*,

- 21(7), 1223-1228.
- Elmanan, M., Al-Assaf, S., Phillips, G. O., & Williams, P. A. (2008). Studies on Acacia exudate gums: Part VI. Interfacial rheology of Acacia senegal and Acacia seyal. *Food Hydrocolloids*, 22(4), 682-689.
- 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.
- Flindt, C., Al-Assaf, S., Phillips, G. O., & Williams, P. A. (2005). Studies on acacia exudate gums. Part V. Structural features of Acacia seyal. *Food Hydrocolloids*, 19(4), 687-701.
- Gianfreda, L., Xu, F., & Bollag, J-M. (1999). Laccases: a useful group of oxidoreductive enzymes. *Bioremediation Journal*, 3, 1-25.
- Guillon, F., & Thibault, J. -F. (1988). Further characterization of acid- and alkali-soluble pectins from sugar beet pulp. *Lebensmittel Wissenschaft und Technologie*, 21, 198-205.
- Kirby, A. R., MacDougall, A. J., & Morris, V. J. (2008). Atomic force microscopy of tomato and sugar beet pectin molecules. *Carbohydrate Polymers*, 71(4), 640-647.
- 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.
- Leroux, J., Langendorff, V., Schick, G., Vaishnav, V., & Mazoyer, J. (2003). Emulsion stabilizing properties of pectin. *Food Hydrocolloids*, 17(4), 455-462.
- 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.
- May, C. D. (1990). Industrial pectins: Sources, production and applications. *Carbohydrate Polymers*, 12(1), 79-99.

- McClements, D. J. (2007). Critical review of techniques and methodologies for characterization of emulsion stability. *Critical Reviews in Food Science and Nutrition*, 47(7), 611-649.
- McClements, D. J. (2005). *Food emulsions : principles, practices, and techniques* (2nd ed.). Boca Raton CRC Press.
- 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.
- Nakauma, M., Funami, T., Noda, S., Ishihara, S., Al-Assaf, S., Nishinari, K., & Phillips, G. O. (2008). Comparison of sugar beet pectin, soybean soluble polysaccharide, and gum arabic as food emulsifiers. 1. Effect of concentration, pH, and salts on the emulsifying properties. *Food Hydrocolloids*, 22(7), 1254-1267.
- 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.
- Ralet, M. C., Thibault, J.F., Faulds, C. B., & Williamson, G. (1994). Isolation and purification of feruloylated oligosaccharides from cell walls of sugar-beet pulp. *Carbohydrate Research*, 263, 227-241.
- 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.
- Rombouts, F. M., & Thibault, J. F. (1986). Enzymatic and Chemical Degradation and the Fine-Structure of Pectins from Sugar-Beet Pulp. *Carbohydrate Research*, 154, 189-203.
- 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.
- Siew, C. M., & Williams, P. A. (2008). Role of protein and ferulic acid in the emulsification

- properties of sugar beet pectin. *Journal of Agricultural and Food Chemistry*, 56(11), 4164-4171.
- Synytsya, A., Copikova, J., Jankovska, P., Matejka, P., & Machovic, V. (2003). Spectroscopic estimation of feruloyl groups in sugar beet pulp and pectin. *International Sugar Journal*, 105(1258), 481-488.
- Thurston, C. F. (1994). The Structure and Function of Fungal Laccases. *Microbiology-Uk*, 140, 19-26.
- Voragen AGJ, P. W., Thibault JF, Axelos MAV, Renard CMGC. (1995). Pectins. In *Food polysaccharides and their Applications*, (pp. 287-339). New York: Marcel Dekker Inc.
- Williams, P. A., Sayers, C., Viebke, C., Senan, C., Mazoyer, J., & Boulenguer, P. (2005). Elucidation of the Emulsification Properties of Sugar Beet Pectin. *Journal of Agricultural and Food Chemistry*, 53(9), 3592-3597.

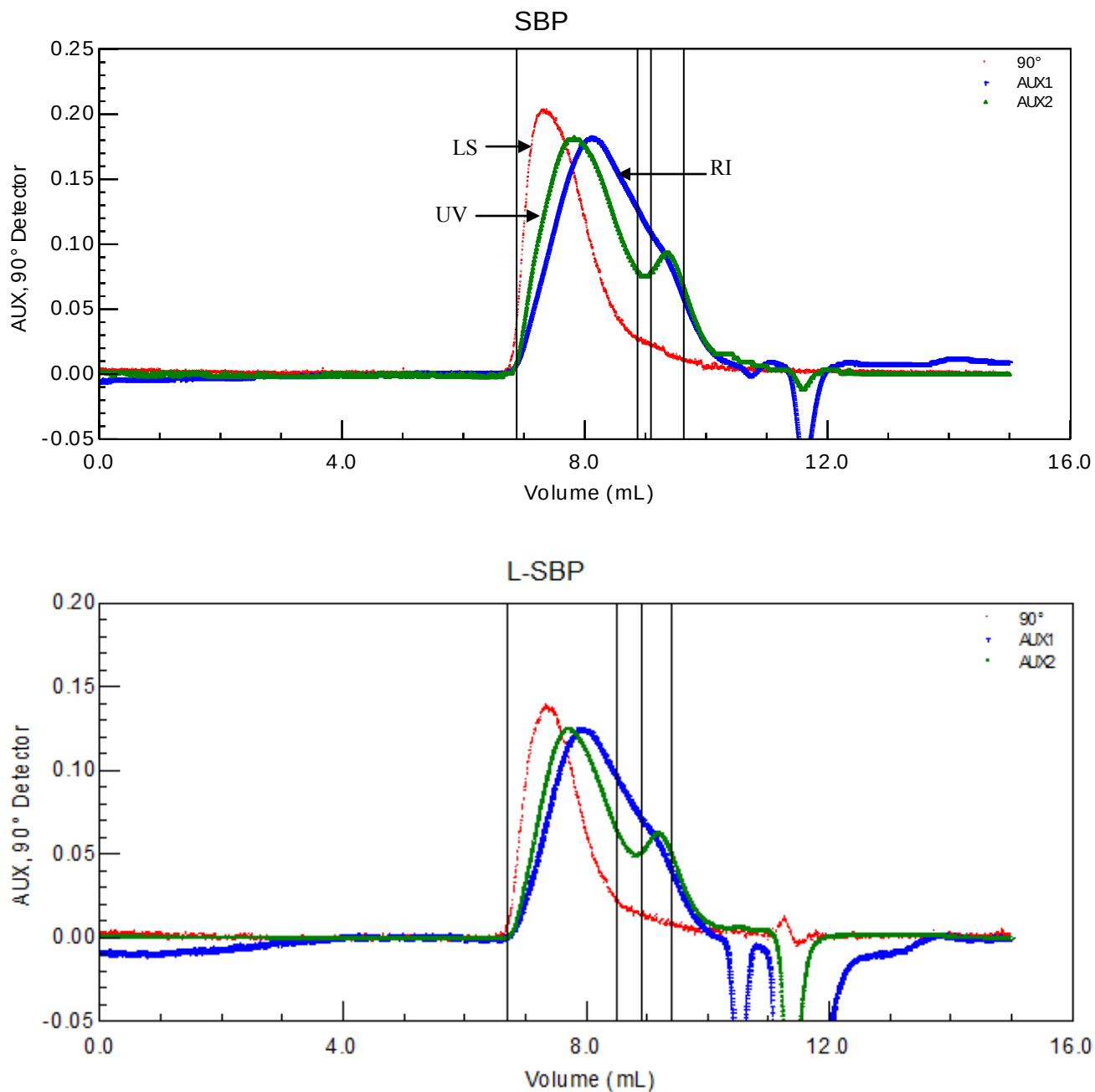


Fig.3-1. Elution profiles of representative SBP and L-SBP samples monitored by light scattering, refractive index and UV at 325 nm. The y-axis scale is for LS (light scattering) and two concentration detectors (UV, RI) were set to 90 %. Therefore, the peak heights do not give the true value.

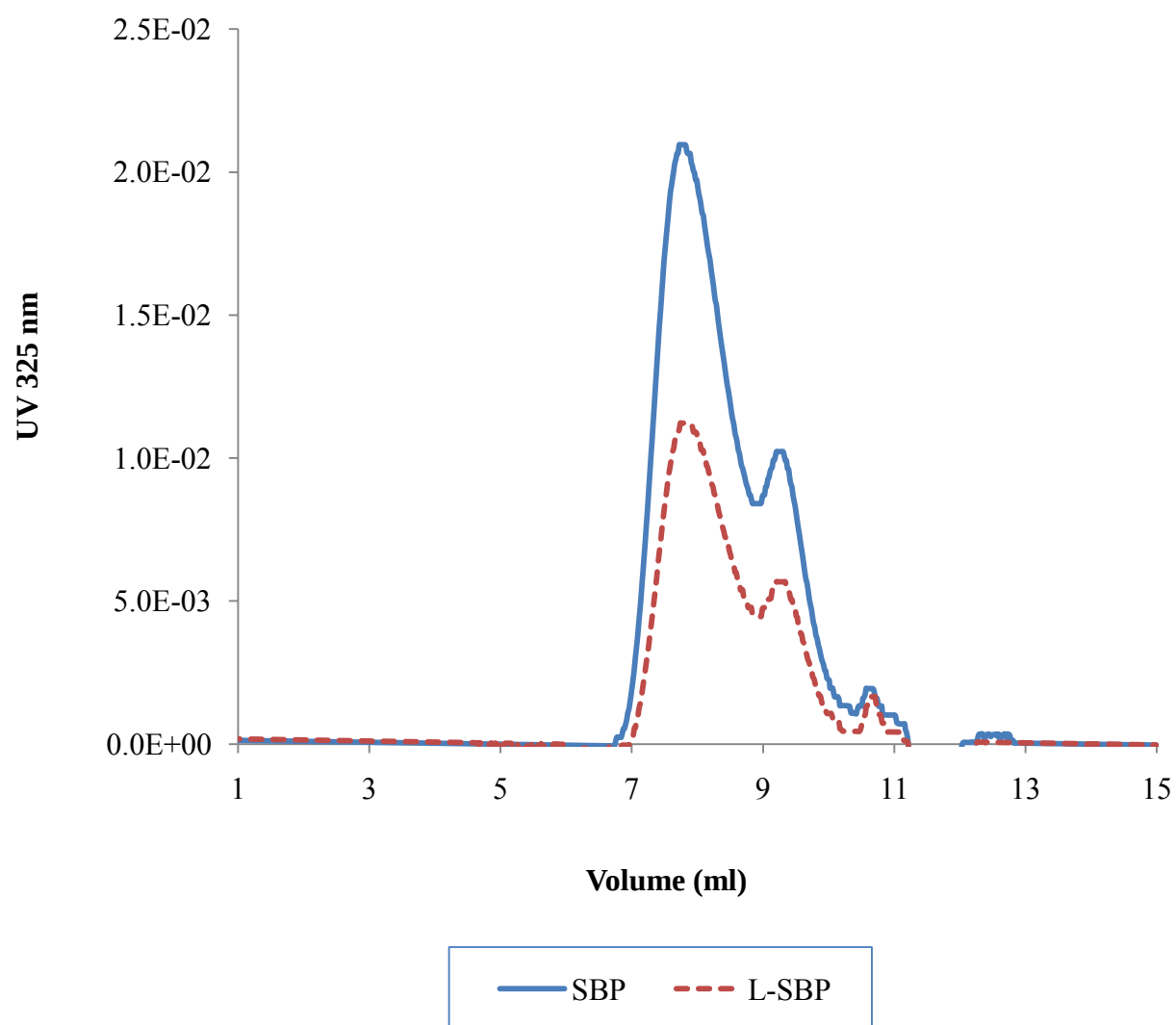


Fig.3-2. UV elution profiles of SBP (solid blue line) and L-SBP (dashed red line) at 325 nm.

Table 3-1. Mw, RMS, recovery % and ζ –potential of SBP and L-SBP samples.

	Peak	Mw (kDa)	Rw (nm)	Rz (nm)	Recovery %	ζ –potential (mV)
SBP	1 st	372.7 $\pm$ 14.1 ^a	28.4 $\pm$ 1.6 ^a	30.4 $\pm$ 0.4 ^a	43.4 $\pm$ 7.0 ^a	-40.5 $\pm$ 3.0 ^a
	2 nd	65.7 $\pm$ 13.2 ^a	36.7 $\pm$ 2.3 ^a	36.6 $\pm$ 2.2 ^a	9.7 $\pm$ 1.1 ^a	
L-SBP	1 st	403.2 $\pm$ 23.7 ^b	23.3 $\pm$ 1.7 ^b	28.1 $\pm$ 2.7 ^b	32.2 $\pm$ 4.5 ^b	-32.5 $\pm$ 2.6 ^b
	2 nd	83.2 $\pm$ 24.3 ^b	28.3 $\pm$ 2.2 ^b	27.8 $\pm$ 2.2 ^a	6.8 $\pm$ 1.5 ^a	

Data are presented as means $\pm$ SD of more than triplicate.

^{a,b} : Same letters are not significantly different between SBP and L-SBP within the same peak in the same column (p<0.05).

Mw: Weight average molecular weight.

RMS: Root mean square radius.

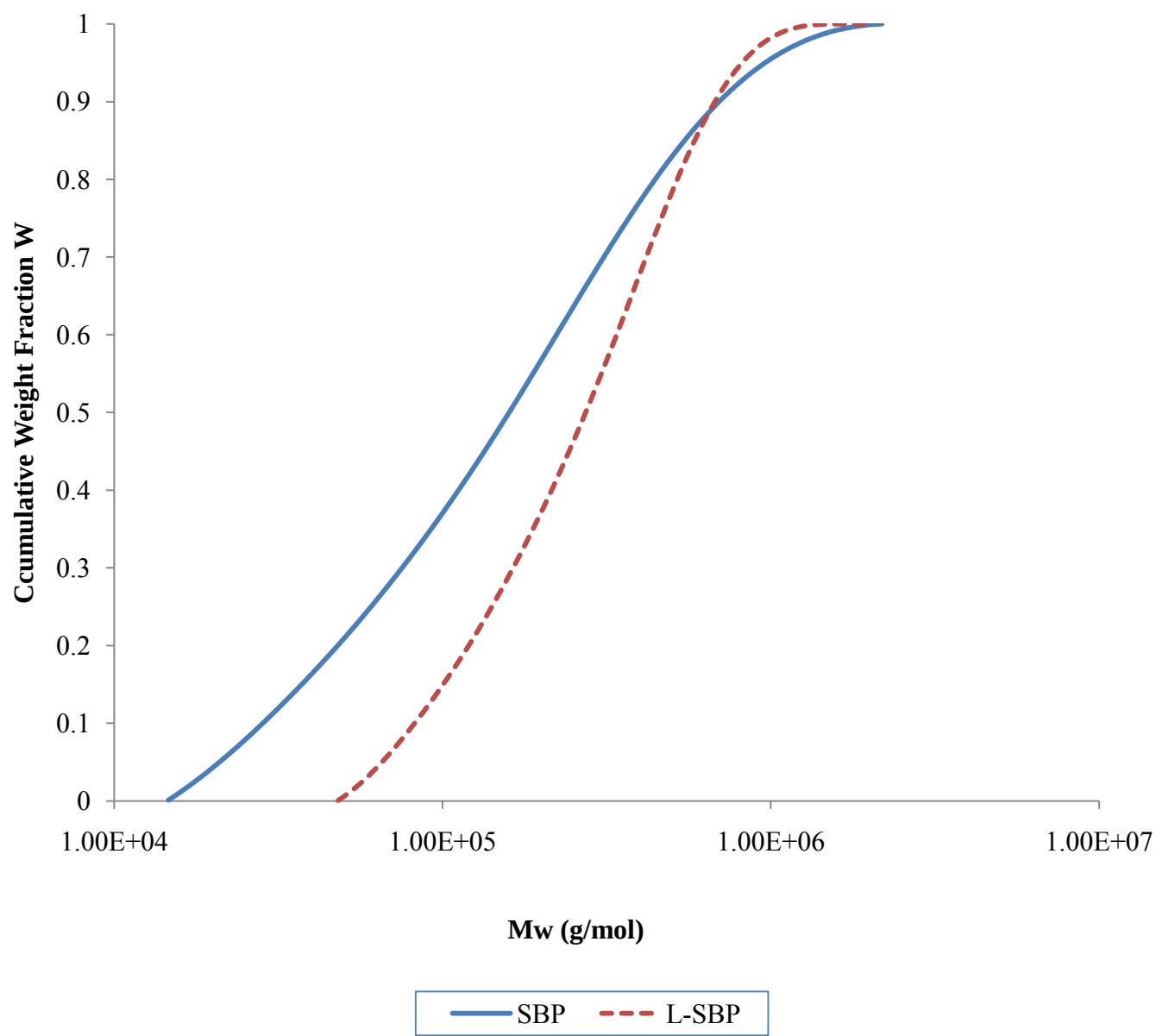


Fig.3-3. Cumulative molecular weight fractions of SBP and L-SBP samples.

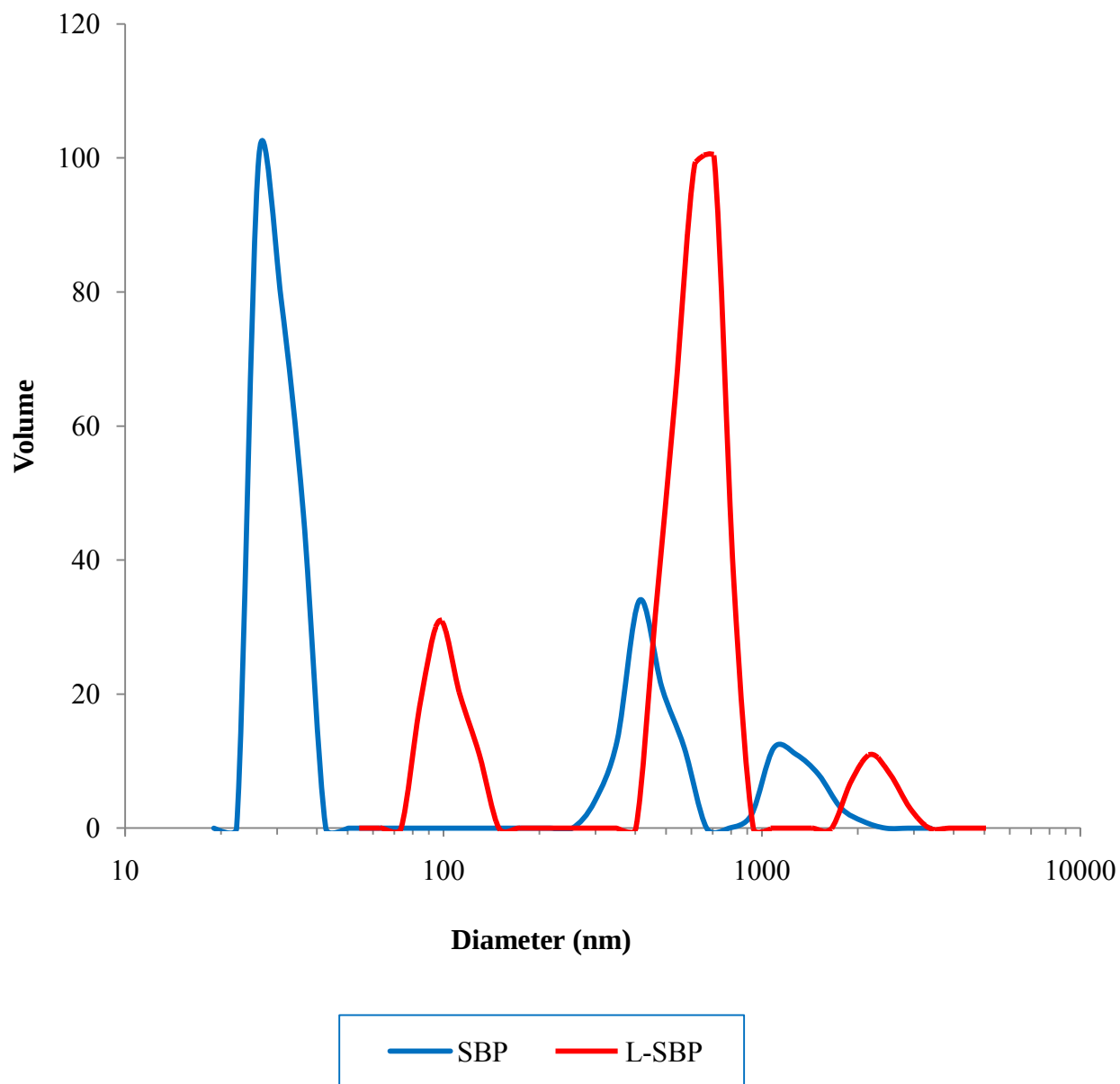


Fig. 3-4. Size distributions of SBP (blue line) and L-SBP (red line) samples.

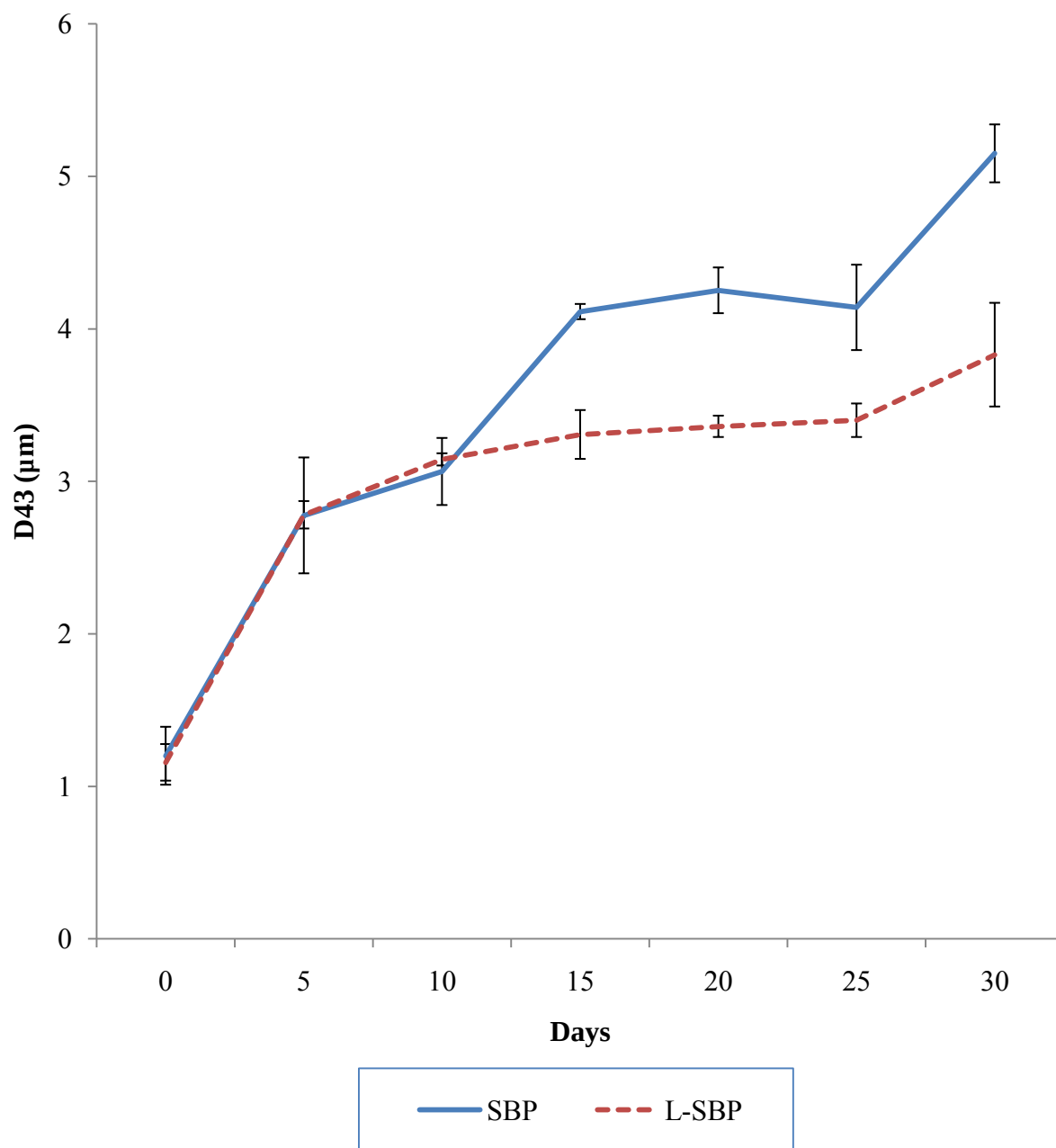


Fig. 3-5. *d*₄₃ diameer of SBP and L-SBP emulsions for 30 incubation days.

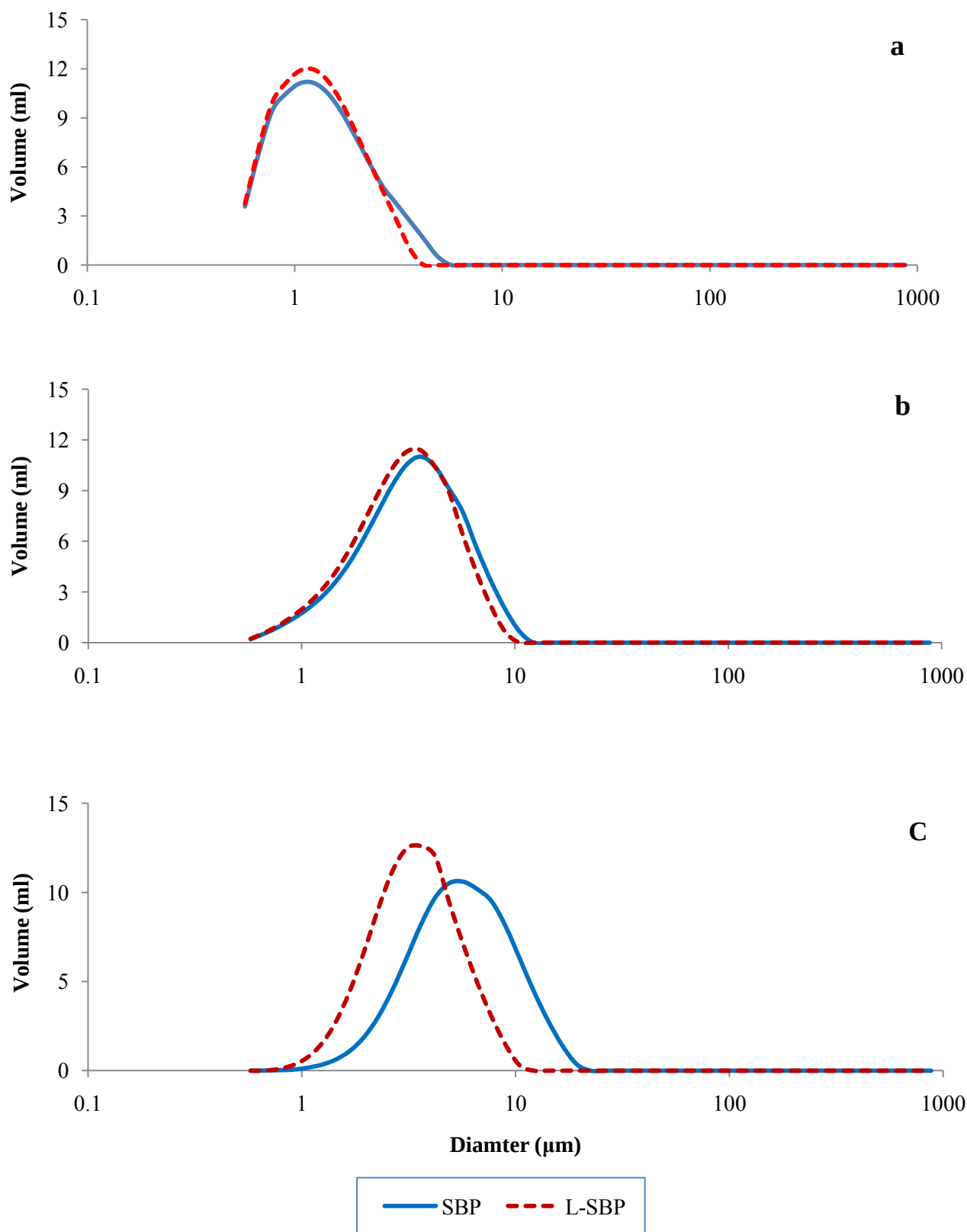


Fig. 3-6. *d*₄₃ size distribution of SBP and L-SBP emulsions at 0 day (a), 10 day (b) and 30 day (c)

Table 3-2. ζ -potential of SBP and L-SBP emulsions for 30 incubation days.

Sample Days	SBP	L-SBP
0	-36.5 ± 2.5^a	-36.8 ± 1.1^a
5	-30.2 ± 2.9^a	-34.2 ± 1.2^a
10	-29.2 ± 3.6^a	-33.5 ± 3.2^b
15	-34.7 ± 1.8^a	-36.3 ± 0.7^a
20	-31.0 ± 2.9^a	-34.0 ± 1.7^b
25	-29.2 ± 1.1^a	-35.2 ± 1.7^b
30	-28.5 ± 3.0^a	-34.5 ± 3.2^b

- Data are presented by mean $\pm$ SD.
- ^{a,b}: Same letters are not significantly different between SBP and L-SBP within the same incubation day (row).

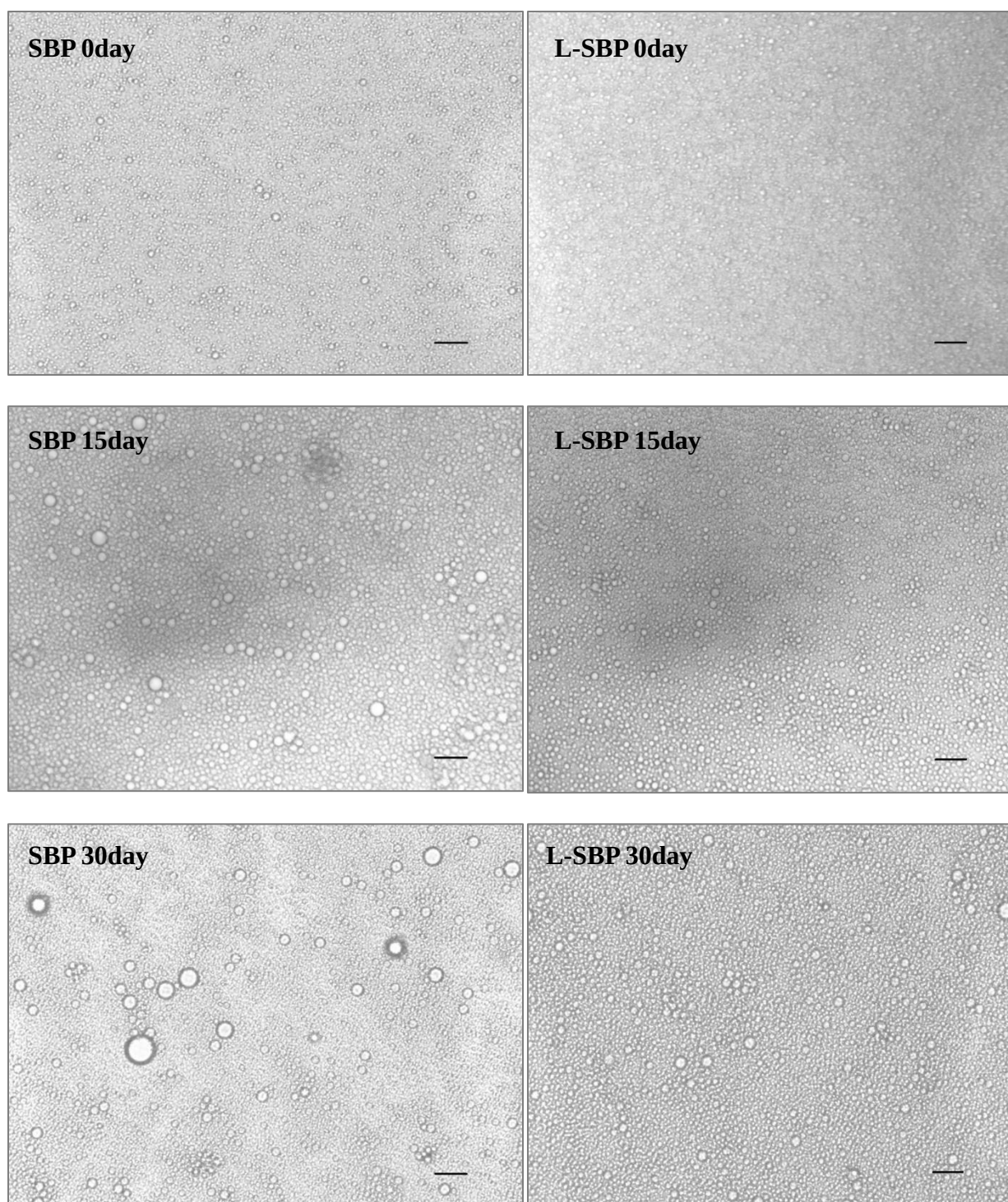


Fig 3-7. Light microscopy images of SBP, L-SBP emulsions at 0, 15 and 30 day.

Size bar = 20 µm.

CHAPTER 4

LACCASE MEDIATED CONJUGATION OF β -LACTOGLOBULIN
AND SUGAR BEET PECTIN¹

¹ Jung, J., & Wicker, L., To be submitted *Food hydrocolloids*, 2011

Abstract

β -lactoglobulin (BLG) dispersions (3%, w/v) prepared in 100 mM sodium phosphate buffer (pH 6.5), were equilibrated with and without laccase at 30 °C for 20 hours and heated at 80 °C for 15 min. The characteristics of BLG were investigated by size exclusion chromatography combined with multi angle laser light scattering (HPSEC-MALLS), refractive index (RI) and UV detection, by particle size and ζ -potential analysis. Homo conjugation was not observed in BLG via laccase, though conformational change in BLG was confirmed as indicated by a smaller and broader RMS distribution, slope increase from 0.35 to 0.72 in a log-log RMS vs Mw plot, and increased particle size from 222 nm to 1278 nm. Upon heat treatment, free thiol group (SH) and tyrosine residues increased, followed by increased molecular weight. Laccase treated conjugates of BLG with SBP, had greater molecular weight corresponding with a reduction in overall concentration, and lower ferulic acid than non-treated BLG-SBP.

INDEX WORDS: Sugar beet pectin, β -lactoglobulin, Laccase, HPSEC-MALLS, Conjugation

Introduction

Whey protein is widely used as a food ingredient in the food industry, due to its nutritional value, and its effective physical functionality, such as gelling, foaming, emulsifying and stabilizing abilities. Chemical, thermal, and enzymatic methods as well as complexation with polysaccharides have been attempted to enhance protein functionality and stability (Chen, Embree, Brown, Taylor, & Payne, 2003; Einhorn-Stoll, Ulbrich, Sever, & Kunzek, 2005; Flanagan & Singh, 2006; Guzey & McClements, 2007; Peyron, Mouecoucou, Fremont, Sanchez, & Gontard, 2006; Reddy, Kella, & Kinsella, 1988; Santipanichwong, Supphantharika, Weiss, & McClements, 2008; Weinbreck, Minor, & De Kruif, 2004).

Enzymatic cross-linking of polymers is a preferred protein modification method due to specificity and minimal risk of possible toxic by-products that result from chemical cross-linking (James & Simpson, 1996). Transglutaminase is the most common enzyme to produce cross-linkages and to improve functionality, attributed to broad applicability to most food proteins with available primary amines, such as glutamine and lysine residues (Faergemand, Otte, & Qvist, 1997; Flanagan & FitzGerald, 2002; Kato, Wada, Kobayashi, Seguro, & Motoki, 1991; Walsh, Cleary, McCarthy, Murphy, & FitzGerald, 2003). Additionally, oxidative cross-linking through oxidoreductases, such as peroxidase, horseradish peroxidase, laccase and monoamine oxidase have been studied as well (Faergemand, Otte, & Qvist, 1998; Figueroa-Espinoza & Rouau, 1998; Matheis & Whitaker, 1984; Oudgenoeg, Hilhorst, Piersma, Boeriu, Gruppen, Hessing, et al., 2001). Laccase is a type of polyphenol oxidase (p-diphenol oxidase, E.C. 1.10.3.2) that oxidizes phenolic compounds with broader specificity than polyphenol oxidase or peroxidase. Laccase improves the gelling ability of SBP via ferulic acid (Micard & Thibault, 1999; Oosterveld, Beldman, Schols, & Voragen, 1996) and enhances the mixing properties of wheat flour dough

(Gianfreda, 1999; Labat, Morel, & Rouau, 2000). In addition, laccase mediated covalent cross-linked gels of feruloylated arabinoxylan protected embedded protein against pepsin proteolysis (Vansteenkiste, Babot, Rouau, & Micard, 2004).

Tyrosine, cysteine and tryptophan residues are substrates for laccase in protein, and the oxidation of SH groups is accelerated by the addition of FA (ferulic acid), phenolic substances (Figuerola-Espinoza, Morel, & Rouau, 1998; M.-L. Mattinen, Kruus, Buchert, Nielsen, Andersen, & Steffensen, 2005; M. L. Mattinen, Hellman, Permi, Autio, Kalkkinen, & Buchert, 2006). Laccase mediated polymerization of casein and WPI (whey protein isolate) is enhanced in the presence of low molecular weight phenolic substances (Faergemand, Otte, & Qvist, 1998; Selinheimo, Lampila, Mattinen, & Buchert, 2008). However, Mattinen et al. (2006) suggested that proteins, such as BSA and BLG are poor substrates for laccase. Even though BSA and BLG have sufficient tyrosine residues, buried tyrosine residues are inaccessible to laccase in 3-dimensional structure of BSA and BLG (Uhrinova, Smith, Jameson, Uhrin, Sawyer, & Barlow, 2000).

BLG is the most abundant protein in whey protein and its structure and mechanism are well known. BLG exists as a dimer at neutral pH with 36kDa Mw, two disulfide groups, and one free, buried cysteine residue (Walstra, Wouters, & Geurts, 2006). The dimer dissociates into a monomer, and hydrophobic groups are exposed by partial unfolding if heated to temperatures over 70 °C (Fox & McSweeney, 2003; Qi, Holt, McNulty, Clarke, Brownlow, & Jones, 1997; Relkin, 1998). Thermal treatment has been considered as a method to induce dramatic changes in BLG structure, particle shape and side amino acid chain alterations, which can be controlled by heating temperature, pH adjustment and ionic strength (Hoffmann, Roefs, Verheul, VanMil, & DeKruif, 1996; Ikeda, 2003; Relkin, 1998; Sava, Van der Plancken, Claeys, & Hendrickx, 2005;

Schmitt, Bovay, Rouvet, Shojaei-Rami, & Kolodziejczyk, 2007). Controlled modification of BLG structure by cross-linkages between BLG and SBP may offer a new application of whey protein for various food industries. In this research, we evaluated the polymerization of BLG by laccase, the role of heat pre-treatment on conjugation, and the conjugation of laccase pre-treated BLG with SBP.

Materials & Methods

Materials

β -lactoglobulin (BLG) (93.6%) was provided by Davisco Foods International (Le Sueur, MN) and contained 93.6% BLG content of total protein as stated by the manufacturer. Sugar beet pectin (SBP) was donated by Herbstreith & Fox KG (Elmsford, NY). Laccase (*Rhus vernificera*, EC 1. 10. 3. 2, p-diphenol oxidase) and syringaldazine were purchased from Sigma – Aldrich Co. (St. Louis, MO).

Stock preparation

SBP and BLG dispersions were prepared by dispersing 3% (w/v) dry power in 100 mM sodium phosphate buffer (pH 6.5) separately, stirred at room temperature for at least 2 hours and stored overnight at 4 °C for complete hydration. The dispersions were centrifuged for 30 min at 5,000 g, 4 °C to remove insoluble materials (Sorvall RC 6Plus, Thermo scientific products).

Laccase mediated SBP & heat treated BLG conjugation preparation

The procedure of sample preparation is presented in Fig. 4-1. The centrifuged SBP and BLG dispersions were equilibrated with and without laccase in a water bath (30 °C) for 20 hours with mild agitation on an orbital shaker (PR 70 Red Rotar Hoffer Scientific, MO). An aliquot of 1 unit laccase/mg BLG solids was added to the each dispersion. The activity of the laccase was verified just before addition. An aliquot of water was added to the controls. Laccase treated and

non-laccase treated BLG dispersions were mixed with laccase treated SBP and without laccase treated SBP at the same ratio. These mixtures were equilibrated for another 20 hours at ambient temperature. The samples were diluted 10 fold to slow down laccase activity or analyzed immediately to reduce complications from further enzymatic activity. Further diluted laccase showed very low activities enough to inhibit further enzymatic actions. Negligible laccase activity in diluted samples was confirmed by activity assay.

Laccase activity measurement

Laccase activity was measured by modified procedure of previously described method using syringaldazine as substrate at 30 °C (Ride, 1980). Laccase was dissolved in cold, deionized water and stirred for 10 min, filtered through 5 µm PVDF (polyvinylidene fluoride) syringe filters (Millipore, Bedford, MA). After 2.2 ml sodium phosphate buffer (100 mM, pH 6.5) was mixed with 0.5ml of diluted laccase solution, 0.3 ml syringaldazine (0.216 mM in methanol) was added and measurement promptly followed. The loss in absorbance at 530 nm per unit of time was used to assess activity.

Particle size measurement

Particle size was performed using a Particle Size Analyzer with the BI-Zeta option (90 Plus, Brookhaven Instruments Corporation, Holtsville, NY) with a 50 mV diode laser (90° angle) and a BI-9000AT correlator. The samples were diluted to 0.05 wt% with deionized water prior to analysis. Particle size measurements were performed in duplicate with 5 measurements in each run. Average effective diameter was reported as individual particle size.

Zeta (ζ) potential measurement

Each sample was diluted to 0.05 wt% with deionized water prior to analysis. The ζ-potential was performed subsequently after the particle size determination for all samples. The

measurements were carried out in duplicate with 5 runs. The ζ -potential is calculated from the average of ten readings.

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. The separation was achieved by a PL-Aquagel-OH guard column (50×7.5mm) and PL-Aquagel-OH mix separating column (300 × 7.5 mm) (Polymer Laboratories, Inc., Amherst, MA) with a flow rate of 0.5 ml/min, with 10 mM of Na-Phosphate and 100 mM of Na-Nitrate, pH 7 as elution buffer. Mixed samples (3 mg/ml) and individual SBP (1.5 mg/ml) or BLG (1.5 mg/ml) were filtered through 0.8 μ m MCE (mixed cellulose ester) syringe filters (Millipore, Bedford, MA) and the injection volume was 0.05 ml. Specific refractive index increment (dn/dc) values of 0.131 and 0.180 were used for SBP and BLG, respectively (Corredig & Wicker, 2001; Van Dijk & Smit, 2000). Elution of BLG was monitored with UV detector at 280 nm and ferulic acid was detected at 325 nm. Elution data was processed using ASTRA software (vs. 4.73.03).

Determination of sulfhydryl (SH) groups

Surface SH groups were determined using Ellman's reagent [5,5'-dithio-bis2-nitrobenzoic acid; DTNB] previously described method (Sava, Van der Plancken, Claeys, & Hendrickx, 2005). All the samples were diluted to 0.1 % protein (w/v) with standard buffer (0.086 M Tris, 0.09 M glycine, 4 mM Na₂EDTA; pH 8) and then total diluted 1500 μ l sample was mixed with 40 μ l DTNB (4 mg/ml in standard buffer). In order to determine surface SH groups, absorbance was measured at 412 nm after 15 min (surface SH groups) and 13,600 M⁻¹cm was used as molar extinction coefficient (Labat, Morel, & Rouau, 2000).

Determination of tyrosine

The procedure to determine tyrosine in samples were adapted from a previous method (Hassan, 1975). A mixture of 0.9 ml samples (protein 0.1% w/v) and 1 ml 16 M nitric acid was heated in a water bath for 15 min at 50 °C. After cooling to room temperature, 4 ml sodium hydroxide (5 M) and 4 ml ethyl alcohol were mixed, and absorbance was measured at 360 nm and 430 nm using UV-visible spectrophotometer (UV-1700, Shimadzu scientific instrument, Columbia, MD USA). The concentration of tyrosine (µg/ml) was determined by the following equation.

$$\frac{260 A_{430\text{nm}}}{225 \times 260} - \frac{150 A_{360\text{nm}}}{110 \times 150} = 0.5357 A_{430\text{nm}} - 0.35714 A_{360\text{nm}}$$

Statistical analysis

Values reported result from at least duplicate conjugations. Statistical differences estimated by one-way analysis of variance and student's t-test.

Results

Influence of laccase on BLG

The elution profile of BLG at UV₂₈₀ is presented in Fig. 4-2. The Mw (molecular weight), RMS (root mean square) and slope exponent data, calculated under UV detector by HPSEC-MALLS are presented in Table 4-1. Untreated BLG eluted as a single peak between 9.6 ml and 10.3 ml with a Mw of $\sim 3.8 \times 10^4$ g/mol, representing a dimeric structure. The Mw and RMS of LBLG (laccase treated BLG) decreased significantly ($p < 0.05$) from 37,512 to 29,230 g/mol and 41 to 36 nm, respectively (Table 4-1). However, LBLG had almost the same UV elution profile (Data not shown) as BLG. It was expected that Mw of LBLG would be greater than BLG or have an additional, earlier eluting peak, indicating polymerization of BLG. The slight decrease in Mw indicated that homo cross-linking of BLG by laccase did not occur. In fact, this result is in

agreement with Faergemand et al. (1998) who studied cross-linking of whey protein with several oxidoreductases including laccase. They reported that laccase induced polymerization in α -lactalbumin, while BLG showed almost the same Mw before and after laccase treatment (Faergemand, Otte, & Qvist, 1998). Non polymerization in BLG might be attributed to lack of available phenolic substrate in BLG. According to Mattinen et al. (2006), tyrosine residues in native BLG are buried in the globular structure with other hydrophobic amino acids. Laccase might have limited access to tyrosine when it is applied to large protein substrates, such as BLG and BSA (bovine serum albumin) that contain numerous amino acids arranged within 3 dimensional structure (M.-L. Mattinen, Kruus, Buchert, Nielsen, Andersen, & Steffensen, 2005). However, they reported that tyrosine and tyrosine containing peptides formed oxidative cross-links via laccase, and hetero-conjugation was enhanced in the presence of small molecular weight phenolic compounds, such as ferulic acid (M.-L. Mattinen, Kruus, Buchert, Nielsen, Andersen, & Steffensen, 2005).

In this study, conformational change catalyzed by laccase was identified in BLG. The Mark Houwink analysis that used to determine polymer structure, is described as $[\eta] = K \cdot MW$ and is obtained from plotting logarithmic plot of intrinsic viscosity against Mw (Brandrup, Immergut, Grulke, Abe, & Bloch, 1999). The conformational change in polymer structure can be estimated from light scattering by plotting log RMS against log Mw. The slope may vary from 0 to 1 depending on polymer chain shape. Typically, a slope of 0.33 reflects a sphere and slopes of 0.50-0.60 or close to 1.0 indicate a random coil or rigid rod, respectively. Theoretically, smaller values, close to 0, indicate a more compactly structured polymer, and as the slope value moves closer to 1, a less condensed conformation results. Since two or more shapes may exist in the solution at the same molecular weight and volume, a typical, single shape could not be

designated in the sample. In this study, the slope exponent in the log RMS & log Mw plot changed to ~ 0.72 from ~ 0.35 with the addition of laccase to BLG. The results suggested that BLG became less compact and more rod like in structure with laccase treatment (Table 4-1). Another prominent change was found in the particle size measured by dynamic light scattering, which increased more than five folds, from 222nm to 1278nm, with the addition of laccase (Table 4-1). Dynamic light scattering (DLS) measures the Brownian motion, particle movement suspended in a solution, which is proportional to size. Normally, larger particles diffuse slowly and will give slower Brownian motion, and the velocity of the Brownian motion is known as the translational diffusion coefficient. Theoretically, non-spherical particle size is calculated based on the equivalent sphere that has the same translational diffusion speed in the dynamic light scattering (DLS). Thus, the dramatically enlarged particle size might be occurred from conformational change in BLG by the addition of laccase. The RMS distribution obtained from HPSEC-MALLS is depicted in Fig. 4-3. LBLG exhibited smaller and wider RMS distribution profile than BLG (Fig. 4-3).

Influence of heat treatment on BLG and LBLG

While native and dimeric BLG eluted as a single peak, heated BLG (HBLG) eluted as two peaks (Fig.4-2). An additional peak eluted earlier between 8.0 ml and 9.6 ml volume and the second, dimeric peak eluted between 9.6 and 10.3 ml on HBLG. Heat greatly reduced the peak height of the second, dimeric peak, presumably due to the formation of large, earlier eluting aggregates with decrease concentration (Fig.4-2). The Mw of HBLG were $\sim 2.6 \times 10^6$ and $\sim 0.7 \times 10^6$ g/mol for the first and second peaks, respectively (Table 4-2). Intermediate size aggregates (e.g., trimer, tetramer, oligomer) were eluted as separate peaks based on their Mw in the previous works (Bauer, Carrotta, Rischel, & Ogendal, 2000; Schokker, Singh, Pinder, Norris, & Creamer,

1999). In this study, the second HBLG peak eluted where unheated dimer BLG eluted, but had a larger Mw and lower concentration. It is likely that the native BLG co-eluted with intermediate aggregates as a single peak, not as resolved by the linear mix column.

Heated BLG with laccase treatment (HLBLG) showed almost the same shape in the elution profile with HBLG. However, HLBLG eluted earlier and had a larger first peak than HBLG with corresponding reduction in the second peak (9.6 -10.1 ml) (Fig.4-2). Additionally, HLBLG exhibited significantly a greater Mw on both the first and the second peak than HBLG ($p < 0.05$) (Fig.4-2, Table 4-2). The second peak of HLBLG had a greater Mw although the height of the peak was smaller than the second peak of HBLG. It might be due to small molecules agglomerated together forming larger aggregates, and the same observation was monitored in the study where whey protein aggregates induced by the different heating temperature (Beaulieu, Corredig, Turgeon, Wicker, & Doublier, 2005). On the contrary, the first peak exhibited a larger peak (more amounts of protein) and Mw also increased compared to HBLG. It indicates that more protein polymerization was induced by the presence of laccase.

RMS was not significantly different between HLBLG and HBLG (Table 4-2). Also, RMS distribution was not different between HBLG and HLBLG (Data not shown). In a previous study, Mw and RMS increased as aggregation of BLG increased more by heat treatment (Bauer, Carrotta, Rischel, & Ogendal, 2000). These results showed that laccase treatment induced cross-linking with heat, but the shape of aggregates was not affected by cross-linking.

Surface thiol (SH) groups and tyrosine determination

BLG has one free thiol (SH) group located at position Cys121, out of four cysteine residues, but it is hidden within the hydrophobic interior of the native BLG (Shimada & Cheftel, 1989). Most tyrosine residues in BLG are buried with other hydrophobic side chains in 3-

dimensional structure (Uhrinova, Smith, Jameson, Uhrin, Sawyer, & Barlow, 2000). The free SH groups and some hydrophobic groups are exposed and become reactive as the protein partially unfolds with dissociation of dimer by heat treatment. The amount of SH groups increased when temperature and treatment time increased (Sava, Van der Plancken, Claeys, & Hendrickx, 2005). Since free surface SH groups are reactive by heating leading to the aggregation through the disulfide interchange reaction, it is an important tool to investigate the structure of BLG (Manderson, Creamer, & Hardman, 1999; Roefs & De Kruif, 1994). The changes of surface SH groups and tyrosine residues in BLG, resulting from laccase and heat treatment are presented in Fig. 4-4 and 4-5. The surface SH groups increased significantly ($p < 0.05$) with heat treatment and in the presence of laccase with and without heat (Fig. 4-4). Laccase alone affected the protein surface, unfolding, and resulted in more exposed surface SH. HLBLG showed the greatest increase in accessible SH groups (Fig. 4-4). Tyrosine also residues increased as a result of heat treatment but were not significantly affected by the laccase treatment ($p < 0.05$) (Fig. 4-5).

Influence of laccase on SBP and BLG complexes

The RI elution profiles of complexes of SBP and BLG, treated with and without laccase are presented in Fig. 4-6. Two peaks were observed and the first peak eluted between 6.7 ml to 9 ml and the second one was shown between 9.6 ml to 10 ml. The height of peaks was reduced in the mixed sample of SBP and laccase treated BLG (LBLG) on both peaks. Note that the overall concentration decreased in response to the addition of laccase; LBLG-S had the lowest peak height among samples (Fig. 4-6). The reduction in overall concentration might result from production of conjugation as two and more molecules were cross-linked by laccase. In addition, it was observed that the apex of the first peak in LBLG-S shifted toward earlier elution volume, around 7.7 ml from 7.9 ml (Fig. 4-6). This result indicated that LBLG-S had greater M_w than

other samples. The increasing in Mw among mixed samples was more clearly demonstrated in a cumulative Mw distribution plot (Fig.4-7). Cumulative Mw distribution curve presented that laccase treated samples had more amount of higher Mw fractions reducing in smaller Mw fractions than non-laccase treated sample (Fig.4-7). BLG ($\sim 4 \times 10^4$ g/mol) or relatively smaller Mw fractions in SBP might be preferentially incorporated into polymer conjugates. Documentation of conjugation between molecules is typically reported by increased Mw (Flanagan & Singh, 2006; Selinheimo, Lampila, Mattinen, & Buchert, 2008). Flanagan et al. (2006) reported the covalent cross-linking induced by transglutaminase between gum arabic and sodium caseinate was confirmed by shortened elution time and increased Mw measured by HPSEC.

BLG tends to be a poor substrates for laccase because most tyrosine residues are buried inside protein structure, and only Tyr99 has exposed OH group on the surface (Qin, Bewley, Creamer, Baker, Baker, & Jameson, 1998; Uhrinova, Smith, Jameson, Uhrin, Sawyer, & Barlow, 2000). Hetero coupling catalyzed by laccase was not observed between BSA and WEAX (wheat water extractable arabinoxylan) in a previous study (Vansteenkiste, Babot, Rouau, & Micard, 2004). They suggested that BSA was embedded in a WEAX gel network rather than forming hetero coupling. Typical evidences of conjugation, e.g. increased Mw and decreased polymer concentration detected by RI, were found in this study especially in laccase treated samples (LBLG-S, LBLG-LS) (Fig.4-6, Fig.4-7). However, the amount of tyrosine residues was not significantly affected by laccase (Fig.4-5). LBLG-LS showed an almost identical protein profile in UV₂₈₀ chromatograph with BLG-S, and also LBLG-S showed a reduced peak only where BLG eluted (data not shown). Laccase might work on SBP predominantly inducing cross-links in SBP molecules, and BLG might be embedded within conjugated SBP rather than forming hetero-

conjugation with SBP through tyrosine and ferulic acid.

The changes of ferulic acid monitored by UV₃₂₅ are displayed in Fig. 4-8. The two poorly resolved peaks eluted between 6.8 ml and 9.7 ml, and most of the ferulic acid in SBP eluted in the first peak between 6.8 ml and 8.6 ml. As shown in RI chromatograph, the apex of the SBP shifted toward earlier elution volume on mixed sample with LBLG (LBLG-S and LBLG-LS). Also, the mixed samples showed lower ferulic acids than SBP alone. It suggested that ferulic acid contents were influenced not only by the laccase addition but also by the presence of BLG. Laccase treated sample, LBLG-S and LBLG-LS showed lower ferulic acids than the BLG-S sample, and LBLG-LS showed lower ferulic acid contents than LBLG-S (Fig. 4-8). Since ferulic acid is the substrate for laccase in SBP (Carunchio, Crescenzi, Girelli, Messina, & Tarola, 2001; Oosterveld, Beldman, & Voragen, 2000; Ralet, Andre-Leroux, Quemener, & Thibault, 2005), any decrease in ferulic acid is an evidence that ferulic acid is consumed to create cross-links study (Littoz & McClements, 2008).

In this study, BLG was pre incubated with laccase for 20 hours to modify the BLG, next, it was equilibrated with SBP or SBP pre-treated with laccase (LS) for another 20 hours. Since SBP was equilibrated with laccase in advance before mixing with LBLG, homo cross-linking within SBP might be produced in LBLG-LS sample, mainly. This result could be supported by the observation which LBLG-LS had the lowest ferulic acid contents (Fig. 8) but LBLG-S showed greater Mw (Fig. 7) and less concentration than LBLG-LS (Fig. 6).

Conclusions

This study presents the effect of laccase and heat treatment on BLG, and the conjugation of pre-treated BLG with SBP. Mw was not increased in BLG by laccase treatment. However, a conformational change was observed in LBLG, and was confirmed by RMS distribution and

conformation plot, particle size and ζ -potential. LBLG showed smaller and broader RMS distribution, and changed into less condensed structure as results of conformation plot. In addition, enlarged particle size was monitored by DLS particle analyzer. Upon heat treatment, HLBLG induced greater polymerization than HBLG, which was attributable to the result of more revealed SH groups and tyrosine residues by heat. The conjugation formed between BLG and SBP with and without laccase was examined. Laccase treated mixed samples exhibited increased Mw, primarily resulting from conjugation of smaller Mw polymers. Moreover, ferulic acid contents detected at UV₃₂₅ reduced in this order BLG-S>LBLG-S> LBLG-LS, and increased Mw was confirmed by observing peak apex which was shifted toward earlier elution volume at UV₃₂₅ elution profile, once again. However, hetero-conjugation through tyrosine in BLG and ferulic acid in SBP might be doubted since the amount of tyrosine residues did not change even when conformational change was confirmed in BLG by laccase treatment.

Therefore, these results suggest that laccase did not induce the homo-conjugation in BLG but caused the conformational change in BLG. Also, modified BLG enhanced the polymerization by heat treatment. BLG might be trapped within SBP cross-linked conjugation rather than forming hetero-conjugation with SBP through phenol groups.

References

- Bauer, R., Carrotta, R., Rischel, C., & Ogendal, L. (2000). Characterization and isolation of intermediates in beta-lactoglobulin heat aggregation at high pH. *Biophysical Journal*, 79(2), 1030-1038.
- Beaulieu, M., Corredig, M., Turgeon, S. L., Wicker, L., & Doublier, J.-L. (2005). The formation of heat-induced protein aggregates in whey protein/pectin mixtures studied by size exclusion chromatography coupled with multi-angle laser light scattering detection. *Food*

- Hydrocolloids*, 19(5), 803-812.
- Brandrup, J., Immergut, E. H., Grulke, E. A., Abe, A., & Bloch, D. R. (1999). *Polymer Handbook* (4th ed.). New York: John Wiley & Sons.
- Carunchio, F., Crescenzi, C., Girelli, A. M., Messina, A., & Tarola, A. M. (2001). Oxidation of ferulic acid by laccase: identification of the products and inhibitory effects of some dipeptides. *Talanta*, 55(1), 189-200.
- Chen, T. H., Embree, H. D., Brown, E. M., Taylor, M. M., & Payne, G. F. (2003). Enzyme-catalyzed gel formation of gelatin and chitosan: potential for in situ applications. *Biomaterials*, 24(17), 2831-2841.
- Corredig, M., & Wicker, L. (2001). Changes in the molecular weight distribution of three commercial pectins after valve homogenization. *Food Hydrocolloids*, 15(1), 17-23.
- Einhorn-Stoll, U., Ulbrich, M., Sever, S., & Kunzek, H. (2005). Formation of milk protein-pectin conjugates with improved emulsifying properties by controlled dry heating. *Food Hydrocolloids*, 19(2), 329-340.
- Faergemand, M., Otte, J., & Qvist, K. B. (1997). Enzymatic cross-linking of whey proteins by a Ca²⁺-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.
- Figueroa-Espinoza, M. C., Morel, M. H., & Rouau, X. (1998). Effect of lysine, tyrosine, cysteine, and glutathione on the oxidative cross-linking of feruloylated arabinoxylans by a fungal laccase. *Journal of Agricultural and Food Chemistry*, 46(7), 2583-2589.
- Figueroa-Espinoza, M. C., & Rouau, X. (1998). Oxidative Cross-Linking of Pentosans by a

- Fungal Laccase and Horseradish Peroxidase: Mechanism of Linkage Between Feruloylated Arabinoxylans¹. *Cereal Chemistry*, 75(2), 259-265.
- Flanagan, J., & FitzGerald, R. J. (2002). Physicochemical and nitrogen solubility properties of *Bacillus* proteinase hydrolysates of sodium caseinate incubated with transglutaminase pre- and post-hydrolysis. *Journal of Agricultural and Food Chemistry*, 50(19), 5429-5436.
- 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.
- Fox, P. F., & McSweeney, P. L. H. (2003). *Advanced dairy chemistry* (3rd ed.). New York: Kluwer Academic/Plenum.
- Gianfreda, L., Xu, F., & Bollag, J-M. (1999). Laccases: a useful group of oxidoreductive enzymes. *Bioremediation Journal*, 3, 1-25.
- 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.
- Hoffmann, M. A. M., Roefs, S., Verheul, M., VanMil, P., & DeKruif, K. G. (1996). Aggregation of beta-lactoglobulin studied by in situ light scattering. *Journal of Dairy Research*, 63(3), 423-440.
- Ikeda, S. (2003). Atomic force microscopy and Raman scattering spectroscopy studies on heat-induced fibrous aggregates of beta-lactoglobulin. *Spectroscopy-an International Journal*, 17(2-3), 195-202.
- James, J., & Simpson, B. K. (1996). Application of enzymes in food processing. *Critical Reviews*

- in Food Science and Nutrition*, 36(5), 437-463.
- 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.
- Labat, E., Morel, M. H., & Rouau, X. (2000). Effects of laccase and ferulic acid on wheat flour doughs. *Cereal Chemistry*, 77(6), 823-828.
- 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.
- Manderson, G. A., Creamer, L. K., & Hardman, M. J. (1999). Effect of heat treatment on the circular dichroism spectra of bovine beta-lactoglobulin A, B, and C. *Journal of Agricultural and Food Chemistry*, 47(11), 4557-4567.
- Matheis, G., & Whitaker, J. R. (1984). Modification of Proteins by Polyphenol Oxidase and Peroxidase and their products. *Journal of Food Biochemistry*, 8(3), 137-162.
- 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.
- 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.
- 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., Schols, H. A., & Voragen, A. G. J. (1996). Arabinose and ferulic

- acid rich pectic polysaccharides extracted from sugar beet pulp. *Carbohydrate Research*, 288, 143-153.
- 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.
- Oudgenoeg, G., Hilhorst, R., Piersma, S. R., Boeriu, C. G., Gruppen, H., Hessing, M., Voragen, A. G. J., & Laane, C. (2001). Peroxidase-mediated cross-linking of a tyrosine-containing peptide with ferulic acid. *Journal of Agricultural and Food Chemistry*, 49(5), 2503-2510.
- 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.
- Qi, X. L., Holt, C., McNulty, D., Clarke, D. T., Brownlow, S., & Jones, G. R. (1997). Effect of temperature on the secondary structure of beta-lactoglobulin at pH 6.7, as determined by CD and IR spectroscopy: A test of the molten globule hypothesis. *Biochemical Journal*, 324, 341-346.
- Qin, B. Y., Bewley, M. C., Creamer, L. K., Baker, H. M., Baker, E. N., & Jameson, G. B. (1998). Structural basis of the tanford transition of bovine beta-lactoglobulin. *Biochemistry*, 37(40), 14014-14023.
- Ralet, M. C., Andre-Leroux, G., Quemener, B., & Thibault, J. F. (2005). Sugar beet (*Beta vulgaris*) pectins are covalently cross-linked through diferulic bridges in the cell wall. *Phytochemistry*, 66(24), 2800-2814.
- Reddy, I. M., Kella, N. K. D., & Kinsella, J. E. (1988). Structural and conformational basis of the resistance of beta-lactoglobulin to peptic and chymotryptic digestion. *Journal of Agricultural and Food Chemistry*, 36(4), 737-741.

- Relkin, P. (1998). Reversibility of heat-induced conformational changes and surface exposed hydrophobic clusters of beta-lactoglobulin: their role in heat-induced sol-gel state transition. *International Journal of Biological Macromolecules*, 22(1), 59-66.
- 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.
- Roefs, S. P., & De Kruif, K. G. (1994). A model for the denaturation and aggregation of beta-lactoglobulin. *European Journal Of Biochemistry / FEBS*, 226(3), 883-889.
- Santipanichwong, R., Suphantharika, M., Weiss, J., & McClements, D. J. (2008). Core-shell biopolymer nanoparticles produced by electrostatic deposition of beet pectin onto heat-denatured beta-lactoglobulin aggregates. *Journal of Food Science*, 73(6), N23-N30.
- Sava, N., Van der Plancken, I., Claeys, W., & Hendrickx, M. (2005). The kinetics of heat-induced structural changes of beta-lactoglobulin. *Journal of Dairy Science*, 88(5), 1646-1653.
- Schmitt, C., Bovay, C., Rouvet, M., Shojaei-Rami, S., & Kolodziejczyk, E. (2007). Whey protein soluble aggregates from heating with NaCl: Physicochemical, interfacial, and foaming properties. *Langmuir*, 23(8), 4155-4166.
- Schokker, E. P., Singh, H., Pinder, D. N., Norris, G. E., & Creamer, L. K. (1999). Characterization of intermediates formed during heat-induced aggregation of beta-lactoglobulin AB at neutral pH. *International Dairy Journal*, 9(11), 791-800.
- 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.
- Shimada, K., & Cheftel, J. C. (1989). Sulfhydryl-group Disulfid bond Interchange Reactions during Heat-induced Gelation of Whey-Protein Isolate. *Journal of Agricultural and Food*

- Chemistry*, 37(1), 161-168.
- 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.
- Van Dijk, J. A. P. P., & Smit, J. A. M. (2000). Size-exclusion chromatography-multiangle laser light scattering analysis of beta-lactoglobulin and bovine serum albumin in aqueous solution with added salt. *Journal of Chromatography A*, 867(1-2), 105-112.
- Vansteenkiste, E., Babot, C., Rouau, X., & Micard, V. (2004). Oxidative gelation of feruloylated arabinoxylan as affected by protein. Influence on protein enzymatic hydrolysis. *Food Hydrocolloids*, 18(4), 557-564.
- 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.
- Walstra, P., Wouters, J. T. M., & Geurts, T. J. (2006). *Dairy science and technology* (2nd ed.). Boca Raton: CRC/Taylor & Francis.
- Weinbreck, F., Minor, M., & De Kruif, C. G. (2004). Microencapsulation of oils using whey protein/gum arabic coacervates. *Journal of Microencapsulation*, 21(6), 667-679.

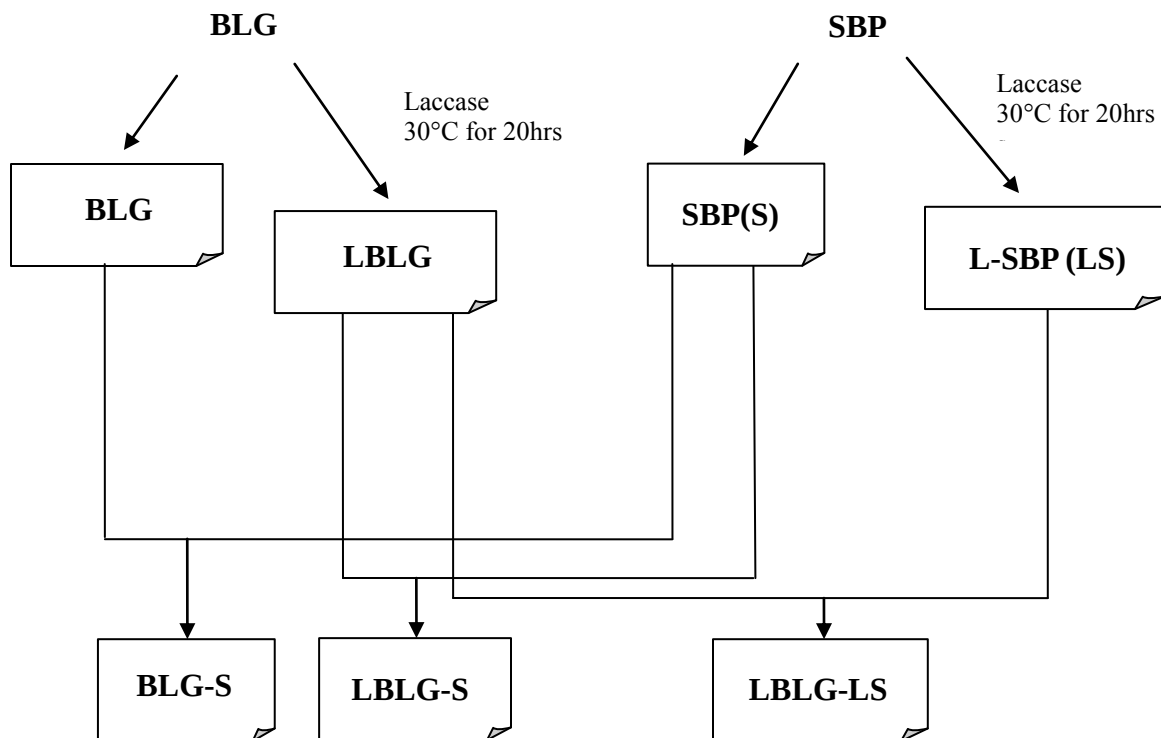


Fig. 4-1. Flow diagram of laccase mediated SBP and BLG sample preparation.

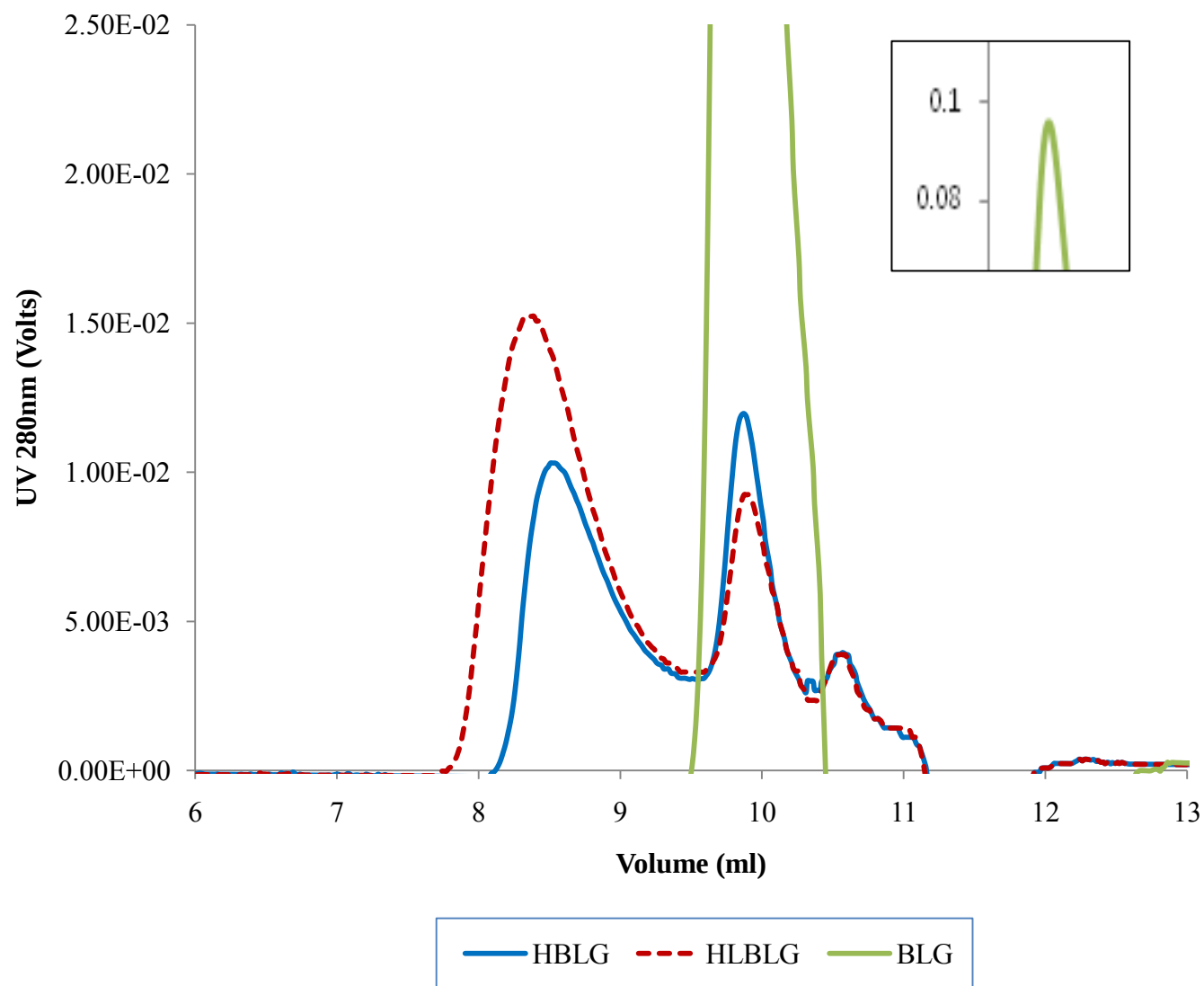


Fig. 4-2. UV elution profile of BLG, heat treated BLG (HBLG) and laccase pre-treated BLG with heat treatment (HLBLG) monitored at 280nm.

Table 4-1. Physical properties of BLG and LBLG (laccase treated BLG).

	Mw (g/mol)	RMS (nm)	Slope	Particle size (nm)	ζ-potential (mV)
BLG	37511.6 ± 5517.9 ^a	41.2 ± 4.3 ^a	0.35 ± 0.09 ^a	222.2 ± 61.8 ^a	-25.7 ± 3.9 ^a
LBLG	29230.0 ± 1636.4 ^b	36.5 ± 2.7 ^b	0.72 ± 0.18 ^b	1278.4 ± 161.2 ^b	-7.3 ± 0.9 ^b

^{a,b} : Same letters are not significantly different between BLG and LBLG within the same column (p<0.05).

Mw: Weight average molecular weight

RMS: Root Mean Square radius determined by HPSEC-MALLS

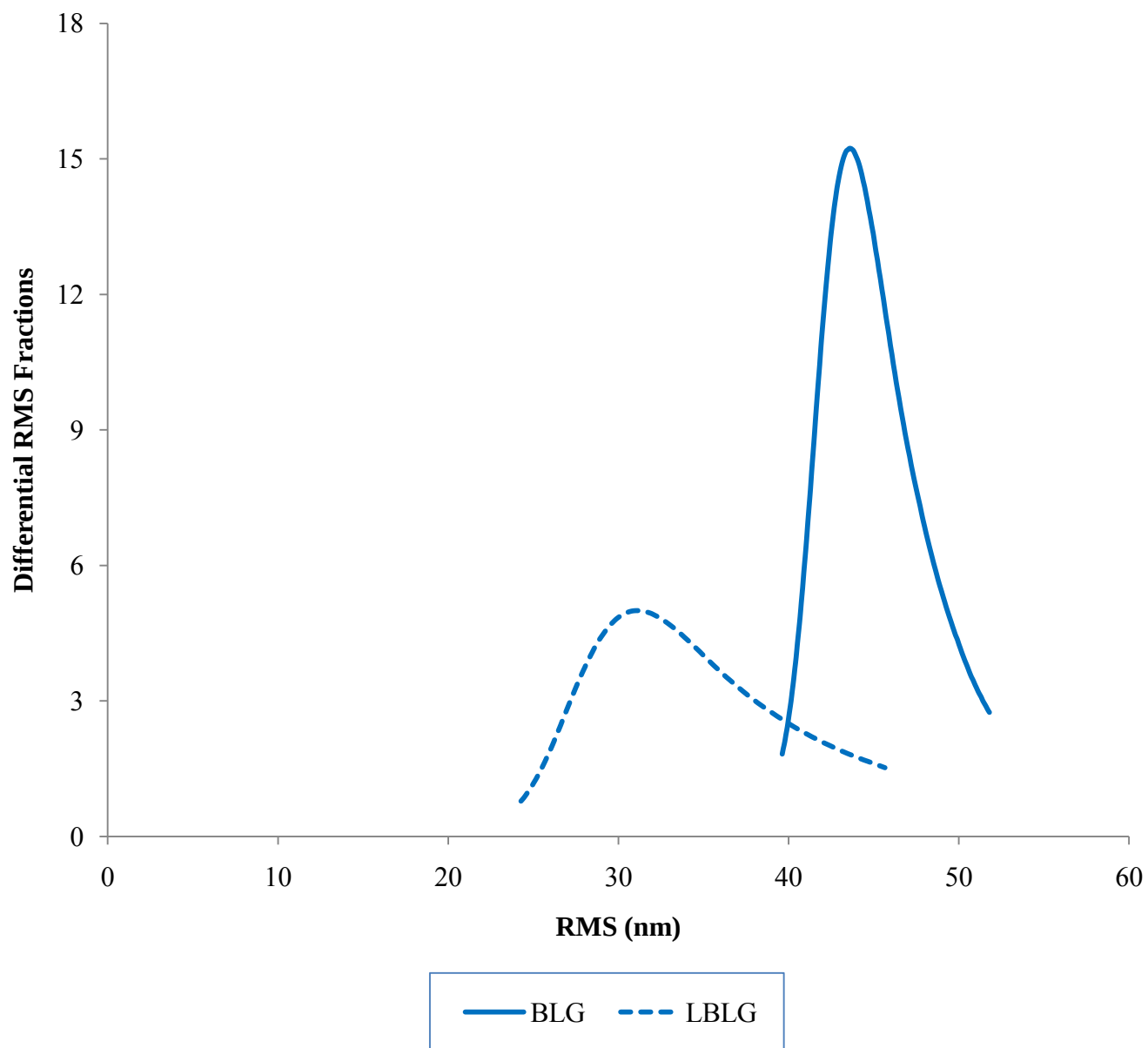


Fig. 4-3. Differential RMS distribution of BLG and LBLG.

Table 4-2. Physical properties of HBLG and HLBLG

		Mw (g/mol)	RMS (nm)	Particle size (nm)	ζ-potential (mV)
HBLG	1st	$2.9 \times 10^6 \pm 3.5 \times 10^{5a}$	20.3 ± 2.7^a	72.6 ± 11.0	-41.7 ± 7.4^a
	2nd	$0.7 \times 10^6 \pm 2.2 \times 10^{5A}$	23.6 ± 3.3^A		
HLBLG	1st	$4.4 \times 10^6 \pm 8.6 \times 10^{5b}$	23.4 ± 5.7^a	85.5 ± 5.4	-28.6 ± 4.0^b
	2nd	$1.4 \times 10^6 \pm 2.0 \times 10^{5B}$	27.4 ± 3.1^A		

^{a,b} : Same letters are not significantly different between HBLG and LHBLG within the same column ($p < 0.05$) on the 1st peak.

^{A,B} : Same letters are not significantly different between HBLG and LHBLG within the same column ($p < 0.05$) on the 2st peak.

Mw: Weight average molecular weight

RMS: Root Mean Square radius

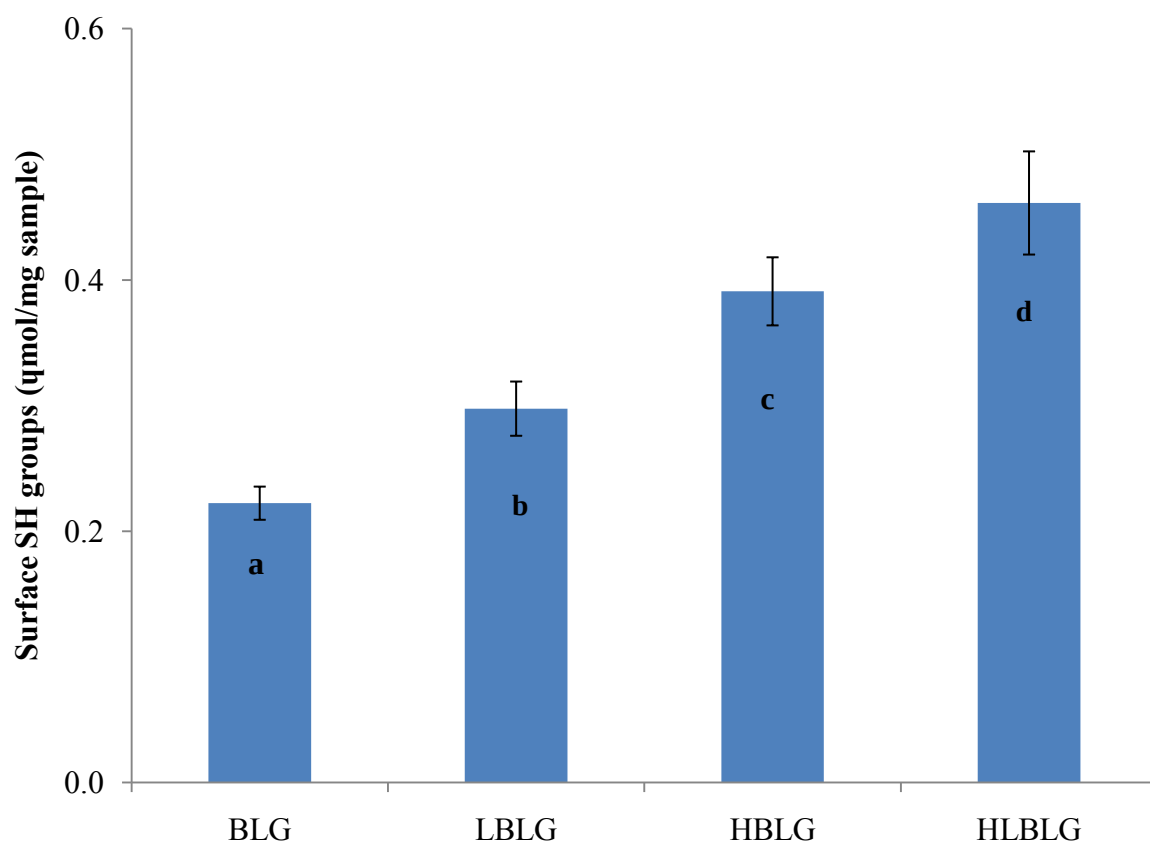


Fig. 4-4. Changes of Surface SH groups of BLG treated with laccase and heat treatment.

^{a,b,c,d} : Same letters are not significantly different among samples ($p < 0.05$).

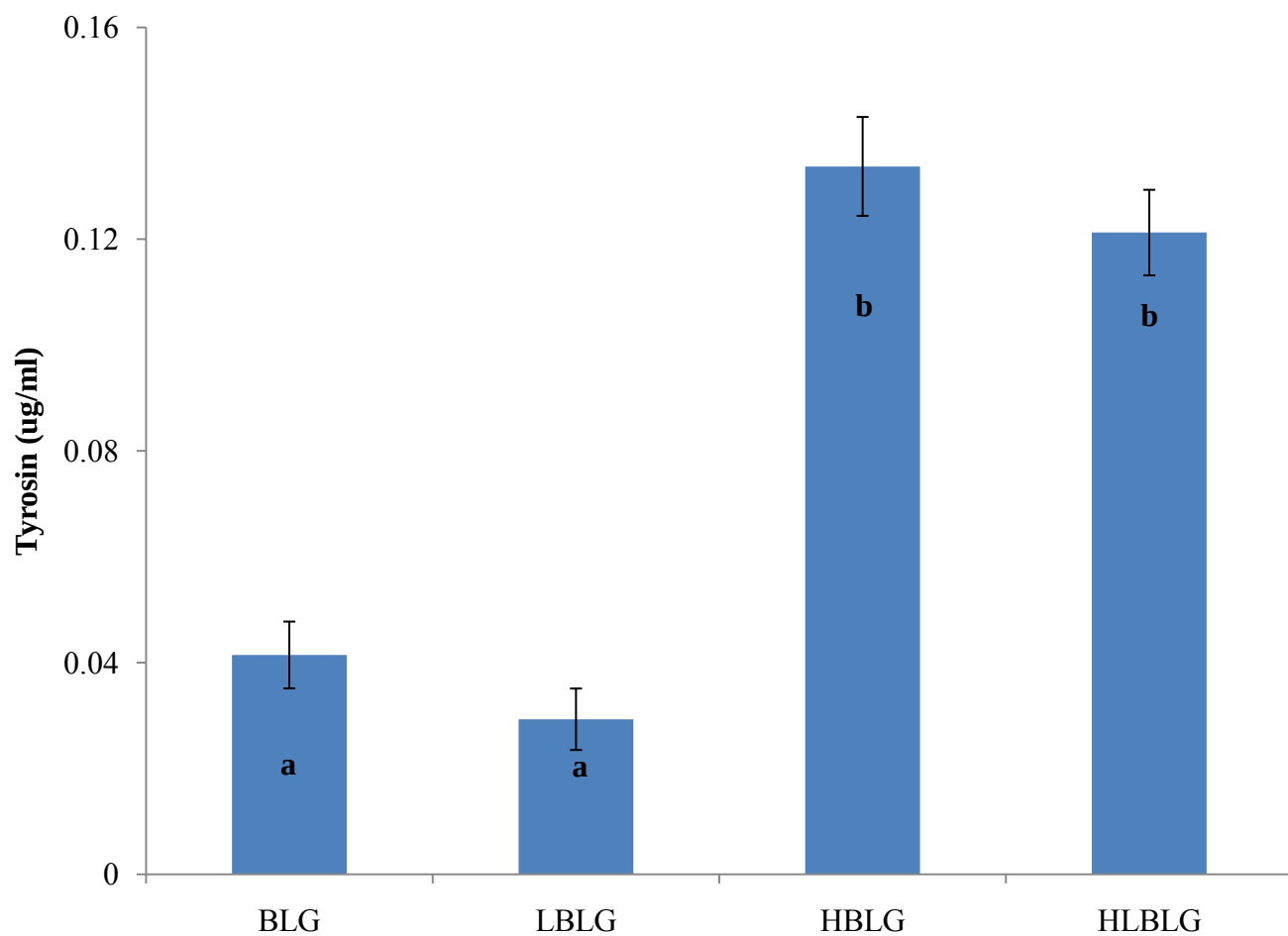


Fig. 4-5. Changes of Tyrosine residue of BLG treated with laccase and heat treatment.

^{a,b} : Same letters are not significantly different among samples ($p < 0.05$).

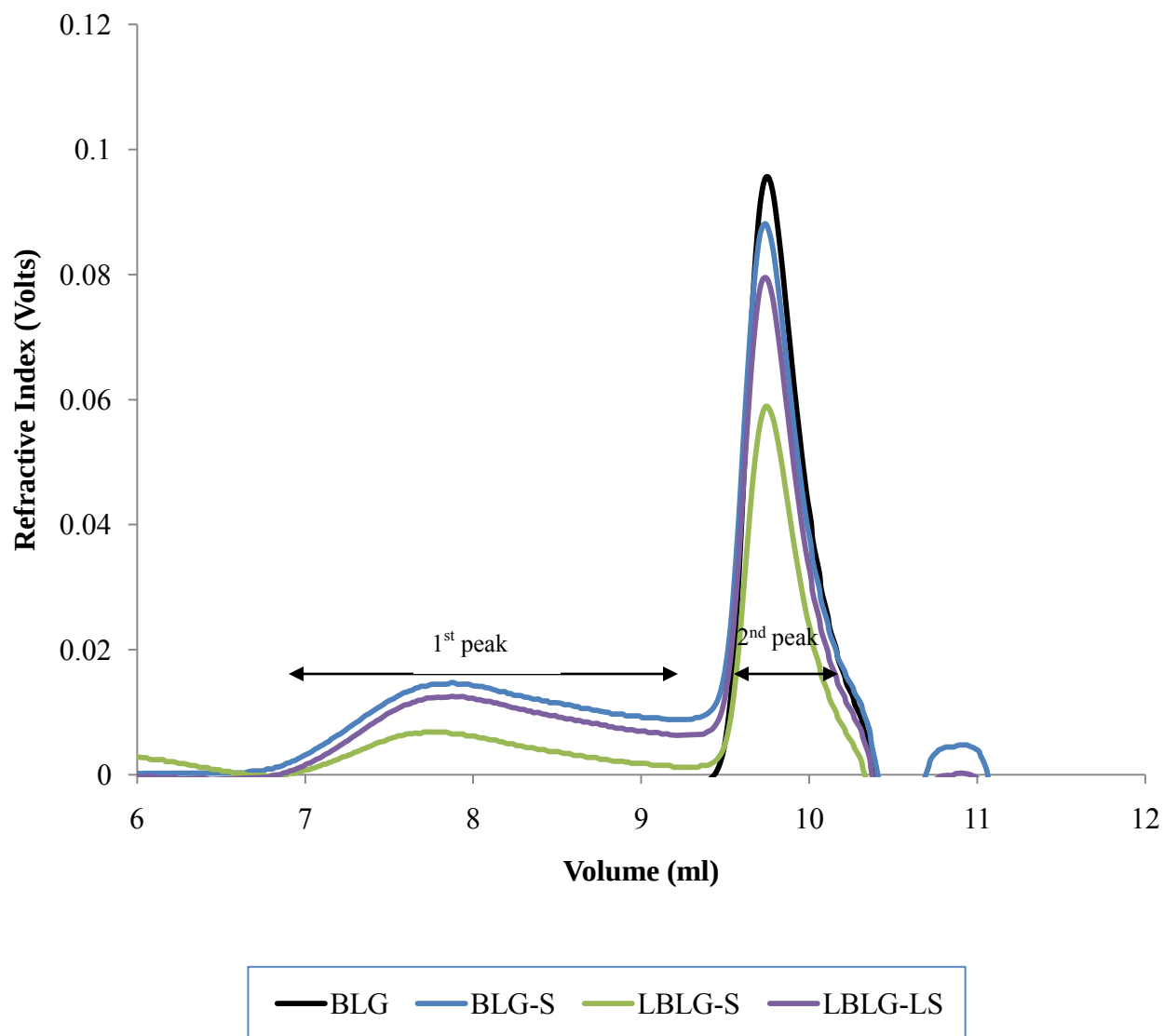


Fig. 4-6. RI (Refractive Index) elution profile of complexes of SBP and BLG pre-incubated with and without laccase.

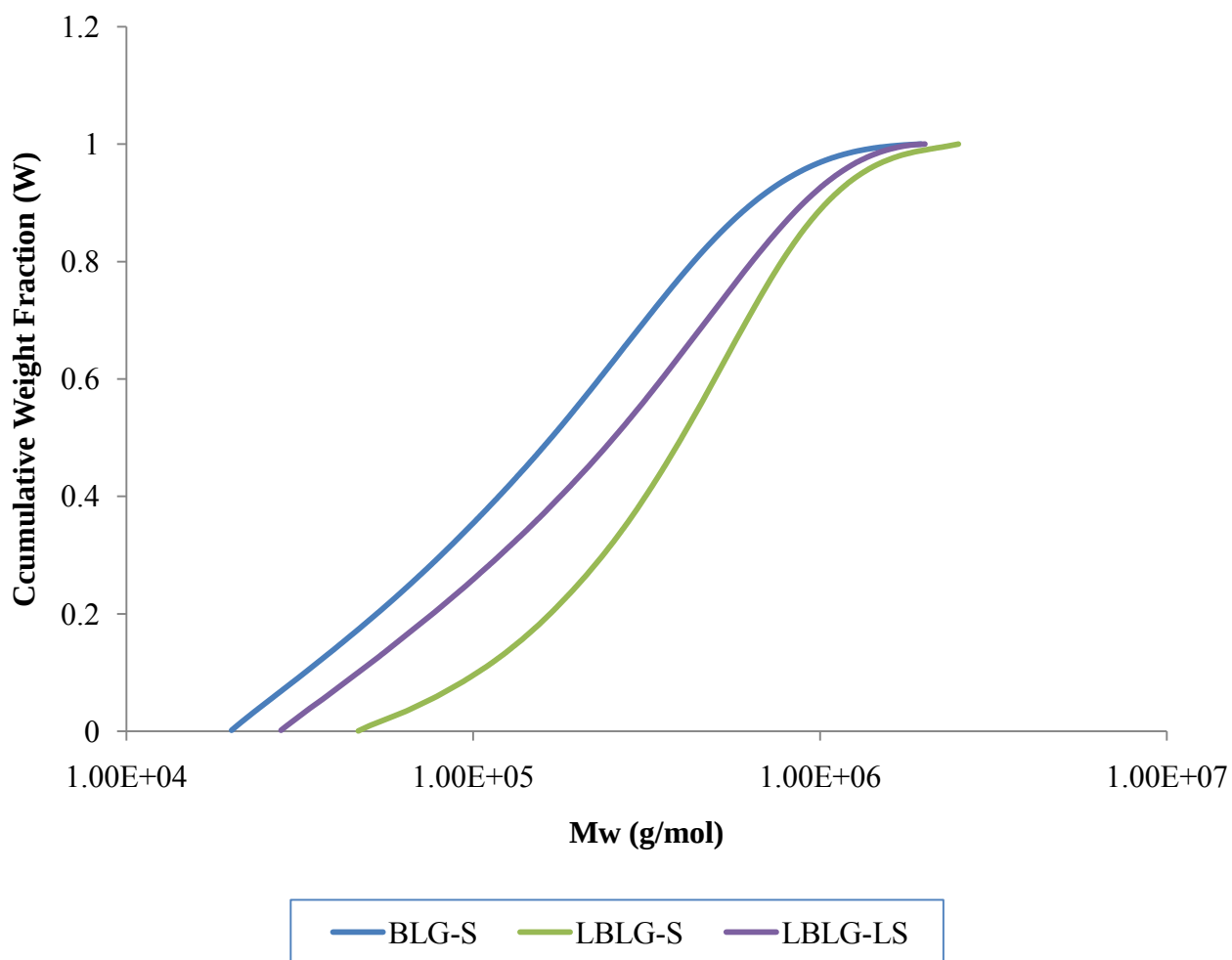


Fig 4-7. Cumulative Mw distribution of complexes between SBP and BLG pre-incubated with and without laccase

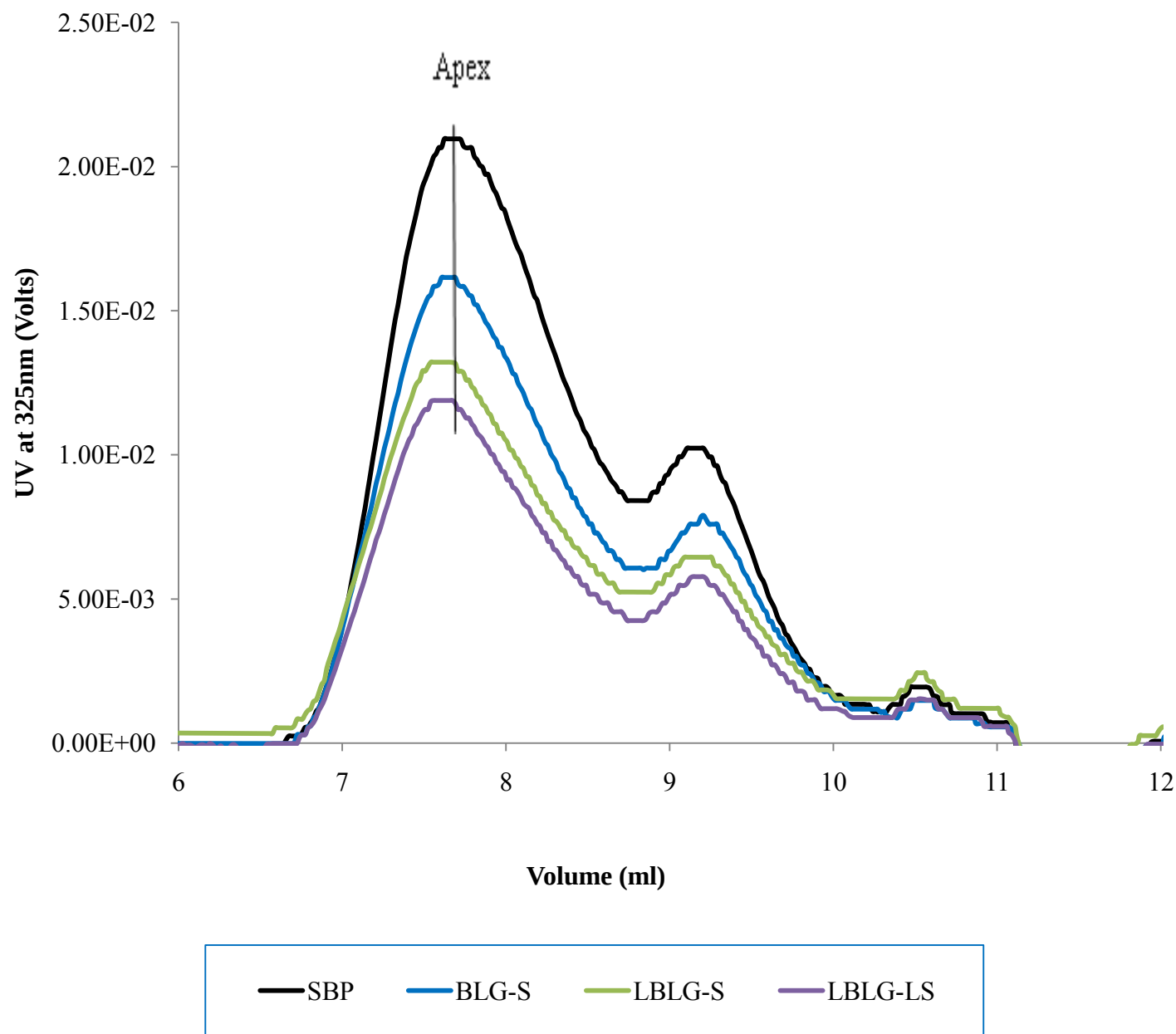


Fig.4-8. UV elution profile monitored at 325 nm of SBP and SBP-BLG interacted sample with and without laccase.

CHAPTER 5

LACCASE MEDIATED CONJUGATION OF HEAT TREATED

β -LACTOGLOBULIN AND SUGAR BEET PECTIN¹

¹ Jung, J., & Wicker, L., To be submitted *Food hydrocolloids*, 2011

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 β -lactoglobulin (BLG). The conjugation of SBP and heated BLG was confirmed by peak position using size exclusion chromatography, multi angle laser light scattering, refractive index, and UV detection. Pre-treated BLG with laccase eluted at an earlier volume with greater absorbance than the untreated dispersions. Tyrosine decreased in BLG that contained laccase treated SBP samples. The results suggest that heat enhanced exposure of tyrosine in BLG and improved conjugation with SBP by laccase. BLG-SBP conjugates had ~40 % higher solubility at pH values near the isoelectric point of BLG and showed less shear thinning behavior.

INDEX WORDS: Sugar beet pectin, β -lactoglobulin, Laccase, Conjugation, Solubility

Introduction

Much interest has focused on the interacted complexes between proteins and polysaccharides because these interacted complexes enhance functional properties, such as solubility and gelling as well as forming and emulsifying stability better than that of lone polymer (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 as well as food industry, such as fat replacer, cream substitute, and oil substitute (Goldberg, Langer, & Jia, 2007; Kloser & Sheuring, 1954; Madene, Jacquot, Scher, & Desobry, 2006; Pettersson, Johnsson, Brannas, & Pickova, 2009; Renard, Robert, Lavenant, Melcion, Popineau, Gueguen, et al., 2002).

A complex formation between proteins and polysaccharides could be achieved mainly 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, Okada, & Takahashi, 2000; Kato, 2002; Kato, Wada, Kobayashi, Seguro, & Motoki, 1991; 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 enzyme is safe, non-toxic food ingredient and also improves food nutritional quality (Flanagan & Singh, 2006; James & Simpson, 1996).

Various enzymes have been studied to form conjugation and improve functionality of proteins and polysaccharides conjugation (Boeriu, Oudgenoeg, Spekking, Berendsen, Vancon, Boumans, et al., 2004; T. H. Chen, Vazquez-Duhalt, Wu, Bentley, & Payne, 2001; Faergemand, Otte, & Qvist, 1997, 1998; Kuuva, Lantto, Reinikainen, Buchert, & Autio, 2003; Littoz & McClements, 2008; Selinheimo, Lampila, Mattinen, & Buchert, 2008; Tianhong Chen, 2002; Walsh, Cleary, McCarthy, Murphy, & FitzGerald, 2003). Transglutaminase is used to work on protein molecules by catalyzing acyl transfer between a λ -carboxyamide of a protein bound glutamine (as a donor) and lysine (as an acceptor) (Fork, 1977). It has been reported that transglutaminase induced the covalent cross-linking successfully between gum arabic containing protein and sodium caseinate (Flanagan & Singh, 2006). Laccase and tyrosinase were also used to form conjugates between phenolic compounds present in proteins and polysaccharides (T. H. Chen, Vazquez-Duhalt, Wu, Bentley, & Payne, 2001; Flanagan & Singh, 2006; Selinheimo, Lampila, Mattinen, & Buchert, 2008). It has been found that enzyme mediated conjugation showed improved functionality. Emulsion oil droplets coated with cross-linked SBP through laccase showed greater stability than non cross-linked SBP at pH 4.5 with NaCl addition (B. C. Chen, McClements, Gray, & Decker; Littoz & McClements, 2008). Furthermore, the conjugation of BLG and cationic polysaccharide which was catalyzed by microbial transglutaminase, improved the emulsifying properties (Ikeuchi, Aoki, Yoshida, Takahashi, & Hattori, 2008).

The aim of this study was to determine a covalent conjugation between SBP and heat denatured BLG with the addition of laccase as well as improved functional properties. Laccase (p-diphenol oxidase, EC 1.10.3.2) can oxidize various phenolic compounds including tyrosine

containing peptides, cysteine and tryptophan in whey protein (Gianfreda, 1999; M.-L. Mattinen, Kruus, Buchert, Nielsen, Andersen, & Steffensen, 2005; M. L. Mattinen, Hellman, Permi, Autio, Kalkkinen, & Buchert, 2006; Micard & Thibault, 1999). Therefore, formation of homo and hetero cross-link between phenol compounds, tyrosine in BLG and ferulic acid or protein residue in SBP through laccase could be suggested, theoretically (Boeriu, et al., 2004; Neukom & Markwalder, 1977). As described in an earlier study, heated BLG showed more tyrosine than native BLG, as a result, the heated BLG could enhance the conjugation with SBP and modify the functionalities of the conjugation.

Materials & Methods

Dry powder β -lactoglobulin (BLG) (93.6%) was provided by Davisco Foods International (Le Sueur, MN) and contained 93.6% BLG content of total protein as stated by the manufacturer. Sugar beet pectin (SBP) was donated by Herbstreith & Fox KG (Elmsford, NY). 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).

Stock preparation

SBP and BLG dispersions were prepared by dispersing 3% (w/v) dry power in 100 mM sodium phosphate buffer (pH 6.5) separately, stirred at room temperature for at least 2 hours and stored overnight at 4 °C for complete hydration. Each dispersion was centrifuged for 30 min at 5000 g, 4 °C to remove insoluble materials (Sorvall RC 6Plus, Thermo scientific products).

Laccase activity measurement

Laccase activity was measured through a modified procedure of the previously described method using syringaldazine as substrate at 30 °C (Ride, 1980). The laccase was dissolved in

cold, deionized water and stirred for 10 min, filtered through 5 μ m PVDF (polyvinylidene fluoride) syringe filters (Millipore, Bedford, MA). After 2.2 ml sodium phosphate buffer (100 mM, pH 6.5) was mixed with 0.5 ml of diluted laccase solution, 0.3 ml syringaldazine (0.216 mM in methanol) added and measurement was followed promptly. The loss in absorbance at 530 nm per unit of time was used to assess activity.

Laccase mediated SBP & BLG conjugation

The sample preparation is presented in Fig. 5-1. The centrifuged SBP and BLG dispersions were equilibrated with and without laccase in a water bath (30 °C) for 20 hours with mild agitation on an orbital shaker (PR 70 Red Rotar 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. An aliquot of water was added to the controls. The laccase treated and non-laccase treated BLG dispersions were heated at 80 °C for 15 min and then mixed with laccase treated SBP or without laccase treated SBP at the same ratio. These SBP and BLG mixtures were equilibrated for another 20 hour at ambient temperature. The samples were diluted by 10 fold to slow down laccase activity or were analyzed immediately to reduce complications from further enzymatic activity. Further diluted laccase showed very low activities enough to inhibit further enzymatic action and it was confirmed by measurement of activity substrate with syringaldazine described below in the activity methodology.

Particle size measurement

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

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. The separation was achieved by a PL-Aquagel-OH guard column (50×7.5 mm) and PL-Aquagel-OH mix columns (300 × 7.5 mm) (Polymer Laboratories, Inc., Amherst, MA) with a flow rate of 0.5 mL/min, with 10 mM of Na-Phosphate and 100 mM of Na-Nitrate, pH 7 as elution buffer. Mixed samples (3 mg/ml) and heated BLG (1.5 mg/ml) were filtered through 0.8 µm MCE (mixed cellulose ester) syringe filters (Millipore, Bedford, MA) and the injection volume was 0.05 ml. Specific refractive index increment (dn/dc) values of 0.131 and 0.180 were used for SBP and BLG, respectively and 0.961 g⁻¹cm⁻¹ was used as extinction coefficient value of BLG (Corredig & Wicker, 2001; Van Dijk & Smit, 2000). Elution and molecular weight of BLG was monitored with a UV detector at 280 nm and SBP was detected at 325 nm.

Determination of tyrosine

The procedure to determine tyrosine in samples was adapted from a previous method (Hassan, 1975). A mixture of 0.9 ml samples (protein 0.1% w/v) and 1 ml 16 M nitric acid was heated in a water bath for 15 min at 50 °C. After cooling to ambient temperature, 4 ml sodium hydroxide (5 M) and 4 ml ethyl alcohol were added, and absorbance was measured at 360 nm and 430 nm using UV-visible spectrophotometer (UV-1700, Shimadzu scientific instrument, Columbia, MD USA). The concentration of tyrosine (µg/ml) was determined by the following equation.

$$\frac{260 A_{430\text{nm}} - 150 A_{360\text{nm}}}{225 \times 260 - 110 \times 150} = 0.5357 A_{430\text{nm}} - 0.35714 A_{360\text{nm}}$$

Solubility

The samples were diluted with Milli-Q water at 1:10 ratio and then pH was adjusted to the pH2, 4, 5, 7 per each sample with 1 M, 0.5 M HCl and 0.1 NaOH solutions as required. The pH adjusted samples were under moderate stirring at 37 °C for 1hour. The pH was re-checked and re-adjusted as needed in the middle of stirring. The samples were then centrifuged (Sorvall RC 6Plus, Thermo scientific products) for 30 min at 10,000 g at 20 °C. The supernatant were collected and the soluble protein quantification 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 contents in supernatant from the untreated protein contents.

Rheological measurement

The rheological property was investigated by a Rheometric Scientific SR5000 Controlled Stress Dynamic RheometerTM (Rheometrics, Piscataway, NJ, U.S.A) equipped with cone and plate apparatus (60 mm, 0.0385, 0.5 mm gap) and were maintained at 20 °C on all measurement. A dynamic stress test was performed with a 0.1 Hz frequency.

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 ($p < 0.05$).

Results

Conjugation properties

The UV₂₈₀ elution profiles for the presence of protein in mixed samples are presented in Fig. 5-2. Heated BLG (HBLG) alone eluted as two peaks between 8.2 ~ 9.0 ml and 9.5 ~ 10.1 ml volume; the first peak was attributed to aggregation of BLG and the second peak was attributed to dimeric BLG. BLG mixed with SBP with and without laccase eluted, between 6.8 and 10.1 ml, covering a broader and more extended elution volume than HBLG (Fig. 5-2). This elution profile results indicate that protein was eluted with SBP at 6.8 ~ 8.4 ml corresponding to a reduction of absorbance where HBLG eluted (8.2 ~ 10.1 ml) (Fig. 5-2). The peak of SBP spreads from 6.8 to 9.6 ml, and is shown in fig. 5-3 (UV₃₂₅ elution profile). It suggests that some parts of HBLG were dissociated and eluted at an earlier elution volume due to forming 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 is not likely to occur between BLG and SBP. Nevertheless, some non-covalent adsorption was noticed by the increased absorbance in the elution volume between 6.8 and 8.4 ml in HBLG-S sample without laccase (Fig. 5-2). 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. 5-2).

As observed in the UV₂₈₀ elution profile, HLBLG-S, the mixture of laccase treated HBLG and SBP, had the biggest peak in the 6.8 ~ 8.2 ml elution range (Fig. 5-2) where SBP elutes (Fig. 5-3, SBP at UV₃₂₅). The smallest peak was shown where HBLG eluted in 8.4 ~ 10.1 ml elution volume (Fig. 5-2). It indicated that heat and laccase treatments promoted interaction

between BLG and SBP, and interacted complexes mostly concentrated in the first peak. HLBLG-LS, in which BLG and SBP were independently incubated with laccase prior to mix, showed a bigger peak at 8.2 ~ 8.9 ml, indicating oligomer was more dominant compared to other mixture samples, HLBLG-S and HBLG-S. Furthermore, the peak height of HLBLG-LS at 6.8 ~ 8.2 ml was about the same height peak with HBLG-S but the first peak was more polydisperse at 6.8 ~ 7.0 ml. These results suggested that homo oxidative cross-linking within SBP molecules through the ferulic acids was favored since laccase was incubated with SBP prior to mixing with HLBLG (Fig.5-2). 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 et al. (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 UV₃₂₅ elution profile denoted the elution of SBP that contains ferulic acid (Fig. 5-3), and is the site for oxidative coupling between ferulic acid via laccase (Kuuva, Lantto, Reinikainen, Buchert, & Autio, 2003; M. L. Mattinen, Hellman, Permi, Autio, Kalkkinen, & Buchert, 2006). Ferulic acid was eluted as two peaks near 6.9 ~ 8.7 ml and 9.0 ~ 9.6 ml (Fig. 5-3). Untreated SBP alone showed the highest peak and the peak height was reduced when mixed with heated BLG with laccase and without laccase treatment (Fig. 5-3). The first peaks in HLBLG-LS and HLBLG-S showed extended width on the left part of the peak (6.8 ~ 7.0 ml), suggesting large aggregates were formed. All mixed samples showed lower ferulic acid contents than SBP alone sample, and HLBLG-S had the highest ferulic acid contents among mixed samples. A larger peak in HLBLG-S indicated that oxidative coupling between ferulic acids was interrupted due to hetero cross-linking between ferulic acid in SBP and tyrosine in BLG, or

hetero cross-linking between tyrosine in protein moiety of SBP and tyrosine in BLG, were favored. More homo-conjugations in HLBLG-LS 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 incubated with laccase prior to mixing with BLG.

The amount of tyrosine residues in HBLG was 0.13 $\mu\text{g/ml}$ and decreased when mixed with SBP and laccase (Fig. 5-4). The differences in the tyrosine residues among samples were significant ($p < 0.05$). Tyrosine in HLBLG-S and HLBLG-LS were reduced as much as $\sim 26\%$ and $\sim 41\%$, respectively compared to HBLG-S, and HLBLG-LS showed the lowest concentration, 0.058 $\mu\text{g/ml}$ (Fig.5-4). This indicated that dityrosine between two tyrosine residues or intermolecular cross-links via dityrosine were formed whether they occurred within homo-polymer or hetero-polymer (Gross & Sizer, 1959). 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.5-4). The detected tyrosine residue was no more than 0.01 $\mu\text{g/ml}$ in SBP (Data not shown), and thus may not be a dominant factor for cross-linking. However, Boerie et al (2004) reported that tyrosine residues in polysaccharide could be catalyzed by the oxidoreductase enzyme, so tyrosine residue existing in SBP could not be excluded as one of the substrates for laccase. The contribution of phenolic groups (-OH) 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 (M.-L. Mattinen, Kruus, Buchert, Nielsen, Andersen, & Steffensen, 2005). It could be believed that tyrosine was consumed by oxidative coupling via laccase forming cross-linking whether homo- or hetero- conjugation (Fig. 5-4). The lowest tyrosine concentration in HLBLG-LS could be additional evidence that the homo-

conjugation was more favored within heated BLG through tyrosine (Fig. 5-4). RI detects the concentration of all substances found in the sample whereas UV monitors the concentration of the specific substances detected at a given wavelength. In this study, RI elution profile showed a similar trend with the UV₂₈₀ profile. HBLG-S had a higher peak at 6.8 ~ 8.4 ml and HBLG-LS 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 (Fig. 5-2, 5-5).

In Fig. 5-6, the particle size distribution of HBLG, and mixed samples of SBP and heat treated BLG are presented. HBLG had a single peak around 150 μ m, and an additional smaller size population between 10 μ m and 100 μ m was produced as HBLG mixed with SBP and laccase (Fig. 5-6). Moreover, mixed samples showed the smaller peak than HBLG alone at the second peak, eluted near 150 μ m, and the reduction in the second peak was enhanced with laccase treatment (Fig. 5-6). It has been reported that pectin helps prevent protein aggregation with increasing protein hydrolysis (Cooper, Dubin, Kayitmazer, & Turksen, 2005; Peyron, Mouecoucou, Fremont, Sanchez, & Gontard, 2006). Thus, some aggregates of HBLG might have dissociated by the presence of SBP inducing complexes with SBP and led to a smaller size population. Laccase treated sample, HBLG-S and HBLG-LS showed two clearly separated peaks compared to HBLG-S. HBLG-S exhibited more volume % on the first peak, smaller size population, while showed the smallest peak in the second population peak than HBLG-S and HBLG-LS. HBLG-LS had smaller peak in the large size population than HBLG-S but d_{43} was bigger than other mixed sample (Data not shown). Therefore, the protein polymerization was accelerated within HBLG-LS, and hetero conjugation was more strengthened in HBLG-LS.

Functional properties

The solubility at different pH values (pH 2, 4, 5, 7) are presented in Fig. 5-7 and expressed as soluble protein concentration in the supernatant after centrifugation. Soluble protein % was calculated based on the total protein measured in unheated BLG. Heated BLG showed significantly lower solubility ~ 40 % at pH values close to the isoelectric point (pI) 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. 5-7). Chevalier et al. (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. HLBLG, BLG pre-treated with laccase, had a higher solubility over the entire range of pH than HBLG (Fig. 5-7). In addition, the amount of soluble protein increased significantly more in the presence of SBP at pH above 4 (Fig. 5-7). Glycated BLG with sugars had improved solubility in the range of pI of BLG (Chevalier, Chobert, Popineau, Nicolas, & Haertl, 2001). Protein aggregates might be dissociated by the presence of pectin, and some soluble complexes between BLG and SBP were produced (Jones, Decker, & McClements, 2009; Peyron, Mouecoucou, Fremont, Sanchez, & Gontard, 2006). However, lower solubility was observed in samples mixed with SBP than HBLG alone at pH 2. The negative charge of SBP might form insoluble complexes with positively charged BLG by strong electrostatic interaction (Jourdain, Leser, Schmitt, Michel, & Dickinson, 2008). Laccase treated mixed samples exhibited a higher solubility than HBLG and HBLG-S at pH greater than 4 (Fig. 5-7). At pH 7, solubility was more than 90 % but there was no significant differences between HLBLG-S and HLBLG-LS ($p < 0.05$). 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.

To measure mechanical properties, oscillatory shear force was used. Since samples used in this study were too weak and had more liquid like behavior, viscous (loss) modulus (G''), dissipated energy is presented (Fig. 5-8). In this study, all samples showed shear thinning behavior, exhibiting decreasing G'' with stress. Especially, G'' for HBLG decreased markedly compare to other samples as soon as stress was added to sample. HBLG-S had relatively constant G'' at all stresses when compared with laccase treated samples. HLBLG-S and HLBLG-LS, showed greater G'' than HBLG-S at low shear forces, but G'' was similar at stresses over 1 Pa. Cross-linked complexes through dityrosine or ferulic acid and tyrosine by laccase, exhibited almost 10 fold less liquid properties than non laccase treated sample (Fig. 5-8). However, they did not have strong solid like gel properties. G' showed a similar trend with G'' but was smaller than G'' (data not shown) which indicated those samples in this study had more liquid like and relatively weak gel. Rheological properties could be controlled by various conditions such as ionic strength, pH, polymer concentration and temperature (Clark, 1998). At neutral pH, particulate gels are formed by aggregates induced by intermolecular hydrophobic and electrostatic interaction within partially exposed amphoteric molecules and disulfide cross-linking (Alting, Hamer, de Kruif, & Visschers, 2000; Ikeda & Li-Chan, 2004; Manderson, Creamer, & Hardman, 1999). Ikeda et al. (2004) confirmed that tyrosine residue showed stronger hydrogen bond in particulate gels by raman spectroscopy. In contrast to our result, Figueroa-Espinoza et al. (1998) reported that laccase had no effect on gelation in tyrosine and feruloylated arabinoxylan conjugation even though dimer was produced and ferulic acid was reduced when tyrosine presented. It might be attributed to forming polymerization or cross-linking through more exposed tyrosine and disulfide generated by heating.

Conclusion

The aim of this study was to examine the effect of laccase and heat treatment to form conjugates between BLG and SBP. The conjugation was confirmed by the peak location detected at UV₂₈₀ and UV₃₂₅, and RI detectors. Laccase treated samples eluted earlier and more protein was eluted with SBP as observed in UV₂₈₀ and RI elution profile. Laccase mediated hetero-conjugation through ferulic acid in SBP and tyrosine in BLG was confirmed in HLBLG-S eluting higher protein contents where SBP eluted and more ferulic acids were detected at UV₃₂₅ than HBLG-S and HLBLG-LS. It indicated that laccase mediated cross-linking through the ferulic acid, was interrupted by interacting with tyrosine followed by forming hetero-conjugation. However, homo-conjugation was more dominant in HLBLG-LS within SBP through ferulic acids and BLG through tyrosine residue. It was confirmed by the result of the lowest ferulic acid and tyrosine residue. Conjugated samples showed improved solubility all over range of pH and had ~ 40 % better solubility than HBLG at pH 5, around isoelectric point. It was likely protein precipitation was prevented forming soluble conjugation between SBP and BLG. In addition, conjugated samples, laccase pre-treated showed higher viscous modulus (G'') than non-laccase treated samples at lower shear force but they were disrupted at high shear force. This result supported soluble conjugates formed via laccase treated BLG and SBP showed a relatively liquid like weak gel behavior.

References

- Alting, A. C., Hamer, R. J., de Kruif, G. G., & Visschers, R. W. (2000). Formation of disulfide bonds in acid-induced gels of preheated whey protein isolate. *Journal of Agricultural and Food Chemistry*, 48(10), 5001-5007.
- Boeriu, C. G., Oudgenoeg, G., Spekking, W. T. J., Berendsen, L. B. J. M., Vancon, L., Boumans, H., Gruppen, H., vanBerkel, W. J. H., Laane, C., & Voragen, A. G. J. (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. 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 glycosylated through the Maillard reaction is related to the nature of the sugar. *International Dairy Journal*, 11(3), 145-152.
- Clark, A. H. (1998). *Gelation of globular proteins*. Gaintherburg: Aspen Publishers Inc.
- 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.
- Corredig, M., & Wicker, L. (2001). Changes in the molecular weight distribution of three commercial pectins after valve homogenization. *Food Hydrocolloids*, 15(1), 17-23.
- 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.
- Ikeda, S., & Li-Chan, E. C. Y. (2004). Raman spectroscopy of heat-induced fine-stranded and particulate beta-lactoglobulin gels. *Food Hydrocolloids*, 18(3), 489-498.

- 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 transglutaminase (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.
- 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.
- Manderson, G. A., Creamer, L. K., & Hardman, M. J. (1999). Effect of heat treatment on the circular dichroism spectra of bovine beta-lactoglobulin A, B, and C. *Journal of Agricultural and Food Chemistry*, 47(11), 4557-4567.
- 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.
- 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.
- 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.
- Neukom, H., & Markwalder, H. H. (1977). Oxidation gelation of wheat-flour pentosans-new way of crosslinking polymers. *Cereal Foods World*, 22(9), 458-458.
- 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., Duclairoir, C., Nakache, E., Sanchez, C., & Schmitt, C. (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., Li-Qun Wu Gregory F. Payne. (2002). In vitro protein-polysaccharide conjugation: Tyrosinase-catalyzed conjugation of gelatin and chitosan. *Biopolymers*, 64(6), 292-302.
- Van Dijk, J. A. P. P., & Smit, J. A. M. (2000). Size-exclusion chromatography-multiangle laser light scattering analysis of beta-lactoglobulin and bovine serum albumin in aqueous solution with added salt. *Journal of Chromatography A*, 867(1-2), 105-112.
- 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.

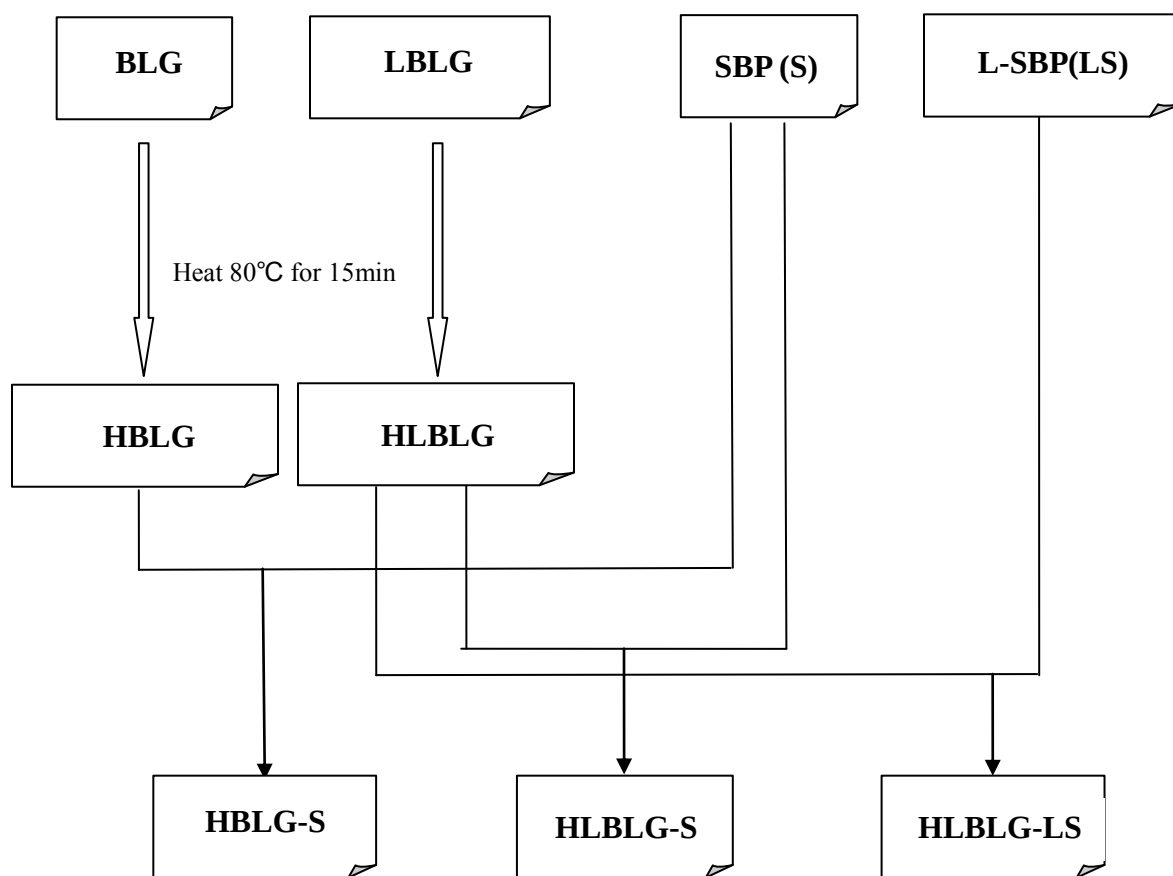


Fig.5-1. Flow diagram of laccase mediated HBLG and SBP mixture preparation.

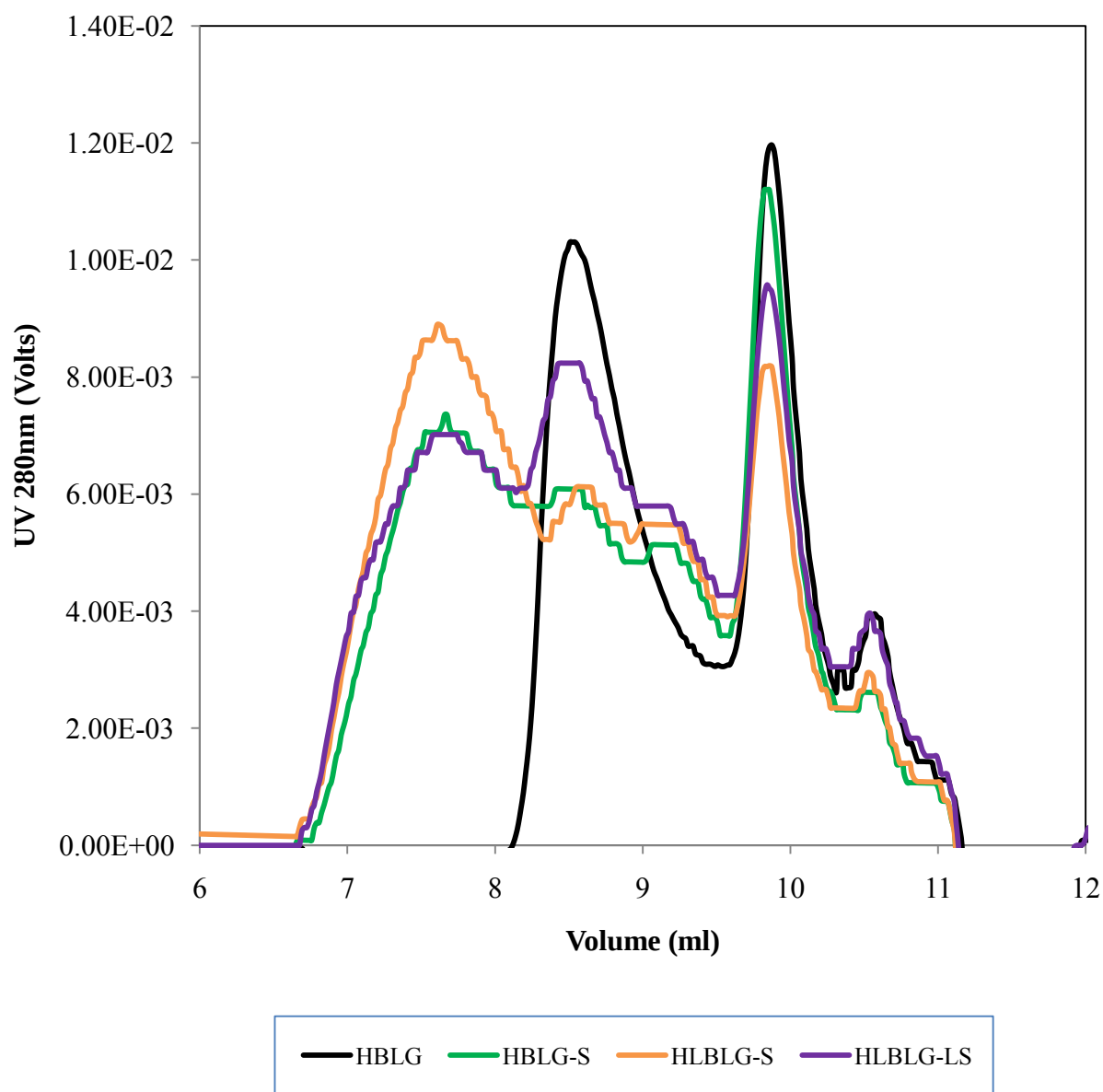


Fig.5-2. UV elution profile of HBLG and HBLG & SBP with and without laccase monitored at 280nm.

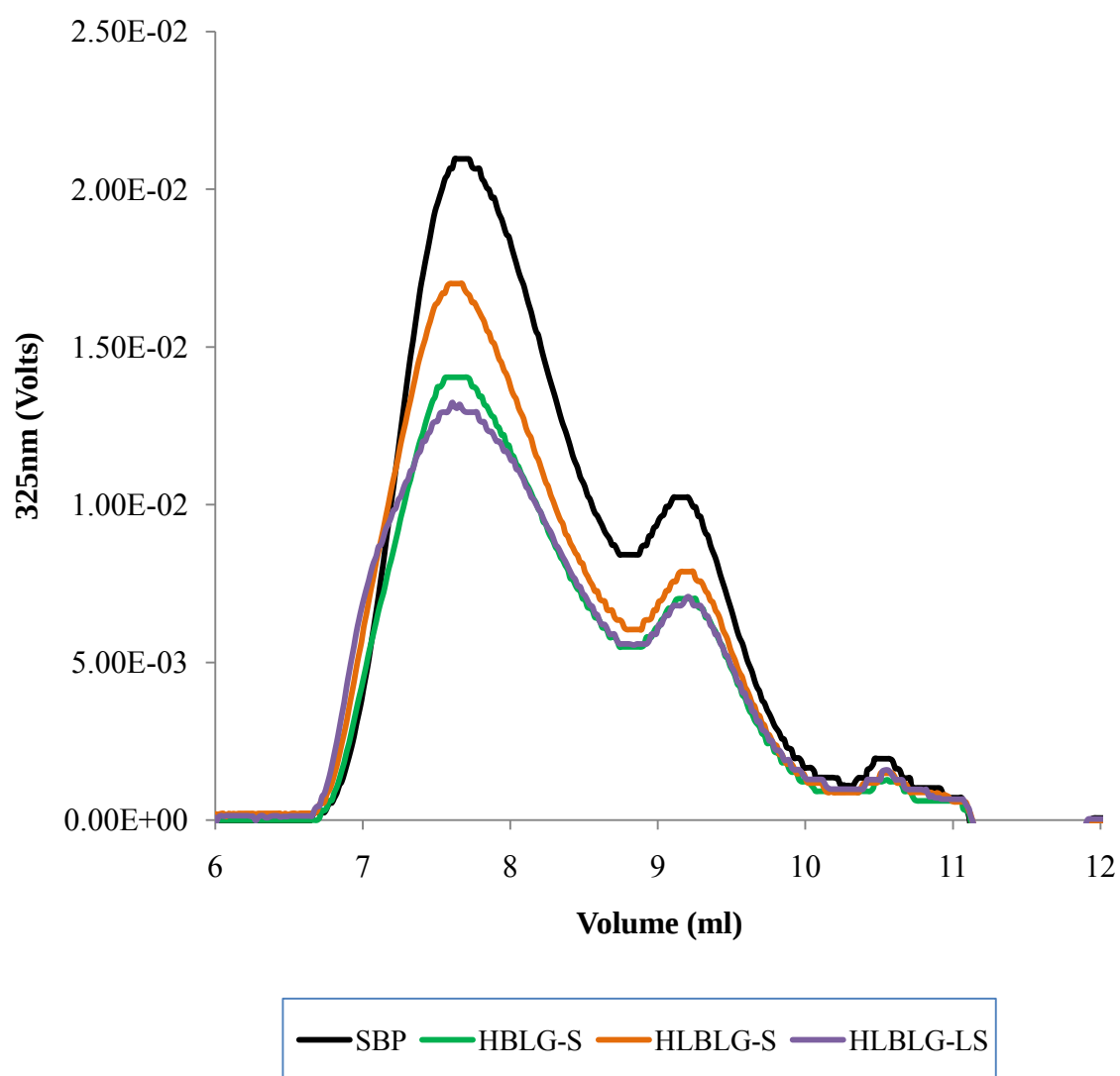


Fig.5-3. UV elution profile of SBP, and HBLG & SBP with and without laccase monitored at 325nm

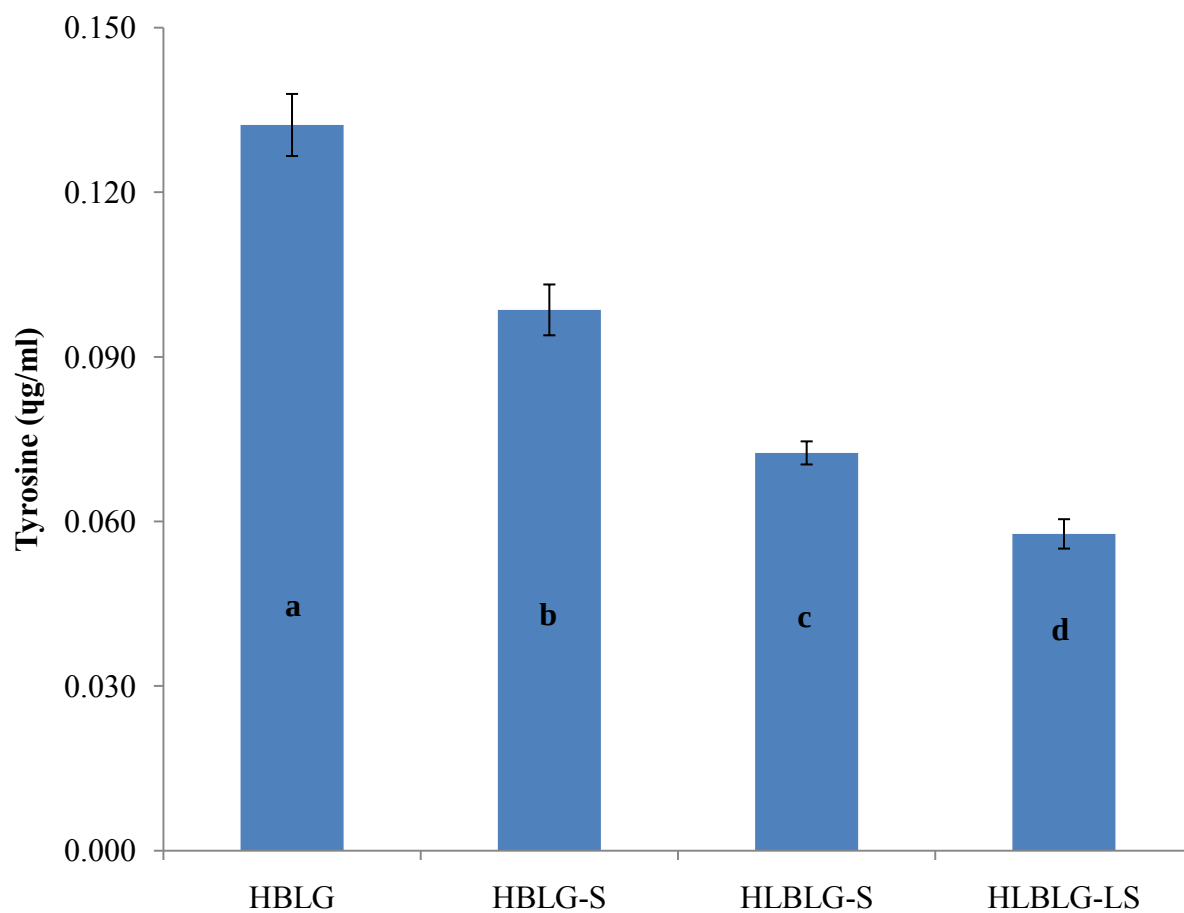


Fig.5-4. Changes of tyrosine residue in HBLG, and HBLG & SBP with and without laccase.

^{a,b,c,d} : Same letters are not significantly different among samples ($p < 0.05$).

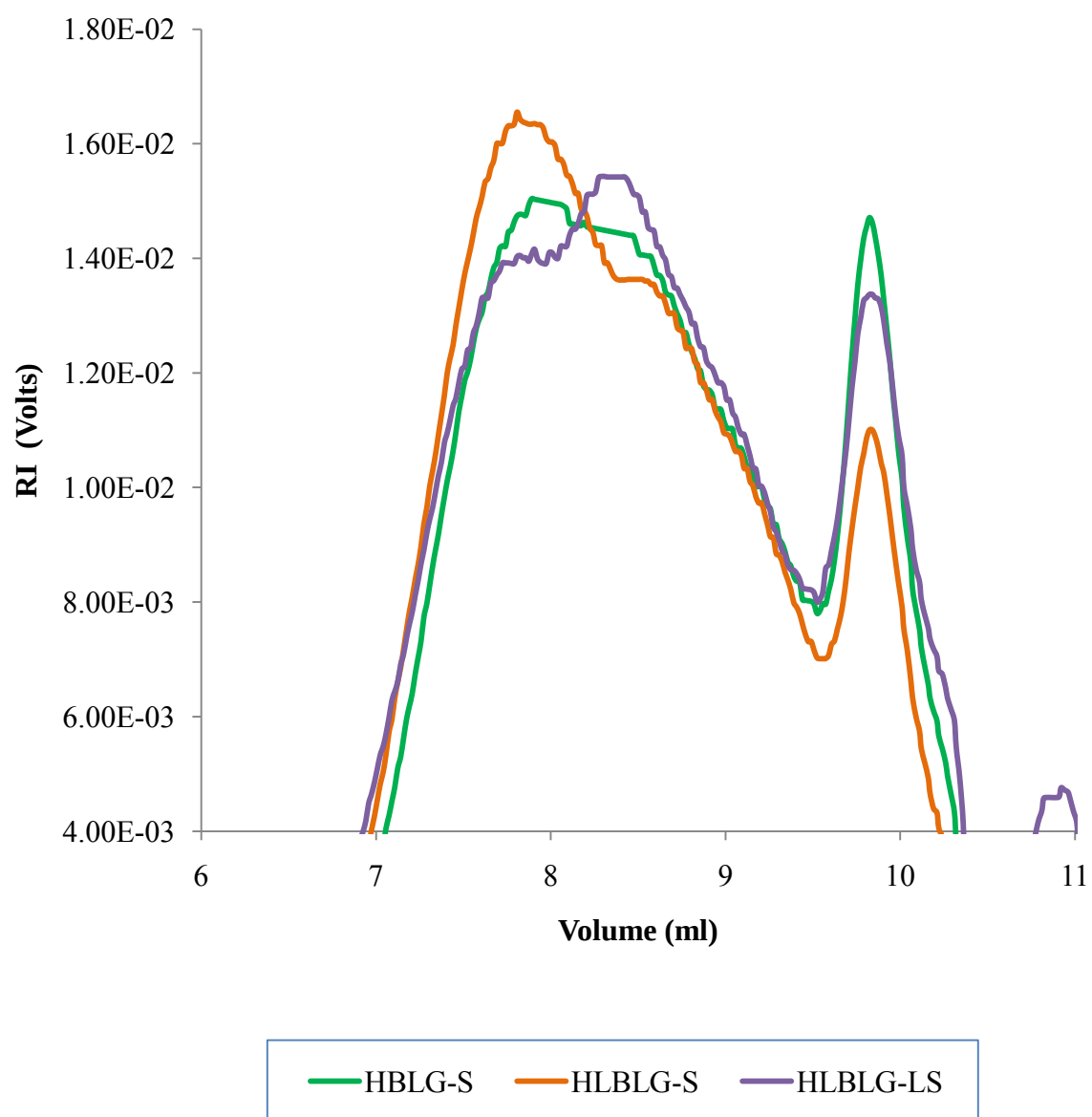


Fig.5-5. RI elution profile of HBLG & SBP with and without laccase.

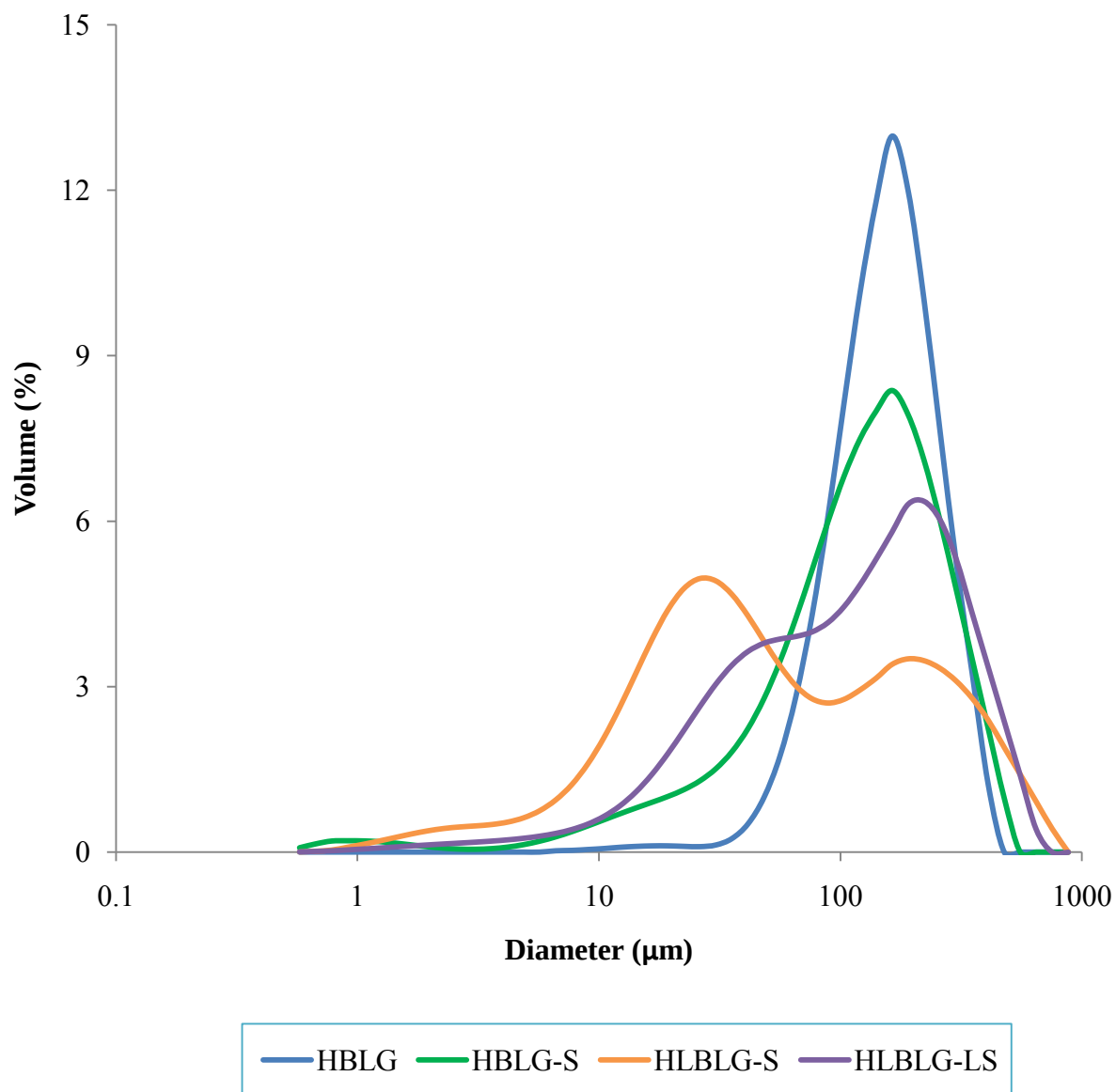


Fig.5-6. Particle size distribution of HBLG, and HBLG & SBP with and without laccase.

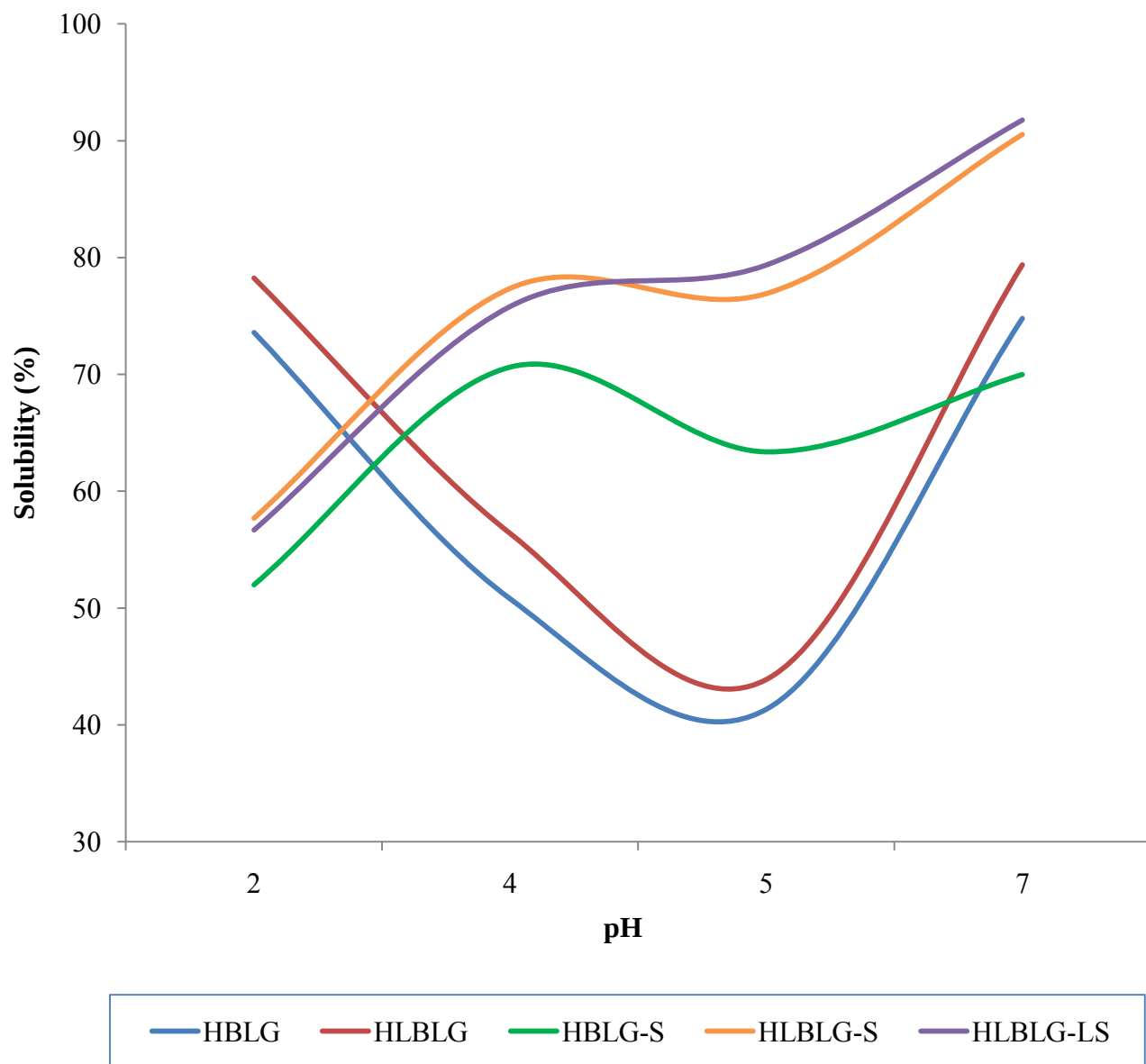


Fig.5-7. Soluble protein concentration of HBLG, and HBLG & SBP with and without laccase.

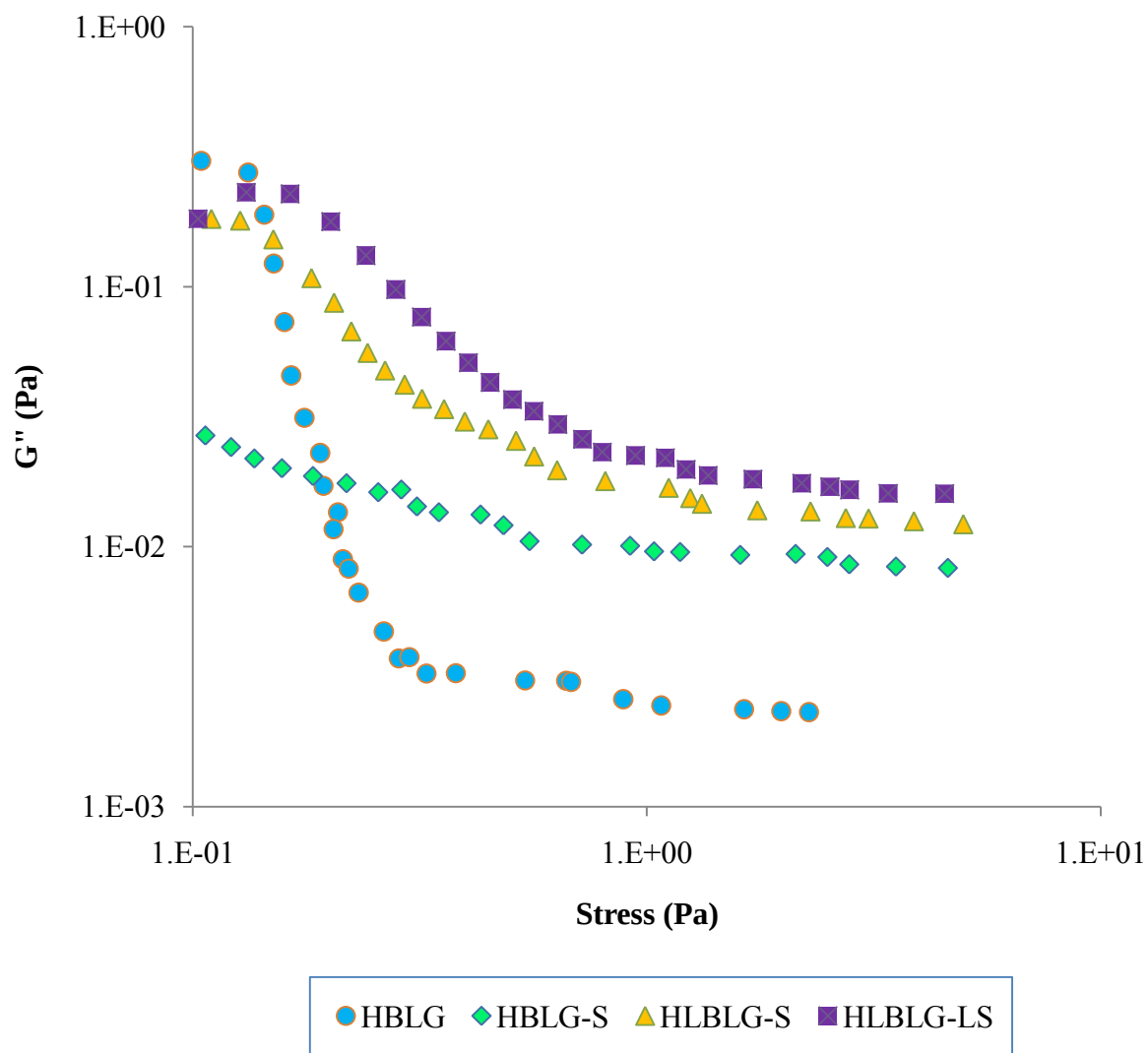


Fig. 5-8. G'' on shear force for HBLG, and HBLG & SBP with and without laccase at 0.1HZ frequency.

CHAPTER 6

CONCLUSION

Sugar beet pectin (SBP), β -lactoglobulin (BLG) and their conjugations as well are potential functional components in the encapsulation and the delivery system for bioactive materials with wider range of functional properties. In this study, laccase which oxidizes phenolic compounds was used to produce homo- and hetero- conjugation in SBP and BLG. It was hypothesized that conjugation would be generated through phenol compounds present in SBP (ferulic acid) and BLG (tyrosine) by laccase, an oxidative enzyme. The purpose of this research was to determine if laccase could catalyze oxidative coupling in SBP and BLG, individually or corporately, and to measure the improvement of functional properties of homo- and hetero-conjugates.

Laccase induced cross-links within SBP moieties through ferulic acids. Cross-linked SBP had reduced ferulic acids and had greater molecular weight, observed by HPSEC-MALLS and UV detector. Laccase mediated SBP conjugations had a smaller RMS against larger molecular weight leading more packed and highly branched polymer structure. Moreover, this study found that an emulsion prepared with conjugated SBP exhibited better emulsion stability than untreated SBP, showing significantly a smaller d_{43} diameter and uniformly dispersed oil droplets in microscope image. Laccase did not create cross-linking in BLG, but the conformational change was observed based on the following results: 1) a smaller and broader in RMS distribution 2) a change in polymer chain structure 3) bigger particle size (222 nm to 1278 nm). The mixture of modified BLG via laccase and SBP presented greater molecular weight corresponding to a

reduction of overall concentration. However, hetero-conjugation between BLG and SBP was unlikely produced because the protein detected at UV₂₈₀ was almost the same before and after laccase treatment. Upon heating, polymerization occurred in BLG treated laccase with greater molecular weight. It was assumed that exposed tyrosine and cysteine groups induced homo-conjugation by laccase. In order to determine the effect of heat and laccase treatment on conjugation, BLG with and without laccase was heated for 15 min at 80 °C and mixed with laccase treated SBP and non-treated SBP. The results of UV₂₈₀ to follow protein revealed that laccase treated samples with heat treatment eluted earlier and showed a higher peak where SBP normally eluted. It was proved that more hetero-conjugation was preferred in HLBLG-S by observing more proteins eluted with SBP at UV₂₈₀ and more ferulic acids at UV₃₂₅. On the contrary, HLBLG-LS showed the lowest ferulic acid and tyrosine contents. It indicated that homo-conjugation within SBP through ferulic acids and BLG through tyrosine residue was dominant in HLBLG-LS. Cross-linked conjugates were confirmed by particle size distribution. HLBLG-S and HLBLG-LS exhibited additional population at smaller size with reduction of bigger size population indicating heated BLG aggregates. Moreover, the conjugated samples demonstrated better solubility over the all range of pH than non laccase treated samples, HBLG-S and HBLG. In addition, the conjugated samples showed quiet weak gel behavior by observing the loss modulus against shear force in a mechanical experiment.

It was verified that SBP induced homo-conjugation through oxidative coupling by laccase and also produced hetero-conjugation by combining with laccase treated BLG with heat. Those conjugated samples had better functionality in emulsion stability, solubility and potential gelling ability. Laccase caused conformational change rather than inducing homo-conjugation in BLG alone. However, polymerization in heated BLG was encouraged by the presence of laccase

and it might be caused since tyrosine residues, substrate for laccase, were exposed on the surface of BLG through heat.